

Spatial Atlas of Diabetic Kidney Disease Reveals a B-Cell-Rich Subgroup

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Any redactions in this file are there to maintain patient confidentiality, the confidentiality of unpublished data, or to remove third-party material.

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

Version 0:

Reviewer comments:

Referee #1

(Remarks to the Author)

The manuscript by Dumoulin et al. titled "Spatial Human Kidney Map Highlights B-cell Driven Kidney Disease Subgroup" aims to map kidney cells using spatial transcriptomics, focusing on identifying disease-relevant spatial niches. The authors generated data from 1.7 million cells across 16 human kidney samples and integrated it with publicly available single-cell RNA sequencing data. In addition, they created an immune atlas of the kidney from 143 patients, which revealed a B-cell predominant immune microenvironment near the injured loop of Henle. Spatial signatures were then used to discover a blood biomarker that correlates with kidney health and mortality.

However, there are various major concerns:

This is a very preliminary study that analyzes only a small number of patients with spatial data (n=13), despite the seemingly large number of cells. While the cell count appears substantial, the actual number of patients is very low, and spatial analyses typically yield high cell counts. I feel that the authors are overstating their data by highlighting the cell count without mentioning the low patient count in the abstract. The small number of patients is especially significant when comparing different conditions within the spatial cohort.

Additionally, the computational methods are not clearly defined. The authors need to provide a more robust and transparent description of the computational pipelines used and their choice of parameters. Regarding the discovery of niches in their spatial data, is the number of samples sufficient to account for inter-individual and inter-disease variability? Was any power analysis conducted prior to the study?

The biomarker discovery should also be compared to current standard markers for predicting kidney outcomes, such as eGFR and proteinuria. Furthermore, many of the findings presented lack novelty, as outlined below.

It is also unclear what the distinction is between a niche and a microenvironment. Can the authors provide a clearer definition of these terms?

Regarding the calculation of differentially expressed (DE) genes: Why was the Wilcoxon test chosen for DE analysis? Doesn't this test lead to a higher rate of false positives compared to MAST or pseudo-bulk approaches?

The identification of niches (Fig. 2) in this paper doesn't offer any novel insights, as B cell accumulation is common in most chronic diseases.

What is the overlap in gene expression between the niche signatures? How are these spatial niche signatures determined? Is it based on some variation of marker gene analysis at the niche level? If so, how do the authors account for the different cell types present in each niche when comparing them to other niches?

Figure 2a: The niche identification method needs to be described in more detail. For instance, are different KNNs (k-nearest neighbors) constructed for different radii, or do the authors use a single optimal radius? Additionally, are overlapping

elements in each neighborhood taken into account? Would it be possible to differentiate between long- and short-range interactions based on the chosen radii?

Figure 2i: “Change in relative niche fraction in DKD vs healthy controls” → How is the fraction calculated? The fibrosis fraction is listed as 245. Does this represent a 245-fold change in DKD compared to controls?

Figure 3, Line 333: “This clustering uses K-means clustering of the 20, 40, 60, and 80µm neighborhood fraction” → This is not clearly defined. Is the microenvironment (ME) and the niche different depending on the identity of the center cell? This also relates to line 247 in Figure 2.

The authors provide some statistical evidence of the glomerular niche, such as podocyte loss and mesangial cell expansion, but these insights are not novel. Did the authors identify any new interactions or features across the 10 niches they studied?

It appears that the calculation of the spatial niche signature does not take any of the spatial features such as the spatial organization of the niche into account. Is this correct?

The biomarker expression of iLOH ME1 and the biomarker in Figure 5g do not seem to overlap. How strong is the overlap? This is unclear from the presented data. The authors need to clearly demonstrate whether the signatures overlap or specify which cell types express them.

SPP1 and MMP7 are not novel biomarkers and are not specific to this disease. Their clinical relevance is uncertain. The authors should reference the numerous studies, including reviews, on SPP1+ macrophages in chronic diseases across different organs.

For the biomarker discovery, it is critical to compare the new biomarkers with the current gold standards, such as proteinuria and eGFR at baseline, to demonstrate that the newly identified biomarkers outperform these simple measures. Additionally, a comparison with other reported biomarkers, such as urinary EGF, should be included.

Although the authors claim this is the largest dataset available, the same lab recently published a spatial atlas in Nature Genetics covering more patient samples and a greater number of cells (though these were not included in this study).

The authors should also compare their findings to previous KPMP studies, which have already identified iLOH as an injury phenotype.

The discovery of TNFRSF19 is interesting, but it remains unclear how this gene mechanistically contributes to the progression of CKD.

Since the authors used a label transfer approach with existing cell-type annotations to label their spatial data, how can they detect previously unidentified cell types or states that were not present in the reference datasets?

Is there any overall correlation between kidney and plasma proteomics (Fig 5)?

Minor comments:

Generalized additive model – Figure 1f, predicted gene expression. This procedure is not clearly defined. What is the overall performance of this GAM based method for inferring gene-expression per cell?

Line 143: “Additionally, fewer immune cells were detected “ → can this be quantified and presented with p-values.

Page 4, lines 116 and 119: how are the low quality cells defined in these two steps. Is this done sample per sample or on aggregated data?

Extended Figure 4a → “65 genes were selected for the COVET”. How were these genes selected? and how is the number of genes determined?

Extended Figure 4g → what does each dot represent? Is it the low dimension representation of a niche times samples? So is it 130 dots?

Line 225: “and an increase in mesangial cells” → podocyte loss and increase in mesangial cells in the Glom Niche. Is there any association between these observations?

Figure 2j and 2m: are the genesets reported in these figures statistically significant?

Figure 3: are the differences reported in figure 3c and 3b statistically significant? Why is 20µm radius chosen here? while the ME is defined over 200µm?

Figure 3a: what is the cellular composition of each ME?

Figure 3d, and 3e: how are the DE genes calculated? are they driven by the major cell-type in each ME?

Line 360: data integration needs to be described in greater detail - are these all healthy kidneys? are the datasets comparable to each other in terms of their QC related properties such as percentage of genes mapped to mitochondrial genes? ribosomal genes?, UMI and gene counts? What batch variable was used to mitigate technical batch-effects during integration? How does the integrated map compare to individual studies? Are the cell-states identified observed in cells and samples from each state?

Figure 4b: how is the immune-center and the neighborhood of the center defined in figure 4b?

Figure 5a: are the correlation values provided in figure 5a statistically significant?

(Remarks on code availability)

Referee #2

(Remarks to the Author)

Dumoulin B and colleagues present a comprehensive work on the integration and analysis of healthy and diseased human kidneys. This is an impressive effort and it can be seen that a lot of work went into it. A particular aspect of the study that I enjoy is the visionary translational medicine aspect of the B-cell niche to classify kidney disease. However, some shortcomings require addressing.

Major Comments:

The authors' integration methodology, whilst comprehensive, would benefit from rigorous validation through established benchmarking metrics, such as scIB-metrics (<https://scib-metrics.readthedocs.io/en/stable/>). The implementation of scIB metrics would be particularly valuable to demonstrate the preservation of biological signals whilst accounting for batch effects in their scVI/scANVI integration.

The approach to niche identification with Mini Batch k-means clustering and COVET is sound, the incorporation of contemporary methodologies such as NicheCompass (<https://nichecompass.readthedocs.io/en/latest/>) would substantially strengthen their analysis. NicheCompass's integrated workflows for multi-sample analysis and superior batch effect handling could provide additional validation whilst potentially unveiling subtle niche variations across disease states.

The COVET implementation gives valuable insights but does not fully exploit the method's capabilities. The authors might consider examining gene-gene covariance matrices within niches to identify coordinated gene programmes, analyse spatial correlation structures to reveal niche-specific regulatory networks, and investigate transition zones between niches. Such analyses would provide deeper mechanistic insights into niche organisation and regulation, which would be a fantastic addition to their translational aim.

Can the authors elaborate on the selection process for the cell-cell communication database? The cell-cell interaction analysis, currently limited to CellPhoneDB v5, would be considerably enhanced through integration with OmniPath database (<https://omnipathdb.org>). This would facilitate validation across multiple interaction datasets whilst providing valuable pathway context. Moreover, extending the analysis beyond iPT/iLOH-fibroblast interactions to encompass all identified niches would offer a more complete understanding of cellular communication within the kidney architecture.

The spatial data quality control requires more thorough documentation. The authors should delineate their criteria for filtering spatial coordinates, assessment of tissue processing artifacts, and validation of spatial patterns across replicates. Additionally, quality metrics specific to the CosMx platform should be explicitly stated.

The current GitHub repository would benefit from detailed workflows, preprocessing protocols, and clear parameter justifications. The inclusion of validation steps, example outputs, and quality control metrics would ensure reproducibility. At the moment, it looks like a collection of scripts to generate the figures. Formalising them into notebooks would facilitate reproducibility and aid with clarity. Moreover, there is no information on the environments used for each step of the analysis.

The manuscript would benefit from a more thorough discussion of spatial resolution limitations. The authors should address the CosMx platform's resolution constraints, their impact on niche boundary definition, and potential artifacts in densely packed regions. Validation of spatial patterns at different resolution scales would strengthen their conclusions.

The construction of the reference space for annotation transfer warrants a more detailed explanation. Specifically, the selection criteria for anchor genes, validation of transfer accuracy, and assessment of reference space stability should be thoroughly documented. This ties back to point #1.

Minor Comments:

The visualisation of spatial data is informative but could be enhanced through multi-scale representations of niches, three-dimensional reconstructions where feasible, and explicit demonstration of transition zones between niches. The inclusion of validation visualisations would strengthen the presentation of results.

The reproducibility framework would benefit from the provision of a containerised analysis environment, comprehensive package versioning, and documented parameter sensitivity analyses. The inclusion of validation datasets would facilitate independent verification of the authors' findings.

In the opinion of this reviewer, these enhancements would substantially strengthen this otherwise commendable study of kidney tissue architecture and disease progression.

(Remarks on code availability)

The reproducibility framework would benefit from the provision of a containerised analysis environment, comprehensive package versioning, and documented parameter sensitivity analyses. The inclusion of validation datasets would facilitate independent verification of the authors' findings.

Referee #3

(Remarks to the Author)

Summary

Dumoulin and colleagues utilize advances in spatial transcriptomic to build a single-cell atlas of the human kidney in health and chronic kidney diseases, with the focus of the latter upon diabetic kidney injury. The authors integrate spatial transcriptomic analysis with matched snRNAseq dataset and mapped the snRNAseq annotations to the spatial transcriptomic dataset, generating a high-resolution spatial map that captures the finely-tuned changes in gene expression across numerous cell types in different regions of the kidney. Using this atlas, the group defines specific parenchymal and immune cell niches via their cellular composition that are present in healthy kidneys as well as niches that are specific to disease. They go on to define the cellular microenvironments surrounding these presumably injured regions of the kidney, reporting cell types associated with disease, highlighting increased B cells, and report markers associated with pathogenesis. Most importantly, the work defines patient subgroups based on associations with niche composition, fulfilling an unmet need of classification of chronic kidney diseases that are highly heterogenous in their clinical presentation and course. Lastly, the authors combine serum and kidney proteomics to generate potential biomarkers derived from the aforementioned niches and validate these markers with prediction of disease course. x

Significance

Understanding the complex cellular landscape of the kidney is crucial for the mechanistic understanding of renal physiology in health and disease. The authors provide the largest spatial single cell atlas of the kidney yet, performing analysis of large regions rather than previously-reported spot areas. Previously, the Susztak lab reported broad cellular environments within the kidney but now go on to further classify these microenvironments as distinct niches, with unique cellular composition where gene expression varies depending on specific location within the kidney, highlighting disease-specific niches. While it remains to be seen how these niches hold true in other settings of other kidney diseases, such as acute, autoimmune, and inflammatory injury, the authors make advances including the documentation of gene expression changes in the same cell type along the corticomedullary axis which reflect cell type changes to meet tissue and organ function, the integration of nuclear/cellular RNAseq data with matched spatial transcriptomic data, and the identification of unique cellular niches that are associated with disease. Furthermore, the largest conceptual advance comes from their use of the spatial niches to subdivide patients as this may bode for personalized treatment strategies. The combination of niche composition with kidney and serum proteomics to map disease states to specific cellular locations is impressive as it provides a framework in which spatial transcriptomics can be combined with proteomics (especially serum considering ease-of-sampling) and can be used to not only to identify patients with complex and heterogenous diseases into specific subgroups, but also to investigate the underlying cellular and molecular drivers of disease. While the paper falls somewhat short in providing mechanistic link underpinning what the authors describe, the paper is impactful as it provides a detailed roadmap for using these types of omics studies to stratify patients with complex/heterogeneous diseases.

Points for the authors to consider

- 1) While the identification of niches corresponding to "injured" cells is crucial, it would be informative to know how these injured niches (iPT and iLOH) compare to normal niches in their gene expression differences. While the supplementary tables contain what the cluster defining genes are, it should be stated in the text how the authors define iPT and PT and what the differentially expressed genes between iPT vs PT and iLOH and LOH. This is an analysis that should be stated.
- 2) Figure 1: Validation of spatially variable genes would be helpful, showing the differences in protein expression along the cortical and medullary sides (panel f).
- 3) Figure 2: The presence of iPT in control samples and diabetic samples without kidney involvement warrants further validation of the injured niches and is not mentioned in the text (panel h). The authors show fraction increases in DKD but it would also be informative to show what niches decrease in DKD (panel i).
- 4) Figure 3: While the authors define microenvironments surrounding the injured niches, it would be interesting to see if and how microenvironments around the other niches change in DKD patients. In 3j, the authors should consider further dissection of the immune cell composition shared between iPT ME4 and iLOH ME1. What are the predominant immune cell types shared between these microenvironments?

5) Figure 4: The resident kidney immune cell microenvironment is insufficiently defined. Because these cells were not seen around injured tubular areas does not mean they are typical of the kidney. Further characterization and validation of this resident kidney immune cell microenvironment should be performed. In panel c, the immune cell zonation as characterized by the predominance of immune cells is insightful, yet the presence of CD8+ T cells in all microenvironments except the B cell microenvironment is interesting. Further interrogation of this observation may be beyond the scope of the manuscript but is worth noting. Only two patients show B cell-predominant immune microenvironments (panel d). The authors might consider not generalizing these findings. Furthermore, CD4+ predominant environments seem to be more consistently seen across diseased patients. The authors suggest a causal role of B cells in kidney progression but it is unclear what effects the B cells have in progressive kidney disease. Furthermore, have the authors ruled out recent viral infections? B cell TLS are seen in acute kidney injury as well as ageing so it is unclear whether the observation reported is a microenvironment unique to chronic kidney disease or kidney injury (PMID: 27699223). At some point in the future, the authors might consider examining clonal expansion within these populations. This would help our understanding the presence of B cells in these microenvironments.

6) Figure 5: iPT does not correlate with disease severity as strongly as the iLOH and immune niche. Does this call into question the validity of the iPT niche? In panel b, statistical analysis should be performed for the survival curves.

Minor comments

- 1) Sample origin: 13 total samples: 3 control (relatively young males) vs 10 diseased: 5 with Diabetic kidney disease, 1 CKD, and 1 DM
- 2) Figure 1a: Missing scale bar on PAS panel
- 3) Figure 1e and 4a: It may be more helpful to place the cluster labels on each population rather than on a legend as some of the colors are difficult to distinguish from one another.
- 4) Fig 2K, 2L: Stats missing from the plots themselves.
- 5) Figure 3: The case is made that fibroblasts are consistently seen in close proximity to iPT and iLOH; however, the representative images shown in Figure 2f are not so convincing. PAS staining of these niches would also be beneficial.
- 6) Figure 4b: The labeling scheme used here is rather confusing as similar colors are used between the outer and inner circle although they refer to different cell types.

(Remarks on code availability)

Referee #4

(Remarks to the Author)

Comments to authors

Thank you for the opportunity to review the manuscript "Spatial Human Kidney Map Highlights B-cell driven Kidney Disease Subgroup" by Dumoulin and colleagues. This body of work applies subcellular spatial transcriptomics using the CosMx platform to profile the complex architecture of the Kidney in the context of 4 healthy kidney samples compared to tissue from 4 patients with diabetes (DM), 2 samples from a patient with chronic kidney disease (CKD) and 7 samples from 5 patients with diabetic kidney disease (DKD).

Using their analysis pipeline, the authors describe the kidney architecture spatial in reference to ssRNA seq data including spatially variable genes (figure 1). By performing "neighbourhood" analysis at a range of sizes (varying from 20-80um), niches are defined relative to central cells based on the cell type composition and local gene expression. Using this approach, the authors can characterise local microenvironments (ME) relative to structures (such as the proximal tubule and loop of Henle in) either a healthy or disease/injured state (figures 2 and 3). Based on this analysis, both the cell composition of the niche as well as the genes that are differentially expressed, and potential drivers of ligand-receptor interactions can be inferred. Based on this approach, cell types profiled at either a per sample (healthy versus disease) or local ME, outline an association with immune cell expansion/infiltration (with a particular focus on B cells in Diabetic Kidney Disease) in patients with kidney disease compared to healthy controls (Figure 4). While the findings of the study are interesting, I do have some concerns.

1. Biological insight.

The major conclusions drawn in this paper appear to confirm well-known features of diabetic renal disease rather than demonstrating novel ones. It is concluded, for example, that inflammatory changes correlate with DKD, as does an increase in mesangial cells and reduced podocytes in the glomerulus. There is a large literature on the role of inflammation on the pathogenesis of diabetic nephropathy. Similarly, the classic histological features of DKD include mesangial hypercellularity and associated glomerular (and therefore podocyte) destruction. Neither are referenced. The observation that tubular injury is associated with disease and its progression (p 13) is also well known. These observations therefore demonstrate that the technology being used in this study can replicate findings using more conventional approaches – a useful "positive control" for the approach rather than a demonstration that it can discover important new features of disease.

The description of a microenvironment in which B cells predominate is interesting, and a more detailed analysis of it would be of interest. The finding that there is an increase in immune infiltrate including B cells in diabetic kidney disease has been reported previously, and a correlate with infiltrate and disease progression is not unexpected. In the absence of applying

CosMx to refine B cell subtypes via gene signatures at single cell resolution (outside of inflammatory pathways consistent with DKD), it is difficult to determine the additional value of this analysis compared to existing studies. What subset of B cells are present, what are their isotypes, are they associated with other features of tertiary lymphoid structures etc.. This is a potentially interesting finding that needs to be described in more detail, validated, and correlated with disease in a larger cohort. That a “large number” of renal function/disease associated genes identified in a GWAS are expressed in B cells is inconclusive. Have the authors compared the expression of GWAS-implicated genes across all kidney-resident immune cells to determine whether B cells uniquely express these genes at higher levels?

2. Sample size

The techniques and analyses used appear appropriate. One concern, however, is the very low sample number, with only five individuals with DKD being compared with four healthy control samples. It is also not always clear which diseases are being compared with healthy. The initial analysis appears to include all three disease subcohorts together with the healthy one. It is not clear if the replicate samples (that expand the DKD cohort from 5 to 7, for example) are treated as independent values during statistical analysis – this would not seem appropriate and given the cohort sizes their exclusion might make a difference to conclusions.

3. Comparison with previous work.

Many findings in the study are incremental in reference to a similar study recently published (July 2024) by the same authors in Nature Genetics (<https://doi.org/10.1038/s41588-024-01802-x>). In this study a similar sized cohort (including an overlap of samples analysed in this submitted manuscript) are profiled spatially using Visium genome sequencing at 50um resolution. Using the same custom gene panel in this study, CosMx was previously performed on two samples (1 Healthy, 1 disease) to train methodology to resolve, or infer, cell types at single cell resolution. Therefore, while only 2 samples are analysed at subcellular resolution in the previously published manuscript (compared to 16 here), there is significant overlap in the kidney structures that can be identified, and possibly meta-analysis that could have been performed to reach the same conclusions. For example, expression of immune signatures (such as B cells) that are not reported in the previous manuscript could be analysed from this data without increased resolution as opposed to the fibrotic niche/microenvironment. Thus, while this manuscript does report 14 more samples with increased resolution, there is significant overlap between the two studies in design, methodology and patient cohorts.

4. Literature

Little attempt is made to compare the findings of this study to the literature (incl ref 15). There is, for example, a literature on the association of diabetic nephropathy with inflammation and immune dysfunction. Comparisons should be made between the niches described and conventional histological features that correlate with them (e.g. comment on mesangial hypercellularity above). Some previous single cell and spatial studies of kidney disease are dismissed out of hand (see refs 15 and 21-26 in the discussion – mouse studies might well be very relevant) or not discussed in detail (e.g. Lake BB Nature 2023). These studies need to be compared to the present one in more detail to avoid the inadvertent implication of novelty.

5. Other issues

a) The term “kidney disease” needs to be used with care. It is not possible to generalise to all kidney disease from this small study focused on diabetic nephropathy, and yet this is implied on occasion, including in the title. Care should also be taken to avoid implying causation where association is described – again one reading of the title would imply this (another would imply that DKD is B cell-driven).

b) The clinical cohorts are not well described. Disease course and stage, past and current treatment and ethnicity could all impact on the data collected – but this information is not provided or not discussed. How is DKD, DM and CKD defined? How is variable renal function taken into account? How are the repeat specimens related to each other (some seem contemporaneous - as GFR is the same for each - others not)? Such clinical detail is important in a disease-focussed study, especially one with small patient numbers.

c) The study analysed spatiotemporal gene expression patterns along the corticomedullary axis, linking these patterns to physiological roles such as water resorption, calcium handling, and bicarbonate transport. The authors make a number of points regarding spatial gene expression (Figure 1f) all of which are inferred based on computational prediction. Have any of these been experimentally validated? Later injured proximal tubule and loop of Henle disease-specific niches suggested a role for fibrosis-related markers and signalling pathways in disease progression. Have any of the computationally predicted receptor-ligand interactions been functionally validated?

d) The analysis offers a snapshot of kidney niches, that lacks temporal dynamics to understand how niches evolve over time or under disease progression. A small number of the FFPE samples analysed come from the same individual. Were these taken at distinct times and have they been assessed for temporal changes in niche composition that may be associated with disease?

e) The authors then went on to generate a spatially resolved kidney immune cell atlas using seven (1x single cell (sc) RNA-Seq, 5x snRNA-Seq and 1x CosMx) datasets with 143 patients and 165,161 kidney immune cells. This approach is not without its pitfalls. Differences in experimental conditions, platforms (snRNA-seq, scRNA-seq, CosMx), and sample preparation methods could introduce batch effects that distort biological signals. The use of three and five-layer neural

network models in SCVI/scANVI with 30 latent dimensions may overfit or underfit depending on the dataset size and complexity. Fixed parameters (e.g., 3,000 highly variable genes) might not be optimal for all datasets, potentially excluding important genes or introducing bias. Data quality across sources (e.g., KPMP and CosMx datasets) might vary, and lower-quality datasets could disproportionately influence integration outcomes. Finally, as the authors describe cell dissociation artifacts can lead to selective loss of fragile cell types which might distort cell type representation. How have the authors addressed these potential concerns?

f) Finally, the authors explored the relationship between the cellular niches they identified and kidney disease severity. The iLOH ME1 niche was found to be strongly associated with interstitial fibrosis (Figure 5a) and clustering of patients from the TRIDENT study using this spatial gene signature defined three groups of patients, iLOH-, iLOH+ and iLOH+ B+. These three groups differ in baseline kidney function with eGFR being 49.5, 33 and 19 cc/min/1.72m² in the three groups, respectively. Unsurprisingly, patients with the lowest baseline eGFR, and by definition most severe disease, reached the renal composite endpoint fastest (Figure 5b). Do any of the other spatial gene signatures stratify patients in a manner that correlates with renal outcome but doesn't simply reflect baseline disease severity?

g) Kidney and blood proteome analyses identified 394 significantly altered proteins in kidney tissue and 217 altered proteins in blood samples from patients with the iLOH+ gene signature. An overlap of 111 proteins was observed (Figure 5e), suggesting these proteins may serve as biomarkers of tubular damage and kidney disease risk. Comparing the degree of overlap based on p value thresholds will result in an underestimation of the degree of overlap due to differences in the number of proteins measured and relative sample sizes. What is the correlation between effect sizes for proteins measured in both studies? This is likely to produce a better indication of the degree of overlap between the two modalities.

h) Differentially expressed proteins found to be common to both modalities showed a strong positive correlation with clinical outcome, measured as either a kidney-specific or composite outcome. The hazard ratios (HR) for both outcomes appear identical (Figure 5f) suggesting that the association with combined outcome is driven predominantly by the association with kidney outcome. Do any of the protein biomarkers show an association with death alone? In addition, the HR observed for all patients are broadly similar to those seen for the patient groups with the lowest eGFRs. Are similar HR seen for the patients that don't have hypertension, diabetes or chronic kidney disease and who are likely to have preserved eGFR? Finally, can a sparse multivariate predictive model be developed from this collection of potential biomarkers that might have utility in predicting kidney disease outcome in an unseen cohort of patients?

(Remarks on code availability)

Version 2:

Reviewer comments:

Referee #1

(Remarks to the Author)

The study has substantially improved by clarifying previous issues, adding 64 new samples and clearly explaining the computational methods.

The niche concept has been nicely validated using three independent methods. I thank the authors for their excellent work on revising the manuscript. Overall, my remaining concern is with the spatial relevance of the gene-gene correlations, spatial relevance of the LRs reported from cellphoneDB cell-cell communication analyses and multiple hypothesis correction of the p-values reported in most sections of the paper.

Extended Figure 15 to Figure 25 shows spatially correlated genes in different niches and a comparative differential correlation analysis then identifies gene correlations increased in disease (DKD) over controls. This is a really interesting way to find unique and common gene-gene patterns. The niches are found in a spatially constrained manner. The question now is: are any of these gene-gene correlations obtained convey any spatial relationship? Can these gene-gene correlations be used to identify long and short-range interactions?

In addition to the above point in line 246-251, are these correlations reported after adjusting for multiple hypothesis correction?

Figure 2r - are these ligand-receptors spatially constrained? It would be interesting to show some close distance proximity of these LR pairs and their corresponding cell types.

Figure 3o shows really robust correlation with a nice narrow confidence interval. However, what is not clear is - given that these are findings reported from the spatial data, are these also spatially co-localised or in close proximity?

Figure 4u and 4w, I am concerned about the statical relevance after controlling for multiple hypothesis correction.

Line 478 "organized B cell-predominant microenvironment that case be revealed by spatial transcriptomics." is not clear. Do

the authors want to say “can” instead of “case”

(Remarks on code availability)

Referee #3

(Remarks to the Author)

Dumoulin, et al., Spatial single-cell atlas of diabetic kidney disease reveals a B-cell-rich disease subgroup

Summary

The authors strengthen their findings by quadrupling the sample size, combining two different spatial transcriptomic platforms, and identifying eleven distinct and reproducible spatial niches that can be generalized across humans, not heretofore done at this scale and resolution. Using unbiased discovery, they identified distinct spatial niches, shared across both patients and healthy controls, that were orthogonally validated with different computational methods and inked to changes in eGFR. They go on to define injured tubular and immune cell microenvironments according to their putative roles in driving disease pathogenesis, by defining the nature of their interactions with surrounding cells. Lastly, they identify a B cell-rich microenvironment that identified a patient subgroup more likely to progress to renal failure. In toto, the work details a largesspatial atlas at the single-cell resolution for a human disease, with clinically-relevant findings for DKD that sheds light on potential therapeutic targets.

The authors have addressed concerns raised at time of original review, as detailed below.

An additional point: while the authors' demonstration that cell therapy mediated B-cell depletion in lymphoma leads to (quite modest) improvement in kidney function(extended figure 34), this observation does not support their contention that kidney-infiltrating B cells are associated with poor outcome in DKD. There are multiple possible explanations why renal function improves following such therapy, for example, improvement in overall organismal physiology. Moreover, since control kidneys (of the type found in lymphoma, given that such patients lacked intrinsic evidence of kidney disease) lacked significant B-cell infiltration, it is an overreach to use such modest data to validate a central thesis of the paper, which is otherwise supported.

In response to original review:

1) While the identification of niches corresponding to “injured” cells is crucial, it would be informative to know how these injured niches (iPT and iLOH) compare to normal niches in their gene expression differences. While the supplementary tables contain what the cluster defining genes are, it should be stated in the text how the authors define iPT and PT and what the differentially expressed genes between iPT vs PT and iLOH and LOH. This is a analysis that should be stated.

The authors sufficiently address this point by defining the transcriptomic differences between healthy and injured niches, demonstrating that key markers of damage were upregulated across both niches and across platforms. Furthermore, the authors provide a comprehensive and robust analysis that supports disease-specific spatial niches present in DKD.

2) Figure 1: Validation of spatially variable genes would be helpful, showing the differences in protein expression along the cortical and medullary sides (panel f).

Response: We thank the reviewer for requesting additional protein-level validation. After careful consideration we concluded that we cannot generate a uniformly convincing protein map for every spatially variable gene within the scope of this revision. To avoid presenting an incomplete or potentially misleading validation we have removed the spatially variable gene panel altogether and have withdrawn the related text.

While removing the data entirely sidesteps the question, validating the cell types that comprise the individual niches with protein staining (1-2 markers per stain) would suffice but understandable that not every spatially variable gene could be validated with protein confirmation.

3) Figure 2: The presence of iPT in control samples and diabetic samples without kidney involvement warrants further validation of the injured niches and is not mentioned in the text (panel h). The authors show fraction increases in DKD but it would also be informative to show what niches decrease in DKD (panel i).

The authors provide a reasonable biological explanation for the presence of certain “injury” niches in control samples and highlight a finding that may explain subclinical injury / aging. Furthermore, the authors added the requested analysis and demonstrate a shift from homeostatic to damaged tissue states.

4) Figure 3: While the authors define microenvironments surrounding the injured niches, it would be interesting to see if and how microenvironments around the other niches change in DKD patients. In 3j, the authors should consider further dissection of the immune cell composition shared between iPT ME4 and iLOH ME1. What are the predominant immune cell types shared between these microenvironments?

The authors sufficiently address these points by examining niche-specific regulatory networks and demonstrating changes

in gene-gene correlations in DKD. Specifically, they highlight that changes that are associated with disease and perform this analysis on all eleven niches. The authors also go further by generating a kidney immune atlas to identify seven distinct immune microenvironments and their cellular composition. Furthermore, they perform extensive analysis including ligand-receptor interaction analysis to probe how nearby immune cells are interacting with parenchymal cells.

5) Figure 4: The resident kidney immune cell microenvironment is insufficiently defined. Because these cells were not seen around injured tubular areas does not mean they are typical of the kidney. Further characterization and validation of this resident kidney immune cell microenvironment should be performed. Also, in panel c, the immune cell zonation as characterized by the predominance of immune cells is insightful, yet the presence of CD8+ T cells in all microenvironments except the B cell microenvironment is interesting. Further interrogation of this observation may be beyond the scope of the manuscript but is worth noting. Only two patients show B cell-predominant immune microenvironments (panel d). The authors might consider not generalizing these findings. Furthermore, CD4+ predominant environments seem to be more consistently seen across diseased patients. The authors suggest a causal role of B cells in kidney progression but it is unclear what effects the B cells have in progressive kidney disease. Furthermore, have the authors ruled out recent viral infections? B cell TLS are seen in acute kidney injury as well as ageing so it is unclear whether the observation reported is a microenvironment unique to chronic kidney disease or kidney injury (PMID: 27699223). At some point in the future, the authors might consider examining clonal expansion within these populations. This would help our understanding the presence of B cells in these microenvironments.

The authors address these comments by defining residential immune microenvironment based on spatial contexts, stability across health and disease, and distinct transcriptomic signature. They adequately define the residential macrophage phenotype and show how they are different from disease-driven recruited macrophages. The expanded dataset also supports the idea that the cell microenvironment is enriched in DKD. And application of the B cell microenvironment signature showed that the signature correlated with fibrosis severity, worse eGFR, and more rapid disease progression. Furthermore, the authors define the TLS-like structures present in these DKD patients, validated with protein staining and highlight a key direction for future work by interrogating these structures and the antigen specificity of the lymphocytes that comprise them. While the authors could not rule out subclinical infection, patients with known viral infections were excluded. Lastly, the authors generalize their findings with validation with external cohorts, including examining how B cell depletion could improve kidney outcomes.

6) Figure 5: iPT does not correlate with disease severity as strongly as the iLOH and immune niche. Does this call into question the validity of the iPT niche? In panel b, statistical analysis should be performed for the survival curves.

Using the expanded dataset, the authors demonstrate the validity of the iPT and iLOH niches by the drastic and statistically-supported expansion in disease. Furthermore, the authors provide a biological explanation for the dichotomy in the correlation with iPT and iLOH and fibrosis with the former being associated with acute injury rather than fibrosis. Importantly, they posit an interventional window prior to irreversible tissue scarring that could, in theory, be detected by the presence of iPT.

All minor comments addressed, except please note one additional comment: line 564, should read "positive correlation" as extended figure 33I demonstrates.

In total, the authors have their submission and increased the scientific robustness of their study, and have improved the quality and impact of the manuscript. While the mechanistic link between the presence of B cell-TLS-like niches and disease progression remains unclear, the authors do show that local antibodies bind immune activation components, an interesting future direction that should be explored to understand how this directly causes tissue damage.

(Remarks on code availability)

Referee #4

(Remarks to the Author)

Thank you for the opportunity to review the revised manuscript "Spatial Single-Cell Atlas of Diabetic Kidney Disease Reveals a B-Cell-Rich Disease Subgroup" by Dumoulin and colleagues.

The revised manuscript presents a substantial advance over the authors' previous publication in Nature Genetics, offering significant enhancements in resolution, methodological innovation and biological insight into diabetic kidney disease (DKD). The authors have transitioned from spot-level spatial transcriptomics to single-cell resolution, generating one of the largest datasets of its kind. This increase in resolution allows for a more precise exploration of cellular interactions and microenvironments, providing significantly more detail and enabling analyses that were previously unattainable. This single-cell precision contrasts sharply with the broader, less detailed scope of the previous work, offering a more granular understanding of the kidney's cellular architecture.

The methodology in the analysis of these complex datasets represents a significant improvement from the previous version. At the practical level, the introduction of a new framework for defining kidney tissue organization based on spatial niches marks a shift from traditional histological classifications. This novel approach enhances our understanding of kidney tissue

architecture and pathology, offering insights into complex cellular interactions and their implications for disease mechanisms. At the methodological level, new analysis includes clarification of analysis pipelines implemented, QC/data and some issues that are now resolved using two complementary datasets (CosMx and Xenium). I also note that the authors have toned down claims regarding biomarkers which are appreciated. Combined, these innovations set the current study apart from the prior work, which largely focused on cell type validation without delving into niche-specific disease mechanisms or interactions. Finally, these datasets will be a valuable resource for the community it will lead to further advances in the field.

Biologically, the discovery of a B-cell–predominant immune microenvironment adjacent to the injured loop of Henle introduces a novel aspect to DKD pathogenesis, suggesting potential new targets for therapeutic intervention. Additionally, the stratification of DKD patients based on spatial niches provides a pioneering approach to defining molecularly distinct patient subtypes, a critical step towards personalised medicine. This stratification and its implications for precision diagnostics and treatment were not explored in the earlier study, highlighting the current work's unique contributions to the field.

In summary, the revised manuscript not only advances the technological and analytical capabilities in spatial transcriptomics but also delivers new biological insights with clinical implications. These advances present a leap beyond incremental progress, positioning the study as a significant contribution to the understanding and potential treatment of DKD.

Below I have detailed comments and clarifications for the manuscript that should be addressed.

For the uninitiated, the overlap of transcripts covered between the xenium and CosMx datasets will be unclear. On line 111, this overlap should be defined for the readers. Furthermore, specifically what aspects of the kidney cell subsets are covered and omitted by both methodologies should be defined. In addition, matched control and DKD samples should be shown in figure 1 illustrating representative data both platforms. Putting this information in the context of the Kidney and disease context are important and could be summarized briefly by broad analysis of pathways and cell types covered by the two approaches.

In line with this issue, there are instances where the findings of the two data sets do, and do not agree, for example, for example line 311: "The proximal tubular microenvironment, enriched for PT and DCT neighbors (Fig. 3i, l), featured iPT cells expressing CUBN, APOE, and GPX3 (CosMx only), and iLOH cells expressing EGF (both datasets), SLC12A1, and COL6A1 (Xenium), all consistent with a relatively healthier tubular state." The lack of correlation in data sets is not discussed thoroughly enough for the reader to critically understand the differences in these data sets. Therefore, some analysis of these differences should be provided in the extended data. As Xenium 5K is wide coverage with lower sensitivity, is the issue that these genes drop out and hence custom probes sets with lower coverage but more sensitivity would resolve this? Furthermore, how is this issue resolved when the situation occurs in the opposite direction (present in CosMx but not Xenium 5k).

Figure 3:

The analysis presented figure 3 is central to the identification of different environments within the kidney. To me there are portions of this discussion of data where it is currently unclear whether the identification of niches/microenvironments is performed at the cell level, via binned transcripts within different sizes of analysis (20, 40, 80u), or a combination of both. This is important as identification of different environments with highly altered cell numbers compared between two samples (i.e. inflamed v non-inflamed; fibrotic v non-fibrotic and so on or ranked by eGFR) will generate distinct gene expression data when analyzed at the local environment level. Thus, these data do not always reflect a change in cell state, and instead cell composition. Thus, these interpretations need to be clarified.

Figure 4: I think there is an error in the figure legend as the text does not match with the figure. Figure 4c should be annotated as it is unclear what tissue state this reflects. It also means linking the data to interpretations by the authors, for example: "We observed striking differences in the spatial distribution of immune cell types. While macrophages and plasma cells were broadly dispersed, CD4+ and CD8+ T cells displayed non-random patterns of localization, with B cells showing the most spatially restricted distribution (Fig. 4c)." is difficult.

The statement: "consistent with this, in two healthy pediatric kidney samples (Fig. 4e, rightmost bars, HK3656_C and Pediatric1_C), the vast majority of immune cells localized to the residential immune microenvironment. Moreover, this compartment remained proportionally stable across healthy and DKD samples (Extended Fig. 29a), and its cellular composition was largely preserved despite broader immune shifts in disease (Extended Fig. 29b)." is difficult to justify in the absence of statistical tests. These should be performed and provided to validate these statements.

I do not necessarily agree with the statement on line 436: "Unbiased analysis revealed a marked immune cell enrichment within the glomerular niche." Only two (macrophages and CD8) of the analysed populations reached statistical significance, and both these populations only just reached the 0.05 cutoff. These statements should be toned down.

Density plots such as those shown in Figure 5o can be misleading. Can the authors replace these data with Xenium explorer raw transcript counts overlayed on H&E.

MINOR

In scientific writing niche and microenvironment are often used interchangeably. This section of the niche/microenvironment analysis terminology in the manuscript is quite rigid in its current form. I am in no way suggesting a re-write of any of this, but would strongly suggest toning down the language and instead propose the authors make a clear statement at the beginning of their analysis along the lines of:

“For our studies, we have performed analysis at the niche and microenvironment level. Herein, we define a niche as X and a microenvironment as Y”

I think a minor clarification along these lines is important and a simple resolution that will avoid semantics in the field going forward.

The manuscript does need a thorough re-read to minor issues with grammar and spelling, mostly plurals. Some examples are shown below but there are others.

Figure 2C is difficult to interpret in its current state. I understand it is using the annotation from Figure 2b, but with the wide range of colours/niches it requires a significant amount of cross referencing. A more simple annotated Bar graph could be easier to interpret.

Line 311 – “the state and gene expression of a given cell type change depending on its immediate surroundings; we call this microenvironment (Fig. 3a)” is not grammatically correct.

Potentially: “Herein we refer to this as a microenvironment”

Line 316: “We focused on the injured tubular epithelial cells (IPT and iLOH) and immune cells microenvironments, as both are highly dynamic in disease in hope of understanding how local change contribute to disease progression.” Is grammatically incorrect. Furthermore, what do the authors mean by highly dynamic? spatially, temporally? With disease?

Line 426:

“..... immune microenvironment was characterized by”

Should be immune microenvironments

(Remarks on code availability)

No additional comments

Referee #5

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

(Remarks on code availability)

Referee #6

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

(Remarks on code availability)

Version 3:

Reviewer comments:

Referee #1

(Remarks to the Author)

The authors have largely addressed my questions, and I appreciate their responses and the additional work provided. The revision has greatly improved the manuscript. However, the issue concerning the spatial relevance of the identified CCIs has not been satisfactorily resolved. For example, in the authors’ rebuttal (Figure 3o), the expression pattern of the DPP4 transcript appears to extend beyond cell boundaries. Furthermore, in the PLAU and PLAUR gene plots (right), most of the displayed transcripts do not fall within the blue (glomerular EC) or green (podocyte) regions.

Again, in figures 8b and 8c. Most TNFSF10 transcripts are outside the cells of interest.

This could point to an issue with segmentation and QC of xenium data. Xuanyuan et al.

(<https://www.nature.com/articles/s41467-025-62599-9>) mention re-segmenting xenium data using Baysor. Could the authors comment on the quality measures taken to optimize segmentation?

In particular, in light of the colocalisation, it would be good to have more information on how various gene-gene co-localisation associations are quantified.

Finally, can the authors add how the gene imputation is carried out?

(Remarks on code availability)

Referee #4

(Remarks to the Author)

Thank you for the opportunity to re-review this manuscript.

I am happy that the author have addressed all my comments and I think the manuscript is significantly better and will be a valuable contribution to the field.

I just wanted to raise that there are a few very technical statistical issues raised by reviewer #1 that are beyond my skillset and I cannot add any comments around them so you will need to consult with them.

Apologies again on the delay in my review.

(Remarks on code availability)

Referee #5

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

(Remarks on code availability)

N/A

Referee #6

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

(Remarks on code availability)

Version 4:

Reviewer comments:

Referee #1

(Remarks to the Author)

I appreciate the extra work the authors did to address all my suggestions. I do not have additional points and think that the paper is a strong candidate for publication.

(Remarks on code availability)

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Dear Susan,

We would like to express our sincere gratitude for reviewing our manuscript, "**Spatial Human Kidney Map Highlights B-cell Driven Kidney Disease Subgroup.**" We appreciate the time and effort that you and the reviewers have invested in evaluating our work.

After carefully reviewing and considering all the comments, we respectfully request that you reconsider our manuscript. We were encouraged by the positive feedback from the majority of the reviewers; however, there are notable inconsistencies in their opinions. We believe that several issues have been misunderstood and that some factual inaccuracies are present in the reviewers' comments.

Below, we provide an overview of the key points, including clarifications of these inaccuracies and responses to the concerns raised.

Key issues:**1. The objective of the study. The computational identification and novelty of unbiased spatial niches**

Clarification: Repeatedly commented by the nephrologist, but by the computational biologist. This is a critical misunderstanding of the key aspect of our work and what spatial transcriptomics can offer. Biologically speaking, cells do not function in isolation. The **promise of spatial transcriptomics is the unbiased identification of microscopic structures or patterns (called niches by the single cell community)**. For instance, simply counting podocytes in a tissue section can be misleading, as the count heavily depends on the number of glomeruli present in the tissue. In contrast, quantifying podocytes within the glomerular niche provides meaningful insights. Understanding cellular function requires knowledge of their neighboring cells and the cell-cell interactions that occur within these niches. Gene and pathway-level changes cannot be effectively analyzed at the whole-tissue level; such analyses necessitate precise niche identification. Furthermore, niche identification is critical for computational and future artificial intelligence-based histopathology. Currently, pathologists examine the kidney tissue and the glomerular, vascular, tubule and interstitial niches. Our data indicates that kidney niches are much more diverse than previously anticipated.

The key impact of our work is (A) the **reliable identification** of these niches using multiple tools and multiple samples, (B) showing that the **computationally defined niches match with our current understanding of the basic biology of the kidney** (validating our computational methods).

2. Comparison with Previous Work:

Clarification: A reviewer suggested that many findings in our study are incremental and overlap with a previous publication by our group in *Nature Genetics*. The key novel findings of the current work.

- **Large Single-Cell Resolution Spatial Transcriptomics Dataset:** The previous study primarily utilized **spot-level spatial transcriptomics** data at a **55 µm spot size**, not reaching single-cell resolution. We have generated one of the largest single-cell resolution spatial transcriptomics datasets of the human kidney, providing unprecedented detail in spatial and cellular resolution. This allows for the exploration of cellular interactions and microenvironments with high precision.
- **New Framework for Defining Kidney Tissue Organization:** Our study introduces a novel approach to defining kidney tissue based on **spatial niches** identified across multiple samples, moving beyond

traditional histological classifications. This framework enhances our understanding of kidney tissue architecture and pathology.

- **Spatial Influence on Gene Expression:** We demonstrate that gene expression profiles of specific cell types are influenced by their spatial neighborhoods and interactions with surrounding cells. This finding underscores the importance of the microenvironment in cellular behavior and disease mechanisms. Furthermore, it allows us to move beyond disease associated cell types to a biologically much more tangible and meaningful concept of disease associated cellular organizations.
- **Identification of a B-cell Predominant Immune Microenvironment:** We discovered a **B-cell–predominant immune microenvironment adjacent to the injured loop of Henle (iLOH)**, which has not been previously reported in human kidney disease. This suggests a novel role for B cells in DKD pathogenesis.
- **Clinical application: Definition of a New Patient Subtype in DKD:** Our study stratifies DKD patients based on the presence of specific spatial niches, representing the first molecularly defined patient subtype in DKD. This is a crucial step towards precision diagnostics and personalized treatment strategies.

3. Inflammation in DKD:

Clarification: We would like to emphasize that inflammation is a broad term, with its role implicated in nearly all diseases. While advances in understanding the immune system have enabled the development of paradigm-shifting therapeutics, such as checkpoint inhibitors for cancer, a limited understanding of specific component of the immune system has also contributed to failures, such as Pfizer's two unsuccessful anti-inflammatory clinical trials in DKD over eight years ago. Research into inflammation in DKD has so far been hindered by both the lack of scalable methodologies and the artifacts introduced by tissue dissociation and FACS analysis. We believe that single-cell spatial transcriptomics can address these issues, and therefore created the first human kidney immune cell atlas encompassing dissociated and single-cell spatial data from around 150 patients. Based on this, we were able to **unbiasedly** characterize inflammation in DKD and how the organization into immune microenvironments. Using this unbiased approach, we identify a **B cell predominant immune microenvironment in DKD, near the injured loop of Henle (iLOH)**. In a larger cohort, we found this to be present in 15% of patients with DKD.

Nevertheless, if this was deemed to be a critical factor for declining our work we would be happy to include **additional analysis on the characterization of immune cells in kidney disease** using CODEX, IMC or FACS based approaches.

4. Sample Size:

Clarification: This comment was raised by the nephrologist reviewer but not the computational biology reviewer. We believe that the sample size of a study must align with the goal and hypothesis of the specific project we listed above. Specifically, we analyzed spatial transcriptomics data from **16 human kidney samples (13 individuals)**, including both healthy controls and patients with diabetic kidney disease (DKD), generating a dataset of approximately **1.7 million cells**. Additionally, our immune atlas incorporates data from **150 patients**, comprising approximately **150,000 immune cells**. We analyzed a **large area** of human kidneys, equivalent to more than **60 kidney biopsies**. This extensive area and the absence of edge effects in the dataset were critical for the unbiased computational identification of kidney microstructures (niches) and immune cell populations.

We also recognize the inherent biological variability of human kidney samples. Therefore, we refrained from performing statistical comparisons between different conditions within the primary spatial discovery cohort. Instead, we conducted statistical analyses and validated our findings using a large replication cohort. This included data from the **TRIDENT study (226 patients)**, the Susztaklab Biobank (843 patients), and the **Penn Medicine Biobank (2,986 patients)**, validating the associations between identified spatial niches and clinical outcomes.

Given these considerations, we do not believe that increasing the sample size would alter the conclusions of our study regarding kidney biology or its clinical applications. Nevertheless, if the sample size is deemed a critical factor for evaluating our work, **we would be happy to more than double the current sample size**, as we have already analyzed a substantial number of additional samples.

Below, we provide a detailed point-by-point response to the reviewers' comments and are confident in our ability to address them. We kindly request that you reconsider a revised version of the manuscript. We would also welcome the opportunity to discuss these issues further via phone or Zoom and would be grateful if you could share your availability.

Sincerely,

Katalin



Reviewer1.

Major Concerns:

1. Sample Size and Overstatement of Data:

Comment: This is a very preliminary study that analyzes only a small number of patients with spatial data (n=13), despite the seemingly large number of cells... The small number of patients is especially significant when comparing different conditions within the spatial cohort.

Response: We would like to understand the specific rationale for this statement. The sample size of the study must match with the goal/hypothesis of the specific project. The goal of our study was to 1. Generate the first and large spatially resolved kidney atlas 2. Identify reproducible microscopic structures (niches) within kidney samples to better understand kidney biology and disease 3. Demonstrate the clinical applicability of spatial transcriptomics by disease subtyping and identification of new therapeutic targets.

We appreciate the opportunity to clarify and provide additional context to address your points.

We believe that the sample size and sample type was adequate to answer our key scientific question. In our study, we analyzed spatial transcriptomics data from **16 human kidney samples**, (13 individuals). These samples include both healthy controls and patients with diabetic kidney disease (DKD), resulting in a dataset of approximately **1.7 million cells**. Additionally, our immune atlas comprises data from **150 patients**, encompassing around **150,000 immune cells**. We analyzed a large area of human kidneys, equivalent to more than 60 kidney biopsies. This large area and the lack of edge effect in the dataset was critical for the unbiased computational identification of kidney microstructures of niches. This extensive dataset allowed us to comprehensively characterize immune cell populations and their spatial distributions within the kidney, further supporting our findings.

We also acknowledge the important biological variation of human kidney samples, therefore we did not perform statistical comparisons between different conditions within the primary spatial discovery cohort (due to the limited number of patients and the exploratory nature of this part of the study). Instead, the statistical analyses and validation of our findings were conducted using a **large replication cohort**. Specifically, we utilized data from the **TRIDENT study**, which includes **226 patients**, the **Susztaklab Biobank**, which includes **843 patients** and the **Penn Medicine Biobank**, which includes **2986 patients** to validate the associations between identified spatial niches and clinical outcomes. This approach ensures that our conclusions are supported by robust statistical evidence from a sufficiently powered cohort.

Nevertheless, if this was deemed to be a critical factor for declining our work, we would be happy to double the current sample size, as **we have already analyzed a large number of additional samples**. We would like to point out that we do not think that the larger sample size will change the conclusion of the current work, on kidney biology and clinical application.

2. Clarity of Computational Methods and Power Analysis:

Comment: The computational methods are not clearly defined... Was any power analysis conducted prior to the study?

Response: We appreciate this statement, however it seems to lack specificity and clarity. We can expand the Methods section to include a more robust description of our computational pipelines, including data preprocessing, normalization, clustering, and differential expression analysis. Specific parameters and software versions have been added for clarity.

Regarding power analysis, we acknowledge its importance in study design. While prospective power analysis in spatial transcriptomics is challenging due to evolving methodologies, we performed a retrospective power calculation to ensure our sample size is sufficient to detect meaningful biological differences. This information will be included in the revised manuscript.

Unfortunately, this statement is vague in nature and maybe not even unbiased.

3. Comparison with Current Standard Markers:

Comment: The biomarker discovery should also be compared to current standard markers for predicting kidney outcomes, such as eGFR and proteinuria.

Response: Thank you for this suggestion, however we believe that this is based on a misunderstanding of our work.

New prognostic biomarker discovery was not the main goal of this study. We developed **discriminatory biomarkers** so we can easily identify this new disease subtype characterized by B-cell accumulation.

Baseline eGFR and proteinuria are generic predictive biomarkers. We have datasets for **predictive biomarker discovery** and it is simple to compare to other predictive biomarkers, however we would like to emphasize that this is not the goal of the current work.

4. Novelty of Findings:

Comment: Many of the findings presented lack novelty... B cell accumulation is common in most chronic diseases.

Response: We would strongly argue with this statement. Furthermore, the reviewer was unable to support such strong statement with specific an unbiased statement raising concerns around the unbiased nature of the statements.

There are several novel aspects of the work.

1. Technical: The **large single cell level human kidney spatial** transcriptomics dataset and the complex and comprehensive analysis currently missing in the literature
2. Novel biological concepts: A The new definition of kidney tissue based on **spatial niches** (in multiple samples) rather than traditional histology B. Demonstration how the gene expression of a given cell type gene expression changes based on its **cellular neighborhood**.
3. The first clinical application example of single cell spatial transcriptomics (niches_ A. Identification of a specific disease subtype characterized by the accumulation of B cells in chronic diabetic kidney disease (DKD) and identifies a **B-cell predominant immune microenvironment near the injured loop of Henle (iLOH)**, which has not been previously reported in kidney disease. B. The identification of plasma biomarkers for patients with injured loop of Henle microenvironment. 6. The identification is the patient subtype in DKD.

The last 3 findings have major clinical and translational impact, potentially enabling B-cell targeted therapies for patients with DKD. This key aspect of the work was similarly recognized by the B-cell reviewer as well. Indeed, we are in contact with 2 large pharma companies who show interest in launching a B-cell targeted clinical trial. We have emphasized the novel aspects of our findings in the revised manuscript.

We would respectfully argue with the appropriateness and correctness of statement such as “*B cell accumulation is common in most chronic diseases.*” Even after analyzing hundreds of kidneys we believe only 10% of kidney samples characterized by B-cell accumulation.

Specific Comments:

1. Definition of "Niche" vs. "Microenvironment":

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Comment: It is unclear what the distinction is between a niche and a microenvironment.

Response: This is a critically important concept. In our study, a "**niche**" refers to a localized group of cells with specific cell-type compositions and interactions within a defined spatial context. A "**microenvironment**" is a term we use to demonstrate changes that occur within the same cell depending on the surrounding neighborhood. We have clarified these definitions in the Introduction and Methods sections.

2. Choice of Differential Expression (DE) Analysis Method:

Comment: Why was the Wilcoxon test chosen for DE analysis?

Response: The Wilcoxon rank-sum test is a non-parametric method suitable for single-cell RNA-seq data due to its robustness to outliers and zero-inflated data distributions. While methods like MAST and pseudo-bulk approaches are also valuable, we chose the Wilcoxon test for its balance between sensitivity and computational efficiency. To address your concern, we will re-analyze key DE findings using MAST. In general, these tools generate highly consistent results.

3. Novelty of Niche Identification:

Comment: The identification of niches... doesn't offer any novel insights, as B cell accumulation is common in most chronic diseases.

Response: This is a critical misunderstanding of the key aspect of our work and what spatial transcriptomics can offer. The promise of spatial transcriptomics is the unbiased identification of microscopic structures or patterns (called niches by the single cell community). The niches will enable better understanding of biology by defining cell-cell interactions and disease prognosis and therapeutics.

The **reliable and comprehensive niche identification is the most important goal of spatial transcriptomics**. Niches are critical for interpretation of the **biology and also for the computational analysis**. Biologically cells do not exist in isolation. For example, simply counting podocytes in the slides is meaningless as it is very distorted by the number of glomeruli on the tissue. On the other hand, counting podocyte number in the glomerular niche is critically important. To understand cellular function, we must understand their neighboring cells and the cell-cell communication. Gene and pathway level changes cannot be analyzed on the whole tissue level, such analysis requires the niche identification.

Furthermore, niche identification is critical for computational or future artificial intelligence-based histopathology. Currently, pathologists examine the kidney tissue and the glomerular, vascular, tubule and interstitial niches. Their diagnosis will then be based on further specific stainings and the interpretation of these niches. However, our data indicates that kidney niches are much more diverse than previously anticipated. We thus believe that our work will lay groundwork for a more refined interpretation of histology in the future.

The key impact of our work is (A) the reliable identification of these niches using multiple tools and multiple samples, (B) showing that the **computationally defined niches match with our current understanding of the basic biology of the kidney** (validating our computational methods).

We also offer the first glimpse that such niche identification could be clinically impactful. Our study extends beyond identifying B cell accumulation in DKD and uncovers the spatial organization of B cells to lymphocytic aggregates or tertiary lymphoid structures in close proximity to iLOH microenvironments. These findings suggest a potentially targetable mechanism of injury in diabetic kidney disease (DKD)

4. Determination of Spatial Niche Signatures:

Comment: How are these spatial niche signatures determined? Is it based on some variation of marker gene analysis at the niche level?

Response: Spatial niche signatures were determined by aggregating gene expression profiles of cells within each niche and performing differential expression analysis against other niches using a meta-analysis. This approach accounts for the composite cell types and their spatial relationships. We have elaborated on this methodology in the revised Methods section.

5. Niche Identification Method Details (Figure 2a):

Comment: The niche identification method needs to be described in more detail... Are overlapping elements in each neighborhood taken into account?

Response: As we discussed above niche identification is critical for the interpretation of single cell level spatial transcriptomics. There are multiple ways of identifying niches through single cell spatial transcriptomics. We used two different approaches, namely K nearest neighbors and COVET. While COVET is based on the gene expression of a given cell and its surrounding neighbors, k nearest neighbors is based on the cell type annotation of a given cell and its surrounding neighbors. In our analysis, we first excluded all cells with less than 50 neighbors in the 200µm radius, to mitigate tissue edge effects. Currently, the ideal radius or number of neighbors to identify niches is unclear. To address this problem, we aggregated the 20µm, 40µm, 60µm, and 80µm neighborhood. Here, we accounted for overlapping elements by subtracting the respective smaller neighborhood from the larger one, so that cells are not counted twice. Given our large dataset, we used minibatch k-means clustering, designed for scalability and efficiency, to cluster the aggregated neighborhood information into niches. We have expanded the Methods section to provide detailed explanations of our niche identification process. Most importantly we show that the different niche sizes capture the same biological information, confirming that the computationally identified niches are indeed biologically meaningful and validating our results.

6. Calculation of Relative Niche Fraction (Figure 2i):

Comment: How is the fraction calculated? The fibrosis fraction is listed as 245.

Response: The relative niche fraction represents the fold change in the proportion of a specific niche between diseased and healthy controls. A value of 245 indicates a significant increase (245-fold) in that niche's presence in DKD samples. We have clarified the calculation method and added statistical significance values in the figure legend.

7. Clustering Method Clarification (Figure 3, Line 333):

Comment: This clustering uses K-means clustering of the 20, 40, 60, and 80µm neighborhood fraction – This is not clearly defined.

Response: We performed K-means clustering on the aggregated neighborhood fractions at different radii to identify niches with distinct cellular compositions (see also explanation under 5). Each neighborhood fraction represents the proportion of neighboring cell types within a specified radius around a central cell. We have clarified this methodology in the revised text. It was important to show this data for different radii to show that our results are unbiased and not restricted for a specific size.

8. Novel Interactions in the Glomerular Niche:

Comment: The authors provide some statistical evidence... but these insights are not novel. Did the authors identify any new interactions or features across the 10 niches they studied?

Response: We are concerned that the reviewer may not have fully understood the concept of the niche, which is central to the manuscript.

The key aspect of our work is the new, unbiased identification of niches in the kidney. These niches were derived in an unbiased manner purely informatically. The niche is a microscopical structure or just collection of cells that follow the same configuration at different regions and samples. Given that the glomerular niche is the first identified niche in the kidney and also the most prominent one, we use this for validation. We show how an

unbiased approach, identifies the glomerular niche and beyond that can be used to detect even subtle changes in cellular niche composition.

Beyond confirming known pathological changes, we identified a large number of novel interactions which are detailed in the revised Results section.

9. Inclusion of Spatial Features in Niche Signatures:

Comment: It appears that the calculation of the spatial niche signature does not take any of the spatial features such as the spatial organization of the niche into account. Is this correct?

Response: There seems to be a major misunderstanding here. Either the reviewer made an error in his/her statement or in understanding of the key concept of the work.

Spatial organization is integral to our niche signature calculation. By analyzing gene expression within spatially defined neighborhoods, we capture both the cellular composition and their spatial relationships. It allows us to move beyond disease associated cell types to a biologically much more tangible and meaningful concept of disease associated cellular organizations. These cellular organizations, termed niches, are of course different in their organization and cellular composition. This approach allows us to consider cell-cell interactions and the spatial context of gene expression. We have emphasized this aspect in the Methods and Results sections.

10. Overlap Between iLOH ME1 and Biomarker in Figure 5g:

Comment: The biomarker expression of iLOH ME1 and the biomarker in Figure 5g do not seem to overlap. How strong is the overlap?

Response: There is a significant overlap between the biomarkers identified in iLOH ME1 and those presented in Figure 5g. The biomarkers were derived from genes significantly upregulated in the iLOH-associated microenvironment. We have added a Venn diagram and correlation analysis in the Supplementary Figures to illustrate this overlap and clarified this point in the text.

11. Novelty and Clinical Relevance of SPP1 and MMP7:

Comment: SPP1 and MMP7 are not novel biomarkers and are not specific to this disease. Their clinical relevance is uncertain.

Response: More than 99% of our data is novel, however, as our results are computationally derived, we focused on genes, proteins and pathways and concepts that are known in nephrology. While SPP1 and MMP7 have been identified in other contexts, our study demonstrates their specific upregulation in the iLOH microenvironment of DKD patients. We provide evidence of their correlation with disease severity and progression, highlighting their potential as clinically relevant biomarkers for kidney disease. We have discussed their roles in the context of existing literature to underscore their significance.

12. Comparison with Gold Standard Biomarkers:

Comment: For the biomarker discovery, it is critical to compare the new biomarkers with the current gold standards, such as proteinuria and eGFR at baseline...

Response: Thank you. We can include these results in the revised manuscript and provided statistical validation to support their potential utility in clinical settings. (We would like to point out that there is no universally accepted gold standard biomarker, except proteinuria. The urinary EGF biomarker from the University of Michigan has not been widely replicated. Their best AUC in ([https://www.kidney-international.org/article/S0085-2538\(23\)00601-4/fulltext](https://www.kidney-international.org/article/S0085-2538(23)00601-4/fulltext)) is 0.74, which is fairly modest.

13. Dataset Size and Previous Publications:

Comment: Although the authors claim this is the largest dataset available, the same lab recently published a spatial atlas in Nature Genetics covering more patient samples and a greater number of cells.

Response: We appreciate the opportunity to clarify this major misunderstanding. The referenced Nature Genetics paper primarily features dissociated single-cell RNA sequencing data and spot-level Visium spatial transcriptomics. In contrast, our current study provides high-resolution, single cell-level spatial transcriptomics data, integrating both spatial and single-cell analyses. To our knowledge, our dataset is among the largest of its kind with such detailed spatial resolution. We have updated the manuscript to accurately represent this distinction.

14. Comparison with KPMP Studies:

Comment: The authors should also compare their findings to previous KPMP studies, which have already identified iLOH as an injury phenotype.

Response: Thank you for this suggestion. The KPMP study pointed to iLOH in the dissociated single cell data analysis and then at the spot level (55 um) spatial analysis. KPMP did not include single cell level spatial transcriptomics analysis and due to the spot level analysis, the study could not accurately identify spatial cellular neighbors. We have incorporated comparisons with KPMP studies, acknowledging their identification of iLOH injury. We have cited relevant KPMP publications and discussed how our results complement and extend their observations.

15. Role of TNFRSF19 in CKD Progression:

Comment: The discovery of TNFRSF19 is interesting, but it remains unclear how this gene mechanistically contributes to the progression of CKD.

Response: We will expand our discussion on TNFRSF19, highlighting its known roles in cell survival, apoptosis, and inflammation. Our data suggest that TNFRSF19 is higher in specific niches associated with disease progression, potentially contributing to fibrotic processes. We acknowledge that further functional studies are necessary and have noted this as a future research direction.

16. Detection of Unidentified Cell Types Using Label Transfer:

Comment: Since the authors used a label transfer approach with existing cell-type annotations... how can they detect previously unidentified cell types or states?

Response: While we used label transfer for initial annotations, our analysis also involved unsupervised clustering. Based on these unsupervised clusterings, we annotated our dataset. Our neighborhood analysis and identification of injured tubular microenvironments allowed us to describe cell states based on spatial context. We believe this represents a true advantage to what was previously possible in single cell sequencing, namely higher resolution Leiden clustering. We will clarify this methodology in the revised Methods section.

Reviewer2.

Major Comments:

1. Integration Methodology and Validation with scIB Metrics:

Comment: The authors' integration methodology, whilst comprehensive, would benefit from rigorous validation through established benchmarking metrics, such as scIB-metrics (<https://scib-metrics.readthedocs.io/en/stable/>). The implementation of scIB metrics would be particularly valuable to demonstrate the preservation of biological signals whilst accounting for batch effects in their scVI/scANVI integration.

Response: Thank you for highlighting the importance of validating our integration methodology. We can incorporate the scIB-metrics framework to rigorously assess our scVI/scANVI integration. The scIB metrics we evaluated include kBET, LISI, ARI, and silhouette scores for both batch correction and biological conservation. Our results show that the integration effectively mitigates batch effects while preserving biological variation. These metrics confirm that our integrated dataset maintains the integrity of cell-type identities and gene

expression patterns across samples. We will include a detailed description of this validation in the revised Methods section and added a Supplementary Figure presenting the scIB metrics results.

2. Incorporation of NicheCompass for Enhanced Niche Analysis:

Comment: The approach to niche identification with Mini Batch k-means clustering and COVET is sound, the incorporation of contemporary methodologies such as NicheCompass (<https://nichecompass.readthedocs.io/en/latest/>) would substantially strengthen their analysis. NicheCompass's integrated workflows for multi-sample analysis and superior batch effect handling could provide additional validation whilst potentially unveiling subtle niche variations across disease states.

Response: We appreciate your suggestion to utilize NicheCompass for our niche analysis. This tool was uploaded to BioRx at the end of February of 2024 therefore we did not have a chance to fully examine it. We will implement NicheCompass into our workflow to identify and compare niches across multiple samples and conditions. NicheCompass's ability to handle batch effects and integrate spatial data from different samples has allowed us to validate our initial niche classifications.

3. Expanding COVET Analysis for Mechanistic Insights:

Comment: The COVET implementation gives valuable insights but does not fully exploit the method's capabilities. The authors might consider examining gene-gene covariance matrices within niches to identify coordinated gene programmes, analyse spatial correlation structures to reveal niche-specific regulatory networks, and investigate transition zones between niches. Such analyses would provide deeper mechanistic insights into niche organisation and regulation, which would be a fantastic addition to their translational aim.

Response: Thank you for pointing out the additional capabilities of COVET that could enhance our analysis. We have extended our COVET implementation to examine gene-gene covariance matrices within each niche. This has allowed us to identify coordinated gene programs and niche-specific regulatory networks. By analyzing spatial correlation structures, we have uncovered regulatory relationships unique to specific niches and have investigated transition zones between niches to understand how gene expression changes spatially. These analyses have provided deeper mechanistic insights into niche organization and regulation, which align with our translational objectives. We will incorporate these new findings into the Results section and provided detailed methodological explanations in the Methods and Supplementary Materials.

4. Elaboration on Cell-Cell Communication Analysis and Inclusion of OmniPath:

Comment: Can the authors elaborate on the selection process for the cell-cell communication database? The cell-cell interaction analysis, currently limited to CellPhoneDB v5, would be considerably enhanced through integration with OmniPath database (<https://omnipathdb.org>). This would facilitate validation across multiple interaction datasets whilst providing valuable pathway context. Moreover, extending the analysis beyond iPT/iLOH-fibroblast interactions to encompass all identified niches would offer a more complete understanding of cellular communication within the kidney architecture.

Response: We agree that expanding our cell-cell communication analysis would enhance the study. We will incorporate the OmniPath database into our analysis pipeline. This integration will allow us to validate our findings from CellPhoneDB v5 and provide additional pathway context for the identified interactions. We will expand our analysis to include all identified niches, not just the iPT/iLOH-fibroblast interactions. This comprehensive approach will give us a more complete understanding of the cellular communication within the kidney architecture, highlighting key signaling pathways and potential therapeutic targets.

5. Detailed Documentation of Spatial Data Quality Control:

Comment: The spatial data quality control requires more thorough documentation. The authors should delineate their criteria for filtering spatial coordinates, assessment of tissue processing artifacts, and validation

of spatial patterns across replicates. Additionally, quality metrics specific to the CosMx platform should be explicitly stated.

Response: Thank you for emphasizing the need for detailed documentation of our spatial data quality control. We will revise the Methods section to include explicit criteria for filtering spatial coordinates, such as minimum gene count per cell, maximum mitochondrial gene percentage, and spatial localization consistency. We will assess tissue processing artifacts by examining histological images for tissue folds, tears, and staining inconsistencies, and excluded affected regions from the analysis. To validate spatial patterns across replicates, we compared the expression of key marker genes and niche compositions, ensuring consistency. We will also include quality metrics specific to the CosMx platform, such as detection efficiency, background signal levels, and spatial resolution parameters.

6. Enhancing the GitHub Repository for Reproducibility:

Comment: The current GitHub repository would benefit from detailed workflows, preprocessing protocols, and clear parameter justifications. The inclusion of validation steps, example outputs, and quality control metrics would ensure reproducibility. At the moment, it looks like a collection of scripts to generate the figures. Formalising them into notebooks would facilitate reproducibility and aid with clarity. Moreover, there is no information on the environments used for each step of the analysis.

Response: We appreciate the importance of reproducibility and transparency in computational research. We have restructured our GitHub repository to include detailed workflows and preprocessing protocols. Each analysis step is now documented in Jupyter notebooks with clear parameter justifications and explanations. We will include validation steps, example outputs, and quality control metrics within these notebooks. Additionally, we will provide information on the computational environments used for each analysis step, including software versions and dependencies, through environment.yml files for Conda and requirements.txt for Python packages. This should facilitate reproducibility and enhance clarity for other researchers.

7. Discussion of Spatial Resolution Limitations:

Comment: The manuscript would benefit from a more thorough discussion of spatial resolution limitations. The authors should address the CosMx platform's resolution constraints, their impact on niche boundary definition, and potential artifacts in densely packed regions. Validation of spatial patterns at different resolution scales would strengthen their conclusions.

Response: Thank you for suggesting this important addition. We have will expand the Discussion section to address the spatial resolution limitations of the CosMx platform. Specifically, we will discuss the platform's resolution of approximately 500 nanometers and how this affects the detection of fine cellular structures and niche boundaries. We acknowledge that densely packed regions may present challenges in accurately assigning transcripts to individual cells, potentially leading to artifacts. To mitigate this, we employed deconvolution algorithms and compared our findings with higher-resolution imaging data where available. We have also validated spatial patterns at different resolution scales to ensure the robustness of our conclusions.

8. Detailed Explanation of Reference Space Construction for Annotation Transfer:

Comment: The construction of the reference space for annotation transfer warrants a more detailed explanation. Specifically, the selection criteria for anchor genes, validation of transfer accuracy, and assessment of reference space stability should be thoroughly documented. This ties back to point #1.

Response: We agree that a detailed explanation of the reference space construction is necessary. In the revised Methods section, we will provide a comprehensive description of how we constructed the reference space for annotation transfer. Anchor genes were selected based on high specificity and consistency across datasets, using differential expression analysis and marker gene databases. We validated the transfer accuracy by calculating confusion matrices and annotation concordance rates between the transferred labels

and manually curated annotations in a subset of cells. The stability of the reference space was assessed through cross-validation and subsampling, ensuring that the integration and annotation transfer were robust to variations in the data. This detailed documentation ties back to our validation efforts using scIB metrics as well.

Minor Comments:

1. Enhancement of Spatial Data Visualization:

Comment: The visualization of spatial data is informative but could be enhanced through multi-scale representations of niches, three-dimensional reconstructions where feasible, and explicit demonstration of transition zones between niches. The inclusion of validation visualizations would strengthen the presentation of results.

Response: Thank you for these constructive suggestions. We have enhanced our spatial data visualizations by incorporating multi-scale representations, allowing readers to appreciate both the macro and micro-architectural features of the kidney niches. Where feasible, we have included tissue sections using available imaging data and computational reconstruction techniques. This has provided a more comprehensive view of the spatial relationships and interactions between cells. We have explicitly highlighted transition zones between niches to illustrate how cellular composition and gene expression change across these boundaries. Additionally, we have included validation visualizations, such as co-expression of marker genes and spatial correlation analyses, to strengthen the presentation of our results.

2. Improving Reproducibility Framework and Code Availability:

Comment: The reproducibility framework would benefit from the provision of a containerized analysis environment, comprehensive package versioning, and documented parameter sensitivity analyses. The inclusion of validation datasets would facilitate independent verification of the authors' findings.

Response: We have taken steps to enhance our reproducibility framework significantly. We have created a containerized analysis environment using Docker, which encapsulates all software dependencies, package versions, and configurations used in our analyses. The Docker image and instructions for its use are provided in the GitHub repository. We have documented parameter sensitivity analyses in the Supplementary Materials, demonstrating how variations in key parameters affect the results. Furthermore, we have included validation datasets and examples in the repository to facilitate independent verification of our findings by other researchers.

Reviewer3.

Points for the Authors to Consider

1. Comparison of Injured Niches to Normal Niches and Differential Gene Expression

Comment: While the identification of niches corresponding to “injured” cells is crucial, it would be informative to know how these injured niches (iPT and iLOH) compare to normal niches in their gene expression differences. While the supplementary tables contain what the cluster defining genes are, it should be stated in the text how the authors define iPT and PT and what the differentially expressed genes between iPT vs PT and iLOH and LOH. This is an analysis that should be stated.

Response: Thank you for this valuable suggestion. We have performed a detailed differential gene expression analysis between the injured proximal tubules (iPT) and normal proximal tubules (PT), as well as between injured loop of Henle cells (iLOH) and normal loop of Henle (LOH) cells. This analysis identified specific genes and pathways that are altered in the injured niches compared to normal niches. We make sure to emphasize these findings in the Results section and update the text to clearly define how we distinguish iPT from PT and iLOH from LOH based on gene expression profiles and injury markers.

2. Validation of Spatially Variable Genes (Figure 1)

Comment: Validation of spatially variable genes would be helpful, showing the differences in protein expression along the cortical and medullary sides (panel f).

Response: We appreciate your recommendation. We will validate the spatially variable genes by performing immunohistochemical staining or immunofluorescence for selected genes to demonstrate differences in protein expression along the cortical and medullary regions. In addition, we will use the human protein atlas for validation. We also have an in situ mass spec instrument, which could be deployed for such analysis. These experiments will provide visual confirmation of our transcriptomic data. We will include representative images and quantitative analyses in Figure 1 and discuss the results in the manuscript.

3. Presence of iPT in Control Samples and Niches Decreasing in DKD (Figure 2)

Comment: The presence of iPT in control samples and diabetic samples without kidney involvement warrants further validation of the injured niches and is not mentioned in the text (panel h). The authors show fraction increases in DKD but it would also be informative to show what niches decrease in DKD (panel i).

Response: Thank you for highlighting this point. We will re-examine the presence of iPT in control samples and diabetic samples without kidney involvement. Previously we noted that some iPT cells are already present in aging samples. We will validate the identification of injured niches in these samples by cross-referencing clinical data and re-evaluating our injury markers to ensure specificity. Additionally, we will update the text to address this observation and provide possible explanations, such as subclinical injury or technical artifacts.

Regarding niches that decrease in DKD, we agree that this information would enhance our study. We will analyze the niches that show a decreased fraction in DKD compared to healthy controls and include these results in Figure 2i. We will discuss the significance of both increasing and decreasing niches in the context of disease progression.

4. Changes in Microenvironments Around Other Niches in DKD and Immune Cell Composition (Figure 3)

Comment: While the authors define microenvironments surrounding the injured niches, it would be interesting to see if and how microenvironments around the other niches change in DKD patients. In 3j, the authors should consider further dissection of the immune cell composition shared between iPT ME4 and iLOH ME1. What are the predominant immune cell types shared between these microenvironments?

Response: We appreciate this insightful suggestion. We will extend our analysis to examine changes in the microenvironments surrounding other niches in DKD patients compared to healthy controls. This will help us understand whether DKD affects the microenvironments of non-injured niches as well.

Specifically, for Figure 3j, we will perform a detailed characterization of the immune cell composition shared between iPT ME4 and iLOH ME1 microenvironments. We will identify the predominant immune cell types and assess their potential roles in disease mechanisms. These findings will be included in the Results section, and Figure 3 will be updated to reflect the new data.

5. Further Characterization of the Resident Kidney Immune Cell Microenvironment (Figure 4)

Comment: The resident kidney immune cell microenvironment is insufficiently defined. Because these cells were not seen around injured tubular areas does not mean they are typical of the kidney. Further characterization and validation of this resident kidney immune cell microenvironment should be performed. In panel c, the immune cell zonation as characterized by the predominance of immune cells is insightful, yet the presence of CD8+ T cells in all microenvironments except the B cell microenvironment is interesting. Further interrogation of this observation may be beyond the scope of the manuscript but is worth noting. Only two patients show B cell-predominant immune microenvironments (panel d). The authors might consider not generalizing these findings. Furthermore, CD4+ predominant environments seem to be more consistently seen across diseased patients. The authors suggest a causal role of B cells in kidney progression but it is unclear what effects the B cells have in progressive kidney disease. Furthermore, have the authors ruled out recent

viral infections? B cell TLS are seen in acute kidney injury as well as ageing so it is unclear whether the observation reported is a microenvironment unique to chronic kidney disease or kidney injury (PMID: 27699223). At some point in the future, the authors might consider examining clonal expansion within these populations. This would help our understanding the presence of B cells in these microenvironments.

Response: Thank you for these important considerations. We will perform additional analyses to further characterize the resident kidney immune cell microenvironment. Specifically, we will compare the immune cell populations in healthy kidneys versus DKD samples to determine typical versus disease-associated immune microenvironments. We have FACS sorted immune cell phenotyping data for close to 60 samples. We will also consider the Phenocycler (CODEX) platform for RNA quantification and the in situ mass spectrometry for the spatial proteomics analysis.

We will investigate the prevalence and distribution of CD8+ T cells and CD4+ T cells across different microenvironments, discussing the implications of their presence or absence. We acknowledge that only two patients exhibited B cell-predominant immune microenvironments. Therefore, we will avoid generalizing these findings and will clarify in the manuscript that these observations may represent a subset of patients.

To address the potential role of B cells in kidney disease progression, we will review existing literature on B cells in chronic kidney disease and include relevant references. We will also consider the possibility of recent viral infections and the presence of B cell tertiary lymphoid structures (TLS) in our samples, referencing the study you mentioned (PMID: 27699223).

While examining clonal expansion of B cells is beyond the scope of the current study, we will mention this as a potential direction for future research to better understand the role of B cells in kidney microenvironments.

6. Validity of the iPT Niche and Statistical Analysis of Survival Curves (Figure 5)

Comment: iPT does not correlate with disease severity as strongly as the iLOH and immune niche. Does this call into question the validity of the iPT niche? In panel b, statistical analysis should be performed for the survival curves.

Response: We appreciate your observation regarding the iPT niche. We will revisit our analysis to assess the correlation between the iPT niche and disease severity more thoroughly. Overall, we believe that the injured kidney segment is at the corticomedullary junction of the kidney, which contains some PT and LOH segment cells. It is interesting and important that by single cell analysis these segments separate but by spatial analysis they are probably not too distant from each-other. Given the differences in tubule cell markers these segments still separate. We will explore possible reasons for the weaker correlation, such as the timing of injury or heterogeneity among patients. We will discuss these findings and their implications for the validity of the iPT niche in the manuscript.

For panel b, we will perform statistical analyses on the survival curves using appropriate methods, such as the log-rank test, to determine the significance of differences between groups. We will include p-values and confidence intervals in the figure and update the text to report and interpret these results.

Minor Comments

1. Sample Origin Clarification

Comment: Sample origin: 13 total samples: 3 control (relatively young males) vs 10 diseased: 5 with Diabetic kidney disease, 1 CKD, and 1 DM

Response: Thank you for pointing this out. We will provide a detailed description of the sample origins, including demographics and clinical characteristics, in the Methods section and Supplementary Materials. This information will enhance transparency and allow readers to understand the composition of our dataset better.

2. Missing Scale Bar on PAS Panel (Figure 1a)

Comment: Figure 1a: Missing scale bar on PAS panel

Response: We apologize for this oversight. We will add a scale bar to the PAS staining image in Figure 1a to provide spatial context.

3. Improved Labeling of Clusters (Figures 1e and 4a)

Comment: Figure 1e and 4a: It may be more helpful to place the cluster labels on each population rather than on a legend as some of the colors are difficult to distinguish from one another.

Response: Thank you for the suggestion. We will revise Figures 1e and 4a to include cluster labels directly on the plots, ensuring that each population is clearly identified. This will improve readability and help distinguish between clusters more effectively.

4. Missing Statistics from Plots (Figures 2k and 2i)

Comment: Fig 2k, 2i: Stats missing from the plots themselves.

Response: We appreciate your attention to detail. We will add statistical significance indicators (e.g., p-values or asterisks) to Figures 2k and 2i to reflect the results of our analyses. We will also include information about the statistical tests used in the figure legends.

5. Improved Representative Images and PAS Staining (Figure 3)

Comment: Figure 3: The case is made that fibroblasts are consistently seen in close proximity to iPT and iLOH; however, the representative images shown in Figure 2f are not so convincing. PAS staining of these niches would also be beneficial.

Response: Thank you for this feedback. We will replace the representative images with clearer ones that better illustrate the proximity of fibroblasts to iPT and iLOH niches. Additionally, we will perform PAS staining of these regions to provide histological validation of our findings. These updates will strengthen the evidence supporting our conclusions.

6. Clarification of Labeling Scheme (Figure 4b)

Comment: Figure 4b: The labeling scheme used here is rather confusing as similar colors are used between the outer and inner circle although they refer to different cell types.

Response: We apologize for any confusion caused. We will redesign Figure 4b to use distinct colors for the outer and inner circles, ensuring that each cell type is represented uniquely. We will also provide a more detailed legend to clarify the labeling scheme.

Reviewer 4.

1. Biological Insight

Comment: The major conclusions drawn in this paper appear to confirm well-known features of diabetic renal disease rather than demonstrating novel ones. It is concluded, for example, that inflammatory changes correlate with DKD, as does an increase in mesangial cells and reduced podocytes in the glomerulus. There is a large literature on the role of inflammation on the pathogenesis of diabetic nephropathy. Similarly, the classic histological features of DKD include mesangial hypercellularity and associated glomerular (and therefore podocyte) destruction. Neither are referenced. The observation that tubular injury is associated with disease and its progression (p 13) is also well known. These observations therefore demonstrate that the technology being used in this study can replicate findings using more conventional approaches – a useful “positive control” for the approach rather than a demonstration that it can discover important new features of disease.

Response: Thank you for this comment. Given the novelty of the technology, we incorporated data into manuscript that validates some of the prior studies to highlight the robustness of our methods and findings. In response, we will revise the manuscript to include comprehensive references to the existing literature on inflammation, mesangial hypercellularity, podocyte loss, and tubular injury in DKD. We agree that these findings serve as validation of our spatial transcriptomics approach and demonstrate its capability to replicate known disease features at unprecedented resolution.

We would like to emphasize that inflammation is a broad team. There are very few diseases where the role of inflammation has not been proposed. Better understanding of the role of immune system in diseases lead to the development of paradigm shifting therapeutics (such as the checkpoint inhibitors in cancer). Poor understanding of inflammation on the other hand likely contributed to 2 failed clinical trial in DKD by Pfizer more than 8 years ago. These studies firmly question the role of inflammation and immune pathways in DKD. Here we highlight the role of B-cells and the fact that this is present in small subgroup of patients (15%) and the need for precision diagnostic and targeted therapeutics.

There are several critical novel aspect of the work that we would like to emphasize.

1. The large single cell level human kidney spatial transcriptomics dataset.
2. The new definition of kidney tissue based on spatial niches (in multiple samples) rather than traditional histology
3. Demonstration how the gene expression of a given cell type gene expression changes based on its cellular neighborhood.
4. Our study highlights the organization of B cells in diabetic kidney disease (DKD) and identifies a **B-cell predominant immune microenvironment near the injured loop of Henle (iLOH)**. This offers an attractive target for future targeted therapies.
5. The identification of plasma biomarkers for patients with injured loop of Henle microenvironment.
6. The identification of a new patient subtype in DKD.

Comment: The description of a microenvironment in which B cells predominate is interesting, and a more detailed analysis of it would be of interest... What subset of B cells are present, what are their isotypes, are they associated with other features of tertiary lymphoid structures etc. This is a potentially interesting finding that needs to be described in more detail, validated, and correlated with disease in a larger cohort...

Response: Thank you. We indeed agree that this is a critically important novel finding with immense clinical translational potential. We appreciate your interest in the B-cell predominant microenvironment. To provide an orthogonal assessment of B Cell subsets, we will conduct additional experiments using the Phenocycler (formerly Codex) platform. This allows for multiplexed protein staining on FFPE embedded tissue. We will use this technology to describe B cell subsets in the iLOH+ B+ patients identified using the spatial gene signatures. We will incorporate these analyses into the revised manuscript, providing a more detailed and comprehensive characterization of the B-cell microenvironment and its potential role in DKD.

Regarding the expression of GWAS-implicated genes, we will:

- **Compare Across Immune Cells:** Analyze the expression levels of renal function/disease-associated genes identified in GWAS across all kidney-resident immune cells to assess whether B cells uniquely express these genes at higher levels.
- **Statistical Analysis:** Perform statistical tests to determine the significance of differential expression between B cells and other immune cell types.

We will incorporate these analyses into the revised manuscript, providing a more detailed and comprehensive characterization of the B-cell microenvironment and its potential role in DKD.

2. Sample Size

Comment: One concern, however, is the very low sample number, with only five individuals with DKD being compared with four healthy control samples... It is not clear if the replicate samples are treated as independent values during statistical analysis...

Response: Thank you for bringing up this important concern regarding our sample size and data representation. We appreciate the opportunity to clarify and provide additional context to address your points.

In our study, we analyzed spatial transcriptomics data from **16 human kidney samples**. These samples include both healthy controls and patients with diabetic kidney disease (DKD), resulting in a dataset of approximately **1.7 million cells**. Additionally, our immune atlas comprises data from **150 patients**, encompassing around **150,000 immune cells**. This extensive dataset allowed us to comprehensively characterize immune cell populations and their spatial distributions within the kidney, further supporting our findings.

While we acknowledge that the number of patients in the primary spatial cohort is limited, **we have additional samples available and can effectively more than double primary cohort size to strengthen our findings for a revised manuscript version.**

However, it's important to note that the primary spatial cohort was used primarily for **discovery purposes**. Our key objective was to identify and characterize spatial niches and microenvironments within the kidney tissue at single-cell resolution. The identification of these niches relies on analyzing large tissue areas with high spatial detail, which our samples provide. Each of our spatial samples covers a substantial tissue area, equivalent to what could be obtained from multiple kidney biopsy samples. This extensive coverage allows for reliable identification of spatial niches, which might be challenging with smaller samples.

We did not perform statistical comparisons between different conditions within the primary spatial cohort due to the limited number of patients and the exploratory nature of this part of the study. Instead, the statistical analyses and validation of our findings were conducted using a **large replication cohort**. Specifically, we utilized data from the **TRIDENT study**, which includes **226 patients**, the **Susztaklab Biobank**, which includes **843 patients**, and the **Penn Medicine Biobank**, which includes 2986 patients, to validate the associations between identified spatial niches and clinical outcomes. This approach ensures that our conclusions are supported by robust statistical evidence from sufficiently powered cohorts.

To our knowledge, our study represents one of the **largest single-cell resolution spatial transcriptomics datasets of the kidney**. The focus on single-cell level data (as opposed to spot-level data) provides unprecedented detail in spatial and cellular resolution, which is crucial for identifying and understanding complex spatial niches within the kidney.

3. Comparison with Previous Work

Comment: Many findings in the study are incremental in reference to a similar study recently published (July 2024) by the same authors in Nature Genetics (<https://doi.org/10.1038/s41588-024-01802-x>). In this study a similar sized cohort (including an overlap of samples analysed in this submitted manuscript) are profiled spatially using Visium genome sequencing at 50um resolution. Using the same custom gene panel in this study, CosMx was previously performed on two samples (1 Healthy, 1 disease) to train methodology to resolve, or infer, cell types at single cell resolution. Therefore, while only 2 samples are analysed at subcellular resolution in the previously published manuscript (compared to 16 here), there is significant overlap in the kidney structures that can be identified, and possibly meta-analysis that could have been performed to reach the same conclusions. For example, expression of immune signatures (such as B cells) that are not reported in the previous manuscript could be analysed from this data without increased resolution as opposed to the fibrotic niche/microenvironment. Thus, while this manuscript does report 14 more samples with increased resolution, there is significant overlap between the two studies in design, methodology and patient cohorts.

Response: Thank you for your thoughtful review and for bringing up the comparison with our previous publication in Nature Genetics. We appreciate the opportunity to clarify the distinctions between our current study and the earlier work.

While it is true that both studies involve spatial transcriptomics of the human kidney and include some overlapping samples, the **focus, methodology, resolution, and key findings of the two studies are substantially different.**

1. Resolution and Data Type:

- **Previous Study:** Our Nature Genetics paper primarily utilized **spot-level spatial transcriptomics** data generated using the 10x Genomics **Visium** platform, which provides spatial resolution at a **55µm spot size**. Each Visium spot captures the combined gene expression of multiple cells, resulting in averaged signals that limit single-cell resolution.
- **Current Study:** In contrast, the current work employs the **CosMx Spatial Molecular Imaging (SMI)** platform, which offers **single-cell and even subcellular resolution**. We analyzed **16 samples** at this high resolution, enabling us to map gene expression at the level of individual cells and to discern fine-grained cellular interactions within the tissue microenvironment.

2. Focus of the Study:

- **Previous Study:** The focus was on integrating dissociated single-cell RNA sequencing data with spot-level spatial transcriptomics. The emphasis was on broad tissue architecture and major cell types, without detailed analysis of spatial niches or immune cell interactions.
- **Current Study:** Our present study is centered on the **identification and characterization of specific spatial niches and immune cell populations** within the kidney. We delve deeply into the spatial organization of cells, particularly **B cells**, and their interactions within distinct microenvironments. This allows us to uncover novel insights into the cellular landscapes associated with kidney disease.

3. Identification of Niches and Immune Cells:

- **Previous Study:** Due to the lower resolution of the Visium platform, the previous study could not resolve individual immune cell types or precisely define cellular niches.
- **Current Study:** The single-cell resolution of CosMx SMI enables us to **identify specific immune cell populations**, such as B cells, T cells, and their subtypes, within their precise spatial context. We also define and characterize **distinct spatial niches** (e.g., injured proximal tubules, injured loop of Henle) and their associated cellular microenvironments, which was not possible in the previous study.

4. Methodological Advances:

- **Previous Study:** The methodology was tailored to integrate bulk spatial spots with single-cell data, providing an overview of tissue architecture.
- **Current Study:** We developed and applied advanced analytical methods suited for high-resolution spatial data, including novel approaches for **niche identification, neighborhood analysis, and cell-cell interaction mapping** at the single-cell level. These methods allowed us to explore the tissue microenvironment in unprecedented detail.

5. Key Findings:

- **Previous Study:** The key findings related to the general spatial distribution of major cell types and broad tissue regions.
- **Current Study:** We discovered that **B-cell-driven immune niches** are associated with a subset of kidney disease patients, a finding that was not reported previously. We also identified specific ligand-receptor interactions and signaling pathways that may drive disease progression. These insights have important implications for understanding the pathogenesis of kidney diseases and for developing targeted therapies.

6. Patient Cohorts and Sample Overlap:

- While there is some overlap in patient samples between the studies, the current study includes additional patients and samples, particularly those analyzed at single-cell resolution. The increased number of samples and higher resolution enhance the robustness and generalizability of our findings.

7. Data resolution:

- **Previous Study:** The lower resolution data limits the ability to perform detailed analyses of immune cell populations and their interactions.
- **Current Study:** The high-resolution data allows for comprehensive analyses that were not feasible before. While meta-analysis could theoretically be performed, the insights gained from single-cell spatial data in this study are unique and cannot be fully replicated using lower-resolution datasets.

In summary, while both studies aim to map the human kidney using spatial transcriptomics, the **current work represents a significant advancement** by leveraging single-cell resolution to uncover new biological insights. The focus on **spatial niches, immune cell interactions, and cellular microenvironments** provides a deeper understanding of kidney tissue architecture and disease mechanisms that were not addressed in our previous publication.

We will make sure to clarify these distinctions in the revised manuscript, emphasizing the novel contributions and the importance of high-resolution spatial transcriptomics in uncovering new aspects of kidney biology.

4. Literature

Comment: Little attempt is made to compare the findings of this study to the literature... These studies need to be compared to the present one in more detail to avoid the inadvertent implication of novelty. Little attempt is made to compare the findings of this study to the literature (incl ref 15). There is, for example, a literature on the association of diabetic nephropathy with inflammation and immune dysfunction. Comparisons should be made between the niches described and conventional histological features that correlate with them (e.g., comment on mesangial hypercellularity above). Some previous single-cell and spatial studies of kidney disease are dismissed out of hand (see refs 15 and 21-26 in the discussion – mouse studies might well be very relevant) or not discussed in detail (e.g., Lake BB Nature 2023). These studies need to be compared to the present one in more detail to avoid the inadvertent implication of novelty.

Response:

Thank you for your insightful comments and for emphasizing the importance of situating our findings within the context of existing literature. Unarguably, there is a large body of literature on the role of inflammation in chronic kidney disease. We have rephrased our discussion to include some of this work. However, we would like to point out, that prior investigations on tissue inflammation in diabetic kidney disease were usually performed in a biased manner. The study by Eller K. et al. for example is a good example of such an approach as it nicely investigates the role of Tregs in diabetes and diabetic nephropathy. Obviously, only recent advances such as single-cell spatial transcriptomics have made the ability to investigate tissue inflammation in an unbiased fashion possible. Therefore, while there is a large body of literature on diabetic kidney disease associated inflammation, this work represents a unique, unbiased and tangible assessment of tissue inflammation and its organization in this context.

We have also rephrased the discussion to include, that the KPMP study already pointed to iLOH in the dissociated single cell data analysis and then at the spot level spatial analysis. While KPMP did not include single cell level spatial transcriptomics analysis or iLOH associated biomarkers, we nevertheless incorporated comparisons with KPMP studies, acknowledging their identification of iLOH injury. We have cited relevant KPMP publications and discussed how our results complement and extend their observations. Moreover, recent studies, including our own recently published study and referenced study by Lake BB et al., only allowed for crude investigation of niches and neighborhoods, owing to the low resolution of the Visium

technology. This technology is as discussed above spot-based and therefore far from single-cell resolution. By generating the largest single-cell spatial transcriptomic dataset to date, we introduce a novel framework for defining kidney tissue organization through the identification of spatial niches and neighborhoods across multiple samples.

5. Other Issues

a) Use of the Term "Kidney Disease"

Comment: The term "kidney disease" needs to be used with care...

Response: We agree with your observation. We will revise the manuscript to use more precise terminology, specifying "diabetic kidney disease" or "DKD" when referring to our findings. We will also adjust the title to reflect this specificity, for example: "Spatial Human Kidney Map Highlights B-cell Driven Subgroup in Diabetic Kidney Disease."

b) Clinical Cohort Description

Comment: The clinical cohorts are not well described...

Response: We thank the reviewer for this comment. We have included the information of the clinical cohorts in the supplementary table.

c) Validation of Computational Predictions

Comment: Have any of these been experimentally validated?

Response: We acknowledge the importance of validating computational predictions. Using the high spatial resolution of our atlas, we will validate the spatially variable genes we predicted.

d) Temporal Dynamics and Repeat Specimens

Comment: Were these taken at distinct times and have they been assessed for temporal changes in niche composition that may be associated with disease?

Response: We thank the reviewer for this comment. This is a very interesting idea and worthwhile exploring in the future. However, the samples were taken from the same FFPE block. We therefore did not look at temporal changes in niche composition and their association with disease.

e) Addressing Integration Challenges

Comment: How have the authors addressed these potential concerns?

Response: We thank the reviewer for this comment. To benchmark integration, we will incorporate the scIB-metrics framework to rigorously assess our scVI/scANVI integration. The scIB metrics include kBET, LISI, ARI, and silhouette scores for both batch correction and biological conservation. Our results show that the integration effectively mitigates batch effects while preserving biological variation. These metrics confirm that our integrated dataset maintains the integrity of cell-type identities and gene expression patterns across samples. We have included a detailed description of this validation in the revised Methods section and added a Supplementary Figure presenting the scIB metrics results.

f) Stratification by Other Spatial Gene Signatures

Comment: Do any of the other spatial gene signatures stratify patients in a manner that correlates with renal outcome but doesn't simply reflect baseline disease severity?

Response: We thank the reviewer for this question. Yes, the spatial transcriptomics dataset could identify disease subtypes independent of disease severity. Here we focus on the B-cell subtype as this subtype could have an immediate therapeutic implication.

I think it is worthwhile noting, that our study was primarily focused on understanding the architecture of the human kidney in health and in diabetic kidney disease. By doing this, we identify disease associated niches and microenvironments. These microenvironments help us to approximate the disease state and ligand receptor interactions that are associated with this disease state. Moreover, we investigated how immune cells form different microenvironments, such as the B cell predominant microenvironment. These spatial patterns are disease associated, therefore extraction of spatial gene signatures and clustering of patients into groups based on the expression of these spatial gene signatures will identify patients at different stages of kidney disease. However, this remains the first subgroup identification of diabetic kidney disease and moreover, identifying a subset of patients with DKD and a high abundance of B cells represents a potentially actionable subgroup. A plethora of therapeutics to target B cells have been developed and could be repurposed for these patients.

g) Correlation of Effect Sizes in Proteome Analyses

Comment: What is the correlation between effect sizes for proteins measured in both studies?

Response: We agree that comparing effect sizes will provide an orthogonal assessment of the overlap between kidney and blood proteome analyses and subsequent nomination of biomarkers. To address this, we will compute Pearson correlation between the log-fold changes of proteins measured in both kidney tissue and blood samples.

h) Association with Death and Predictive Modeling

Comment: Do any of the protein biomarkers show an association with death alone?... Can a sparse multivariate predictive model be developed from this collection of potential biomarkers that might have utility in predicting kidney disease outcome in an unseen cohort of patients?

Response: Thank you for this comment. Yes we have two cohorts with plasma proteomics data (Trident and PMBB). In Trident we have matching tissue gene expression and plasma and urine proteomics. A subgroup of these cohorts now have matching single cell spatial transcriptomic information as well.

We have generated proteomics datasets for renal outcomes and death or for combined outcomes. We are happy to present this information, however we feel the goal of the current manuscript is the spatial transcriptomics dataset and the identification of small B-cell enriched patient subgroup, potentially enabling precision diagnostic and therapeutics.

Dear Susan,

We would like to express our sincere gratitude for reviewing our manuscript, "**Spatial Single-Cell Atlas of Diabetic Kidney Disease Reveals a B-Cell-Rich Disease Subgroup**." We appreciate the time and effort that you and the reviewers have invested in evaluating our work. During the last 9 months, we have almost completely rewritten the manuscript to elevate its impact and quality. In addition, we responded to important questions raised by reviewers. The editorial letter has raised four important points:

1. **Sample Size:** To address these concerns, we have now substantially expanded our dataset, increasing the spatial sample size **fourfold** to include **64 spatially profiled samples** using two complementary single cell-resolution platforms—CosMx 1k (n=48) and Xenium 5k (n=16). In addition to the sample number even more important the spatial area covered by our dataset is over 4 million cells. We cleaned and integrated the spatial data with a reference single-nucleus RNA-sequencing atlas from 150 patients (~750,000 cells) to construct a unified atlas of over **5 million cells, including more than 4 million spatially resolved high quality cells and 214 patients**. To the best of our knowledge, this represents one of the largest and most comprehensive spatially resolved single-cell resource to date for any human disease.

We also recognize the inherent biological variability of human kidney samples. We therefore conducted statistical analyses and validated our findings using a large replication cohort. This included data from the **TRIDENT study (226 patients)**, the **Susztaklab Biobank (843 patients)**, the **UK Biobank (3,309 participants** with a diagnosis of diabetes mellitus) and the **CD19-CAR T Lymphoma cohort with reduced eGFR (45 patients)**, validating the associations between identified spatial niches and clinical outcomes. We also note that this comment was raised by the nephrologist reviewer but not the computational biology reviewer.

2. **The objective of the study. The computational identification and novelty of unbiased spatial niches**
We feel this was a critical misunderstanding of the key aspect of our work and what spatial transcriptomics can offer. Biologically speaking, cells do not function in isolation. The **promise of spatial transcriptomics is the unbiased identification of microscopic structures or patterns (called niches** by the single cell community). For instance, simply counting podocytes in a tissue section can be misleading, as the count heavily depends on the number of glomeruli present in the tissue. In contrast, quantifying podocytes within the glomerular niche provides meaningful insights. Understanding cellular function requires knowledge of their neighboring cells and the cell-cell interactions that occur within these niches. Gene and pathway-level changes cannot be effectively analyzed at the whole-tissue level; such analyses necessitate precise niche identification. Furthermore, niche identification is critical for computational and future artificial intelligence-based histopathology. Currently, pathologists examine the kidney tissue and the glomerular, vascular, tubule and interstitial niches. Our data indicate that the kidney's complex cellular architecture is more diverse than previously appreciated and can be resolved into 11 recurrent spatial niches, identified through multiscale spatial transcriptomics (CosMx and Xenium) combined with unsupervised machine learning. We validate these niches across complementary computational approaches, including a graph neural network framework (NicheCompass) and a covariance-based manifold learning method (COVET) that uses approximate optimal transport to compare local gene–gene covariance structures. Moreover, within the glomerular niche, spatially resolved interaction analysis revealed disease-associated signaling changes between glomerular endothelial cells and podocytes that correlate with disease severity. These insights, uniquely enabled by spatial transcriptomics, highlight previously unrecognized alterations in intercellular communication in diabetic kidney disease.
3. **Comparison with Previous Work:** A reviewer suggested that many findings in our study are incremental and overlap with a previous publication by our group in *Nature Genetics*. The key novel findings of the current work.
 - **Large Single-Cell Resolution Spatial Transcriptomics Dataset:** The previous study primarily utilized **spot-level spatial transcriptomics** data at a **55 µm spot size**, not reaching single-cell resolution. We have generated one of the largest single-cell resolution spatial transcriptomics datasets of the human kidney, providing unprecedented detail in spatial and cellular resolution. This allows for the exploration of cellular interactions and microenvironments with high precision.
 - **New Framework for Defining Kidney Tissue Organization:** Our study introduces a novel approach to defining kidney tissue based on **spatial niches** identified across multiple samples, moving beyond

traditional histological classifications. This framework enhances our understanding of kidney tissue architecture and pathology.

- **Spatial Influence on Gene Expression:** We demonstrate that gene expression profiles of specific cell types are influenced by their spatial neighborhoods and interactions with surrounding cells. This finding underscores the importance of the microenvironment in cellular behavior and disease mechanisms. Furthermore, it allows us to move beyond disease associated cell types to a biologically much more tangible and meaningful concept of disease associated cellular organizations.
- **Identification of a B-cell Predominant Immune Microenvironment:** We discovered a **B-cell-predominant immune microenvironment adjacent to the injured loop of Henle (iLOH)**, which has not been previously reported in human kidney disease. This suggests a novel role for B cells in DKD pathogenesis.
- **Clinical application: Definition of a New Patient Subtype in DKD:** Our study stratifies DKD patients based on the presence of specific spatial niches, representing the first molecularly defined patient subtype in DKD. This is a crucial step towards precision diagnostics and personalized treatment strategies.

4. **Inflammation in DKD:** We would like to emphasize that inflammation is a broad term, with its role implicated in nearly all diseases. While advances in understanding the immune system have enabled the development of paradigm-shifting therapeutics, such as checkpoint inhibitors for cancer, a limited understanding of specific component of the immune system has also contributed to failures, such as Pfizer's two unsuccessful anti-inflammatory clinical trials in DKD over eight years ago. Research into inflammation in DKD has so far been hindered by both the lack of scalable methodologies and the artifacts introduced by tissue dissociation and FACS analysis. We believe that single-cell spatial transcriptomics can address these issues, and therefore created the first human kidney immune cell atlas encompassing dissociated and single-cell spatial data with more than 250,000 kidney immune cells. By leveraging the spatial resolution of this kidney immune cell atlas, we were able to identify a stable, tissue-resident immune compartment dominated by anti-inflammatory macrophages, predominantly localized in histologically normal regions. In addition, our data resolved a glomerular immune microenvironment, even in healthy tissue, which expanded in DKD with increased infiltration of CD8⁺ T cells and macrophages, accompanied by altered ligand–receptor interactions correlating with kidney function. We also uncovered an immune microenvironment associated with injured tubules, marked by disease-stage-specific plasma cell accumulation and altered inflammatory signaling. Finally, a distinct B cell–predominant immune microenvironment emerged in DKD, enriched for T follicular helper–like CD4⁺ T cells, class-switching B cells, and proliferating B cells differentiating toward plasma cells—together supporting the presence of tertiary lymphoid structure–like niches in the diseased kidney. Moreover, in a larger cohort, we found this to be present in 9% of patients with DKD and we found these patients to progress to end stage kidney disease more quickly. We investigated whether B cell–targeted therapy could improve kidney outcomes in patients with impaired renal function. We analyzed a cohort of 45 individuals with relapsed or refractory B cell non-Hodgkin lymphoma and an eGFR <60 ml/min/1.73 m² who received CD19-directed CAR T cell therapy. Remarkably, kidney function improved significantly within two months post-infusion, despite the absence of detectable lymphoma involvement in the kidney (Extended Fig. 34). These findings suggest that deep B cell depletion may offer therapeutic benefit in select kidney disease settings and reveal a **previously unrecognized treatment axis** in DKD. Although B cell–directed therapies are widely used in autoimmunity and hematologic malignancies, they remain largely unexplored in the context of DKD. Our results highlight the need for prospective, randomized clinical trials to evaluate the efficacy and safety of B cell–targeted interventions in this disease. Stratification using the spatial gene signature and biomarkers identified here may enable the selection of patients most likely to benefit.

Below, we provide a summary of the key revisions made in response to the comments and suggestions.

1. **Increased sample size:** For the revised manuscript, we have substantially expanded our dataset, increasing the number of spatially profiled samples fourfold to include **64 samples**. This expanded cohort includes both healthy and DKD kidneys across the full range of age, kidney function and encompasses patients of both Black and White racial backgrounds.

2. **Integration of complementary spatial platforms (CosMx and Xenium):** In this revised version, we incorporated two complementary single-cell resolution platforms—CosMx 1k (n=48) and Xenium 5k (n=16)—to achieve both broad cohort coverage and enhanced transcriptomic depth. Importantly, we designed **kidney-specific gene panels** tailored to each platform, which we believe will serve as a valuable resource for the kidney research community. The higher resolution of the Xenium platform enabled the identification of rare cell types, such as lymphatic endothelial cells. This dual-platform approach facilitates cross-validation of findings and helps overcome the gene panel limitations inherent to each technology.
3. **Cross-technology generation of a large-scale spatial kidney atlas:** In the revised manuscript, we leveraged two complementary single-cell resolution spatial transcriptomics platforms—CosMx 1k (n=48) and Xenium 5k (n=16)—to maximize both cohort diversity and transcriptomic resolution. We integrated these data with a reference single-nucleus RNA-sequencing atlas (~750,000 cells from 150 individuals), resulting in a **unified atlas of over 5 million cells**, including more than 4 million high-quality spatially resolved cells. To our knowledge, this represents one of the largest and most comprehensive spatial single-cell resources assembled for any human disease.
4. **Rigorous integration and benchmarking validated by scIB:** To integrate spatial and snRNA-seq data across platforms, we used scVI and scANVI—deep generative models designed for complex batch and modality correction. We accounted for platform, sample identity, data source, and RNA quality metrics as covariates. Importantly, we **benchmarked our integration strategy** using the scIB framework, which quantitatively assesses both batch effect removal and biological conservation. Among six tested methods (unintegrated, Scanorama, Harmony, Liger, scVI, scANVI), scANVI consistently achieved the highest overall scores, confirming its robustness in preserving biological structure while correcting for technical variation (Extended Fig. 4).
5. **High resolution of kidney cell types:** The spatial integration enabled the identification of 20 distinct kidney cell types, including fine-grained annotation of endothelial and stromal subtypes such as glomerular, peritubular, and descending vasa recta endothelial cells, as well as mesangial cells, fibroblasts, and vascular smooth muscle cells—subtypes that are challenging to resolve in dissociated datasets due to overlapping transcriptional profiles. We highlight key marker genes that distinguish these subtypes within each spatial platform (CosMx and Xenium), offering practical guidance for their identification in future kidney spatial transcriptomics studies.
6. **Unbiased identification of 11 spatial kidney niches through multiscale spatial analysis:** Cells operate within complex tissue environments, and their function is shaped by local interactions. Spatial transcriptomics uniquely enables the unbiased identification of such microenvironments—referred to as “niches”—by capturing cell composition and communication in situ. In the revised manuscript, we combined CosMx and Xenium data with multiscale spatial aggregation (20–80 μm radii) and **unsupervised machine learning** to identify 11 reproducible spatial niches across healthy and diseased kidneys. This approach overcomes the limitations of traditional cell-type quantification (e.g., podocyte counts), enabling more biologically meaningful interpretations grounded in anatomical context. Importantly, niche-level resolution is essential for computational and future AI-based histopathology. While current pathology frameworks consider glomerular, vascular, tubular, and interstitial regions, our analysis reveals a more nuanced and reproducible tissue architecture. These niches not only align with known anatomical compartments but also uncover spatially organized injury states, laying the foundation for targeted diagnostics and mechanistic insight into disease progression.
7. **Orthogonal niche validation using covariance-based manifold learning (COVET):** To support the robustness of our niche annotations, we additionally applied COVET, a **gene-gene covariance-based manifold learning** algorithm to our expanded dataset. Inputs to the COVET matrix included: (1) spatially resolved gene expression, (2) 64 highly variable genes (selected via Scanpy’s `highly_variable_genes`), and (3) the number of spatial neighbors ($k = 8$). We computed an adjusted covariance matrix across the 64 genes to capture niche-specific co-expression patterns. Pairwise niche distances were then derived using Approximate Optimal Transport (AOT). Using dimensionality reduction, we demonstrated

clear separation of our annotation-based niches in low-dimensional space—confirming the biological distinctiveness of our spatial niches.

8. **Orthogonal niche validation using NicheCompass:** To complement and validate our unsupervised niche analysis, we applied NicheCompass (Niche Identification based on Cellular graph Embeddings of COMMunication Programs Aligned across Spatial Samples), a **deep learning framework** that models cells as nodes in a spatial graph and integrates gene expression with spatial context. NicheCompass learns interpretable cell embeddings that encode spatial gene program activities by explicitly modeling cellular communication and predicting the molecular profiles of neighboring cells in relation to specific signaling events. This enables pathway usage scoring across microenvironments and facilitates the quantitative identification and characterization of niches. The resulting embeddings revealed eight distinct niches that closely matched our annotation-based niches, reinforcing the biological validity of our approach. Moreover, NicheCompass uncovered niche-specific spatial gene programs—for instance, APOE activity in the proximal tubule niche and IL32-associated signaling in the injured tubule niche—highlighting distinct intra- and intercellular communication states.
9. **Niche composition correlates with kidney function and disease severity:** Our unbiased spatial analysis revealed marked shifts in niche composition between healthy and DKD samples, suggesting that **reorganization of tissue architecture is a hallmark of disease progression**. Several niche fractions significantly correlated with eGFR, a clinical measure of kidney function—for example, DCT ($r = 0.61$) and PT ($r = 0.52$) niches were positively associated with eGFR, while immune ($r = -0.59$) and IPT niches ($r = -0.55$) were negatively associated. These spatially defined changes in cellular neighborhoods provide a quantitative framework to link tissue organization with clinical outcome and may offer novel diagnostic or prognostic markers in DKD.
10. **Validation of disease-associated transcriptional rewiring using Hotspot gene–gene correlation analysis:** We hypothesized that our niche framework could reveal key gene expression changes associated with DKD. As in the initial submission, differential expression analysis across spatially defined niches identified disease-associated transcriptional alterations likely driven by shifts in cellular interactions. To strengthen these findings and move beyond conventional differential expression, we applied Hotspot—a complementary method that identifies spatially co-expressed genes using niche-specific latent representations derived from COVET. This analysis confirmed conserved **gene–gene correlation structures** across control and DKD samples in all niches. Crucially, differential correlation analysis revealed disease-specific transcriptional rewiring in DKD, such as increased coordination between COL1A1–SERPINE1 (matrix remodeling), CXCL12–MHC I (immune signaling), and JUNB–CD74 (antigen presentation) in the vascular niche. Full results are provided for all niches, supporting niche-specific transcriptional remodeling in DKD.
11. **Disease-associated remodeling of the glomerular niche revealed by spatial ligand–receptor analysis:** A key advance of the revised manuscript is the identification of glomerular niche remodeling in DKD at both the cellular and signaling level. Using CellPhoneDB, we uncovered disease-associated changes in ligand–receptor interactions within the glomerular niche, including increased inflammatory signaling such as IL6–IL6R activity on podocytes. This level of resolution—**linking ligand–receptor activity to cell type, spatial context and disease severity**—represents a major conceptual advance that is only achievable through high-resolution spatial transcriptomics. Traditional transcriptomic or histological approaches cannot capture these fine-grained, niche-specific communication patterns. Our findings offer a blueprint for the identification of future therapeutic targeting based on spatially resolved cell–cell interactions.
12. **Microenvironmental context shapes injured tubular epithelial cell states:** Spatial transcriptomics enables analysis not only of multicellular niches but also of microenvironments—defined by the immediate spatial context surrounding a specific cell type. Focusing on injured tubular epithelial cells (IPT and iLOH), we identified five distinct microenvironments - **proximal, perivascular, periglomerular, distal and profibrotic** injured tubular microenvironments - based on local cellular composition. Each was characterized by unique neighboring cell types and transcriptional programs, demonstrating that epithelial injury states are shaped by spatial context. This cell-centric approach reveals

how microenvironmental cues contribute to transcriptional heterogeneity and cellular responses during disease progression.

- 13. High-resolution characterization of the profibrotic microenvironment in DKD:** In the revised manuscript, we identified a profibrotic microenvironment centered around MMP7⁺, HAVCR1⁺ IPT and ITGB6⁺ iLOH cells, characterized by abundant immune and fibroblast neighbors (Fig. 3j, l). This microenvironment was significantly expanded in DKD and showed a strong inverse correlation with kidney function (eGFR). By mapping cell states using our integrated snRNA-seq reference, we found enrichment for inflammatory and fibrotic pathways, including IL1, and focal adhesion signaling. To validate the spatial relevance of this microenvironment, we assessed its colocalization with COL1A1 protein deposition using **Sirius Red staining** on adjacent tissue sections. This analysis confirmed spatial overlap and revealed markedly higher COL1A1 expression in fibroblasts within this microenvironment, supporting its role as a distinct proinflammatory and profibrotic niche closely linked to disease severity.
- 14. Uncovering profibrotic signaling networks through spatial ligand–receptor analysis:** This study provides the first spatially resolved, single-cell analysis of signaling interactions within a profibrotic microenvironment in human kidney tissue. Enabled by high-resolution spatial transcriptomics, we dissected the cellular crosstalk between injured epithelial cells and fibroblasts that underlies fibrosis in DKD—a level of detail unattainable with bulk or dissociated single-cell methods. Focusing on injured proximal tubule cells (marked by HAVCR1 expression), we identified ligand–receptor interactions such as TNFSF10–TNFRSF10B, which correlated strongly with injury severity and suggest epithelial–stromal communication via death receptor signaling. To explore pathways associated with extracellular matrix production, we analyzed COL1A1-associated signaling and uncovered enrichment of interactions such as PDGFB–PDGFRB and JAG2–NOTCH, implicating PDGF and Notch signaling in fibroblast activation and matrix deposition.
- 15. Generation of a cross-modality spatial kidney immune cell atlas:** To address longstanding challenges in resolving immune cell heterogeneity and spatial organization, we generated the first comprehensive cross-modality spatial immune cell atlas of the human kidney by integrating high-resolution data from both CosMx and Xenium platforms. Unbiased Leiden clustering of the combined dataset revealed 11 distinct immune populations, including multiple dendritic cell subsets, T cells, NK cells, B cells, plasma cells, macrophages, neutrophils, and basophil/mast cells. While all immune cell types were present across samples, both the absolute number and relative fractions of immune cells were increased in diabetic kidney disease (DKD), with the most pronounced expansion observed in plasma cells (3.1-fold) and B cells (2.4-fold), highlighting immune remodeling in disease at single-cell and spatial resolution. We further observed striking differences in immune cell spatial distribution: while macrophages and plasma cells were broadly dispersed, B cells exhibited the most spatially restricted localization, preferentially clustering within dense immune microenvironments.
- 16. Identification of six distinct immune microenvironments based on local cellular context:** To understand how immune cells are spatially organized within the human kidney, we clustered immune cells based on the composition of their surrounding cellular neighborhoods. This analysis revealed six distinct immune microenvironments, each defined by dominant neighboring cell types. These included fibroblast-rich (fibro–immune), injured tubular (injured tubule–immune), vascular (vascular–immune), and glomerular (glomerular–immune) microenvironments, reflecting spatial alignment with major kidney compartments. Additionally, we identified two dense immune microenvironments: a T cell–predominant niche composed largely of CD8⁺ and CD4⁺ T cells (with some B cells), and a B cell–predominant niche enriched for B cells and a smaller fraction of CD4⁺ T cells. These spatially distinct immune niches provide a framework for understanding how immune cells interact with tissue compartments in health and disease.
- 17. Discovery of a tissue-resident immune microenvironment in the human kidney:** Among the six identified immune microenvironments, one stood out by its relative isolation—lacking neighboring epithelial, stromal, vascular, glomerular, or other immune cells. We termed this the *residential immune microenvironment*, which was predominantly composed of macrophages, with a minor contribution from CD8⁺ T cells. Identifying tissue-resident

immune populations in the human kidney has been challenging due to limited spatial resolution. In the revised manuscript, we demonstrate that immune cells in histologically normal tissue—particularly in two healthy pediatric samples—localized almost exclusively to the residential immune microenvironment, supporting its designation as a tissue-resident compartment. Notably, unlike other immune microenvironments that exhibited substantial shifts in disease, this compartment remained remarkably stable across both healthy and DKD samples in terms of abundance and cellular composition. Transcriptional profiling of macrophages within this niche revealed consistent expression of genes associated with tissue residency and anti-inflammatory function—such as *MRC1*, *CD163*, and *ZFP36* in CosMx, and *MRC1*, *GPR34*, *STAB1*, and *RNASE1* in Xenium—clearly distinguishing them from more inflammatory macrophage populations found in other contexts.

- 18. Disease-associated remodeling of the glomerular immune microenvironment:** In the revised manuscript, we expand our analysis of the glomerular immune microenvironment. Even in healthy kidneys, we observed a small population of immune cells—primarily macrophages—within glomeruli. In DKD, both macrophages and CD8⁺ T cells were significantly expanded, indicating progressive immune involvement. Leveraging our large and diverse cohort, we extended our analysis beyond baseline comparisons to examine ligand–receptor interactions across a continuum of disease severity. This revealed that *FN1–ITGA4B7* signaling between mesangial cells and macrophages negatively correlated with eGFR, linking extracellular matrix remodeling and immune cell recruitment to functional decline. In contrast, *BMP4–BMP2* signaling between podocytes and CD8⁺ T cells positively correlated with eGFR, suggesting a protective, anti-inflammatory role for BMP signaling. Together, these findings highlight the glomerular niche as a dynamic immune microenvironment, with signaling shifts that track with disease progression.
- 19. Immune–epithelial interactions in the injured tubular microenvironment:** In the revised version of the manuscript, we further explored immune cell interactions surrounding injured tubular epithelial cells to better understand local inflammatory dynamics. Given the emergence of a distinct injured tubular–immune microenvironment, we hypothesized that this niche would offer detailed insight into inflammation associated with tubular injury. Even in healthy adult kidneys, injured tubular cells were present, but the surrounding immune context shifted markedly with disease. In DKD, plasma cells surrounding injured tubules were significantly increased, indicating local immune activation. Spatial ligand–receptor analysis revealed disease-associated changes in immune–epithelial signaling, including reduced *ANXA1–FPR3* signaling (implicated in inflammation resolution), and increased *CXCL12–CXCR4* and *TNFSF13–TNFRSF13B* interactions, suggesting enhanced plasma cell recruitment and survival.
- 20. Formation of a TLS-like B cell microenvironment linked to tubular injury and fibrosis in DKD:** In the revised manuscript, we identified a marked increase in B cells in DKD, which exhibited the highest degree of spatial organization among immune cell types. In a subset of patients, B cells clustered into a distinct, DKD-associated B cell–predominant immune microenvironment that spatially overlapped with the profibrotic tubular microenvironment, linking B cell accumulation to areas of injury and fibrosis. Notably, using the Xenium 5k panel, we show that this microenvironment exhibited strong spatial co-expression of *CXCL13* and its receptor *CXCR5*—a chemokine axis critical for lymphoid tissue organization—suggesting the presence of tertiary lymphoid structures (TLSs) within the kidney. Supporting this, CD4⁺ T cells in this niche expressed *CXCR5* and *PCDC1*, consistent with a T follicular helper (Tfh)–like phenotype, while B cells expressed *TNFSF13B* and *TNFRSF13C* (*BAFF–BAFFR*), supporting in situ survival and differentiation. To assess whether these B cells were undergoing functional activation and differentiation into antibody-producing cells, we used Xenium 5k to detect *AICDA* (required for class-switch recombination) and *IRF4* (essential for plasma cell differentiation). These findings suggest that local B cell activation and differentiation into plasma cells may occur within the kidney in DKD.
- 21. Cross-modality integration reveals spatial organization of B cell subtypes in TLS-like B cell microenvironment:** In the revised version of the manuscript, we refined our characterization of the TLS-like microenvironment by integrating spatial B and plasma cell transcriptomes with a recently published single-cell B cell atlas. Leveraging SCVI/SCANVI-based integration and label transfer, we aligned single-cell and spatial data through an

iterative deep generative framework, enabling high-confidence subtype prediction within spatial contexts. This analysis identified distinct B cell subtypes—including naïve B cells, memory B cells, and a smaller population of atypical memory B cells.

- 22. Isotype profiling reveals enrichment of naïve B cells in the B cell–predominant immune microenvironment:** To characterize B cell maturation states across immune niches, we used the CosMx 1k platform to classify B cells into four isotype categories—IgD⁺, IgM⁺, IgA/G⁺, or negative—based on dominant normalized expression of IGHD, IGHM, IGHA1, IGHG1, and IGHG2. The B cell–predominant immune microenvironment exhibited a significantly higher fraction of IgD⁺ ($p = 2.38 \times 10^{-11}$) and IgM⁺ ($p = 1.06 \times 10^{-4}$) B cells compared to other immune microenvironments, while no enrichment was observed for IgA/G⁺ cells. These findings suggest that the B cell–predominant niche is enriched for early-stage or naïve B cells, whereas class-switched B cells are more broadly distributed.
- 23. Validation of TLS-like microenvironment using multiplex imaging mass cytometry:** To validate our transcriptomic findings at the protein level, we performed multiplex imaging mass cytometry (IMC) on adjacent tissue sections. IMC confirmed a high abundance of CD20⁺ B cells and CD4⁺ T cells within the B cell–predominant immune microenvironment, consistent with the presence of a TLS-like structure (Fig. 5c–p). In line with our spatial transcriptomics data, CD8⁺ T cells were sparse, and plasma cells were nearly absent from this compartment. Staining for CD27, CD11c further supported the presence of memory and atypical memory, respectively. Ki67 staining revealed proliferating cells were primarily CD20⁺ B cells, suggesting local expansion, while CD4⁺ T cells showed no evidence of proliferation.
- 24. Proteomic analysis of IgA complexes reveals inflammatory effector proteins in B cell–rich kidneys:** To investigate the downstream consequences of intrarenal B cell aggregation and potential immune complex formation, we generated a spatial gene signature for the B cell–predominant inflammatory niche and applied it to bulk RNA-seq data from the Susztaklab Biobank to identify patients with strong B cell–predominant inflammation. In paired kidney tissue lysates from these patients and matched controls, we performed **IgA immunoprecipitation followed by tandem mass tag (TMT)-based quantitative mass spectrometry** to identify proteins bound to IgA complexes. Samples were lysed under native conditions to preserve protein–protein interactions, and IgA complexes were captured using biotin-conjugated anti-IgA antibodies and streptavidin-coated beads. After on-bead digestion and TMT labeling, samples were analyzed on an Orbitrap Eclipse mass spectrometer with FAIMS Duo for in-depth proteomic profiling. This analysis revealed significant enrichment of immune effector proteins in B cell–rich kidneys, including granzymes—proteolytic enzymes secreted by cytotoxic lymphocytes—and complement components such as C3, C4A, and C8B, suggesting that immune complex formation may contribute to tissue damage in this inflammatory niche.
- 25. B cell–predominant inflammation defines a high-risk molecular subtype of DKD:** To assess the clinical relevance of the B cell–predominant immune microenvironment, we examined its association with kidney disease severity using a bulk RNA-seq dataset from the **Susztaklab Biobank (n = 843)** linked to detailed clinical and histopathological data. Applying the spatial gene signature derived from the TLS-like niche, we observed a strong negative correlation with interstitial fibrosis severity, indicating that this immune compartment is associated with progressive tissue remodeling. To further explore its prognostic significance, we performed unsupervised clustering on bulk transcriptomic data from the **TRIDENT study (n = 226)**, stratifying patients into B⁺ DKD (8.4%) and B[−] DKD (91.6%) subgroups based on the spatial signature. Patients in the B⁺ DKD group had significantly worse baseline kidney function (mean eGFR: 21.05 vs. 35.98 mL/min/1.73 m²) and experienced a more rapid progression to the renal composite endpoint (dialysis, transplantation, or ≥40% decline in eGFR) over three years. This association remained significant in Cox regression models adjusted for age, sex, and race, establishing B cell–predominant inflammation as a marker of high-risk disease.
- 26. CD19-directed CAR T cell therapy improves kidney function in patients with kidney disease:** To investigate whether B cell depletion could improve kidney outcomes, we analyzed a cohort of 45 patients with relapsed or refractory B cell non-Hodgkin lymphoma and impaired kidney function (eGFR < 60 mL/min/1.73 m²) who received CD19-directed CAR T cell therapy. Notably, none of the patients had histological evidence of lymphoma

infiltration in the kidney at the time of treatment. Two months after CAR T infusion, kidney function improved significantly across the cohort, highlighting the potential therapeutic relevance of B cell depletion in settings of B cell-driven kidney inflammation (Extended Fig. 34).

27. RNA-seq profiling reveals candidate biomarkers of B cell-predominant inflammation in DKD: To identify tissue biomarkers specific to the B cell-predominant inflammatory niche, we analyzed bulk RNA-seq data from the TRIDENT study, comparing patients with and without B cell-rich inflammation (B⁺ DKD vs. B⁻ DKD). Differential expression analysis revealed upregulation of canonical B cell markers (CD19, FCRLA, TNFRSF13B, CD79A), immunoglobulin components (IGHM), and the B cell chemoattractant CXCL13. Additionally, we observed elevated expression of PDCD1, CXCR5, LTA, and LTB—key genes involved in T follicular helper cell identity and lymphoid tissue organization—further supporting the presence of TLS-like structures in the kidney and highlighting these molecules as potential biomarkers of intrarenal B cell-driven inflammation.

28. Development of a discriminatory plasma biomarker panel for B⁺ DKD: In the revised manuscript, we leveraged paired plasma proteomics to distinguish B⁺ from B⁻ DKD patients. Using elastic-net logistic regression with the Synthetic Minority Oversampling Technique (SMOTE) to address class imbalance, we trained a classifier optimized via 5-fold cross-validation. Predictive proteins with non-zero model coefficients were subsequently incorporated into a Cox proportional hazards model for kidney survival (death, dialysis, or $\geq 40\%$ eGFR decline). This protein-based model significantly improved risk prediction beyond clinical features alone, as evidenced by a higher mean C-statistic across 1,000 bootstrap resamples, supporting its potential utility in stratifying patients with B cell-driven inflammation. For external validation of the B⁺DKD protein set, we analyzed data from 3,309 UK Biobank participants with a diagnosis of diabetes mellitus and complete clinical information. In this independent cohort, the B⁺DKD protein set, when combined with known clinical predictors (Kidney Failure Risk Equation; KFRE), achieved an AUC of 0.7047, exceeding the performance of the clinical model (KFRE; AUC = 0.6286), thereby supporting its potential utility for risk stratification beyond established clinical predictors (Extended Fig. 38a). Moreover, quartile-based β -coefficients from the Cox model showed that individuals above the highest quartile (Q5) of the B⁺DKD protein set had a markedly increased risk of progression, exceeding that predicted by the KFRE model. This threshold may therefore help to identify a high-risk subgroup, that could be amenable to B cell-directed interventions.

Below, we provide a detailed point-by-point response to the reviewers' comments and are confident in our ability to address them.

We believe that these extensive revisions have raised the impact and the quality of the manuscript and shall be acceptable for the journal. We look forward to hearing back from the reviewers and editorial board.

Sincerely,

Katalin

Referees' comments:

Referee #1 (Remarks to the Author):

The manuscript by Dumoulin et al. titled "Spatial Human Kidney Map Highlights B-cell Driven Kidney Disease Subgroup" aims to map kidney cells using spatial transcriptomics, focusing on identifying disease-relevant spatial niches. The authors generated data from 1.7 million cells across 16 human kidney samples and integrated it with publicly available single-cell RNA sequencing data. In addition, they created an immune atlas of the kidney from 143 patients, which revealed a B-cell predominant immune microenvironment near the injured loop of Henle. Spatial signatures were then used to discover a blood biomarker that correlates with kidney health and mortality.

However, there are various major concerns:

This is a very preliminary study that analyzes only a small number of patients with spatial data (n=13), despite the seemingly large number of cells. While the cell count appears substantial, the actual number of patients is very low, and spatial analyses typically yield high cell counts. I feel that the authors are overstating their data by highlighting the cell count without mentioning the low patient count in the abstract. The small number of patients is especially significant when comparing different conditions within the spatial cohort.

Response: We thank the reviewer for this comment. To address these concerns, we have now substantially expanded our dataset, increasing the spatial sample size **fourfold** to include **64 spatially profiled samples** (Fig. 1) using two complementary single cell-resolution platforms—CosMx 1k (n=48) and Xenium 5k (n=16). In

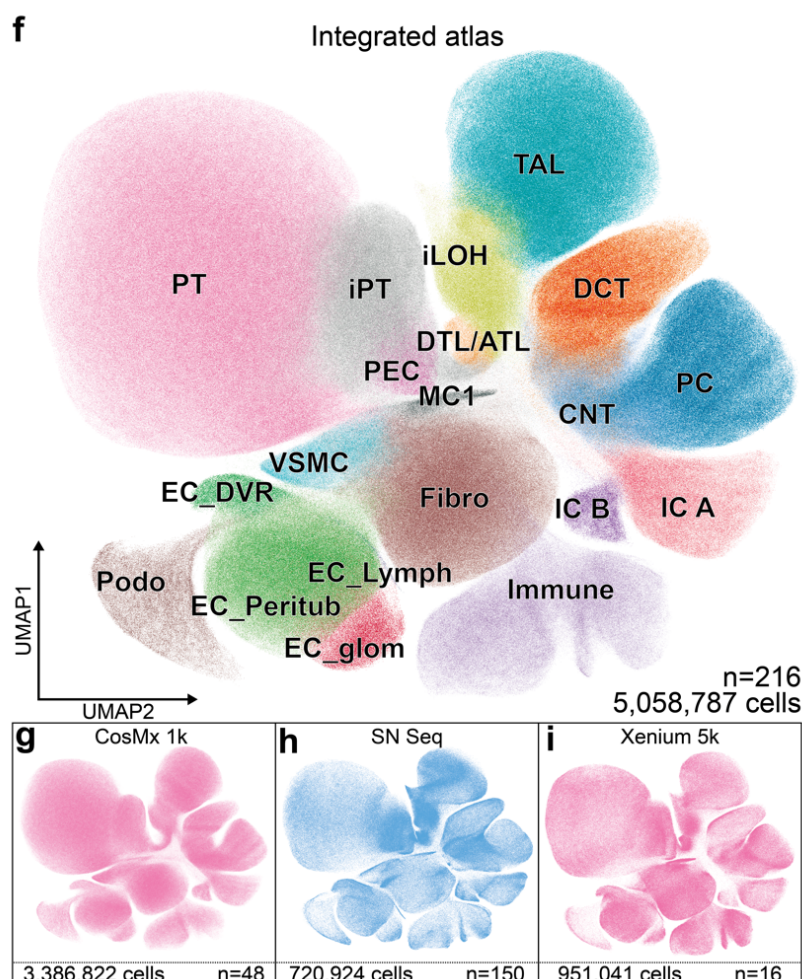


Fig. 1f: The UMAP visualization of the integrated spatial single-cell atlas. The spatial gene expression (CosMx 1k and Xenium 5k data) was integrated with dissociated single nucleus gene expression using scVI and scanVI. Each dot represents a single cell.

g, CosMx 1k spatial gene expression data. 48 samples and 3,386,822 cells were included in this study.

h, snRNA-seq data. 150 samples and 720,924 cells were included in this study.

The color indicates the different cell types such as: CNT, connecting tubular cells; VSMC, vascular smooth muscle cell; PC, principal cell; IC A, IC B, intercalated cells A, B; inj T, injured tubular cells; PT, proximal tubular cells; MC1, mesangial cell; LOH, thick ascending limb cells as well as DTL_ATL, distal thin limb, ascending thin limb cells; Fibro, fibroblasts; Podo, podocytes; DCT, distal convoluted tubular cells; PEC, parietal epithelial cells; EC: EC_Peritub, peritubular endothelial cells as well as EC_DVR, vasa recta endothelial cells; Immune, immune cells; EC_glom, glomerular endothelial cells

addition to the sample number even more important the spatial area covered by our dataset is over 4 million cells. We cleaned and integrated the spatial data with single-nucleus RNA-sequencing data from 150 patients (~750,000 cells) to construct a unified atlas of over **5 million cells, including more than 4 million spatially resolved high quality cells**. To the best of our knowledge, this represents one of the largest and most comprehensive spatially resolved single-cell resource to date for any human disease. To contextualize our work, we conducted a comprehensive literature review of high-impact studies from the past two years (published in *Nature* and *Nature Medicine*) that incorporated dissociative single-cell or spatial transcriptomics. A comparative summary of this research is included below to highlight how our dataset advances the field in both scale and resolution.

- Eriksson et al. conducted a comprehensive single-cell analysis of the human endometrium during the proliferative phase in women with polycystic ovary syndrome (PCOS).¹ They profiled 247,791 nuclei from 27 endometrial biopsies using single-nucleus RNA sequencing (snRNA-seq), including 5 controls, 12 PCOS patients at baseline, 7 after metformin treatment, and 3 after lifestyle intervention. To complement this, spatial gene expression was assessed using Stereo-seq in 6 additional samples. While Stereo-seq adds valuable spatial context, it has a lower resolution compared to other spatial platforms.
- Barnett et al. created an organotypic atlas of human vascular cells by integrating single-cell RNA sequencing data from 19 organs across 67 donors.² In total, they analyzed ~800,000 cells (166 samples), including ~67,000 vascular cells. Of the 19 datasets, 2 were newly generated and 17 were publicly available.
- Mathys et al. generated a single-cell transcriptomic atlas of the aged human brain by profiling six brain regions from 283 post-mortem samples across 48 individuals with and without Alzheimer's disease.³ In total, they analyzed 1.3 million cells to uncover region- and cell-type-specific alterations associated with the disease. While Mathys et al. generated an influential dissociative single-cell atlas of the aging human brain (~1.3 million cells from 48 individuals), our study not only exceeds this in total cell number and donor diversity, but crucially incorporates spatial resolution at subcellular level, preserving tissue architecture and enabling in situ analysis of cellular interactions and microenvironmental structure.
- Wälchli et al. constructed a molecular atlas of the developing and adult human brain vasculature by performing single-cell RNA sequencing on 606,380 freshly isolated endothelial, perivascular, and tissue-derived cells from 117 samples across 68 individuals.⁴ While their study provides valuable insights into vascular development and pathology, it is based entirely on dissociative methods. In contrast, our work integrates spatial transcriptomics with single-nucleus RNA-seq to preserve tissue context and enable in situ analysis of cellular organization.
- Matchett et al. generated a single-cell atlas of human liver regeneration by combining single-nucleus RNA sequencing (snRNA-seq) from 31 liver samples (healthy, APAP-ALF, and NAE-ALF) with spatial transcriptomics using the Visium platform on 7 samples.⁵ While this multimodal approach provides important insights into liver repair, the spatial resolution of Visium is limited and captures transcriptomes from multiple cells per spot. In contrast, our study integrates subcellular-resolution spatial transcriptomics from 64 samples (CosMx 1k, n=48; Xenium 5k, n=16) with single-nucleus RNA-seq profiles from 150 patients (~750,000 cells), enabling precise cell-level mapping and interaction analysis.
- Lai et al. constructed a multimodal cell atlas of aging human skeletal muscle by analyzing 31 individuals (12 adult, 19 older adult) using single-nucleus and single-cell RNA sequencing (sc/snRNA-seq) and single-nucleus ATAC-seq (snATAC-seq), generating 292,423 transcriptomic profiles across 15 major cell populations.⁶ While this study provides valuable insights into age-related changes in muscle biology, the spatial dimension was inferred rather than directly measured. In contrast, our work leverages high-resolution spatial transcriptomics across 64 kidney samples (CosMx 1k, n=48; Xenium 5k, n=16) and integrates them with snRNA-seq from 150 patients (~750,000 nuclei), yielding over 5 million spatially resolved single-cell profiles.
- Farah et al. created a spatial and transcriptional atlas of the developing human heart by combining single-cell RNA sequencing (~143,000 cells from 8 fetal hearts) with high-resolution MERFISH-based spatial transcriptomics (~250,000 cells from 3 hearts).⁷ While this approach enabled mapping of cardiac cell types and spatial organization, the MERFISH panel was limited to 238 genes. In contrast, our study focuses on spatially resolved transcriptomic profiling of human disease using higher-content technologies—CosMx (1,000 genes, n=48) and Xenium (5,100 genes, n=16).
- Han et al. generated an epithelial cell-focused single-cell atlas of lung adenocarcinoma (LUAD) by profiling 246,102 epithelial cells from 16 early-stage LUADs and 47 matched normal lung samples using scRNA-seq.⁸ While this provided important insights into epithelial plasticity in cancer, the analysis was limited to dissociative single-cell profiling and a restricted cellular context. In contrast, our study provides spatially resolved transcriptional data from 64 samples using CosMx (1,000 genes, n=48) and Xenium (5,100 genes, n=16).
- Yao et al. presented a whole-brain cell-type atlas of the adult mouse by integrating ~4 million high-quality scRNA-seq profiles with a spatial transcriptomics dataset of ~4.3 million cells using MERFISH.⁹ While this study delivers unmatched resolution for the mouse brain, the MERFISH profiling was limited to ~500 genes. In contrast, our study focuses on human kidney disease and provides a spatially resolved single-cell atlas of over 5 million cells using two complementary single-cell spatial transcriptomics platforms: CosMx (1,000 genes, n=48) and Xenium (5,100 genes, n=16). These were integrated with single-nucleus RNA-seq data from 150 patients (~750,000 cells).

- Huang et al. performed combinatorial indexing-based single-cell RNA sequencing on over 1.6 million nuclei from 101 E13.5 mouse embryos representing 22 mutant and 4 wild-type genotypes.¹⁰ This whole-embryo approach provides a powerful framework for systematically assessing developmental phenotypes in mouse models. In contrast, our study focuses on human kidney disease and leverages two single-cell spatial transcriptomics platforms—CosMx (1,000 genes, n=48) and Xenium (5,100 genes, n=16)—to profile over 4 million spatially resolved human cells. These datasets were integrated with single-nucleus RNA-seq from 150 human kidney samples (~750,000 cells), resulting in a cross-technology atlas of more than 5 million cells.
- Lake et al. applied multiple single-cell and single-nucleus RNA sequencing assays (>400,000 nuclei or cells) alongside spatial imaging technologies—Visium (22 patients) and Slide-seq2 (6 patients)—to profile healthy (45 donors) and diseased (48 patients) human kidneys, generating a valuable resource for understanding kidney injury and repair.¹¹ However, Visium offers limited spatial resolution, capturing transcriptomes at ~55 µm spots that typically include multiple cells, and Slide-seq2, while higher resolution, still does not achieve true single-cell resolution. In contrast, our study leverages two high-resolution single-cell spatial transcriptomics platforms—CosMx (1,000 genes, n=48) and Xenium (5,100 genes, n=16)—to profile over 4 million spatially resolved human kidney cells at true single-cell resolution. We further integrated these spatial data with single-nucleus RNA-seq from 150 kidney samples (~750,000 cells), generating a cross-technology reference atlas of more than 5 million cells. This makes our work to our knowledge the most comprehensive and spatially resolved single-cell resource available for any human disease to date.
- Harnik et al. constructed a comprehensive spatial expression atlas of the adult human proximal small intestine using Visium spatial transcriptomics (8 patients), spatial proteomics, and single-molecule FISH.¹² While their study provides valuable insights, Visium lacks true single-cell resolution, with each spot covering multiple cells and thus limiting precise cell-type and spatial interaction analyses. By contrast, our study utilizes two single-cell resolution spatial transcriptomics platforms—CosMx (1,000 genes, 48 samples) and Xenium (5,100 genes, 16 samples)—across 64 kidney tissues, profiling over 4 million spatially resolved cells. When integrated with 150 single-nucleus RNA-seq profiles (~750,000 cells), this forms a cross-technology atlas of more than 5 million kidney cells, providing unmatched resolution and scale for dissecting human disease.
- Qiu et al. applied single-cell combinatorial indexing to generate a comprehensive temporal transcriptional atlas of mouse prenatal development, profiling 12.4 million nuclei from 83 embryos across precisely staged intervals from E8 to P0.¹³ While the scale and temporal resolution of this study are impressive, the data are derived exclusively from nuclei and mouse embryos, without spatial resolution. In contrast, our study focuses on human disease and integrates single-cell spatial transcriptomics from 64 adult human kidney tissues using CosMx (1,000 genes, n=48) and Xenium (5,100 genes, n=16)—platforms that provide true single-cell resolution. Combined with 150 single-nucleus RNA-seq profiles (~750,000 cells), this dataset spans over 5 million cells, forming the most comprehensive spatially resolved human disease atlas to date.
- Zhang et al. generated a spatially resolved single-cell atlas of the entire adult mouse brain by profiling ~10 million cells using MERFISH targeting ~1,100 genes.¹⁴ This study provides an impressive spatial transcriptomic overview at the whole-organ scale in mouse, though it remains limited to a predefined gene panel and is not focused on human disease. In contrast, our study creates a human disease-focused spatial atlas using two true single-cell spatial transcriptomics platforms—CosMx (1,000 genes, n=48) and Xenium (5,100 genes, n=16)—in combination with 150 snRNA-seq samples (~750,000 cells), totaling over 5 million human kidney cells. This integrative approach offers higher gene coverage than MERFISH and yields the most comprehensive single-cell spatial dataset yet available for any human disease.
- Shi et al. constructed a spatial atlas of the adult mouse central nervous system by applying STARmap PLUS, an in situ sequencing method, to profile 1,022 genes across 1.09 million high-quality cells in 3D at subcellular resolution.¹⁵ While this provides remarkable spatial precision in the mouse CNS, the study is limited to a predefined gene panel and does not address human disease. In contrast, our study focuses on human pathology, integrating two true single-cell spatial transcriptomics platforms—CosMx (1,000 genes, n=48 samples) and Xenium (5,100 genes, n=16 samples)—with 150 snRNA-seq datasets (~750,000 cells), totaling over 5 million human kidney cells. This enables high-resolution spatial mapping with broader gene coverage and direct relevance to human disease, making it the largest and most comprehensive spatial single-cell resource for any human pathology to date.
- Qian et al. generated a spatial atlas of the developing human fetal cortex by applying MERFISH with a 300-gene panel to over 18 million cells across 39 fetal brain samples, complemented by Visium (n=2) and snRNA-seq (n=3).¹⁶ While the study provides valuable insight into cortical arealization, it is limited to fetal tissue, uses a restricted gene panel, and employs only a single single-cell spatial technology. It also does not address adult human disease. In contrast, our study focuses on adult human disease—specifically diabetic kidney disease—and employs two single-cell spatial transcriptomics platforms: CosMx (1,000 genes, n=48 samples) and Xenium (5,100 genes, n=16 samples). These were integrated with snRNA-seq data from 150 samples (~750,000 cells), resulting in a dataset of over 5 million spatially resolved human cells.
- Pei et al. constructed spatial maps of metastatic pancreatic cancer using Visium spatial transcriptomics on 49 biopsies from 13 patients¹⁷, profiling the tumor microenvironment and clonal architecture. While informative, the study is limited by the low spatial resolution of Visium. In contrast, our study focuses on diabetic kidney disease in the adult human, leveraging two high-resolution single-cell spatial transcriptomics platforms—CosMx (1,000 genes, n=48 samples) and Xenium (5,100 genes, n=16 samples)—alongside snRNA-seq data from 150 samples (~750,000 cells). This integration generated a spatial atlas of over 5 million human cells, offering superior cellular resolution, broader gene coverage, and multimodal insight into disease-related microenvironments, establishing the most comprehensive spatial single-cell resource in human disease to date.
- Sun et al. generated a spatially resolved single-cell brain atlas in mice, profiling 4.2 million cells across 20 ages and two rejuvenation interventions using MERFISH with a 300-gene panel.¹⁸ While this study provides valuable insights into aging, it is

limited to mouse tissue, uses a restricted gene panel, and relies on a single spatial transcriptomics technology. In contrast, our study focuses on human diabetic kidney disease, combining two high-resolution single-cell spatial transcriptomics platforms—CosMx (1,000 genes; 48 samples) and Xenium (5,100 genes; 16 samples)—integrated with snRNA-seq data from 150 human samples (~750,000 nuclei). This integration yields a spatial atlas of over 5 million human cells, enabling in-depth exploration of disease-associated microenvironments at unprecedented spatial and transcriptional resolution.

- Yapon et al. constructed a spatial human thymus cell atlas across fetal development by integrating multimodal single-cell data, spatial transcriptomics, and multiplex imaging from 29 donors.¹⁹ The spatial data was generated using the Visium platform, which provides limited spatial resolution and lacks single-cell precision. In contrast, our study focuses on human diabetic kidney disease and applies two high-resolution, single-cell spatial transcriptomics technologies—CosMx (1,000 genes; 48 samples) and Xenium (5,100 genes; 16 samples)—combined with single-nucleus RNA-seq from 150 samples (~750,000 cells). This integrated atlas of over 5 million spatially resolved human cells enables fine-scale mapping of disease-associated cellular niches and intercellular interactions.
- To et al. created a multi-omic atlas of human embryonic skeletal development by combining paired transcriptional and epigenetic profiling of ~336,000 nuclei with spatial transcriptomics from 12 donors, focusing on joint and cranial development between 5 and 11 weeks post-conception.²⁰ Spatial transcriptomics was limited to in situ sequencing (ISS) using the 10x Genomics CARTANA platform, which targets only 150 genes, and Visium, performed on just two samples, both of which offer limited gene coverage and spatial resolution. In contrast, our study of diabetic kidney disease utilizes two high-resolution single-cell spatial transcriptomics platforms—CosMx (1,000 genes; 48 samples) and Xenium (5,100 genes; 16 samples)—integrated with single-nucleus RNA-seq from 150 patients (~750,000 cells). This results in a dataset of over 5 million spatially resolved human cells, making it the most comprehensive single-cell spatial atlas assembled for any human disease to date.
- He et al. compiled an integrated transcriptomic cell atlas of human neural organoids by merging 36 publicly available single-cell transcriptomic datasets spanning 26 different protocols, totaling over 1.7 million cells.²¹ No new data was generated, and the atlas represents a meta-analysis of dissociated scRNA-seq data. In contrast, our study generates entirely new primary data from human kidney tissue using two single-cell spatial transcriptomics platforms—CosMx (1,000 genes; 48 samples) and Xenium (5,100 genes; 16 samples)—and integrates this with publicly available single-nucleus RNA-seq data from 150 patients (~750,000 cells). Altogether, our atlas comprises over 5 million spatially resolved human cells, providing the most comprehensive, experimentally generated single-cell spatial resource for any human disease to date.
- Oliver et al. present a systematic integration of 25 scRNA-seq datasets from 385 samples across 189 healthy individuals, covering the entire gastrointestinal tract during development and adulthood.²² Using a standardized pipeline (scAutoQC), they assembled a healthy reference atlas of ~1.1 million dissociated single cells. In contrast, our study generates primary spatially resolved single-cell data from diseased human kidney tissue, using two distinct spatial transcriptomics platforms—CosMx (1,000 genes; 48 samples) and Xenium (5,100 genes; 16 samples)—and integrates it with single-nucleus RNA-seq from 150 patients (~750,000 cells). This results in a multi-modal atlas of over 5 million cells from more than 200 patients, offering high-resolution insight into disease-associated cellular neighborhoods and molecular states
- Muñoz-Blat et al. employed a multi-omic approach to investigate decidualization resistance (DR) in the endometrium of women with a history of severe preeclampsia (sPE).²³ Their study combined morphological analysis with single-cell RNA sequencing (scRNA-seq) on endometrial samples from 23 patients (11 sPE, 12 controls) to characterize cellular and molecular disruptions. In contrast, our study goes beyond dissociative profiling by generating spatially resolved single-cell data from 64 human kidney tissue samples using two cutting-edge spatial transcriptomics platforms—CosMx (1,000 genes, 48 samples) and Xenium (5,100 genes, 16 samples). These datasets were integrated with single-nucleus RNA-seq profiles from 150 patients (~750,000 cells), creating a multi-modal kidney atlas of over 5 million spatially mapped cells.
- Ounadjela et al. present a spatial multiomic atlas of the human placenta during early gestation.²⁴ They combined paired snRNA-seq and snATAC-seq, spatially resolved snATAC-seq and RNA-seq, and in situ sequencing/hybridization to map the epigenomic and transcriptomic landscape of placental development. This work was performed on tissue from eight donors at early gestational time points. In contrast, our study establishes a spatially resolved single-cell atlas of human kidney disease using a much larger cohort—64 samples profiled by two high-resolution platforms, CosMx (1,000 genes, 48 samples) and Xenium (5,100 genes, 16 samples). We further integrated these data with single-nucleus RNA-seq from 150 patients (~750,000 cells), generating a multi-modal reference of over 5 million cells.

This work is not a small or incremental dataset contribution. It comprises over four million spatially resolved cells, integrates two leading single-cell spatial transcriptomics platforms (CosMx and Xenium), and harmonizes them with single-nucleus RNA sequencing from more than 150 individuals. To our knowledge, this is the first study to achieve cross-platform spatial integration at this scale and resolution in any human organ. The dataset enables the discovery of clinically actionable, spatially defined niches that transcend descriptive pathology and offer mechanistic, prognostically relevant insights into diabetic kidney disease. Importantly, we identify a B cell-enriched fibroinflammatory microenvironment predictive of rapid progression, nominate druggable targets, and nominate tissue and plasma biomarkers across multiple cohorts. This foundational dataset, with immediate implications for diagnostics and therapeutics, is generalizable across organs and diseases. It positions our work not merely as a study, but as a reference framework—on par with the Human Cell Atlas—for spatially resolved, multimodal analysis of human pathology.

Additionally, the computational methods are not clearly defined. The authors need to provide a more robust and transparent description of the computational pipelines used and their choice of parameters.

Response: We thank the reviewer for allowing us to better clarify the computational methods.

- In the revised version of the manuscript, we have expanded the Methods section to provide a more robust and transparent description of our computational pipelines.
- All analysis scripts are publicly available on our [GitHub page] and were executed in containerized environments to ensure full reproducibility. These containers are also available via Docker.
- This study integrates spatial transcriptomic data from 64 samples—CosMx 1k (n=48) and Xenium 5k (n=16)—with a reference of 150 annotated single-nucleus RNA-sequencing (snRNA-seq) profiles. CosMx and Xenium data were integrated separately at first. Expression matrices and metadata were exported from the AtoMx platform and converted into Python objects using Squidpy. All CosMx samples were merged, preprocessed, and analyzed using Scanpy. Cells with fewer than 30 total counts were removed. We performed an inner join with 17 preprocessed and annotated snRNA-seq samples, representing 150 individual donors, retaining 893 overlapping genes. We initially trained a scVI model (4-layer neural network with 30 latent dimensions) on raw counts using a learning rate of 0.001, maximum 600 epochs, and early stopping (patience = 15). After embedding the CosMx data in the scVI latent space, we applied scANVI using the snRNA-seq annotations in a semi-supervised manner to predict cell types. The scANVI model was trained for 50 epochs. After scANVI integration, we filtered out low-quality or ambiguous spatial cells based on their mean distance to snRNA-seq neighbors in the latent space, under the assumption that large distances indicate insufficient transcriptomic information or potential segmentation artifacts. This filtering step was only applied after scANVI, and both scVI and scANVI were retrained on the filtered dataset to improve accuracy and robustness.
- After integrating CosMx and Xenium datasets separately, we then combined both into a unified object and trained a new scVI model to project all spatial cells—CosMx and Xenium—into a shared latent embedding. This integration step allowed us to harmonize data across platforms while accounting for platform-specific variation. Following this, we applied scANVI again, leveraging the annotated snRNA-seq data to refine cell type predictions across the unified spatial dataset. To assign final cell labels across the integrated spatial dataset, we performed Leiden clustering in the scANVI latent space and manually annotated clusters based on the distribution of transferred labels and marker gene expression. Based on the final scanVI latent space, the distance from each spatial cell was calculated to the nearest snRNAseq cell. The expression of 3000 highly variable genes of the snRNA-seq dataset was then inferred by averaging distance² weighted values of the expression of most similar snRNAseq cells. The unified scanVI embedding was used in all downstream analyses, including niche modeling, differential expression, and spatial interaction profiling.
- For CosMx data, spatial plots were generated using custom Python scripts based on the squidpy.pl.spatial_segment function. We applied a dark background style and looped through each field of view (FOV) to create high-resolution (900 dpi) visualizations of segmented cells, colored by their immune_cell_update annotation. Segmentation outlines were shown without background images to emphasize cellular boundaries. Axis labels, frames, and legends were removed for clarity, and plots were exported as PNG files with tightly cropped bounding boxes. For Xenium data, we used the Xenium Explorer V3 software provided by 10x Genomics to generate spatial plots. This platform supports native visualization of gene expression and cell segmentation overlays on high-resolution tissue images.
- To identify spatial niches and microenvironments, we first excluded cells with zero neighbors across all types, removed the sparsest 5% of neighborhoods (based on total neighbor count at 80 μ m) to reduce edge effects. For each cell, we computed the number of neighboring cells within concentric distance shells: 0–20 μ m, 20–40 μ m, 40–60 μ m, and 60–80 μ m. This was achieved by calculating cumulative neighbor counts at 20, 40, 60, and 80 μ m, followed by subtracting counts from the smaller radius to

isolate the number of neighbors in each respective shell (e.g., the 40–60 μm shell = count at 60 μm – count at 40 μm). For each cell, neighbor counts were normalized by the total number of neighbors at that distance, yielding a cell-by-neighbor composition matrix that reflects the relative cellular makeup of the local environment at different spatial scales. This matrix was then scaled and subjected to principal component analysis (PCA), with the top 10 components used as input for MiniBatch K-means clustering. The number of clusters (k) was determined based on the elbow method. Niche clustering was performed on the entire dataset to define global spatial neighborhoods. However, downstream microenvironment classification was restricted to specific subsets of cells: IPT and iLOH cells for injured tubular microenvironments, and immune cells for immune microenvironments, using annotations derived from our kidney immune cell atlas.

- We employed the Covariance Environment (COVET) methodology on all CosMx cells, excluding those with zero neighbors across all types and removing the sparsest 5% of neighborhoods (based on total neighbor count at 80 μm) to reduce edge effects. Due to computational memory constraints, we restricted this analysis to the CosMx dataset, which provided sufficient spatial resolution and cell coverage. For the COVET embedding, we selected the top 64 highly variable genes to construct gene-gene covariance matrices for each cell's local neighborhood. This representation preserves higher-order gene relationships and enables the discovery of spatially organized transcriptional programs. In addition to using COVET for spatial clustering, we also analyzed gene-gene correlation matrices within individual spatial niches to identify disrupted or coordinated expression programs between healthy and disease states using Hotspot.
- In parallel, we applied NicheCompass to the CosMx dataset to further dissect the spatial architecture of the tissue microenvironment. NicheCompass is a graph neural network-based framework that models intercellular communication by integrating spatial proximity with gene expression. It constructs a neighborhood graph where each node represents a cell and edges encode spatial relationships, allowing the model to learn niche-specific latent representations that reflect both cell identity and context. We trained NicheCompass using the CosMx data to learn interpretable embeddings that integrate a cell's intrinsic state with its spatial context. This unsupervised embedding provided an orthogonal approach to COVET.
- To investigate the association between niche abundance and kidney function, we calculated Pearson correlation coefficients between niche fractions and estimated glomerular filtration rate (eGFR), a key clinical marker of renal performance. Differential gene expression (DEG) analysis between niches was performed using the Wilcoxon rank-sum test, enabling identification of niche-specific transcriptional programs. To interpret the biological relevance of the DEGs, we conducted pathway enrichment analysis using GSEAPy with the WikiPathways database using inferred gene expression, which allowed us to identify overrepresented signaling and metabolic pathways within each microenvironment. Additionally, using inferred gene expression we employed CellPhoneDB to perform ligand–receptor interaction analysis, leveraging its curated database of receptor–ligand pairs to infer potential intercellular communication patterns between neighboring cell types within defined spatial niches.
- To further characterize B cell subpopulations in the kidney, we constructed a high-resolution B and plasma cell atlas by integrating our Xenium 5k spatial transcriptomics data with a publicly available single-cell RNA-sequencing atlas of human B and plasma cells. We employed a two-step integration approach using scVI and scANVI. First, we trained a scVI model on the raw count data from both datasets using a four-layer neural network with 30 latent dimensions, a learning rate of 0.001, and early stopping (patience = 15), which enabled the construction of a shared latent space across technologies. We then applied scANVI, a semi-supervised deep generative model, to transfer cell type annotations from the reference atlas to the spatial Xenium dataset. The model was initially trained with early stopping (patience = 15), followed by label transfer based on proximity in the latent space. A proportion of high-confidence labels were used to retrain the model and refine classification. This integration enabled the spatial mapping of fine-grained B cell identities—including naïve, memory, atypical memory, germinal center B cells as well as plasma cells—within the intact tissue context, providing a detailed view of B cell localization and organization.

- For differential expression analysis in the bulk RNAseq dataset, we used the R package limma, which fits linear models to the expression data and applies empirical Bayes moderation to the standard errors, providing robust inference even with small sample sizes.
- Identification of plasma markers of B+ DKD: We trained an elastic-net logistic regression classifier using tidymodels. Because B-cell classes were imbalanced, we applied the synthetic minority oversampling technique (SMOTE) to the training data. Model complexity (α , λ) was tuned via 5-fold cross-validation to maximize the area under the receiver operating characteristic curve (AUROC). Predictors with non-zero coefficients in the refit model were then used as covariates in a Cox proportional hazards model of kidney survival, defined as a composite outcome of death, dialysis, or $\geq 40\%$ decline in eGFR. We compared the C-statistic of a model including clinical characteristics plus these proteins with a clinical-only model. To account for potential overfitting, we performed 1,000 bootstrap resamples and reported the mean C-statistic for each model.

Regarding the discovery of niches in their spatial data, is the number of samples sufficient to account for inter-individual and inter-disease variability? Was any power analysis conducted prior to the study?

Response:

- To our knowledge, no statistical framework currently exists to define sample size requirements for spatial niche discovery in single-cell spatial transcriptomics data. This remains a major methodological gap. To address concerns of inter-individual and inter-disease variability, we deliberately designed our study around scale, technical diversity, and analytic rigor. Specifically, we profiled a large cohort of 64 human kidney tissue samples using **two complementary high-resolution spatial transcriptomics platforms**—CosMx 1k (n=48; ~3.3 million cells) and Xenium 5k (n=16; ~1 million cells)—yielding over 4 million spatially resolved cells. These data were integrated with single-nucleus RNA-sequencing from 150 patients (~750,000 cells), creating a unified, multimodal atlas of more than 5 million cells. To our knowledge, this represents the largest and most comprehensive spatially resolved single-cell dataset to date in any human disease.
- Critically, in combination with our annotation based approach for detection of spatial niches, we also employed **two independent computational frameworks for niche discovery—COVET and NicheCompass**—and identified spatial niches in a reproducible manner across both platforms. The consistency of niche architecture across different technologies, patient cohorts, and analytical approaches underscores the robustness and biological relevance of our findings. Unlike prior studies that relied on a small number of samples or a single spatial modality, our approach leverages **scale, platform diversity, and methodological redundancy** to achieve reproducible and generalizable niche discovery across human tissues.

The biomarker discovery should also be compared to current standard markers for predicting kidney outcomes, such as eGFR and proteinuria. Furthermore, many of the findings presented lack novelty, as outlined below.

Response: We thank the reviewer for their thoughtful comment and the opportunity to clarify our approach.

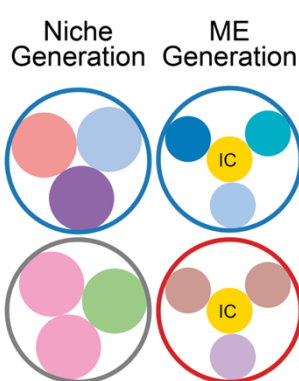
- We thank the reviewer for their thoughtful comment. While eGFR and proteinuria are well-established **predictive biomarkers** for kidney outcomes, they offer limited insight into disease mechanisms or actionable heterogeneity. Our objective in the revised version of the manuscript was not to replace these clinical markers, but to identify **discriminatory biomarkers** that define **mechanistically distinct subtypes** within diabetic kidney disease (DKD).
- A key finding of our study is the identification of a **B cell-rich microenvironment (ME)**—the first mechanistically defined DKD subtype characterized by spatially organized B cell infiltration and features of tertiary lymphoid structures. Although present in only ~8% of patients, this subtype is **clinically significant**, showing markedly faster progression to end-stage kidney disease and unique immunopathology.

- Development of a noninvasive classifier for B cell–predominant DKD:** Because of its low prevalence, a tissue-based classification alone might be insufficient for broader clinical use. Therefore, we identified plasma biomarkers that reflect the presence of this specific microenvironment. These proteins, derived from spatial transcriptomic signatures and validated in independent plasma proteomics datasets (including the UK Biobank), were strongly associated with kidney function decline. In the revised manuscript, we leveraged paired plasma proteomics to distinguish B⁺ from B[−] DKD patients in the TRIDENT cohort (n = 226 patients). Using elastic-net logistic regression with the Synthetic Minority Oversampling Technique (SMOTE) to address class imbalance, we trained a classifier optimized via 5-fold cross-validation. Proteins with non-zero coefficients in the final model were then incorporated into a Cox proportional hazards model assessing kidney survival (death, dialysis, or ≥40% eGFR decline). This protein-based model significantly improved risk prediction beyond clinical features alone, as evidenced by a higher mean C-statistic across 1,000 bootstrap resamples. Notably, when compared to TNFRSF1B—a known biomarker for DKD—our multi-protein classifier demonstrated superior prognostic performance. For external validation of the B+DKD protein set, we analyzed data from 3,309 UK Biobank participants with a diagnosis of diabetes mellitus and complete clinical information. In this independent cohort, the B+DKD protein set, when combined with known clinical predictors (Kidney Failure Risk Equation; KFRE), achieved an AUC of 0.7047, exceeding the performance of the clinical model (KFRE; AUC = 0.6286), thereby supporting its potential utility for risk stratification beyond established clinical predictors (Extended Fig. 38a). Moreover, quartile-based β -coefficients from the Cox model showed that individuals above the highest quartile (Q5) of the B+DKD protein set had a markedly increased risk of progression, exceeding that predicted by the KFRE model (Extended Fig. 38b). This threshold may therefore help to identify a high-risk subgroup, that could be amenable to B cell–directed interventions.

It is also unclear what the distinction is between a niche and a microenvironment. Can the authors provide a clearer definition of these terms?

We thank the reviewer for allowing us to clarify our definitions. We have now included schematics in Fig. 2a, 3a, and 4d to clarify our definitions of *niche* (2a) and *microenvironment* (3a, 4a).

- Conceptually, these two approaches differ in focus and analytical framing. A **niche** refers to a *spatially and molecularly defined tissue region* characterized by a specific ensemble of cell types, their arrangement, and interactions, such as the vessel or the glomerulus. It captures both *cellular composition* and *functional context*, providing a systems-level view of what makes a spatial unit of tissue biologically distinct. This is an integrative concept: it tells us who is there *and* what they are doing collectively (Schematic left side).



Schematic: Schematic of defining niches (left) and microenvironments (right). For niches, all cells were clustered based on their neighborhood fraction after restricting the analysis to those with sufficient local neighborhood density, removing the sparsest 5% of neighborhoods (based on total neighbor count at 80 μ m) to reduce edge effects (n = 4,029,331 cells). For each cell, we computed the number of neighboring cells within concentric distance shells: 0–20 μ m, 20–40 μ m, 40–60 μ m, and 60–80 μ m. This was achieved by calculating cumulative neighbor counts at 20, 40, 60, and 80 μ m, followed by subtracting counts from the smaller radius to isolate the number of neighbors in each respective shell (e.g., the 40–60 μ m shell = count at 60 μ m – count at 40 μ m). For each cell, neighbor counts were normalized by the total number of neighbors at that distance, yielding a cell-by-neighbor composition matrix that reflects the relative cellular makeup of the local environment at different spatial scales. This matrix was then scaled and subjected to principal component analysis (PCA), with the top 10 components used as input for MiniBatch K-means clustering. The number of clusters (k) was determined based on the elbow method. Niche clustering was performed on the entire

- In contrast, a **microenvironment** is defined in a *cell-centric* manner (Schematic right side). It is anchored around a particular *cell type of interest*—in our case, typically immune cells—and describes the immediate spatial context that may influence or be influenced by that central cell type. This approach focuses on the local neighborhood, emphasizing potential signaling and interaction partners from the perspective of that cell type.
- By analyzing both niches and microenvironments, we gain complementary insights: *niches* identify structured, emergent tissue states, while *microenvironments* highlight dynamic, cell-specific

interactions. We believe this dual perspective strengthens the interpretability of spatially resolved data and better captures the complexity of kidney disease pathophysiology.

Regarding the calculation of differentially expressed (DE) genes: Why was the Wilcoxon test chosen for DE analysis? Doesn't this test lead to a higher rate of false positives compared to MAST or pseudo-bulk approaches? Response: We thank the reviewer for this valuable comment. We selected the Wilcoxon rank-sum test for differential expression analysis because it is a non-parametric method that does not assume a specific data distribution. However, to address the reviewers concerns we now calculate our spatial gene signatures by generating pseudobulk counts on a sample level and performing differential expression analysis with DeSeq2.

The identification of niches (Fig. 2) in this paper doesn't offer any novel insights, as B cell accumulation is common in most chronic diseases.

Response: We thank the reviewer for raising this important point. It is true that B cell accumulation is a

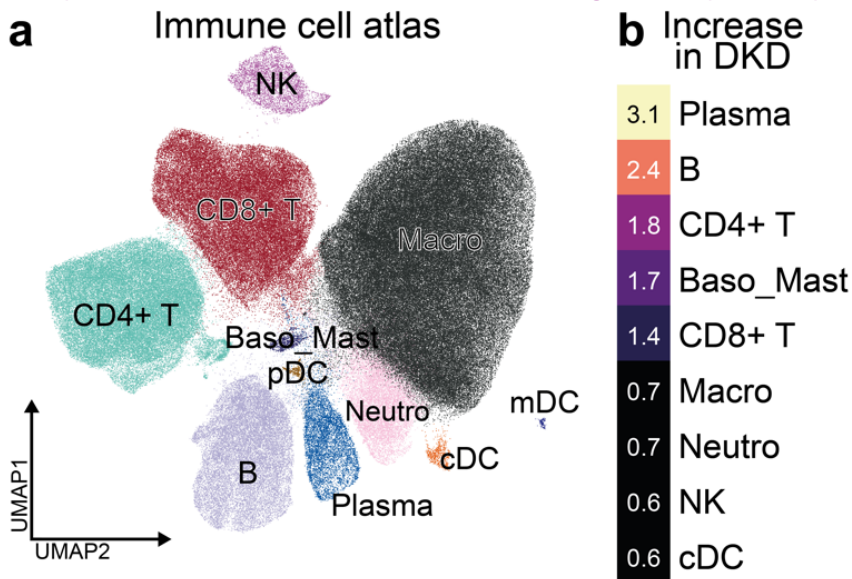


Fig 4a, Human kidney immune cell atlas. UMAP of the integrated datasets colored by cell types. Each dot represents one cell. The color represents the different cell populations: Baso/Mast, basophils and mastcells; Macro, macrophages; Neutro, neutrophils; cDC, classical dendritic cells; mDC, myeloid dendritic cells; pDC, plasmacytoid dendritic cells; B, B cells; Plasma, plasma cells; CD4+ T, CD4+ T cells; CD8+ T, CD8+ T cells; NK, natural killer cells. B, Fold change between immune cell fractions of control vs. DKD patients.

recognized feature in many chronic inflammatory diseases, including lupus nephritis, membranous nephropathy, rheumatoid arthritis, and certain cancers, where tertiary lymphoid structures (TLSs) have been well described. However, we respectfully disagree with the implication that our findings merely reiterate known biology or lack novelty. The presence of B cells alone is not what makes our findings important; rather, it is the **spatial, mechanistic, and clinical resolution** with which we define immune niches—specifically a B cell-rich microenvironment in diabetic kidney disease (DKD)—that distinguishes this work.

To date, **no prior study has systematically characterized the immune landscape of human DKD at single-cell resolution in tissue** or defined the organization of immune cells into discrete and reproducible spatial niches. A PubMed search for “((DKD) OR (diabetic nephropathy)) AND (B cells)” yields only 63 publications, the majority of which are based on peripheral blood analyses or bulk transcriptomic profiling, lacking spatial or cellular specificity. In contrast, we present the **first unbiased, spatially resolved atlas of immune cells in the human kidney and a specific B-cell atlas**, generated from 64 spatial transcriptomics datasets acquired using two orthogonal high-resolution platforms—CosMx and Xenium—along with single-nucleus RNA-seq data from 150 individuals (Fig 4). This dataset enabled the identification and high-resolution mapping of seven immune microenvironments (MEs), including a B cell-predominant ME not previously described in DKD.

Importantly, the **identification of spatial niches itself is a central conceptual advance**. Niches are not defined solely by the presence of particular cell types, but by the **specific spatial arrangement and interaction** of those cells with their microenvironment, including neighboring immune and epithelial cells, cytokine gradients, and structural context. This organization is critical to function. Our approach goes beyond listing immune populations by identifying reproducible spatial configurations that are biologically meaningful and linked to disease mechanisms. In this respect, our identification of niches such as the injured proximal tubule (IPT) niche, the distal convoluted tubule (DCT) niche, and multiple immune niches—including the B cell-rich ME—is not only novel but essential to understanding DKD as a tissue-level disease.

The **B cell–predominant immune microenvironment**, present in approximately 8% of DKD patients, displays classical TLS-like features, including CXCL13–CXCR5 chemokine-receptor expression, colocalization of BAFF and BAFF-R (TNFSF13 and TNFRSF13C), and evidence of local class-switch recombination via AICDA and plasma cell differentiation via IRF4 expression. Through integration with a reference single-cell RNA-seq atlas of human B and plasma cells and validation using imaging mass cytometry, we confirmed the presence of memory, naïve, and atypical memory B cell subsets, as well as proliferating CD20⁺ B cells within the tissue (Fig. 5). The finding that these immune aggregates are transcriptionally distinct, clonally active, and spatially organized confirms that this is not diffuse inflammation or a generic B cell presence, but a **previously unrecognized organized immune niche in DKD**.

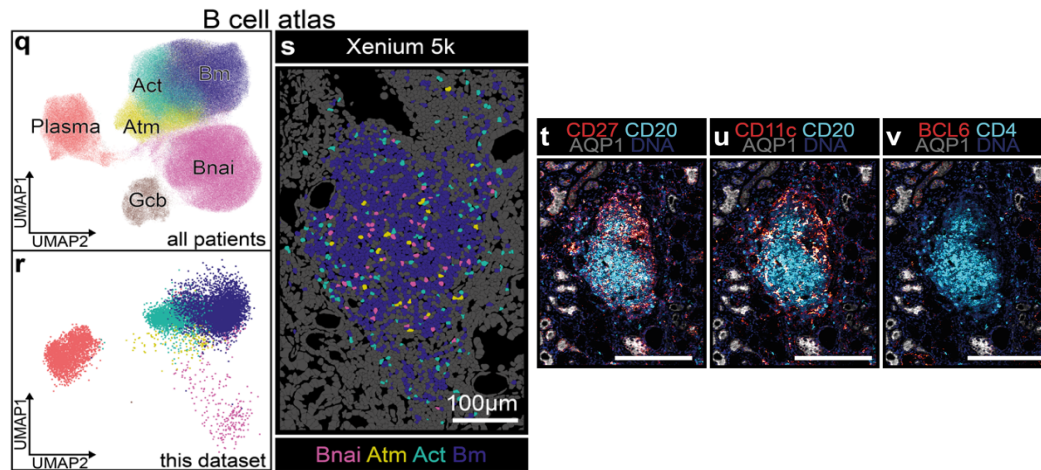


Fig 5: High-resolution spatial B and plasma cell atlas constructed by integrating our Xenium 5k spatial transcriptomics data with a publicly available single-cell RNA-sequencing reference of human B and plasma cells. Integration was performed using a two-step approach with scVI and scANVI. A four-layer scVI model (30 latent dimensions, learning rate = 0.001, early stopping with patience = 15) was trained on raw counts to create a shared latent space. scANVI was then used in a semi-supervised manner to transfer cell type annotations from the reference to the Xenium dataset. Label transfer was based on latent space proximity, followed by retraining on high-confidence annotations to refine classification. q: UMAP of the integrated datasets colored by cell types. Each dot represents one cell. The color represents the different cell populations: Plasma cells (Plasma), atypical memory B cells (Atm), activated B cells (Act), memory B cells (Bm), naïve B cells (Bnai) and germinal center B cells (GcB). r: UMAP of only Xenium cells of this study in the integrated dataset, colored by cell types. s: Spatial plots generated using Xenium Explorer 3 of the B cell subsets in the B predominant immune microenvironment. t-v: Imaging mass cytometry using multiplex protein panel of CD20, AQP1, DNA staining, CD27, CD11c, BCL6.

Beyond tissue analysis, we sought to understand the clinical significance of this B cell-rich microenvironment. In a bulk RNA-seq dataset from the Susztaklab Biobank ($n=843$), we observed a strong correlation between the B cell ME gene signature and interstitial fibrosis, a known marker of irreversible kidney damage (Fig. 5). Applying these gene signatures to an independent cohort from the TRIDENT study, we stratified patients into B^+ and B^- DKD subgroups. Despite being a minority ($\sim 8.4\%$), the B^+ DKD group had significantly faster progression to end-stage kidney disease (Fig. 5).

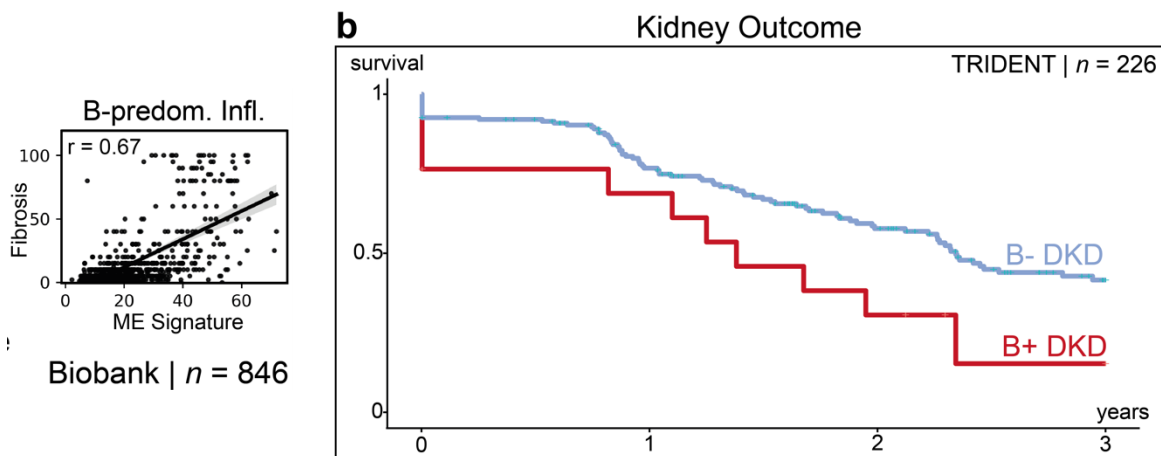


Fig. 5 left side: Correlation between a spatial gene signature derived from the B cell-predominant immune microenvironment (ME) and interstitial fibrosis in a cohort of 846 patients. Spatial signatures were identified by pseudo-bulking spatial transcriptomics data followed by differential expression analysis using DESeq2. The resulting 10-gene signature was correlated against fibrosis scores obtained from histopathological assessment of paired kidney tissue sections by an expert nephropathologist using matched bulk RNA-sequencing data using Pearson correlation ($p<0.0001$). b: Patients were stratified based on the spatial gene signature of the B cell-predominant immune microenvironment. A 10-gene signature was derived from spatial transcriptomics data and applied to bulk RNA-sequencing profiles of kidney tissue from a large patient cohort (TRIDENT). Hierarchical clustering using Ward's method with Euclidean distance separated patients into two groups: those with low expression (B^-) and those with high expression (B^+) of the B ME gene signature. Kaplan-Meier survival analysis was then performed to evaluate time to a renal composite endpoint—defined as progression to dialysis, kidney transplantation, or a $\geq 40\%$ decline in baseline eGFR. The x-axis indicates time (in years), and the y-axis indicates the survival probability ($p<0.05$).

To translate these findings into a clinically actionable framework, we derived and validated **plasma biomarkers reflective of the B cell ME**, including in the UK Biobank plasma proteomics cohort. These biomarkers could

noninvasively identify patients with this immune subtype, providing a path toward prospective patient stratification for precision therapy.

This level of mechanistic and clinical anchoring has not been previously achieved in the study of B cells in DKD or in the field of DKD more broadly. Prior literature has not described organized B cell niches in DKD, has not connected such structures to spatial transcriptional programs or immune crosstalk with tubule cells, and has not shown that they correlate with histologic fibrosis, clinical trajectory, or blood biomarkers. Our study closes this gap and introduces a framework for understanding **DKD as a heterogeneous disease with immunologically defined subtypes**—a concept increasingly embraced in other fields such as oncology and rheumatology but not yet established in DKD.

Moreover, our **findings point directly to therapeutic implications**. The presence of this B cell-driven immune niche raises the possibility that patients with the B⁺ DKD subtype may benefit from **therapies already used in other B cell-mediated diseases**. These include anti-CD20 monoclonal antibodies (e.g., rituximab), BAFF/APRIL pathway inhibitors (e.g., belimumab), and complement inhibitors (e.g., iptacopan). Thus, the translational potential is immediate and actionable.

In conclusion, while the general involvement of B cells in chronic inflammation is well known, our study provides the **first comprehensive, spatial, mechanistic, and clinically validated portrait of B cell-rich immune niches in DKD**. We not only identify a novel immune microenvironment but demonstrate its reproducibility across technologies, its association with disease severity, its detectability in blood, and its potential as a therapeutic target. These findings represent a substantial conceptual advance and open the door to precision immunotherapy in a field where treatment remains largely nonspecific. We believe this contribution meaningfully advances both the understanding and clinical management of diabetic kidney disease.

What is the overlap in gene expression between the niche signatures? How are these spatial niche signatures determined? Is it based on some variation of marker gene analysis at the niche level? If so, how do the authors account for the different cell types present in each niche when comparing them to other niches?

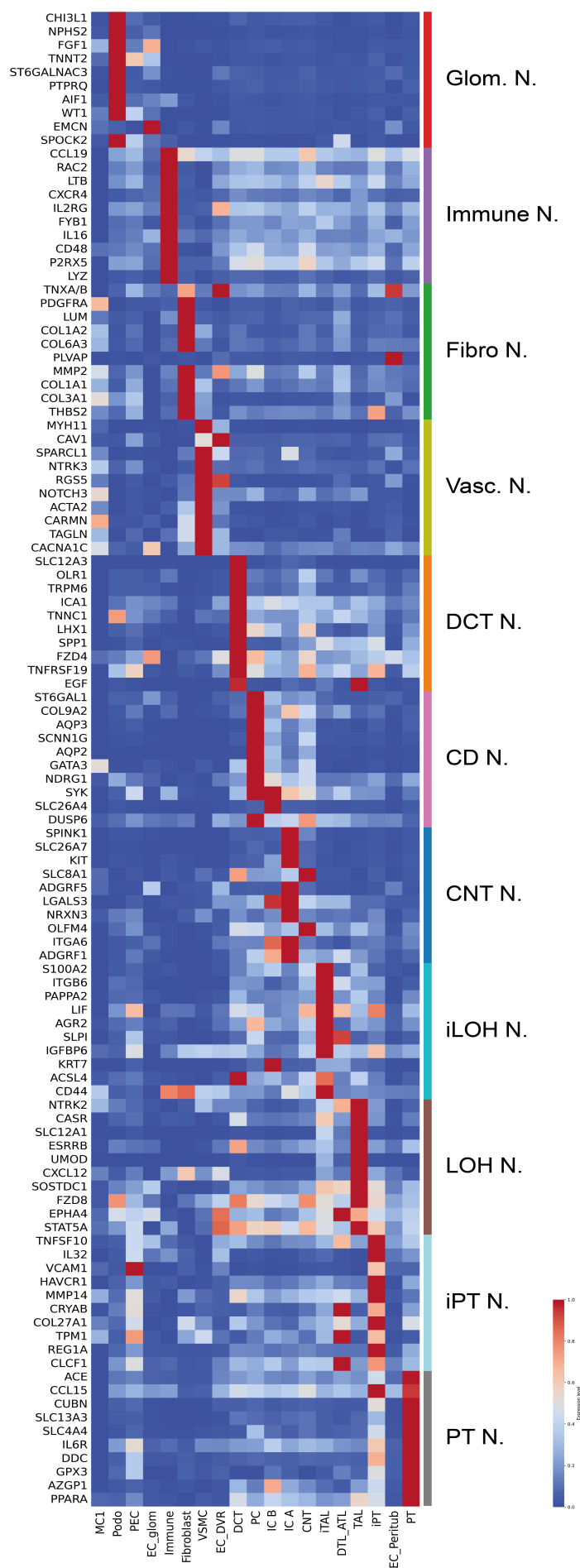
Response: We thank the reviewer for raising these thoughtful questions. The spatial niche signatures in our study were designed to capture emergent transcriptional programs that arise from the **specific cellular composition and spatial organization** of each niche. Our aim was not to define purely cell type-specific or mutually exclusive signatures, but rather to characterize **multicellular environments** as they exist in the tissue, including the transcriptional consequences of their spatial arrangement and cell-cell interactions.

To construct these signatures, we applied a **pseudobulk strategy**, aggregating gene expression across all cells within each niche for each sample in the CosMx dataset. We then performed **differential expression analysis using DESeq2**, comparing each niche to all others. This approach highlights genes that are consistently enriched within a particular spatial context, reflecting both the cell types present and the interactions among them.

Although our method allowed for the possibility of overlapping genes between niche signatures, we found that in practice, the top 10 enriched genes identified for each niche were distinct. Thus, the reported signatures do not share genes, not because of a filtering step to enforce exclusivity, but as a result of the biological and statistical differences captured in the analysis.

To aid visual comparison, we have added a heatmap (Extended Fig. 32) showing representative marker gene expression across all niches. We believe this complements the differential expression analysis and helps clarify both the overlap and the specificity of spatial transcriptional programs.

In summary, the niche signatures were determined to reflect **real multicellular spatial organization**. Our goal was to characterize the transcriptional landscape of **spatially distinct niches**, capturing the complexity of tissue organization in health and disease. For this purpose, we defined a spatial gene signature of 10 distinct transcripts.



Extended Fig. 32: Expression of spatial gene signatures across all cell populations in the CosMx dataset. Gene expression was log-transformed. For each annotated cell type, average gene expression was calculated and normalized using min-max scaling (range 0 to 1) across cell types for each gene. The resulting matrix was visualized as a heatmap (scaled per gene) using the seaborn library, with cell types ordered according to anatomical and functional similarity.

Figure 2a: The niche identification method needs to be described in more detail. For instance, are different KNNs (k-nearest neighbors) constructed for different radii, or do the authors use a single optimal radius? Additionally, are overlapping elements in each neighborhood taken into account? Would it be possible to differentiate between long- and short-range interactions based on the chosen radii?

Response: We thank the reviewer for raising these important technical points. We have now expanded the Methods section to provide additional detail on the niche identification strategy used in Figure 2a.

There are multiple approaches for defining spatial niches in single-cell spatial transcriptomics. In this study—the first of its kind on human clinical kidney samples—we applied and compared **three independent methods**:

1. **K-nearest neighbors (KNN)** based on *cell type annotations* within a fixed spatial radius. To mitigate edge effects and ensure robust niche construction, we first removed the bottom 5% of cells with the fewest neighbors radius, thereby excluding cells with insufficient local context. For each cell, we computed the number of neighboring cells within concentric distance shells: 0–20 μm , 20–40 μm , 40–60 μm , and 60–80 μm . This was achieved by calculating cumulative neighbor counts at 20, 40, 60, and 80 μm , followed by subtracting counts from the smaller radius to isolate the number of neighbors in each respective shell (e.g., the 40–60 μm shell = count at 60 μm – count at 40 μm). For each cell, neighbor counts were normalized by the total number of neighbors at that distance, yielding a cell-by-neighbor composition matrix that reflects the relative cellular makeup of the local environment at different spatial scales. This matrix was then scaled and subjected to principal component analysis (PCA), with the top 10 components used as input for MiniBatch K-means clustering. The number of clusters (k) was determined based on the elbow method.
2. **COVET**, a method that captures *co-variation in gene expression* across spatial neighborhoods (Extended Fig. 13). We employed the Covariance Environment (COVET) methodology on all CosMx cells, excluding the bottom 5% with the fewest neighbors, to ensure sufficient local context for robust computation. Due to computational memory constraints, we restricted this analysis to the CosMx dataset, which provided sufficient spatial resolution and cell coverage. For the COVET embedding, we selected the top 64 highly variable genes to construct gene-gene covariance matrices for each cell's local neighborhood (Extended Fig. 13). This representation preserves higher-order gene relationships and enables the discovery of spatially organized transcriptional programs. In addition to using COVET for spatial clustering, we also analyzed gene-gene correlation matrices within individual spatial niches to identify disrupted or coordinated expression programs between healthy and disease states (Extended Fig. 15-25).
3. In parallel, we applied **NicheCompass** to the CosMx dataset to further dissect the spatial architecture of the tissue microenvironment (Extended Fig. 14). NicheCompass is a graph neural network-based framework that models intercellular communication by integrating spatial proximity with gene expression. It constructs a neighborhood graph where each node represents a cell and edges encode spatial relationships, allowing the model to learn niche-specific latent representations that reflect both cell identity and context.

Importantly, despite the methodological differences between KNN, COVET, and NicheCompass, **all three approaches converged on the same biological interpretations**, underscoring the robustness of our results and validating the biological relevance of the identified niches. This consistency suggests that the spatial patterns we observe are not artifacts of a specific algorithm or radius choice but reflect meaningful and reproducible tissue organization.

While our current analysis aggregates across spatial scales to define stable niche classes, we agree that dissecting long- versus short-range spatial interactions is a promising future direction, particularly as higher-resolution spatial technologies and modeling tools continue to evolve.

Figure 2i: “Change in relative niche fraction in DKD vs healthy controls” → How is the fraction calculated? The fibrosis fraction is listed as 245. Does this represent a 245-fold change in DKD compared to controls?

Response: We thank the reviewer for pointing this out. The values shown in Figure 2i represent the relative fold change in niche frequency between diabetic kidney disease (DKD) patients and healthy controls (Fig. 2). To calculate these values, we first computed the fraction of each niche per condition by dividing the number of cells assigned to a given niche by the total number of cells in each diagnostic group. We then compared the DKD and control groups by taking the ratio of these condition-level frequencies. If a niche was more prevalent in DKD, we report a positive fold change (e.g., 245 means the fibrosis niche was 245 times more common in DKD than in controls). Conversely, if a niche was more prevalent in controls, we report a negative value corresponding to the inverse fold change. We have now added this clarification to the Methods and revised the figure legend for clarity.

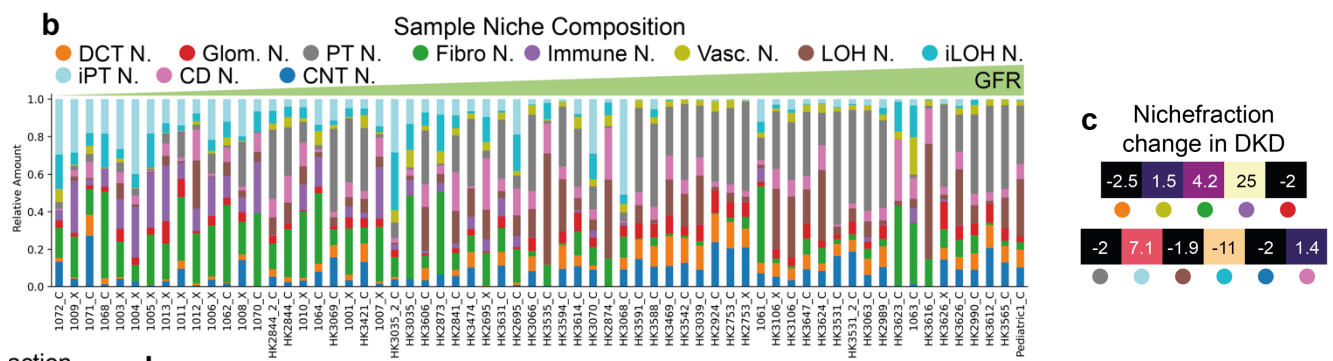


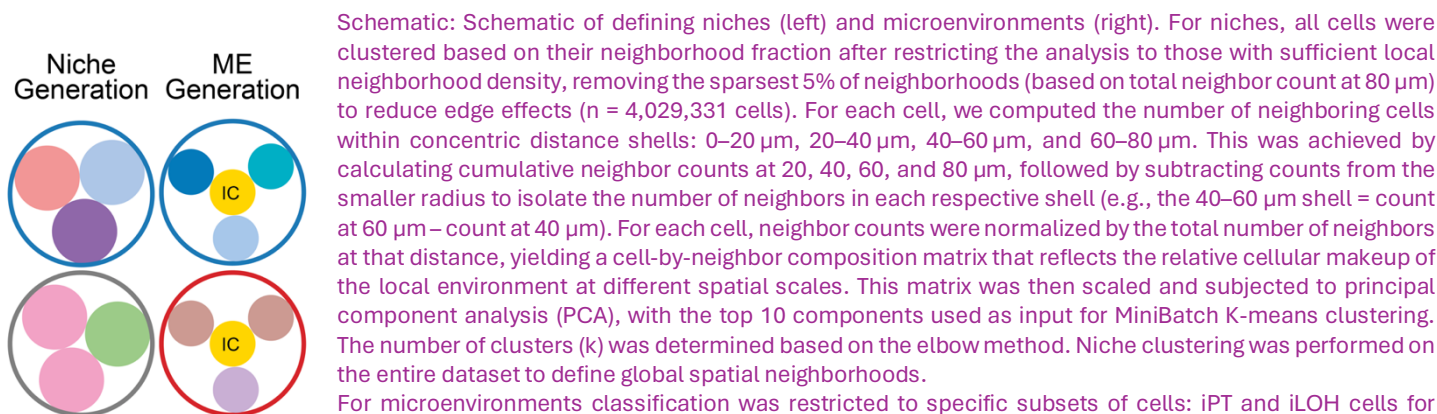
Fig. 2b: Sample niche composition: the fraction of each niche is calculated for all cells within each sample and visualized. The samples are arranged by disease severity (GFR) from left to right. c: Change in relative niche fraction in DKD vs healthy controls. Each value represents the fold change in the average proportion of a given niche between DKD patients and healthy controls. A positive value indicates that the niche is expanded in DKD, while a negative value indicates it is more prevalent in controls. Fold changes were computed by normalizing niche frequencies within each diagnostic group, then taking the ratio of DKD to control values.

Figure 3, Line 333: “This clustering uses K-means clustering of the 20, 40, 60, and 80 μ m neighborhood fraction”
 → This is not clearly defined. Is the microenvironment (ME) and the niche different depending on the identity of the center cell? This also relates to line 247 in Figure 2.

Response: We thank the reviewer for this insightful question. To clarify the distinction between niches and microenvironments (MEs), we have added schematic illustrations to Fig. 2a, 3a, and 4d and expanded our explanations in the Methods section.

Niches are defined as **tissue-level spatial structures composed** of characteristic ensembles of interacting cell types (Schematic, left side). These are not centered on a single cell type but instead reflect recurrent, spatially organized tissue states—such as the glomerulus or vasculature—characterized by distinctive cell compositions, physical arrangements, and shared gene expression programs. The goal is to identify spatial tissue units with emergent biological identity.

Microenvironments (MEs), by contrast, are cell-centric (Schematic, right side). Each ME is constructed by analyzing the local neighborhood composition surrounding a specific cell type, such as an injured tubule or immune cell. We compute cell type fractions within concentric radii (20–80 μ m), subtract overlapping shells to



avoid double-counting, and normalize the resulting fractions. These spatial profiles are then used as input for clustering via MiniBatch K-Means, yielding recurrent contextual patterns around that focal cell type. Thus, MEs capture how a cell is influenced by its immediate surroundings—and how this context varies in health and disease.

Importantly, the definition of an ME depends on the identity of the central cell, whereas niche clustering is agnostic to focal cell identity. We found this dual approach—tissue-centric (niche) and cell-centric (ME)—to be complementary, as it enables both the discovery of spatially structured disease-associated tissue compartments and the dissection of cell-specific interactions that may drive pathology.

We hope this clarification helps distinguish the two frameworks and reinforces their value in interpreting spatially resolved kidney data. We thank the reviewer for this valuable feedback and have now included schematics in Fig. 2a, 3a, and 4d to clarify our definitions of *niche* (2a) and *microenvironment* (3a, 4a).

The authors provide some statistical evidence of the glomerular niche, such as podocyte loss and mesangial cell expansion, but these insights are not novel. Did the authors identify any new interactions or features across the 10 niches they studied?

Response: We thank the reviewer for this thoughtful comment. In our revised manuscript, we provide a detailed characterization of **11 spatially defined kidney niches (Fig. 2a-d)**, enabling the identification of **a vast number of disease-associated alterations in cellular architecture and intercellular signaling**. While some features—such as podocyte loss or mesangial cell expansion in the glomerular niche—are indeed consistent with established DKD pathology, our analysis goes significantly beyond these classical observations. The reproducibility of known findings increases confidence in our approach; however, the real novelty lies in the **scale, resolution, and depth** of interaction-level insights that could not be achieved in previous studies.

Rather than focusing on isolated cell types, our niche framework captures **emergent, spatially organized multicellular structures**—each defined by unique combinations of cell types, transcriptional programs, and physical arrangements. This spatial perspective enables us to map not only which cells are present, but **how they interact, where they interact, and how those interactions change across disease stages**.

Using this approach, we identified **hundreds of previously unreported ligand–receptor interactions** across the 11 niches. While a complete enumeration of these findings is provided in Supplementary Tables 10–11, we emphasize that the full description of these interactions is extensive and overwhelming. Therefore, in the main text, we focused on selected examples that (1) validate known biology, enhancing the interpretability of our data, and (2) uncover **clinically relevant and previously unrecognized signaling circuits** that change with disease progression.

Notably, in the vascular niche, we found enrichment for pathways related to ECM remodeling, senescence, inflammatory signaling, and neovascularization (Fig. 2o) in DKD. As an orthogonal approach, we applied Hotspot to detect spatially correlated genes using niche-specific latent representations derived from COVET. This analysis revealed conserved gene–gene correlations across control and DKD samples in all niches (Extended Data Fig. 15–25). Differential correlation analysis highlighted increased associations between transcript pairs such as COL1A1–SERPINE1, CXCL12–MHC I, and JUNB–CD74—implicated in matrix remodeling, immune signaling, and antigen presentation—in DKD (Extended Fig. 25), consistent with the pathway enrichments observed in Fig. 2o.

Moreover, in the **glomerular niche**, we observed not only shifts in cell type proportions—such as increased mesangial and immune cell presence—but also **new spatially resolved signaling pathways**. Specifically, **IL6–IL6R signaling between glomerular endothelial cells and podocytes** was significantly upregulated in DKD samples, suggesting an inflammatory axis contributing to filtration barrier dysfunction. In addition, **NRG1–ERBB3 interactions** correlated positively with eGFR, while **CXCL9–DPP4** and **PLAU–PLAUR** interactions were negatively associated with kidney function, highlighting potential contributors to injury progression (Fig. 2p-r).

Importantly, the **large sample size in our study (64 spatially profiled samples, >4 million spatially resolved cells)** allowed us, for the first time, to analyze **disease stage–dependent shifts** in both **niche composition** and **intercellular communication**. This temporal dimension is a conceptual advance: interactions that **emerge or intensify with disease progression** are more likely to represent **drivers of pathology**, rather than passive markers of tissue damage. These dynamic changes offer a valuable framework for **target prioritization** and **therapeutic design**.

Furthermore, the spatially anchored nature of our analysis preserves the **physical proximity of signaling partners**, allowing us to determine not just whether a ligand and receptor are expressed in the same tissue, but whether they are **positioned to interact** within a defined anatomical structure. This level of spatial precision is not attainable with bulk transcriptomics or traditional single-cell RNA-seq and represents a core innovation of our work.

In summary, while we acknowledge that some findings confirm known features of DKD, our study introduces a **comprehensive and spatially resolved interaction atlas**, identifies **numerous novel and stage-dependent signaling pathways**, and offers an analytic framework for understanding **how disease unfolds at the tissue architecture level**. We believe this represents a major step forward in both kidney biology and translational nephrology.

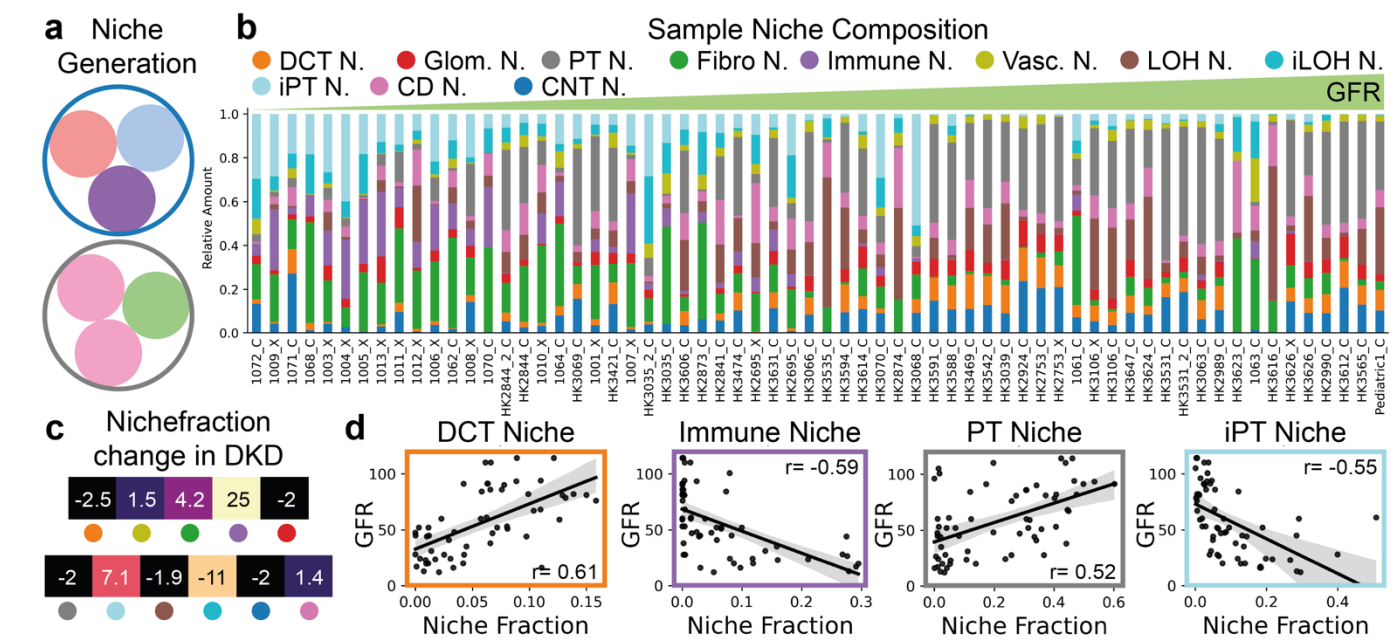


Fig 2a: Schematic of defining niches (left) and microenvironments (right). For niches, all cells were clustered into groups based on their neighborhood fraction after restricting the analysis to those with sufficient local context, removing the sparsest 5% of neighborhoods (based on total neighbor count at 80 μ m) to reduce edge effects ($n = 4,029,331$ cells). For each cell, we computed the number of neighboring cells within concentric distance shells: 0–20 μ m, 20–40 μ m, 40–60 μ m, and 60–80 μ m. This was achieved by calculating cumulative neighbor counts at 20, 40, 60, and 80 μ m, followed by subtracting counts from the smaller radius to isolate the number of neighbors in each respective shell (e.g., the 40–60 μ m shell = count at 60 μ m – count at 40 μ m). For each cell, neighbor counts were normalized by the total number of neighbors at that distance, yielding a cell-by-neighbor composition matrix that reflects the relative cellular makeup of the local environment at different spatial scales. This matrix was then scaled and subjected to principal component analysis (PCA), with the top 10 components used as input for MiniBatch K-means clustering. The number of clusters (k) was determined based on the elbow method. Niche clustering was performed on the entire dataset to define global spatial neighborhoods. b: Sample niche composition: the fraction of each niche is calculated for all cells within each sample and visualized. The samples are arranged by disease severity (GFR) from left to right. c: Change in relative niche fraction in DKD vs healthy controls. Each value represents the fold change in the average proportion of a given niche between DKD patients and healthy controls. A positive value indicates that the niche is expanded in DKD, while a negative value indicates it is more prevalent in controls. Fold changes were computed by normalizing niche frequencies within each diagnostic group, then taking the ratio of DKD to control values. d, correlation of niche fraction with eGFR using Pearson correlation. All correlations with $p < 0.001$.

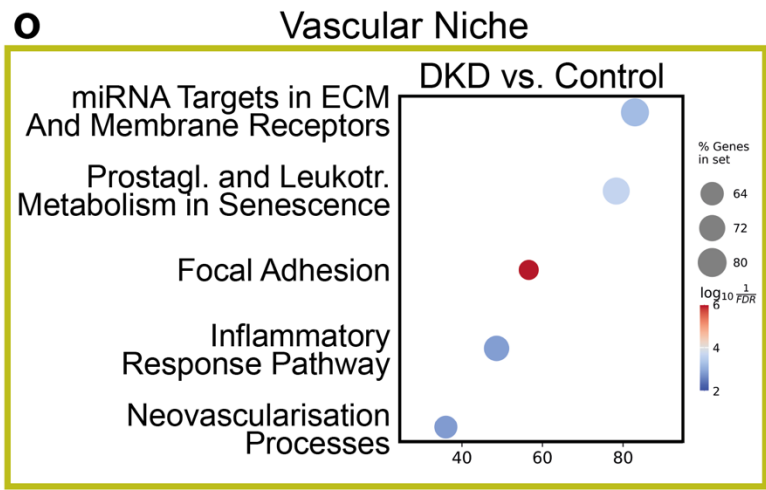


Fig. 2o: Differential gene expression analysis was performed between control and DKD samples specifically within the vascular niche, using the Wilcoxon rank-sum test on imputed gene expression data. Gene set enrichment analysis was subsequently conducted using GSEapy with the Wikipathways 2021 database. Displayed pathways reflect significantly enriched gene sets with an adjusted p -value < 0.01 .

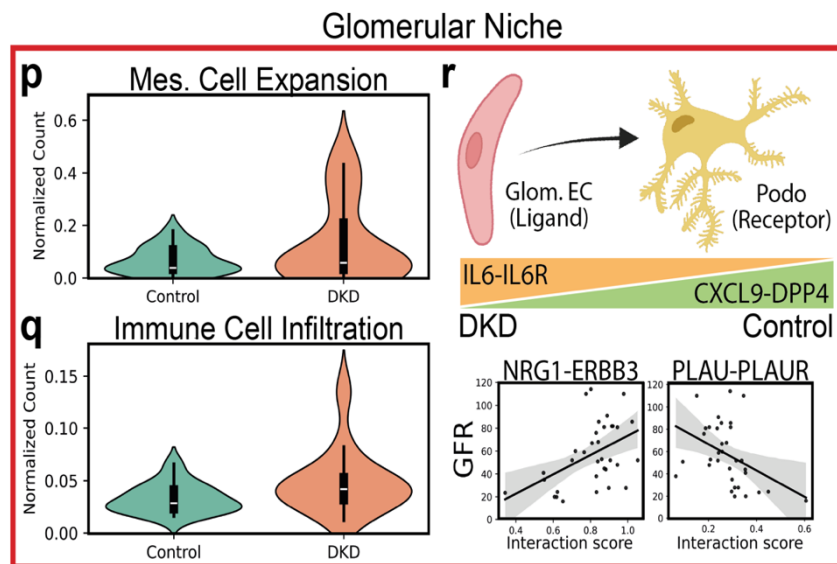


Fig. 2p: analysis of the glomerular niche: The fraction of mesangial cells in the glomerular was calculated and compared between control and DKD patients ($p < 0.05$). q: The fraction of immune cells in the glomerular was calculated and compared between control and DKD patients ($p < 0.05$). r: Ligand–receptor interactions originating from glomerular endothelial cells and targeting podocytes were inferred using CellPhoneDB v5, with microenvironments defined at the sample level. Analyses were performed using imputed gene expression to maximize sensitivity. Only statistically significant ligand–receptor pairs were retained for downstream analysis. Interaction scores were then correlated with estimated glomerular filtration rate (eGFR) across samples.

It appears that the calculation of the spatial niche signature does not take any of the spatial features such as the spatial organization of the niche into account. Is this correct?

Response: We thank the reviewer for the opportunity to clarify this important point. **Spatial organization is indeed central to our definition and calculation of niche signatures.** In our framework, niches are not defined by isolated cell types, but rather as recurrent, spatially organized tissue units—such as the glomerulus or vasculature—that emerge from characteristic ensembles of interacting cell types. **These structures are defined by both their cellular composition and their local spatial arrangement**, allowing us to move beyond traditional cell-type analyses toward a more biologically meaningful understanding of disease-associated tissue architecture.

To calculate spatial niche signatures, we used a pseudobulk approach: for each sample, we aggregated gene expression across all cells belonging to a given niche. Differential expression analysis between niches was then performed using DESeq2. This strategy captures the composite transcriptional identity of each spatially resolved tissue unit, shaped by its constituent cell types and their coordinated molecular programs.

While the final niche signature is based on gene expression, **it is rooted in spatially defined structures** that inherently reflect local tissue organization. Thus, spatial context is not only considered—it is foundational to both niche definition and signature derivation. We hope this expanded explanation clarifies our approach and underscores the critical role of spatial features in calculation of spatial niche signatures.

The biomarker expression of iLOH ME1 and the biomarker in Figure 5g do not seem to overlap. How strong is the overlap? This is unclear from the presented data. The authors need to clearly demonstrate whether the signatures overlap or specify which cell types express them.

Response: We thank the reviewer for the opportunity to clarify our biomarker discovery strategy. In the revised manuscript, we developed spatial gene expression signatures for **the B cell-predominant immune microenvironment**. These signatures were derived independently and directly from our spatial transcriptomic dataset. To construct spatial niche signatures, we employed a pseudobulk approach by aggregating gene expression across all cells assigned to a given niche, on a per-sample basis. We then performed differential expression analysis between niche types using the DESeq2 package to identify genes specifically enriched in

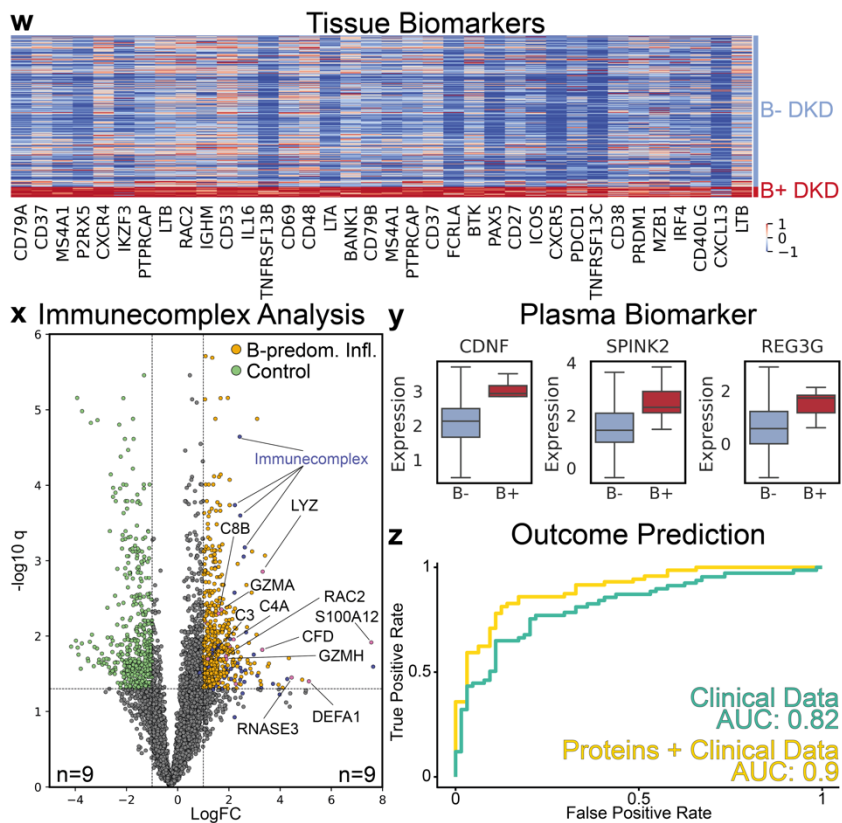


Fig. 5w: To identify candidate protein biomarkers associated with disease-relevant microenvironments, we first derived 10-gene spatial signatures for the B cell-predominant immune microenvironment using high-resolution spatial transcriptomics. These signatures were applied to bulk RNA-sequencing data from the TRIDENT Cohort to stratify patients (n=223) based on spatial microenvironment enrichment. Differential expression analysis was then performed using bulk RNAseq and the Limma R package. Depicted here are a subset of the significant DEGs.

x: Using specific antibodies against IgA, pulldown from 8 controls and 8 patients with B cell predominant inflammation from the Susztaklab Biobank was performed and analyzed using TMT-labeled mass spectrometry. Volcano plot of proteins bound to immune complexes in B-predom. Inflammation vs. Control.

y: Paired plasma proteomic from patients with B+ and B- DKD in the TRIDENT cohort was used to train an elastic-net logistic regression with the Synthetic Minority Oversampling Technique (SMOTE). To address class imbalance, we trained a classifier optimized via 5-fold cross-validation. Depicted are 3 proteins of that model.

z: Proteins with non-zero coefficients in the final model were then incorporated into a Cox proportional hazards model assessing kidney survival (death, dialysis, or $\geq 40\%$ eGFR decline). This protein-based model significantly improved risk prediction beyond clinical features alone, as evidenced by a higher mean C-statistic across 1,000 bootstrap resamples.

each spatial microenvironment. For downstream biomarker discovery, **we applied these spatial signatures to a bulk RNA sequencing dataset of the TRIDENT cohort of patients with diabetic kidney disease (n = 226).** We stratified patients based on expression of the B cell-predominant immune ME signature. To identify candidate biomarkers specific to this B cell-enriched disease state, we conducted differential expression analysis. This analysis, performed using the Limma package, revealed a set of candidate tissue biomarkers specifically elevated in the B cell-associated subset, representing potential biomarkers of immune-driven disease activity. Here, we highlight some of these tissue biomarkers (Fig. 5w). These biomarkers include: CD19, CD79A, PAX5, FCRLA, BTK: Classical markers of B cells, indicating increased B cell infiltration or expansion. CXCL13, LTA, LTB: Chemokines central to the formation of tertiary lymphoid structures (TLSs), guiding B and T cell recruitment. IL16: A cytokine involved in immune cell chemoattraction and activation. MZB1, CD38, PRDM1: Marker associated with antibody-secreting plasma cells, supporting local immunoglobulin production. IGHA1: The alpha chain of IgA, suggesting the presence of local IgA-producing plasma cells and potential immune complex formation. TNFRSF13B (TACI): A BAFF/APRIL receptor crucial for B cell survival and class-switch recombination, highlighting sustained B cell activity within the tissue. Together, these markers indicate the presence of an active, locally organized B cell response with potential implications for immune complex-mediated injury. CXCR5, PDCD1, CD40LG: Canonical markers of T follicular helper (Tfh) cells. CXCR5 enables migration of Tfh cells into B cell follicles, PDCD1 (encoding PD-1) regulates their activity, and CD40LG encodes CD40L, a key costimulatory molecule required for B cell activation, class-switching, and germinal center formation.

To investigate the functional consequences of B cell-predominant inflammation, we performed immune complex analysis by isolating IgA-bound proteins from kidney tissue of 8 controls and 8 patients with a B cell-rich gene signature from the Susztaklab Biobank, using IgA-specific pulldown followed by tandem mass tag (TMT)-labeled mass spectrometry (Fig. 5x). This approach enabled us to identify proteins enriched in immune complexes from B cell-inflamed samples, revealing the presence of effector molecules such as granzymes and components of the complement cascade, which may contribute to local tissue injury.

To find plasma biomarkers that could help discriminate these two patient populations, we used paired plasma proteomic from patients with B+ and B- DKD in the TRIDENT cohort to train an elastic-net logistic regression with the Synthetic Minority Oversampling Technique (SMOTE). To address class imbalance, we trained a classifier optimized via 5-fold cross-validation. The final model included 14 proteins, of which some are depicted in Fig. 5y. Moreover, we then incorporated proteins with non-zero coefficients into a Cox proportional hazards model assessing kidney survival (death, dialysis, or $\geq 40\%$ eGFR decline). This protein-based model improved risk prediction beyond clinical features alone, as evidenced by a higher mean C-statistic across 1,000 bootstrap resamples (Fig. 5y, z, Supplementary Table 26). For external validation of the B+DKD protein set, we analyzed data from 3,309 UK Biobank participants with a diagnosis of diabetes mellitus and complete clinical information. In this independent cohort, the B+DKD protein set, when combined with known clinical predictors (Kidney Failure Risk Equation; KFRE), achieved an AUC of 0.7047, exceeding the performance of the clinical model (KFRE; AUC = 0.6286), thereby supporting its potential utility for risk stratification beyond established clinical predictors (Extended Fig. 38a). Moreover, quartile-based β -coefficients from the Cox model showed that individuals above the highest quartile (Q5) of the B+DKD protein set had a markedly increased risk of progression, exceeding that predicted by the KFRE model (Extended Fig. 38b). This threshold may therefore help to identify a high-risk subgroup, that could be amenable to B cell-directed interventions.

SPP1 and MMP7 are not novel biomarkers and are not specific to this disease. Their clinical relevance is uncertain. The authors should reference the numerous studies, including reviews, on SPP1+ macrophages in chronic diseases across different organs.

Response: We thank the reviewer for highlighting the extensive literature on SPP1⁺ macrophages and MMP7 in chronic inflammatory diseases. We apologize for the misunderstanding. Our goal was not to discover a new gene or a novel prognostic biomarker. Our goal was to perform an unbiased spatial analysis of the human kidney and using this unbiased discovery platform to understand disease heterogeneity. Here we identified a **B-cell rich subgroup**, which is highly interesting as we can repurpose already existing drugs for this disease subgroup. To ease the subgroup identification, we not only developed a spatial gene signature, but also developed tissue and blood biomarker.

For the biomarker discovery, it is critical to compare the new biomarkers with the current gold standards, such as proteinuria and eGFR at baseline, to demonstrate that the newly identified biomarkers outperform these simple measures. Additionally, a comparison with other reported biomarkers, such as urinary EGF, should be included.

Response: We thank the reviewer for their thoughtful comment. While eGFR and proteinuria are well-established biomarkers for predicting kidney outcomes, they offer limited insight into disease mechanisms or actionable heterogeneity. Our objective was not to replace these clinical markers, but to identify **discriminatory biomarkers** that define **mechanistically distinct subtypes** within diabetic kidney disease (DKD).

A key finding of our study is the identification of a **B cell-rich microenvironment (ME)**—the first mechanistically defined DKD subtype characterized by spatially organized B cell infiltration and features of tertiary lymphoid structures. Although present in only ~8% of patients, this subtype is **clinically significant**, showing markedly faster progression to end-stage kidney disease and unique immunopathology.

Because of its low prevalence, **a tissue-based classification alone is insufficient** for broader clinical use. Therefore, we identified **plasma biomarkers** that reflect the presence of this specific ME. These proteins, derived from spatial transcriptomic signatures and validated in independent plasma proteomics datasets (including the UK Biobank), were strongly associated with kidney function decline. This could enable **noninvasive detection** of the B cell-enriched DKD subtype and offers a path toward **precision stratification and targeted intervention**, particularly using therapies directed at B cells, plasma cells, or the complement system.

To find plasma biomarkers that could help discriminate these two patient populations, we used paired plasma proteomic from patients with B+ and B- DKD in the TRIDENT cohort to train an elastic-net logistic regression with the Synthetic Minority Oversampling Technique (SMOTE). To address class imbalance, we trained a classifier optimized via 5-fold cross-validation. The final model included 14 proteins, of which some are depicted in Fig.

5y. Moreover, we then incorporated proteins with non-zero coefficients into a Cox proportional hazards model assessing kidney survival (death, dialysis, or $\geq 40\%$ eGFR decline). This protein-based model significantly improved risk prediction beyond clinical features alone, as evidenced by a higher mean C-statistic across 1,000 bootstrap resamples (Fig. 5z). The addition of protein reached a better AUC, then the clinical parameters alone in predicting kidney survival (Fig. 5z). As urinary EGF data were unavailable in the TRIDENT cohort, we benchmarked our model against TNFRSF1B—a well-established biomarker for DKD progression—and found that it outperformed this comparator (Extended Fig. 37).

Although the authors claim this is the largest dataset available, the same lab recently published a spatial atlas in Nature Genetics covering more patient samples and a greater number of cells (though these were not included in this study).

We thank the reviewer for this thoughtful comment and the opportunity to clarify the differences between our current study and our previous publication in *Nature Genetics*. While both studies investigate the human kidney, they differ substantially in **resolution, scope, methodology, and key findings**.

The *Nature Genetics* study featured broad **dissociated single-cell/nucleus profiling** (338,565 sc/snRNA-seq and snATAC-seq cells from 65 patients) and incorporated **spot-level spatial transcriptomics** using the 10X Visium platform on 14 samples. Each Visium spot (~55–100 μm) captures transcripts from multiple cells, limiting resolution. Although that study included CosMx data from two samples, mostly to verify cell types and gene expression. The spatial profiling was neither the central focus nor performed at the scale or resolution of the current work, such as we could not compare cases and controls in our prior paper.

In contrast, the current study employs two high (single cell)-resolution spatial platforms—CosMx ($n = 48$, ~3.3 million cells) and Xenium ($n = 16$, ~1 million cells)—together profiling over 4 million spatially resolved cells across 64 samples. These data were integrated with single-nucleus RNA sequencing from 150 patients (~750,000 cells), yielding an atlas of over 5 million cells. To our knowledge, this represents the most comprehensive spatially resolved single-cell resource available for any human disease.

Key distinctions include:

- **Resolution:** The previous study used spot-level Visium data; here, we achieve true single-cell and subcellular resolution, enabling fine-grained neighborhood and interaction analyses.
- **Analytical focus:** The prior work spatially validated cell types. This study centers on identifying disease-associated spatial niches, immune microenvironments, and cell–cell signaling circuits—analyses only feasible with single-cell spatial resolution.
- **Methodological innovation:** We employed advanced computational methods, including 3 complementary niche and neighborhood analysis, ligand–receptor inference, and immune microenvironment mapping, tailored to high-resolution spatial data.
- **Sample size:** Given only 2 samples were analyzed previously using the CosMx platform, while 64 samples analyzed using complementary approaches CosMx/Xenium.
- **New biological insights:** The current study reveals B cell–driven microenvironments associated with progressive diabetic kidney disease, defines immune–epithelial cross-talk, and identifies actionable signaling pathways, none of which were addressed in the earlier publication.

To ensure the broader relevance of our findings, we validated our findings across four independent, large-scale cohorts—including the TRIDENT study, Susztaklab Biobank, UK Biobank, and a CD19 CAR T cell lymphoma cohort with impaired kidney function. The consistency of associations across these datasets further underscores the novelty and translational potential of our study. In summary our previous work was mostly about defining cell types, our current work identifies an important and targetable disease subtype that could even be identified based on blood biomarkers. We will ensure these distinctions are clearly stated in the revised manuscript to avoid confusion and to accurately reflect the unique contributions of this high-resolution spatial atlas.

The authors should also compare their findings to previous KPMP studies, which have already identified iLOH as an injury phenotype.

Response: We thank the reviewer for this important suggestion. The Lake et al. study, conducted as part of the KPMP consortium, analyzed **dissociated single-cell and single-nucleus transcriptomic** data and spot level spatial transcriptomics using Visium and some SlideSeq. While this provided valuable insight into injury and

repair programs, the spatial resolution remained limited—Visium spots (~55–100 μm) contain transcripts from multiple cells, and Slide-seq2, though improved, does not achieve true single-cell resolution.

In contrast, our study focuses specifically on high-resolution single-cell spatial transcriptomics using CosMx (n=48 samples; 1,000 genes) and Xenium (n=16 samples; 5,100 genes) across 64 total samples, collectively profiling over 4 million spatially resolved cells. These were integrated with a publicly available single-nucleus RNA-seq dataset from 150 patients (~750,000 nuclei), generating a comprehensive multimodal atlas of over 5 million cells. While the KPMP study identified iLOH at the dissociated and spot level, our approach enables the dissection of cellular microenvironments surrounding injured tubular epithelial cells at true single-cell spatial resolution. We leveraged this to define five distinct tubular injury microenvironments (MEs), including a profibrotic ME enriched for immune and fibroblast neighbors. iLOH cells within this niche expressed MMP7, ANXA2, CLU, ITGB6, and IFITM3, collectively marking a spatially localized, proinflammatory, and profibrotic transcriptional state that was significantly expanded in DKD and correlated with eGFR decline.

Thus, our findings go well beyond the prior KPMP study and extend them by refining spatial localization, neighborhood context, and gene expression programs at single-cell resolution. These comparisons and citations are now included in the revised manuscript to clearly delineate how our work builds upon and advances the KPMP spatial framework.

The discovery of TNFRSF19 is interesting, but it remains unclear how this gene mechanistically contributes to the progression of CKD.

Response: We thank the reviewer for pointing out the mention of TNFRSF19. In the revised version of the manuscript, we have removed TNFRSF19 as a proposed biomarker. Instead, our focus has shifted toward characterizing a clinically and mechanistically relevant immune tissue state: the B cell–predominant microenvironment.

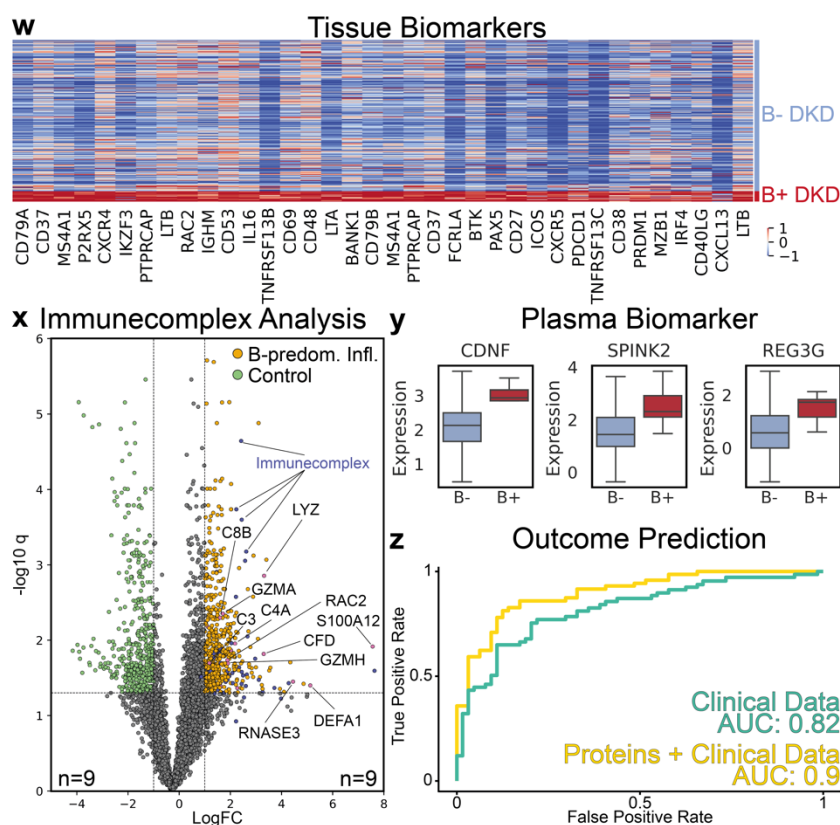


Fig. 5w: To identify candidate protein biomarkers associated with disease-relevant microenvironments, we first derived 10-gene spatial signatures for the B cell–predominant immune microenvironment using high-resolution spatial transcriptomics. These signatures were applied to bulk RNA-sequencing data from the TRIDENT Cohort to stratify patients (n=223) based on spatial microenvironment enrichment. Differential expression analysis was then performed using bulk RNAseq and the Limma R package. Depicted here are a subset of the significant DEGs.

x: Using specific antibodies against IgA, pulldown from 8 controls and 8 patients with B cell predominant inflammation from the Susztaklab Biobank was performed and analyzed using TMT-labeled mass spectrometry. Volcano plot of proteins bound to immune complexes in B-predom. Inflammation vs. Control.

y: Paired plasma proteomic from patients with B+ and B- DKD in the TRIDENT cohort was used to train an elastic-net logistic regression with the Synthetic Minority Oversampling Technique (SMOTE). To address class imbalance, we trained a classifier optimized via 5-fold cross-validation. Depicted are 3 proteins of that model.

z: Proteins with non-zero coefficients in the final model were then incorporated into a Cox proportional hazards model assessing kidney survival (death, dialysis, or $\geq 40\%$ eGFR decline). This protein-based model significantly improved risk prediction beyond clinical features alone, as evidenced by a higher mean C-statistic across 1,000 bootstrap resamples.

These signatures were independently derived from high-resolution spatial transcriptomic data and enabled stratification of patients in the TRIDENT cohort based on their expression profiles in bulk RNA-sequencing data.

For downstream biomarker discovery, we applied the spatially defined signatures to bulk RNA-sequencing data from patients with diabetic kidney disease in the TRIDENT cohort (n = 226). We stratified patients according to expression of the B cell–predominant immune microenvironment (ME) signature. To identify candidate biomarkers specific to this B cell–enriched disease state, we performed differential expression analysis using the Limma package. This analysis revealed a set of tissue biomarkers significantly elevated in the B cell–associated subset, suggesting their potential as indicators of immune-driven disease activity (Fig. 5w).

These biomarkers include:

- **CD19, CD79A, PAX5, FCRLA, BTK:** Classical B cell markers, indicating increased infiltration or expansion of B cells.
- **CXCL13, LTA, LTB:** Chemokines central to the formation of tertiary lymphoid structures (TLSs), promoting B and T cell recruitment.
- **IL16:** A cytokine involved in immune cell chemoattraction and activation.
- **MZB1, CD38, PRDM1:** Markers of antibody-secreting plasma cells, supporting local immunoglobulin production.
- **IGHA1:** The IgA heavy chain, suggesting local IgA production and potential immune complex formation.
- **TNFRSF13B (TACI):** A BAFF/APRIL receptor essential for B cell survival and class-switch recombination, reflecting sustained B cell activity.
- **CXCR5, PDCD1, CD40LG:** Canonical markers of T follicular helper (Tfh) cells. CXCR5 guides Tfh cell migration into B cell follicles; PDCD1 (PD-1) modulates Tfh activity; CD40LG (CD40L) provides critical costimulation for B cell activation, class switching, and germinal center formation.

Together, these markers point to an active, locally organized B cell response with potential for immune complex–mediated injury.

To identify circulating biomarkers distinguishing B+ from B– DKD, we used paired plasma proteomics from TRIDENT patients and trained an elastic-net logistic regression model, applying the Synthetic Minority Oversampling Technique (SMOTE) to address class imbalance. Model performance was optimized via 5-fold cross-validation. The final classifier included 14 proteins, a subset of which is shown in Fig. 5y. We then incorporated the proteins with non-zero coefficients into a Cox proportional hazards model to evaluate kidney survival (defined as death, dialysis, or $\geq 40\%$ decline in eGFR). This protein-based model significantly improved risk prediction beyond clinical features alone, as evidenced by a higher mean C-statistic across 1,000 bootstrap resamples (Fig. 5z). The addition of protein biomarkers also led to a higher AUC compared to clinical parameters alone in predicting kidney survival (Fig. 5z). For external validation of the B+DKD protein set, we analyzed data from 3,309 UK Biobank participants with a diagnosis of diabetes mellitus and complete clinical information. In this independent cohort, the B+DKD protein set, when combined with known clinical predictors (Kidney Failure Risk Equation; KFRE), achieved an AUC of 0.7047, exceeding the performance of the clinical model (KFRE; AUC = 0.6286), thereby supporting its potential utility for risk stratification beyond established clinical predictors (Extended Fig. 38a). Moreover, quartile-based β -coefficients from the Cox model showed that individuals above the highest quartile (Q5) of the B+DKD protein set had a markedly increased risk of progression, exceeding that predicted by the KFRE model (Extended Fig. 38b). This threshold may therefore help to identify a high-risk subgroup, that could be amenable to B cell–directed interventions.

In sum, **rather than focusing on TNFRSF19, the revised manuscript highlights the discovery of a spatially distinct, disease state in DKD**, which we believe has direct implications for patient stratification, and the rationale for B cell–directed therapies such as anti-CD20 or anti-BAFF in a molecularly defined patient subgroup.

Since the authors used a label transfer approach with existing cell-type annotations to label their spatial data, how can they detect previously unidentified cell types or states that were not present in the reference datasets?

Response: We thank the reviewer for this important question. While we used a semi-supervised label transfer approach (scANVI) to annotate spatial cells based on existing snRNA-seq references, **our analysis was not limited to predefined cell types**. To reduce sampling bias and broaden the diversity of states in the reference, we incorporated a larger and more heterogeneous snRNA-seq dataset in the revised manuscript, including profiles from 150 individuals drawn from KPMP, Susztaklab, and Humphreyslab. This expanded reference includes both healthy and diseased kidney samples, which improves the accuracy and coverage of transferred annotations.

Following label transfer, we performed unsupervised Leiden clustering in the scANVI latent space and manually reviewed clusters based on marker gene expression and spatial context. It is important to note that spatial transcriptomic data is inherently more sparse than single-cell or single-nucleus RNA-sequencing, both in terms

of the number of genes detected per cell and the depth of coverage. This sparsity arises from the technical limitations of in situ hybridization methods and impacts the direct resolution of subtle transcriptional differences.

However, the true strength of spatial transcriptomics lies not only in single-cell profiling, but in the ability to **define cell states in their native tissue context—particularly through neighborhood analysis**. By incorporating spatial proximity information, we can identify transcriptional programs that are activated in specific microenvironments, even when individual gene expression signals are weak. Specifically, we leveraged this spatial information to define cell states based not only based on their transcriptional profile, but also on their local cellular neighborhoods. Using this approach, we focused on injured proximal tubule (iPT) and thick

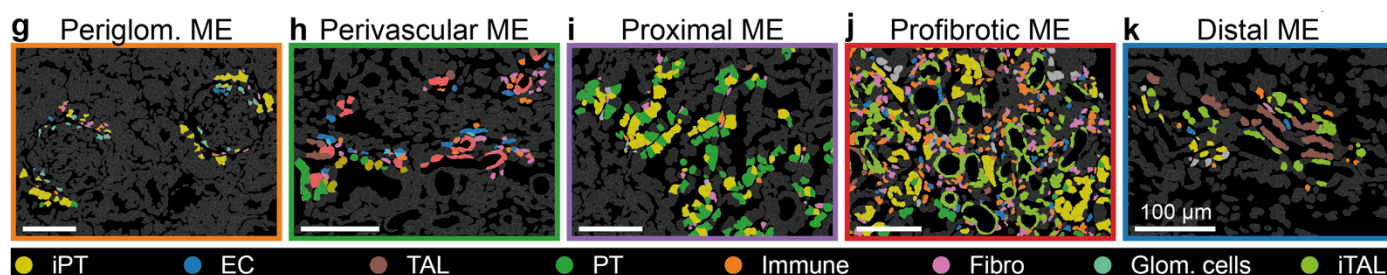


Fig. 3g-k: Representative image of the periglomerular ME. h, Representative image of the perivascular ME. i, Representative image of the proximal ME. j, Representative image of the profibrotic ME. k, Representative image of the distal ME. Spatial plots were generated using Xenium Explorer 3. Celltypes are indicated by their color. iPT: injured PT cells, EC: endothelial cells, including peritubular and distal vasa recta endothelial cells, TAL: thick ascending loop cells, PT: proximal tubular cells, Immune: Immune cells, Fibro: Fibroblast cells, Glom. cells: Glomerular cells, including parietal epithelial cells, podocytes and glomerular endothelial cells. iTAL: injured thick ascending loop cells.

ascending limb (iTAL) cells and clustered their surrounding neighborhoods into five distinct microenvironments (MEs, Fig 3g-k). Among these, the profibrotic ME was particularly notable and characterized by abundant immune and fibroblast neighbors and strongly enriched in diabetic kidney disease (DKD). This ME also showed a robust negative correlation with GFR ($r = -0.6$, $p < 0.001$, Fig 3c), suggesting clinical relevance.

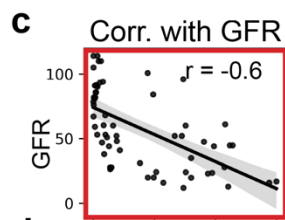


Fig. 3c: For each sample, we calculated the proportion of injured tubular center cells belonging to the profibrotic microenvironment relative to all injured tubular cells. This fraction was then correlated with estimated glomerular filtration rate (eGFR) using Pearson's correlation ($p < 0.001$).

Our analysis was highly robust between different platforms and detection dataset (CosMx 1K, and Xenium 5K). Despite differences in gene panel size between the CosMx dataset (1,000 genes) and the Xenium dataset (5,100 genes), we observed highly consistent transcriptional signatures across platforms (Fig. 3e-f). For example, the iPT compartment of the profibrotic ME exhibited upregulation of HAVCR1 and MMP7 in both CosMx and Xenium datasets, while the iTAI compartment was marked by increased MMP7, ANXA2, ITGB6, and CLU expression (Fig. 3e-f). This neighborhood-based analysis provides a powerful framework for identifying interpretable and

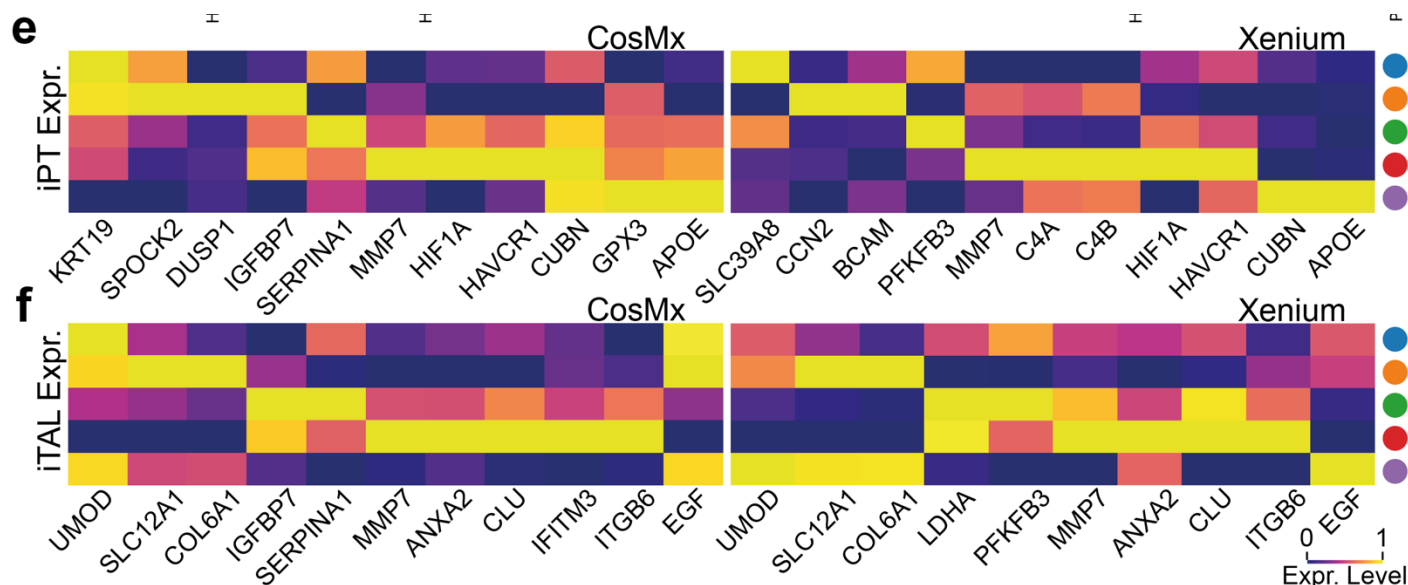


Fig. 3e: Heatmap of key differentially expressed genes of iPT center cells in MEs in the CosMx dataset (left) and Xenium dataset (right). f, Heatmap of key differentially expressed genes of iLOH center cells in MEs in the CosMx dataset (left) and Xenium dataset (right). The differentially expressed (DE) genes were calculated using the Wilcoxon rank-sum test. CosMx and Xenium datasets were analyzed separately due to their differing gene panel sizes.

spatially defined cell states, linking transcriptional reprogramming to specific tissue microenvironments and enabling mechanistic insights that are difficult to achieve with dissociated single-cell data alone.

We have clarified this multi-step approach in the Methods section and emphasized in the Results that our findings build upon—but are not limited to—the reference dataset.

Is there any overall correlation between kidney and plasma proteomics (Fig 5)?

Response: We thank the reviewer for this question, while some individual proteins are detectable in both kidney tissue and plasma, the overall patterns between kidney and plasma proteomics do not correlate. Kidney proteomics capture proteins that may be locally enriched or restricted to specific microenvironments, whereas plasma proteomics reflect a composite of circulating proteins derived from multiple organs and systemic processes. Differences in protein secretion, turnover, and clearance, along with dilution effects in plasma, can further reduce direct correspondence between kidney tissue and circulating levels.

Minor comments:

Generalized additive model – Figure 1f, predicted gene expression. This procedure is not clearly defined. what is the overall performance of this GAM based method for inferring gene-expression per cell?

Response: We thank the reviewer for this suggestion. In the revised manuscript, we have removed this analysis to maintain a clear focus on the spatial tissue architecture and disease-associated cellular interactions in health and diabetic kidney disease.

Line 143: “Additionally, fewer immune cells were detected “ → can this be quantified and presented with p-values.

Response: We thank the reviewer for this suggestion. In the revised manuscript, we have removed this analysis to maintain a clear focus on the spatial tissue architecture and disease-associated cellular interactions in health and diabetic kidney disease.

Page 4, lines 116 and 119: how are the low quality cells defined in these two steps. Is this done sample per sample or on aggregated data?

Response: Low quality cells are defined on aggregate data, we have clarified this in the method section.

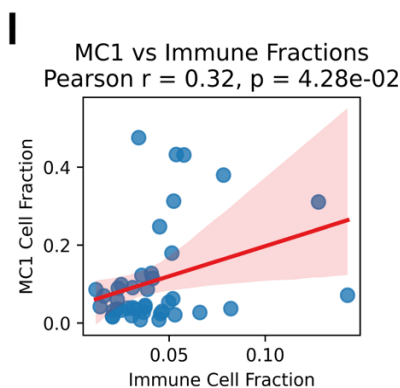
Extended Figure 4a → “65 genes were selected for the COVET”. How were these genes selected? and how is the number of genes determined?

Response: Thank you for pointing this out. The selection of genes for the COVET analysis was guided by both biological relevance and computational feasibility. First, not all 1,000 genes in the CosMx panel are informative for kidney-specific processes. To focus on the most informative features, we selected highly variable genes using the “scanpy.pp.highly_variable_genes” function with the “seurat” flavor, which identifies genes that show significant cell-to-cell variation while accounting for mean expression and dispersion. This ensures that the selected genes capture biologically meaningful heterogeneity within the tissue.

Second, the COVET framework constructs a three-dimensional matrix that incorporates each cell’s gene expression profile alongside that of its spatial neighbors. As this results in large, memory-intensive tensors—particularly in our revised dataset with over 4 million cells—we had to impose a practical limit on the number of genes. We have clarified this in the revised Methods section.

Extended Figure 4g → what does each dot represent? Is it the low dimension representation of a niche times samples? So is it 130 dots?

Response: We thank the reviewer for the opportunity to clarify. Extended Figure 4g of the original manuscript shows a UMAP visualization based on the COVET adjacency matrix. Specifically, diffusion components were



Extended Fig. 12l: For each sample, we calculated the proportion of immune cells and mesangial cells belonging to the glomerular niche. This fraction was then correlated.

computed from the full COVET graph, which captures spatial co-expression relationships across the dataset. These diffusion components were then aggregated per niche and sample—resulting in one vector per niche per sample. In total, this generated 130 points (5 niches × 26 samples), each representing the aggregated diffusion profile of a spatial niche in a given sample. These profiles were then visualized using UMAP to explore global niche relationships across patients. However, due to the computational complexity and memory demands of the diffusion components, this approach is not scalable to the full dataset comprising over 3 million spatially resolved cells of the revised manuscript. In our revised manuscript, we therefore simply calculate UMAP for visualization after PCA, to better examine the relationships within the COVET niche representation.

Hotspot was applied to identify genes with spatially correlated expression patterns based on niche-specific latent representations calculated by COVET analysis. The heatmap displays pairwise gene–gene correlations, with stronger correlations indicated by deeper red coloring. Gene pairs conserved across both conditions are highlighted at the top. Differential correlation analysis revealed condition-specific interactions, with gene pairs significantly increased in diabetic kidney disease (DKD) highlighted (bottom).

However, in our revised manuscript we further analyze the niche-specific latent representations calculated by the COVET analysis using Hotspot. This allows for the identification of gene-gene correlations in the different niches. It also allows for the analysis of differential correlations between Control and DKD patients. The results of this analysis are summarized in extended figures 15 to 25.

Line 225: “and an increase in mesangial cells” → podocyte loss and increase in mesangial cells in the Glom Niche. Is there any association between these observations?

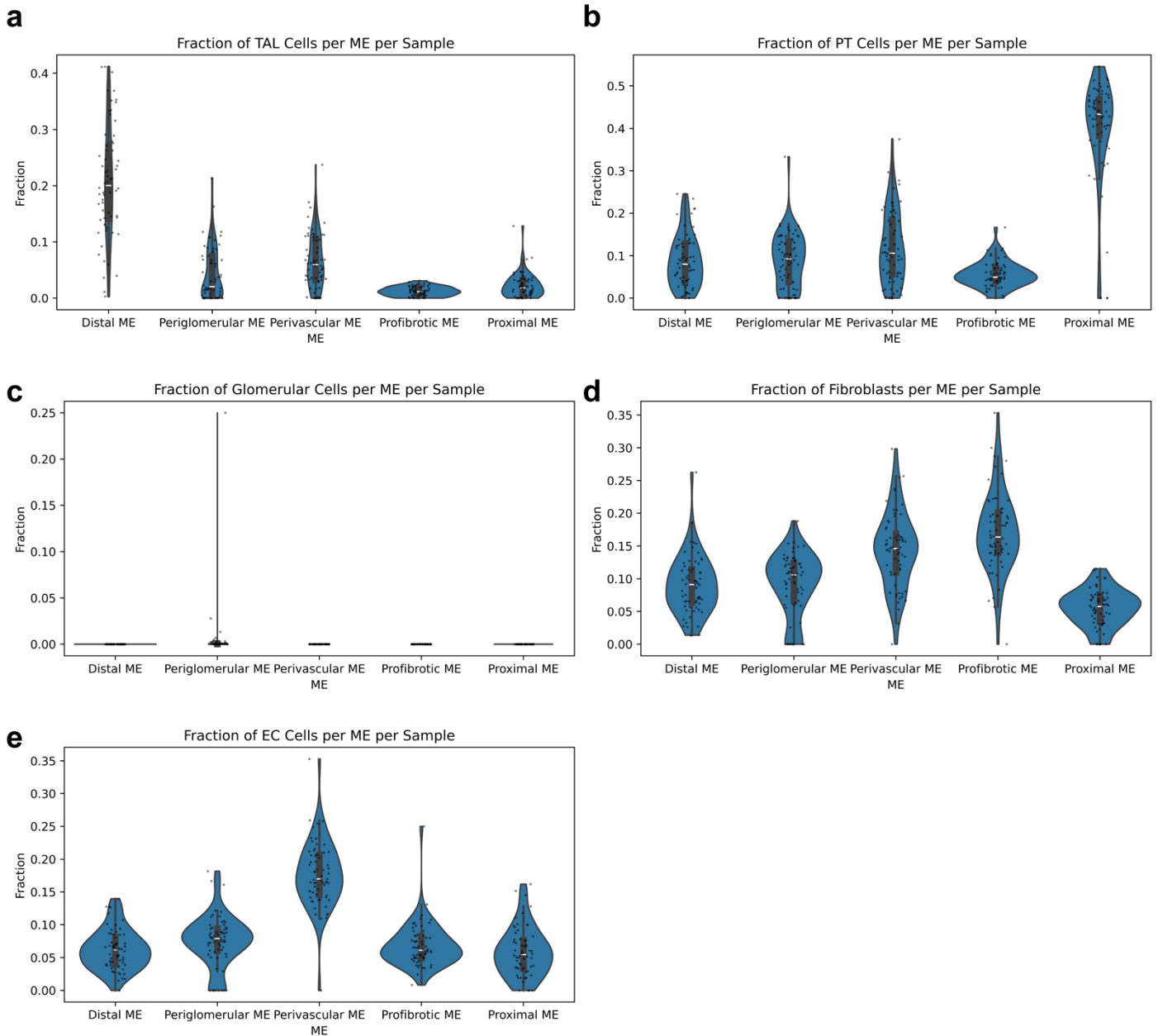
Response: We thank the reviewer for this important question. Indeed, we observed a significant association between the two cell fraction changes within the glomerular niche in diabetic kidney disease—namely, increased immune cell infiltration and expansion of mesangial cells (Extended Fig. 12l). Specifically, samples with greater mesangial cell abundance also showed higher proportions of immune cells, suggesting a potential link.

Figure 2j and 2m: are the genesets reported in these figures statistically significant?

Response: For the calculation of genesets reported in Figure 2 we used gseapy, all genesets are statistically significant (adj. p value <0.05).

Figure 3: are the differences reported in figure 3c and 3b statistically significant? Why is 20um radius chosen here? while the ME is defined over 200um?

Response: We thank the reviewer for this question. For clarification, the injured tubular microenvironment calculation was performed on all injured PT and LOH cells after excluding those with insufficient local neighborhood context and removing the sparsest 5% of neighborhoods (based on total neighbor count at 80 μ m) to reduce edge effects. For each cell, we computed the number of neighboring cells within concentric distance shells: 0–20 μ m, 20–40 μ m, 40–60 μ m, and 60–80 μ m. This was achieved by calculating cumulative neighbor counts at 20, 40, 60, and 80 μ m, followed by subtracting counts from the smaller radius to isolate the number of neighbors in each respective shell (e.g., the 40–60 μ m shell = count at 60 μ m – count at 40 μ m). For each cell, neighbor counts were normalized by the total number of neighbors at that distance, yielding a cell-by-neighbor composition matrix that reflects the relative cellular makeup of the local environment at different spatial scales. This matrix was then scaled and subjected to principal component analysis (PCA), with the top 10 components used as input for MiniBatch K-means clustering. The number of clusters (k) was determined based on the elbow method. Based on the key difference in the composition of these injured tubular microenvironments, we named these proximal (sign. higher amount of surrounding PT cells), distal (sign. higher amount of surrounding TAL cells), periglomerular (sign. higher amount of surrounding glomerular cells), perivascular (sign. higher amount of surrounding vascular cells) and profibrotic (sign. higher amount of surrounding fibroblasts). This analysis is shown in Extended Fig. 35.



Extended Fig. 35a–e: We quantified the relative abundance of selected cell types within injured tubular microenvironments (MEs), excluding cells outside these regions. For each sample, we calculated cell-type fractions across MEs and performed pairwise comparisons using two-sided Mann–Whitney U tests, with Benjamini–Hochberg correction for multiple testing.

a: TAL cells were significantly enriched in the Distal ME compared to the Perivascular ME (adjusted $p < 0.001$), Profibrotic ME (adjusted $p < 0.001$), Proximal ME (adjusted $p < 0.001$), and Periglomerular ME (adjusted $p < 0.001$).

b: PT cells were significantly enriched in the Proximal ME compared to the Perivascular ME (adjusted $p < 0.001$), Profibrotic ME (adjusted $p < 0.001$), Distal ME (adjusted $p < 0.001$), and Periglomerular ME (adjusted $p < 0.001$).

c: Glomerular cells were significantly enriched in the Periglomerular ME compared to the Perivascular ME (adjusted $p < 0.001$), Profibrotic ME (adjusted $p < 0.001$), Proximal ME (adjusted $p < 0.001$), and Distal ME (adjusted $p < 0.001$).

d: Fibroblasts were significantly enriched in the Profibrotic ME compared to the Perivascular ME (adjusted $p < 0.05$), Distal ME (adjusted $p < 0.001$), Proximal ME (adjusted $p < 0.001$), and Periglomerular ME (adjusted $p < 0.001$).

e: Vascular cells (EC_Peritub, VSMC, EC_DVR) were significantly enriched in the Perivascular ME compared to the Distal ME (adjusted $p < 0.001$), Profibrotic ME (adjusted $p < 0.001$), Proximal ME (adjusted $p < 0.001$), and Periglomerular ME (adjusted $p < 0.001$).

Figure 3a: what is the cellular composition of each ME?

Response: We thank the reviewer for this comment. In our revised manuscript, we have added new visualizations to help understanding the cellular composition of the injured tubular microenvironments (Fig. 3l). In these plots the center cell (circle at the center) represents the amount of injured proximal/injured TAL cells. The circle around this represents the neighborhood composition in the 20 μ m radius. As evidenced through analysis of the neighborin cell fractions, the periglomerular microenvironment contains more glomerular cells, the perivascular

Line 360: data integration needs to be described in greater detail - are these all healthy kidneys? are the datasets comparable to each other in terms of their QC related properties such as percentage of genes mapped to mitochondrial genes? ribosomal genes?, UMI and gene counts? What batch variable was used to mitigate technical batch-effects during integration? How does the integrated map compare to individual studies? Are the cell-states identified observed in cells and samples from each state?

Response: We thank the reviewer for the opportunity to elaborate on our data integration strategy. In the revised manuscript, we employed scVI and scANVI, probabilistic deep generative models, to integrate spatial transcriptomics data (CosMx and Xenium) with single-nucleus RNA-sequencing (snRNA-seq) profiles. These models were selected for their capacity to account for technical batch effects while preserving biologically meaningful variation. Moreover, scvi and scanvi are recommended for complex integration tasks due to their ability to scale to large datasets, superior removal of batch effect while preserving cell states.²⁷

As a reference, we used an expanded and heterogeneous snRNA-seq dataset comprising ~750,000 cells from 150 individuals, drawn from three independent sources: KPMP, the Susztak Lab, and the Humphreys Lab as described by Klötzer et al. 2025. This multi-source composition was designed to minimize sampling bias and ensure broad coverage of both healthy and diseased states.

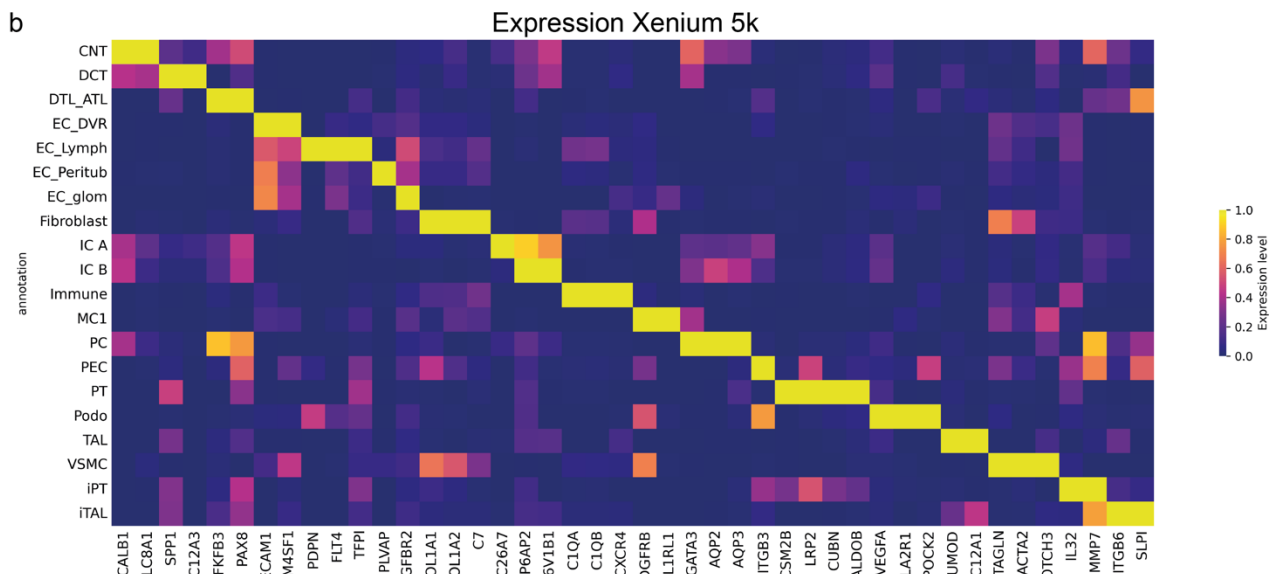
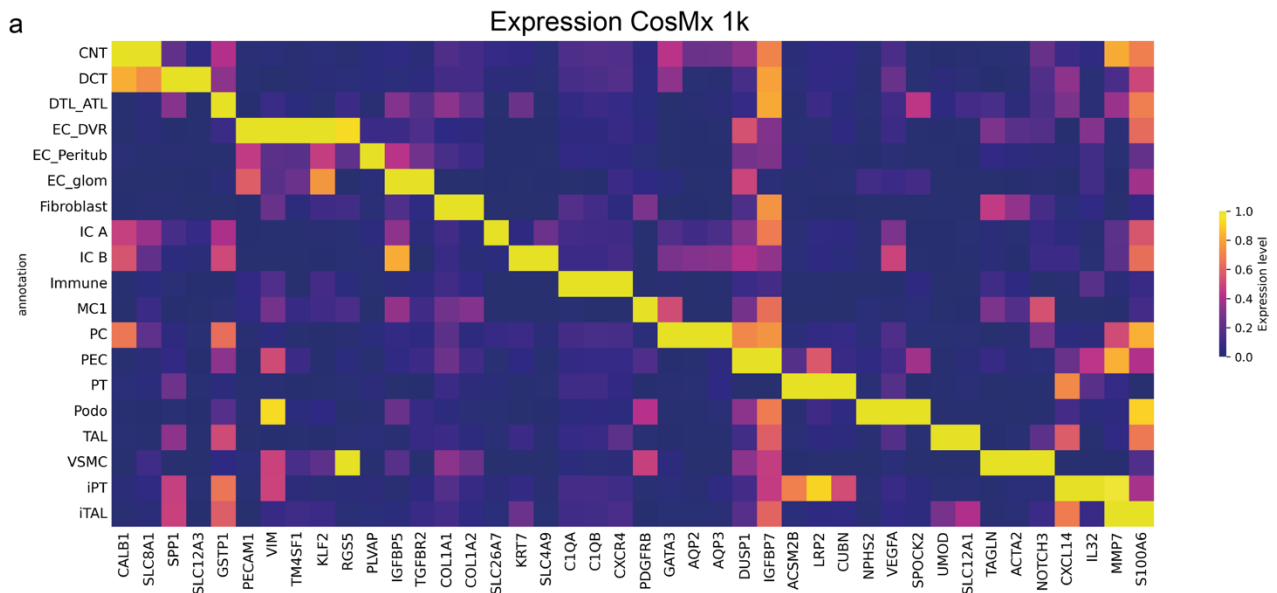
QC metrics—including the percentage of mitochondrial and ribosomal gene expression, and the number of detected genes per cell—were evaluated across all platforms and are summarized in Supplementary Figure 4. For batch correction, we modeled technology (CosMx, Xenium, and SNseq) as the primary categorical batch variable. In addition, we included sample identity and data source (e.g., KPMP vs. Susztaklab vs. Humphreyslab) as categorical covariates, and incorporated continuous covariates such as total RNA counts and mitochondrial gene percentages. This multi-layered correction framework allowed us to address both platform-level and sample-level variability.

Method	Bio conservation					Batch correction					Aggregate score		
	Isolated labels	KMeans NMI	KMeans ARI	Silhouette label	cLISI	Silhouette batch	iLISI	KBET	Graph connectivity	PCR comparison	Batch correction	Bio conservation	Total
scANVI	0.77	0.89	0.63	0.76	1.00	0.89	0.02	0.51	0.99	0.99	0.68	0.81	0.76
scVI	0.59	0.72	0.43	0.59	1.00	0.91	0.03	0.51	0.96	1.00	0.68	0.66	0.67
LIGER	0.55	0.57	0.31	0.55	1.00	0.94	0.08	0.67	0.77	0.99	0.69	0.59	0.63
Harmony	0.53	0.63	0.37	0.54	0.99	0.84	0.23	0.47	0.58	1.00	0.62	0.61	0.62
Scanorama	0.53	0.63	0.36	0.54	1.00	0.93	0.04	0.23	0.66	0.88	0.55	0.61	0.59
Unintegrated	0.53	0.60	0.35	0.54	1.00	0.76	0.00	0.06	0.63	0.00	0.29	0.60	0.48

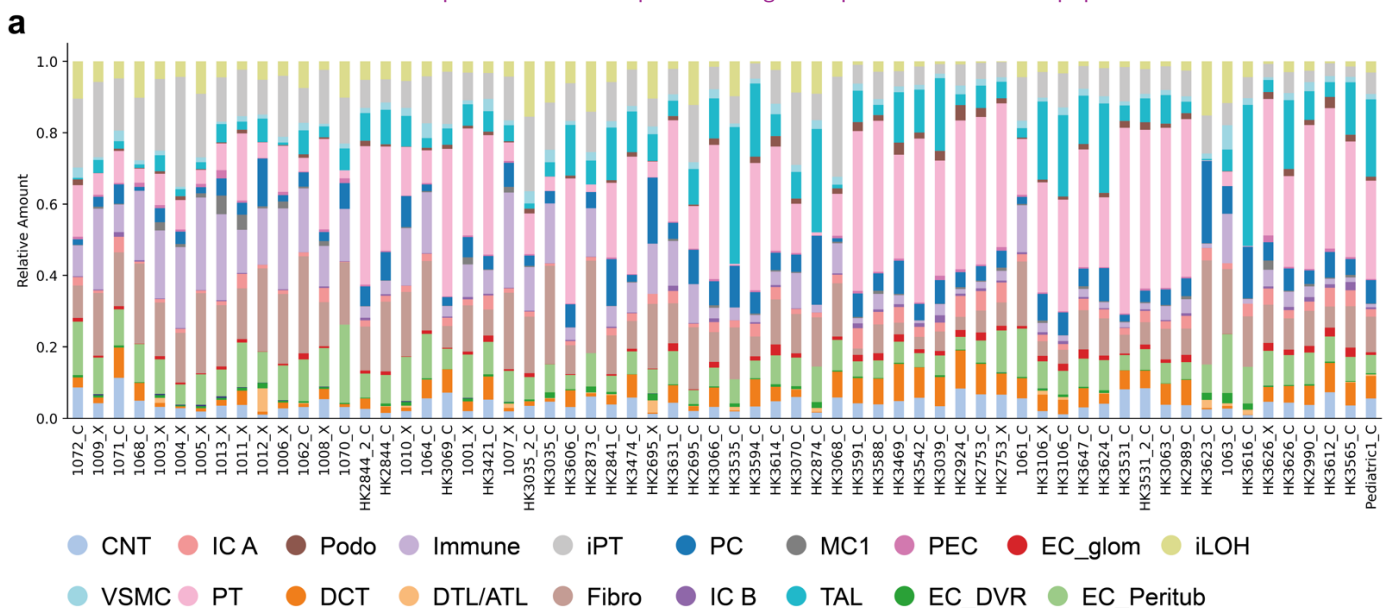
Extended Fig. 1: Benchmarking dataset integration: Methods are sorted by overall score. Overall score is a weighted mean of the batch correction score and the bio conservation score, which in turn are a mean of the individual metrics within the category. Metrics have previously been described (Luecken et al.). Briefly, these metrics were grouped into two broad categories: conservation of biological variance (Bio conservation) and removal of batch effects. All scores are normalized between 0 and 1, where higher values indicate better performance.

To benchmark our integration performance, we implemented the pipeline described in Luecken et al., Nat Methods 2022. We randomly downsampled 10% of the CosMx and SNseq datasets to allow for computationally tractable comparisons, and evaluated five integration methods: unintegrated, Scanorama, Harmony, Liger, scVI, and scANVI. We assessed performance using both batch mixing and biological conservation scores. scANVI achieved the highest aggregate score, demonstrating superior batch effect correction while preserving biological heterogeneity (Extended Fig. 1).

Following integration, spatial transcriptomics data were embedded into a shared latent space, followed by Leiden clustering to identify distinct cell populations. Cell type labels were assigned based on transferred annotations and marker gene expression. To account for differences in gene panel sizes, differentially expressed (DE) genes were calculated separately for CosMx and Xenium datasets using the Wilcoxon rank-sum test. Despite these platform-specific differences, we observed high concordance in marker gene expression between the two datasets. Finally, we assessed the distribution of cell types across samples and found that the majority



Extended Fig. 3a: Marker gene expression of each cell population in the CosMx dataset. Mean expression of each population was calculated and scaled from 0 to 1 and depicted as a heatmap. b: Marker gene expression of each cell population in the Xenium dataset.



Extended Fig. 1a: Celltype fractions per sample: to calculate the cell type fractions for each sample the number of a given celltype was divided by the total number of cells for a given sample. Color indicates different celltype populations: CNT, proximal tubular cells; VSMC, vascular smooth muscle cell; PC, principal cell; IC A, IC B, intercalated cells A, B; inj T, injured tubular cells; PT, proximal tubular cells; MC1, mesangial cell; LOH, thick ascending limb cells as well as DTL_ATL, distal thin limb, ascending thin limb cells; Fibro, fibroblasts; Podo, podocytes; DCT, distal convoluted tubular cells; PEC, parietal epithelial cells; EC: EC_Peritub, peritubular endothelial cells as well as EC_DVR, vasa recta endothelial cells; Immune, immune cells; EC_glom, glomerular

of cell types were consistently detected (Extended Fig. 3a, Extended Fig. 1a). Exceptions were primarily due to

tissue composition—for example, some samples lacked glomerular structures. Spatial transcriptomics offers a unique advantage over dissociated single-cell approaches by capturing not only transcriptional profiles but also the spatial context in which cells reside. This spatial information enables the definition of cell states based not solely on gene expression, but also on local tissue architecture and neighborhood composition. To identify injury-associated cell states in context, we focused on proximal tubule (iPT) and thick ascending limb (iTA) cells that showed transcriptional signatures of injury. We then incorporated spatial proximity data by calculating the composition of each cell’s neighborhood within concentric distance shells (0–20 μm , 20–40 μm , 40–60 μm , and 60–80 μm), normalized to reflect the relative proportions of

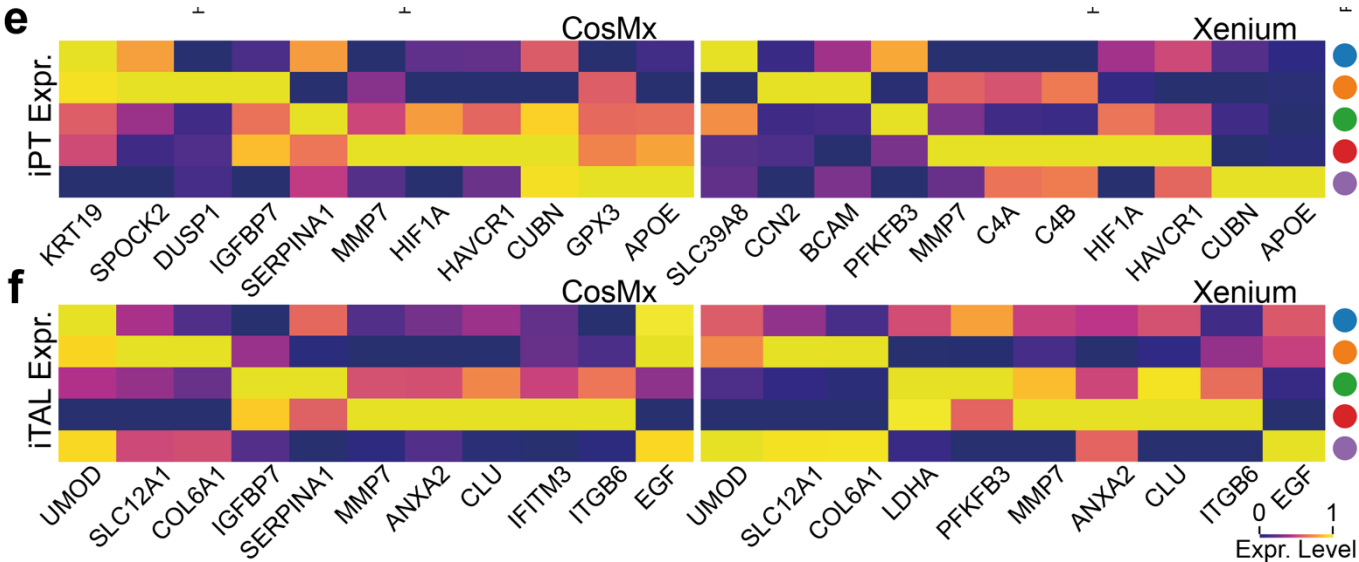


Fig. 3e: Heatmap of key differentially expressed genes of iPT center cells in MEs in the CosMx dataset (left) and Xenium dataset (right). f. Heatmap of key differentially expressed genes of iLOH center cells in MEs in the CosMx dataset (left) and Xenium dataset (right). The differentially expressed (DE) genes were calculated using the Wilcoxon rank-sum test. CosMx and Xenium datasets were analyzed separately due to their differing gene panel sizes.

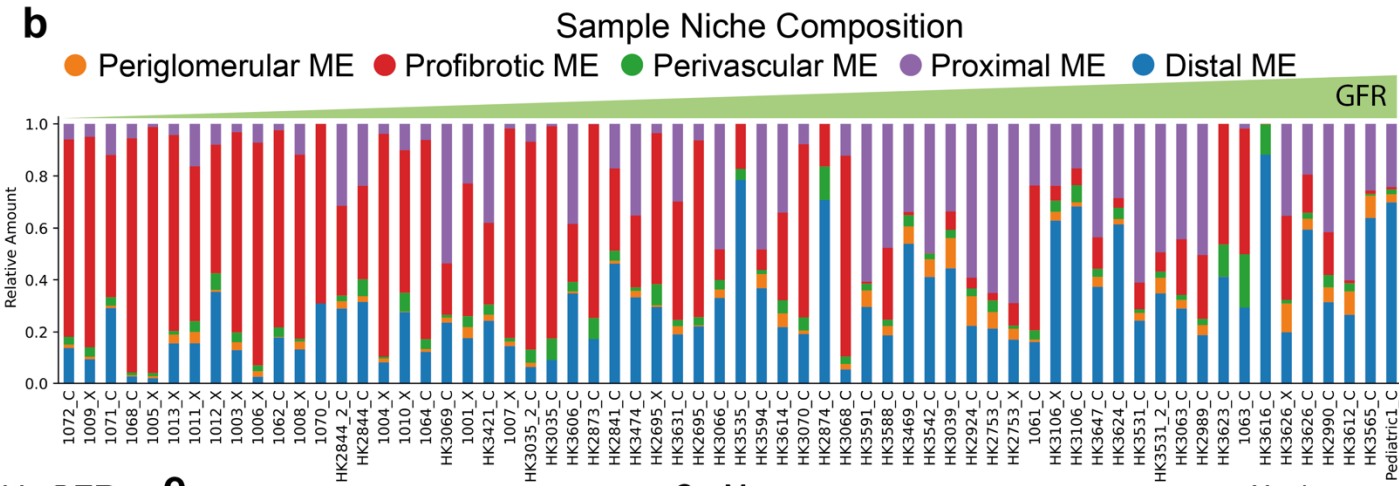


Fig. 3b: The injured tubular ME niche fractions across all samples, sorted by eGFR.

neighboring cell types. This resulted in a cell-by-neighbor composition matrix capturing the microenvironmental context at multiple spatial scales and allowing us to identify five distinct injured tubular microenvironments (MEs, Fig. 3b). Among these, the profibrotic ME was particularly notable, characterized by abundant immune and fibroblast neighbors, strong enrichment in diabetic kidney disease (DKD), and a robust negative correlation with GFR ($r = -0.6$, $p < 0.001$). Despite differences in gene panel sizes between CosMx (1,000 genes) and Xenium (5,100 genes), we observed highly consistent transcriptional signatures across platforms—for example, higher level of HAVCR1 and MMP7 in iPT cells and MMP7, ANXA2, ITGB6, and CLU in iTA cells within the profibrotic ME (Fig. 3e-f). This neighborhood-based framework allowed us to uncover spatially defined and biologically interpretable cell states that are not apparent from transcriptional profiles alone. We confirmed that all five injured tubular MEs were represented across multiple patient samples, demonstrating that these states are reproducible and not confined to individual cases. The only exception was the periglomerular tubular ME, which

was absent in samples that lacked glomerular structures, reflecting expected anatomical variability rather than technical bias.

Figure 4b: how is the immune-center and the neighborhood of the center defined in figure 4b?

Response: We thank the reviewer for the question. The immune microenvironments shown in Figure 4b were defined using a spatially informed clustering approach, (using the microenvironment concept) similar to the one described for niche and injured tubular microenvironment analyses. Immune cells are mobile therefore we proposed that they likely take on different states based on the surrounding microenvironment, which lead to the identification of organ level the immune cell spatial organization pattern.

Specifically, we first selected immune cells—identified using fine immune annotations from our kidney immune cell atlas—that had sufficient local neighborhood density, removing the sparsest 5% of neighborhoods (based on total neighbor count at 80 μ m) to ensure robust context. Each of these immune cells served as a "center" for microenvironment analysis. We then computed the number of neighboring cells within concentric distance shells (0–20 μ m, 20–40 μ m, 40–60 μ m, and 60–80 μ m) and normalized these values to generate a cell-by-neighbor composition matrix, reflecting the relative cellular makeup of the surrounding environment at different spatial scales. For this purpose, we used the annotations from the kidney immune cell atlas.

This matrix was scaled and reduced via principal component analysis (PCA), and the top 10 components were used as input for MiniBatch K-means clustering. The number of clusters (k) was selected using the elbow method. This spatially grounded approach allowed us to classify immune-centered microenvironments based on both immune cell identity and neighborhood composition, while leveraging the granularity of the immune atlas annotations. This allowed us to uncover 7 distinct kidney immune microenvironments (Fig f-s). The revised figures m-s now represent the annotation of the center cell (inner circle) using the fine immune cell annotations

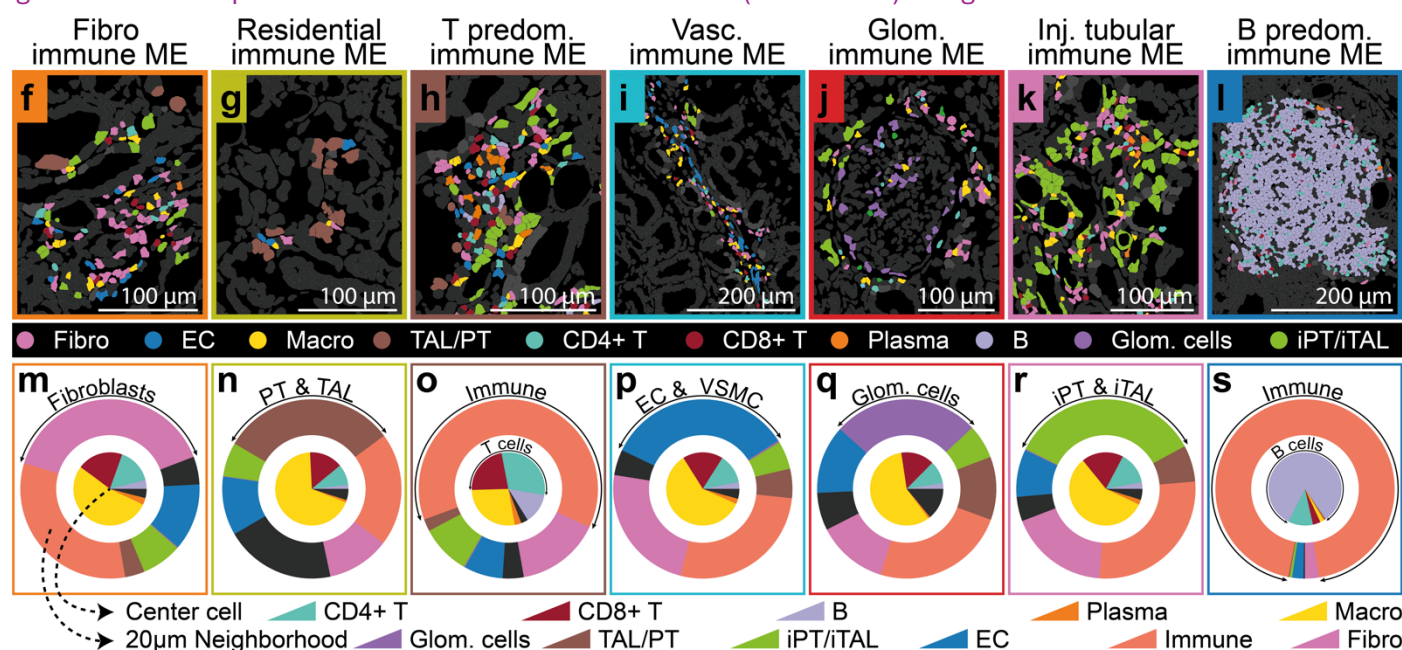
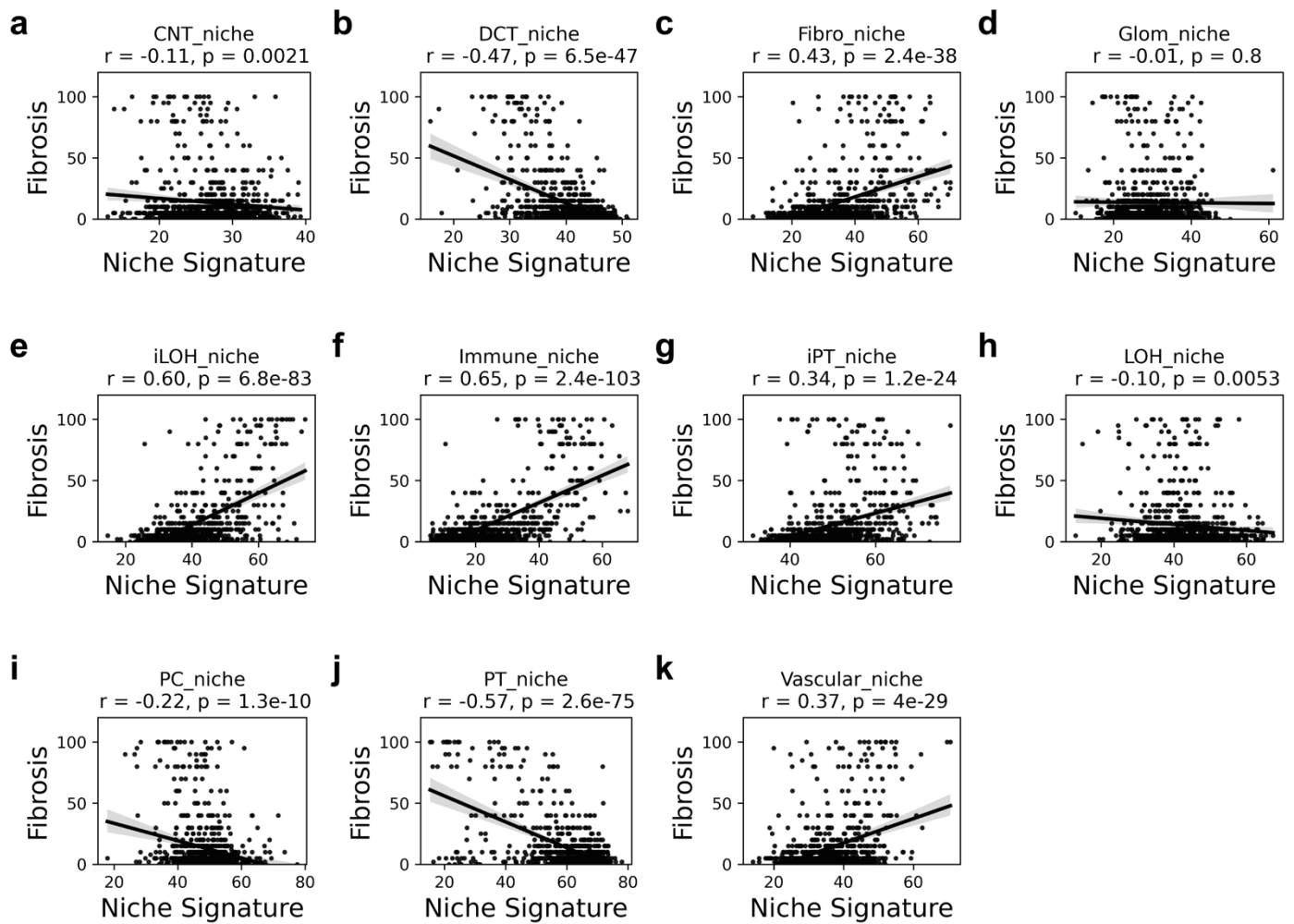


Fig 4: Immune microenvironments: f-l representative spatial plot of the Fibro Immune, Residential immune, T predom. Immune, Vasc. Immune, Glom. Immune, Inj. Tubular immune and B predom. Immune microenvironments. m-s circular plots showing the celltype composition in the 20 μ m surrounding (outer circle) and the annotation of the immune cells at the center (inner circle). Celltypes are colored based on colors shown in the legend. Xenium Explorer 3 was used for the spatial plot visualization.

and the entire 20 μ m using the annotations from the whole spatial kidney atlas. We have clarified these methods in the revised manuscript.

Figure 5a: are the correlation values provided in figure 5a statistically significant?

Response: We thank the reviewer for this question. We have included a supplemental figure for the correlation plots that also includes p values. Yes, PT, iPT, immune cells and several other niche associations are significant even after multiple testing correction (Extended Fig. 33).



Extended Fig 33: The association between spatial gene signature and disease severity: Spatial gene signatures were extracted for each niche with pseudobulk counts on a sample level followed by DeSeq2. 10 genes for each spatial gene signature were used to correlate with interstitial fibrosis scored by a renal pathologist in 843 patients (Susztaklab biobank, microdissected tubule bulk sequencing).

Dumoulin B and colleagues present a comprehensive work on the integration and analysis of healthy and diseased human kidneys. This is an impressive effort and it can be seen that a lot of work went into it. A particular aspect of the study that I enjoy is the visionary translational medicine aspect of the B-cell niche to classify kidney disease. However, some shortcomings require addressing.

Major Comments:

The authors' integration methodology, whilst comprehensive, would benefit from rigorous validation through established benchmarking metrics, such as scIB-metrics (<https://scib-metrics.readthedocs.io/en/stable/>). The implementation of scIB metrics would be particularly valuable to demonstrate the preservation of biological signals whilst accounting for batch effects in their scVI/scANVI integration.

Response: We thank the reviewer for this important suggestion and the opportunity to clarify our data integration and validation strategy. We have now substantially expanded our dataset, increasing the spatial sample size fourfold to include 64 spatially profiled samples using two complementary high-resolution platforms—CosMx 1k (n = 48; ~3.3 million cells) and Xenium 5k (n = 16; ~1 million cells). These were integrated with single-nucleus RNA-sequencing data from 150 patients (~750,000 cells) to construct a unified atlas of over 5 million cells, including more than 4 million spatially resolved cells (Fig. 1). To the best of our knowledge, this represents the largest and most comprehensive spatially resolved single-cell resource to date for any human disease.

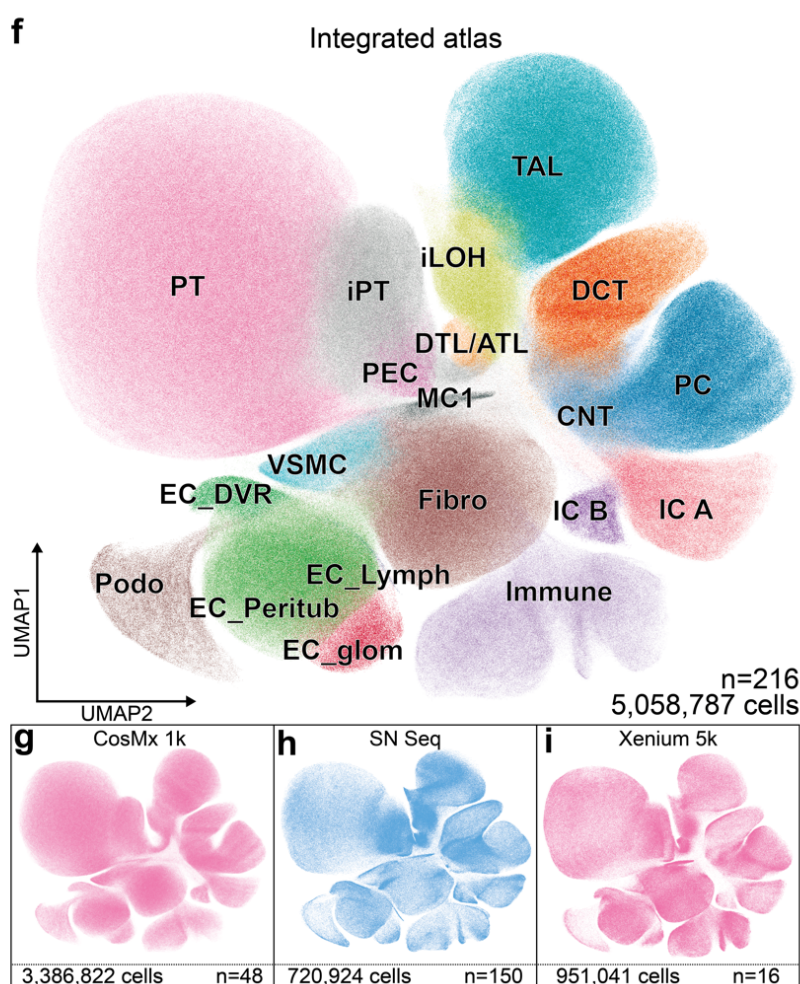


Fig. 1f: The UMAP visualization of the integrated spatial single-cell atlas. The spatial gene expression (CosMx 1k and Xenium 5k data) was integrated with dissociated single nucleus gene expression using scVI and scanVI. Each dot represents a single cell.

g, CosMx 1k spatial gene expression data. 48 samples and 3,386,822 cells were included in this study.

h, snRNA-seq data. 150 samples and 720,924 cells were included in this study.

The color indicates the different cell types such as: CNT, connecting tubular cells; VSMC, vascular smooth muscle cell; PC, principal cell; IC A, IC B, intercalated cells A, B; inj T, injured tubular cells; PT, proximal tubular cells; MC1, mesangial cell; LOH, thick ascending limb cells as well as DTL_ATL, distal thin limb, ascending thin limb cells; Fibro, fibroblasts; Podo, podocytes; DCT, distal convoluted tubular cells; PEC, parietal epithelial cells; EC: EC_Peritub, peritubular endothelial cells as well as EC_DVR, vasa recta endothelial cells; Immune, immune cells; EC_glom, glomerular endothelial cells

In the revised manuscript, we also integrated **2 different high-dimensional spatial transcriptomics data (CosMx 1k and Xenium 5k)** with single-nucleus RNA-sequencing (snRNA-seq) profiles using scVI and scANVI—deep generative models specifically designed to handle complex batch structures while preserving meaningful biological variation. These models were chosen based on their scalability, ability to integrate across modalities, and demonstrated superior performance in recent benchmarking studies (e.g., Luecken et al., Nat Methods 2022).

To ensure robust integration, we modeled the platform (CosMx, Xenium, and SN-seq) as the primary batch variable and further accounted for sample identity, data source (KPMP, Susztak Lab, Humphreys Lab), total RNA counts, and mitochondrial gene percentages as covariates. The snRNA-seq reference dataset comprised ~750,000 cells from 150 individuals across three consortia, chosen to maximize biological diversity and minimize sampling bias. Quality control metrics, including mitochondrial and ribosomal gene content and gene detection rates, were evaluated across all platforms and are summarized in Extended Fig. 8-11.

Importantly, in response to the reviewer’s request, we benchmarked our integration strategy using the scIB framework (<https://scib-metrics.readthedocs.io>), which quantifies both batch correction and biological conservation. We down-sampled 10% of the CosMx and snRNA-seq data for computational feasibility and compared six approaches: unintegrated, Scanorama, Harmony, Liger, scVI, and scANVI. scANVI consistently achieved the highest composite scores across metrics, confirming its ability to remove batch effects while preserving biological structure (Extended Fig. 1).

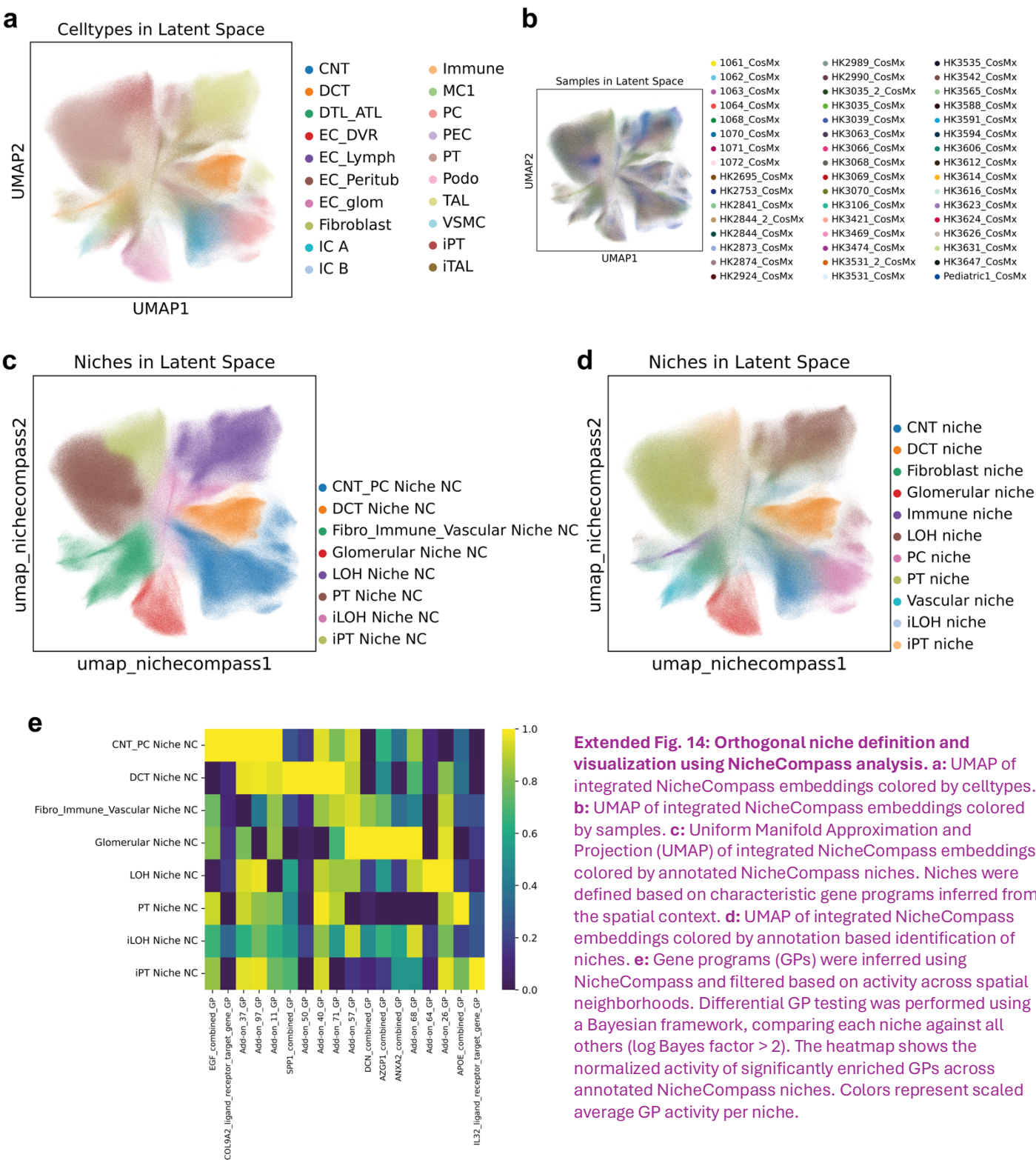
Method	Bio conservation					Batch correction					Aggregate score		
	Isolated labels	KMeans NMI	KMeans ARI	Silhouette label	cLISI	Silhouette batch	iLISI	KBET	Graph connectivity	PCR comparison	Batch correction	Bio conservation	Total
scANVI	0.77	0.89	0.63	0.76	1.00	0.89	0.02	0.51	0.99	0.99	0.68	0.81	0.76
scVI	0.59	0.72	0.43	0.59	1.00	0.91	0.03	0.51	0.96	1.00	0.68	0.66	0.67
LIGER	0.55	0.57	0.31	0.55	1.00	0.94	0.08	0.67	0.77	0.99	0.69	0.59	0.63
Harmony	0.53	0.63	0.37	0.54	0.99	0.84	0.23	0.47	0.58	1.00	0.62	0.61	0.62
Scanorama	0.53	0.63	0.36	0.54	1.00	0.93	0.04	0.23	0.66	0.88	0.55	0.61	0.59
Unintegrated	0.53	0.60	0.35	0.54	1.00	0.76	0.00	0.06	0.63	0.00	0.29	0.60	0.48

Extended Fig. 1: Benchmarking dataset integration: Methods are sorted by overall score. Overall score is a weighted mean of the batch correction score and the bio conservation score, which in turn are a mean of the individual metrics within the category. Metrics have previously been described (Luecken et al.). Briefly, these metrics were grouped into two broad categories: conservation of biological variance (Bio conservation) and removal of batch effects. All scores are normalized between 0 and 1, where higher values indicate better performance.

The approach to niche identification with Mini Batch k-means clustering and COVET is sound, the incorporation of contemporary methodologies such as NicheCompass (<https://nichecompass.readthedocs.io/en/latest/>) would substantially strengthen their analysis. NicheCompass's integrated workflows for multi-sample analysis and superior batch effect handling could provide additional validation whilst potentially unveiling subtle niche variations across disease states.

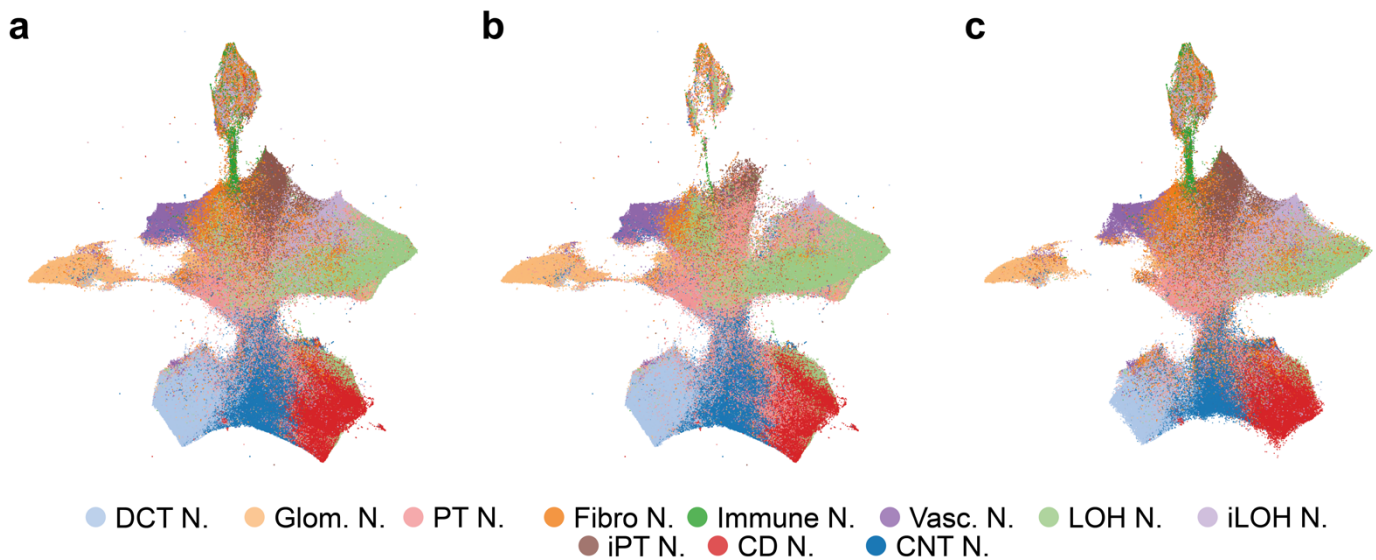
Response: We thank the reviewer for recommending NicheCompass. To infer spatially defined gene programs and cellular niches, we trained the NicheCompass model on all cells in the CosMx dataset, excluding the bottom 5% with the fewest neighbors to ensure sufficient tissue context. The model was initialized with raw count data, sample-specific categorical covariates, and a spatial adjacency matrix to enable edge-aware graph neural network learning. We used a graph attention convolutional layer (GATv2Conv) in the encoder and injected covariates into the decoder. Gene program (GP) activity thresholds were set using a relative cutoff (active_gp_thresh_ratio=0.01). The model was trained for 50 initial epochs and 25 refinement epochs using the following parameters: learning rate (lr=0.001), edge reconstruction penalty ($\lambda_{\text{edge_recon}}=5\times10^5$), gene expression reconstruction penalty ($\lambda_{\text{gene_expr_recon}}=300$), L1 regularization for novel GPs ($\lambda_{\text{L1_addon}}=30$), and edge sampling batch size of 1900. We computed a neighborhood graph and UMAP embedding from the latent representation and applied Leiden clustering, followed by manual annotation to define discrete cellular niches. Clustering revealed eight distinct niches that closely aligned with our prior annotation-based niches (Extended Fig. 14a, b). To identify niche-enriched gene programs, we performed differential testing using a log

Bayes factor threshold of 2. This analysis revealed niche-specific programs, such as *APOE* in the PT niche and *IL32*-associated programs in the iPT niche (Extended Fig. 14e).



The COVET implementation gives valuable insights but does not fully exploit the method's capabilities. The authors might consider examining gene-gene covariance matrices within niches to identify coordinated gene programmes, analyse spatial correlation structures to reveal niche-specific regulatory networks, and investigate transition zones between niches. Such analyses would provide deeper mechanistic insights into niche organisation and regulation, which would be a fantastic addition to their translational aim.

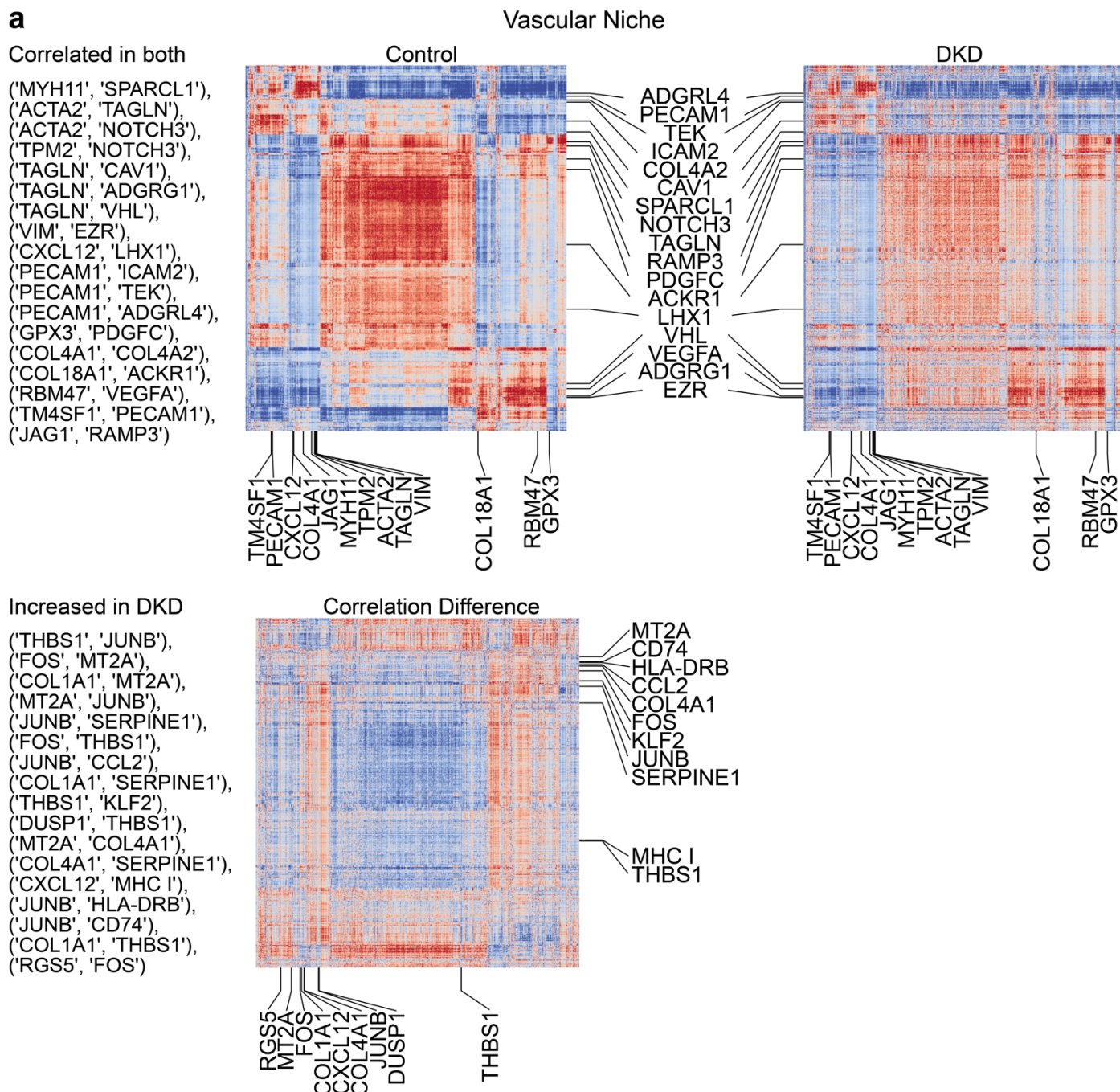
Response: We thank the reviewer for this recommendation. In the revised version of the manuscript, we employed the covariance environment (COVET) methodology on cells in the CosMx dataset, excluding the bottom 5% with the fewest neighbors to ensure sufficient tissue context. This approach incorporates the gene-gene covariate structure with spatial location considerations. Inputs to the COVET matrix included: (1) spatially resolved gene expression, (2) 64 highly variable genes (selected via Scanpy's `highly_variable_genes`), and (3) the number of spatial neighbors ($k = 8$). We computed an adjusted covariance matrix across the 64 genes to capture niche-specific co-expression patterns. Pairwise niche distances were then derived using Approximate Optimal Transport (AOT). Based on this distance matrix, we calculated Uniform Manifold Approximation and Projection (UMAP) for visualization after PCA which help to better examine the relationships within the COVET niche representation (Extended Fig. 13).



Extended Fig. 13: Orthogonal niche definition and visualization using COVET: We applied the recently published COVET method to define cellular niches based on spatial covariance of gene expression. Inputs to the COVET matrix included: (1) spatially resolved gene expression, (2) 64 highly variable genes (selected via Scanpy's `highly_variable_genes`), and (3) the number of spatial neighbors ($k = 8$). For each cell in the CosMx dataset (excluding the bottom 5% with the fewest neighbors to ensure sufficient tissue context), we computed an adjusted covariance matrix across the 64 genes to capture niche-specific co-expression patterns. Pairwise niche distances were then derived using Approximate Optimal Transport (AOT). Based on this distance matrix, we calculated UMAP (Uniform Manifold Approximation and Projection) for visualization after PCA which help to better examine the relationships within the COVET niche representation. Panels show UMAP embeddings of the resulting COVET adjacency matrices, with each point representing a single cell and colored by our annotation based niche annotations. **a:** UMAP for all patient samples combined. **b:** UMAP restricted to control patients. **c:** UMAP restricted to patients with diabetic kidney disease (DKD).

As an extension of this, we analyzed spatially correlated transcripts, using Hotspot for each niche, based on the niche-specific latent representations calculated by COVET analysis. This allowed us to dissect the two points raised by the reviewer. 1) it allowed us to investigate niche-specific regulatory networks: In both DKD patients and control patients, we found an overlap between spatially correlated transcripts. For example, in the vascular niche, which is shown here, we find that key vascular or mural gene pairs like *MYH11-SPARCL1*, *ACTA2-TAGLN*, *ACTA2-NOTCH3*, *PECAM1-TEK* to be correlated in both control as well as DKD patients. Interestingly, when we performed differential correlation analysis, we found increased correlations between transcript pairs such as *COL1A1-SERPINE1*, *CXCL12-MHC I*, and *JUNB-CD74*—implicated in matrix remodeling, immune signaling, and antigen presentation—in DKD (Extended Fig. 25).

In summary, using COVET and subsequently Hotspot, we could further identify conserved gene gene correlations within the defined niches, as well as disease-associated correlation shifts. We have performed this analysis for all niches and illustrated these across the Extended Fig. 15-25.



Extended Fig. 25: Spatially correlated transcripts in the vascular niche. Hotspot was applied to identify genes with spatially correlated expression patterns based on niche-specific latent representations calculated by COVET analysis. The heatmap displays pairwise gene-gene correlations, with stronger correlations indicated by deeper red coloring. Gene pairs conserved across both conditions are highlighted at the top. Differential correlation analysis revealed condition-specific interactions, with gene pairs significantly increased in diabetic kidney disease (DKD) highlighted (bottom).

Can the authors elaborate on the selection process for the cell-cell communication database? The cell-cell interaction analysis, currently limited to CellPhoneDB v5, would be considerably enhanced through integration with OmniPath database (<https://omnipathdb.org>). This would facilitate validation across multiple interaction datasets whilst providing valuable pathway context. Moreover, extending the analysis beyond iPT/iLOH-fibroblast interactions to encompass all identified niches would offer a more complete understanding of cellular communication within the kidney architecture.

Response: We thank the reviewer for this insightful and constructive comment. In our revised manuscript, we have significantly expanded the scope of our spatially resolved ligand-receptor interaction analysis, leveraging

the power of our large dataset and refined niche framework to uncover biologically and clinically relevant signaling networks across the human kidney.

To support this analysis, we selected **CellPhoneDB v5** as our core ligand–receptor reference. While we appreciate the broader pathway integration offered by databases such as OmniPath, we chose CellPhoneDB for its **highly curated, structured, and well-validated interaction list**, which is widely used and optimized for **cell-type-resolved interaction inference** in large-scale single-cell and spatial transcriptomics datasets. Its consistent formatting and broad validation across diverse tissues and species allowed us to perform systematic, reproducible, and interpretable inference across our spatially profiled human kidney samples.

Importantly, the scope of our interaction analysis is not restricted to IPT/iLOH–fibroblast interactions. In the revised manuscript, we have extended our ligand–receptor inference across **all 11 transcriptionally and anatomically defined spatial kidney niches**, each capturing distinct cellular architecture and functional context. This niche-based framework enables us to explore **context-dependent intercellular communication** that reflects the complex multicellular organization of kidney tissue in health and disease.

A major advancement in our revised analysis is the introduction of a **novel correlation-based framework** that correlates **ligand–receptor interaction strength with disease severity**. To our knowledge, this is the **first study to correlate spatially resolved intercellular communication with clinical parameters** such as estimated glomerular filtration rate (eGFR), and with molecular markers of epithelial injury (e.g., **HAVCR1**) and fibrosis (e.g., **COL1A1**). A key limitation of ligand-receptor analysis has been the lack of potential prioritization for disease relevant interaction. This new layer of analysis allows us not only to identify differentially active pathways in disease, but also to **prioritize interactions that track with disease progression**—offering mechanistic and translational insight into the biology of diabetic kidney disease (DKD).

For example, within the **glomerular niche**, we identified elevated **IL6–IL6R** signaling between glomerular endothelial cells and podocytes, suggesting a previously underappreciated inflammatory axis that may contribute to podocyte injury and glomerular dysfunction (Fig. 1p-r). Furthermore, **NRG1–ERBB3** interactions were positively correlated with eGFR, indicating potential homeostatic or reparative signaling, whereas **CXCL9–DPP4** and **PLAU–PLAUR** were strongly negatively associated with eGFR, suggesting roles in

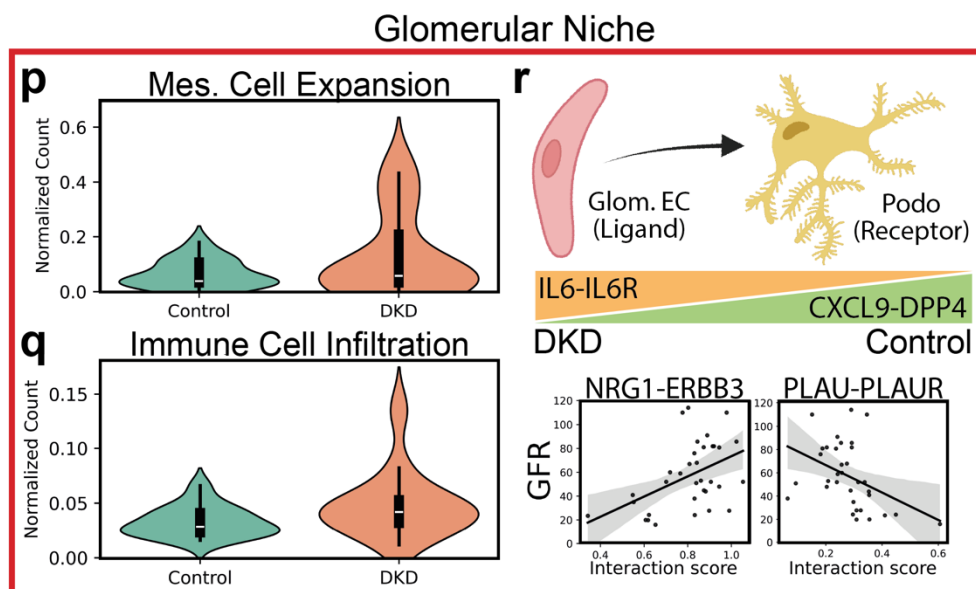


Fig. 2p: analysis of the glomerular niche: The fraction of mesangial cells in the glomerular was calculated and compared between control and DKD patients ($p < 0.05$). q: The fraction of immune cells in the glomerular was calculated and compared between control and DKD patients ($p < 0.05$). r: Ligand–receptor interactions originating from glomerular endothelial cells and targeting podocytes were inferred using CellPhoneDB v5, with microenvironments defined at the sample level. Analyses were performed using imputed gene expression to maximize sensitivity. Only statistically significant ligand–receptor pairs were retained for downstream analysis. Interaction scores were then correlated with estimated glomerular filtration rate (eGFR) across samples.

glomerular injury and DKD progression.

In the **profibrotic niche**, we extended our analysis to assess how epithelial–stromal interactions relate to matrix remodeling. We found that **HBEGF–EGFR** and **TNFSF10–TNFRSF10B** interactions between injured proximal tubule (iPT) cells and fibroblasts correlate strongly with HAVCR1, a marker of epithelial injury (Fig. 3o). Similarly, **JAG2–NOTCH2** and **PDGFB–PDGFRB** interactions were highly correlated with COL1A1 expression, implicating Notch and PDGF signaling in fibroblast activation and fibrosis. These interactions were further validated in an independent bulk RNA-seq DKD cohort, reinforcing their reproducibility and clinical relevance.

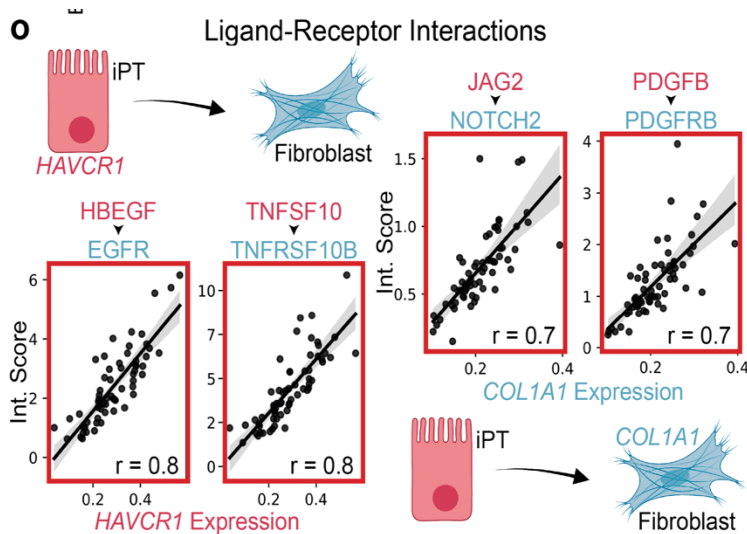


Fig. 3o: Left: Ligand–receptor interactions between injured proximal tubular cells and fibroblasts within the profibrotic microenvironment were inferred using CellPhoneDB v5 and the imputed gene expression. The interaction scores for significantly enriched pairs were then correlated with HAVCR1 expression in proximal tubular cells. Right: The same interaction scores were correlated with COL1A1 expression in fibroblasts. Each dot represents one sample.

We also expanded our analysis of **immune microenvironments**, integrating spatial transcriptomics and snRNA-seq data from over **250,000 immune cells** to define **seven distinct immune MEs**. Within these, we uncovered disease-enriched communication between plasma cells and injured tubular epithelial cells. Notably, we identified increased **TNFSF13–TNFRSF13B (APRIL–TACI)** and **CXCL12–CXCR4** signaling in DKD, pathways known to support plasma cell survival and recruitment (Fig. 4t–w). Correlation with **MZB1** expression in bulk transcriptomes confirmed their relevance to plasma cell localization and potential persistence in the DKD kidney (Extended Fig. 30).

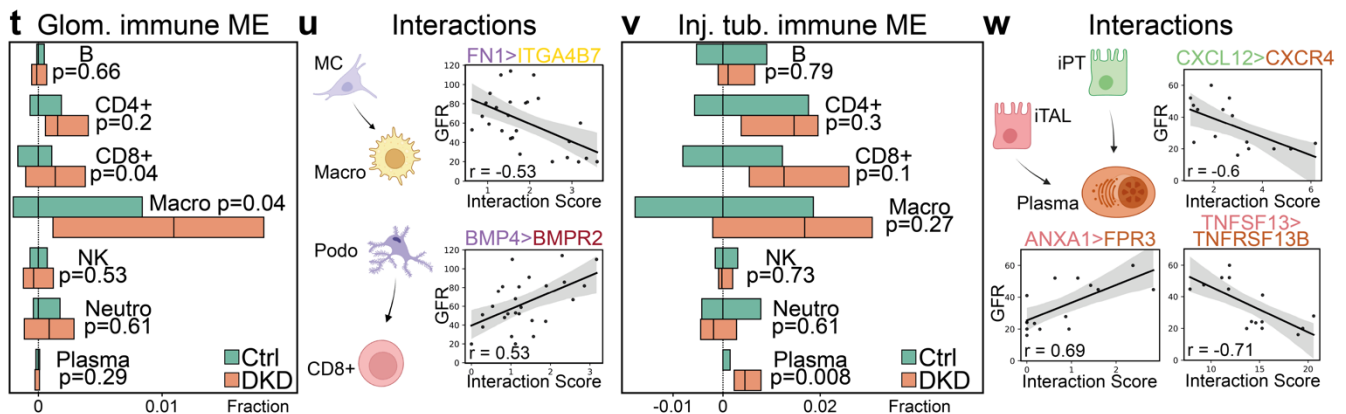


Fig. 4: t, Boxplots showing immune cell subtype frequencies in the glomerular immune microenvironment of control and DKD samples. Fractions were normalized per sample, centered by the control median, and compared using Welch's t-test. Samples with <50 glomerular cells were excluded.

u, Ligand–receptor interactions: For ligand–receptor interactions, we used CellphoneDB v5 with the imputed gene expression and correlated the resulting interaction scores in the indicated celltypes with the eGFR in control and DKD samples. **v**, Immune cell subtype fractions in the injured tubular immune microenvironment of control and DKD samples. Fractions were calculated per sample, centered by the control median, and compared using Welch's t-test. Samples with <50 cells were excluded. Results are shown as violin and boxplots. **w**, Ligand–receptor interactions: For ligand–receptor interactions, we used CellphoneDB v5 with the imputed gene expression correlated the resulting interaction scores in the indicated celltypes with the eGFR in control and DKD samples.

Collectively, this integrated analysis now offers a **comprehensive, spatially resolved map of intercellular signaling across the kidney**, incorporating both tissue-wide niches and focal microenvironments. The ability

to **link interaction dynamics with disease severity**, identify **clinically relevant signaling pathways**, and validate findings across independent datasets represents a substantial advancement over prior work.

While OmniPath may offer expanded pathway annotation for future systems-level modeling, our current approach prioritizes **spatial fidelity, analytic robustness, and clinical anchoring**—features central to translating spatial transcriptomic insights into actionable disease mechanisms.

In conclusion, we believe that our revised ligand–receptor interaction analysis, grounded in a spatial niche framework and strengthened by disease-stage correlation, significantly enhances the depth, rigor, and translational potential of the study. These results highlight the power of spatially anchored interaction profiling to uncover dynamic, disease-relevant signaling pathways across the complex tissue architecture of the human kidney.

The spatial data quality control requires more thorough documentation. The authors should delineate their criteria for filtering spatial coordinates, assessment of tissue processing artifacts, and validation of spatial patterns across replicates. Additionally, quality metrics specific to the CosMx platform should be explicitly stated.

Response: We thank the reviewer for raising this important point regarding spatial data quality control. In our revised manuscript, we have substantially expanded and clarified our quality control (QC) procedures to address pre-analytical, platform-specific, and computational steps applied to ensure the integrity, reproducibility, and biological interpretability of our spatial transcriptomics data.

Tissue Preparation and Pre-analytical Quality Control:

All nephrectomy specimens processed for CosMx and Xenium spatial transcriptomics underwent stringent pre-imaging quality control. Adjacent sections were stained with hematoxylin and eosin (H&E) and reviewed to confirm the presence of intact tissue architecture, absence of necrosis, hemorrhage, or fixation artifacts, and appropriate preservation of cortical and medullary compartments. To assess RNA quality, we extracted total RNA from adjacent sections and evaluated integrity using the Agilent TapeStation system. We applied a **DV200 threshold of >50%**, consistent with CosMx recommendations, ensuring that the majority of RNA fragments exceeded 200 nucleotides in length and were suitable for hybridization-based detection. Only samples passing this threshold were included in spatial profiling.

Field of View (FOV) Selection and Artifact Avoidance:

To minimize the impact of tissue processing artifacts and ensure accurate spatial mapping, for the CosMx runs, **FOVs were manually selected** during imaging setup by trained personnel. Selection criteria included:

- Preservation of nuclear and cytoplasmic morphology
- Even and specific staining without excessive background fluorescence
- Absence of folding, edge tearing, detachment from the slide, or sectioning artifacts
- Visual confirmation of anatomical diversity (e.g., inclusion of glomeruli, tubules, and vasculature)

This manual curation ensured that only morphologically intact and biologically relevant regions were profiled.

Post-segmentation Cell Filtering and Coordinate Cleaning:

Following image processing and transcript assignment, we applied additional filters to remove low-quality cells and spatial artifacts:

- Cells with <30 total transcripts were excluded to eliminate low-complexity detections likely arising from segmentation errors or low RNA content.
- To mitigate edge effects in spatial analyses—including niche detection, microenvironment clustering, and cell–cell interaction modeling—cells in the bottom 5% with the fewest spatial neighbors were excluded to ensure sufficient tissue context.

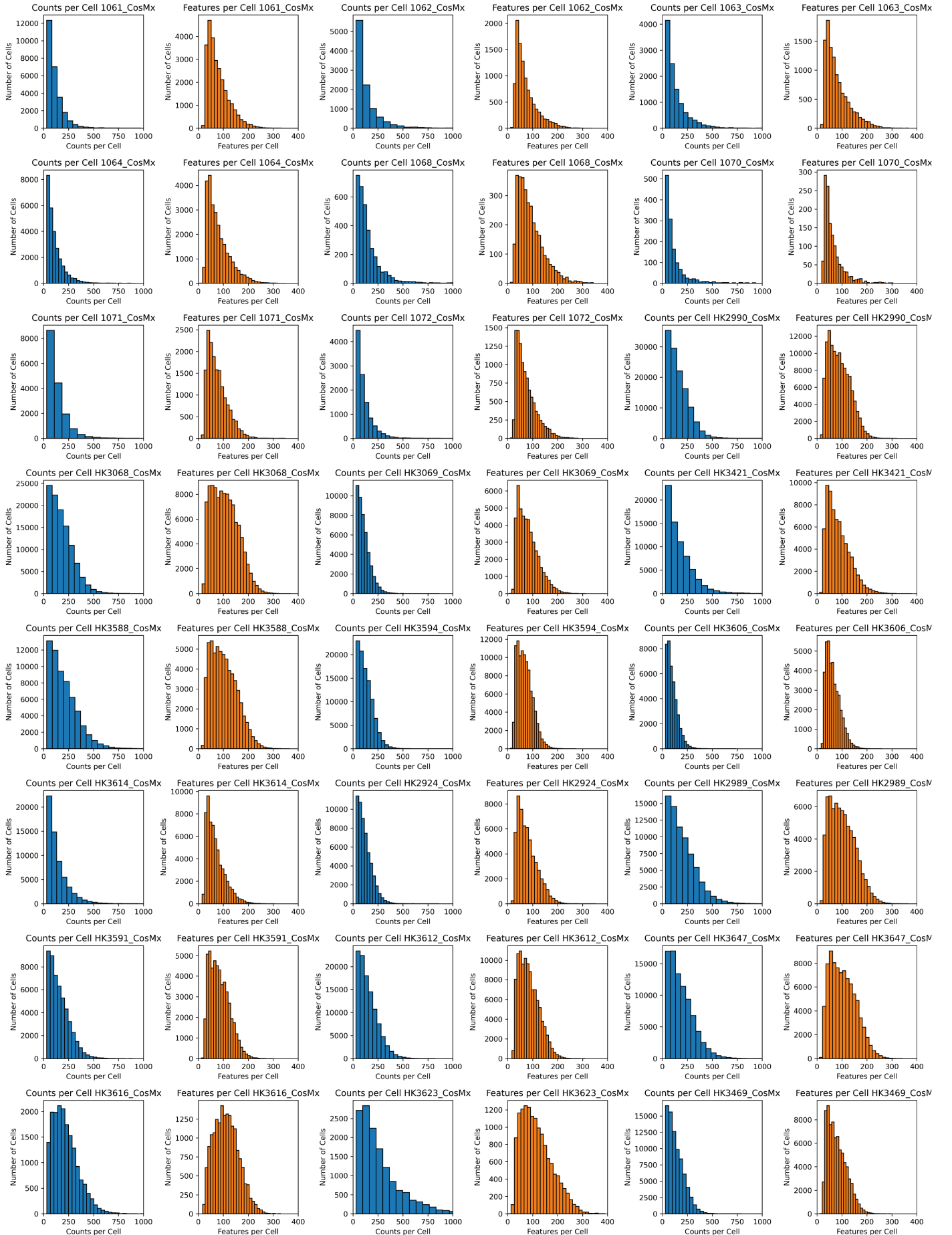
We have added the number of transcripts and features for each of our CosMx as well as Xenium samples (Extended Fig. 5-7)

Cross-platform validation:

After Leiden clustering and annotation of the spatial dataset, we observed that key marker genes are conserved across technologies (Extended Data Fig. 3). Moreover, we compared celltype fractions across three samples that were profiled using Xenium and CosMx. We find that overall, the celltype fractions are similar in both datasets as depicted in Extended Fig. 36a and b. Moreover, we find that niche fractions are similar in both datasets for these samples, too (Extended Fig. 36 c, d), supporting the biological stability and reproducibility of our spatial annotations.

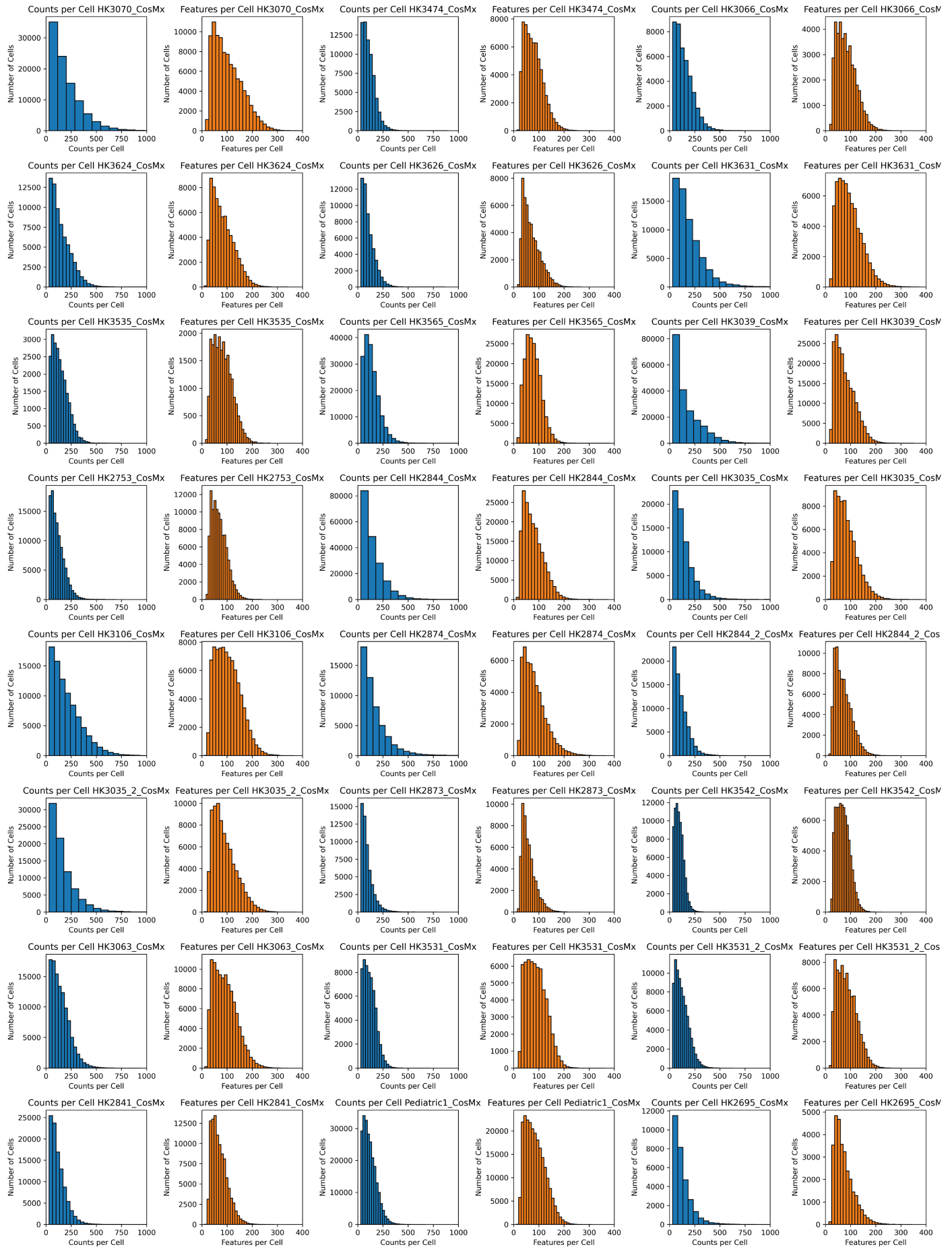
a

Counts and Features CosMx (I/II)



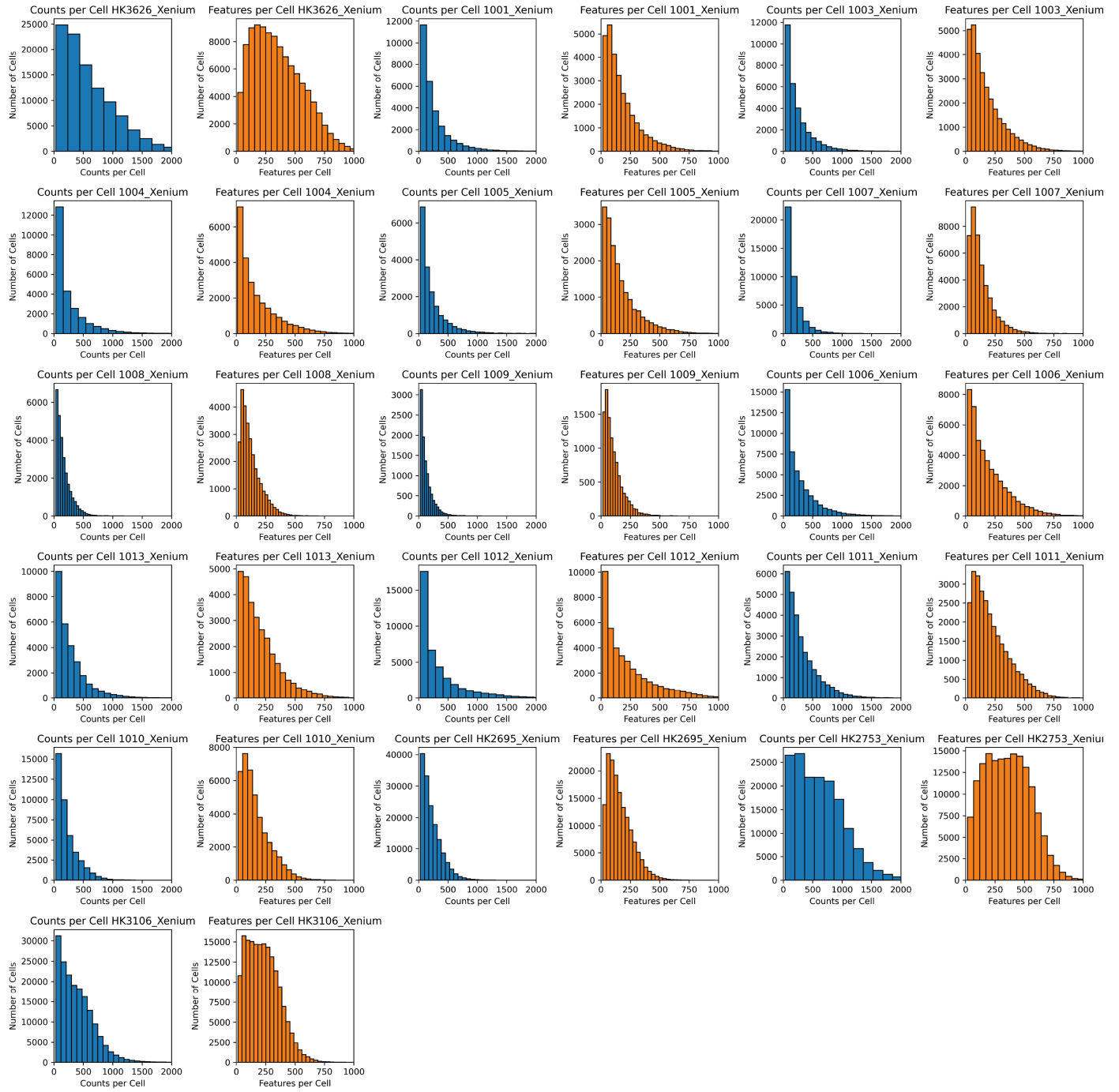
b

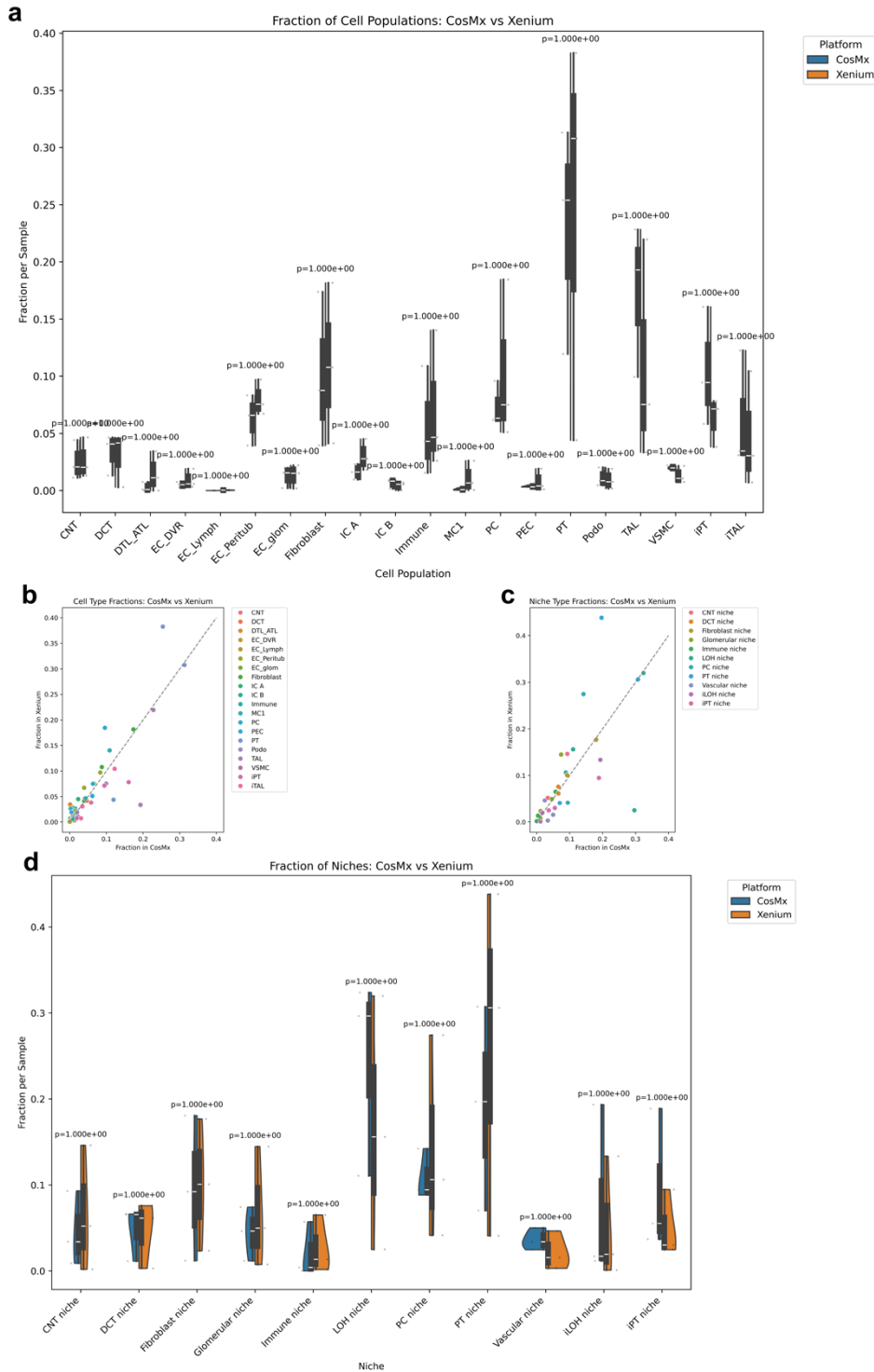
Counts and Features CosMx (II/II)



c

Counts and Features Xenium 5k





Extended Fig. 36: To compare cell population representation across spatial transcriptomics platforms, we selected kidney samples profiled by both CosMx and Xenium (HK2695, HK3626, HK3106). Cells from these samples were subset from the full dataset, and the cell type annotation (annotation_updated) was retained. Each cell was labeled by its corresponding platform and patient identifier. For each sample and technology, we computed the fraction of cells assigned to each cell type by dividing cell type counts by the total number of cells per sample and platform. The resulting fractions were reshaped into a paired format to enable direct comparison between CosMx and Xenium measurements per cell type and donor. Statistical comparisons were performed using the two-sided Wilcoxon signed-rank test for each cell type. Fractions were visualized using split violin plots with overlaid strip plots to show paired values across platforms. Adjusted p-values were annotated for each cell population comparison. c, d: To compare niche representation across spatial transcriptomics platforms, we selected kidney samples profiled by both CosMx and Xenium (HK2695, HK3626, HK3106). Cells from these samples were subset from the full dataset, and the cell type annotation (annotation_updated) was retained. Each cell was labeled by its corresponding platform and patient identifier. For each sample and technology, we computed the fraction of cells assigned to each cell type by dividing cell type counts by the total number of cells per sample and platform. The resulting fractions were reshaped into a paired format to enable direct comparison between CosMx and Xenium measurements per cell type and donor. Statistical comparisons were performed using the two-sided Wilcoxon signed-rank test for each cell type. Fractions were visualized using split violin plots with overlaid strip plots to show paired values across platforms. Adjusted p-values were annotated for each cell population comparison.

The current GitHub repository would benefit from detailed workflows, preprocessing protocols, and clear parameter justifications. The inclusion of validation steps, example outputs, and quality control metrics would ensure reproducibility. At the moment, it looks like a collection of scripts to generate the figures. Formalising them into notebooks would facilitate reproducibility and aid with clarity. Moreover, there is no information on the environments used for each step of the analysis.

Response: We thank the reviewer for this comment, we have now included all scripts for the analysis and a readme as well as links to download the docker images that were used for the analysis (<https://github.com/Woopsydaisy/Spatial-Human-Kidney-Map>).

The manuscript would benefit from a more thorough discussion of spatial resolution limitations. The authors should address the CosMx platform's resolution constraints, their impact on niche boundary definition, and potential artifacts in densely packed regions. Validation of spatial patterns at different resolution scales would strengthen their conclusions.

Response: We thank the reviewer for this important comment regarding spatial resolution limitations. We agree that resolution is a critical consideration in interpreting spatial data, particularly when defining niche boundaries or analyzing densely packed regions. While the CosMx platform offers high spatial resolution (~1 μ m), limitations persist—especially in highly cellular or fibrotic regions—due to potential segmentation errors, cell overlap, and proximity artifacts.

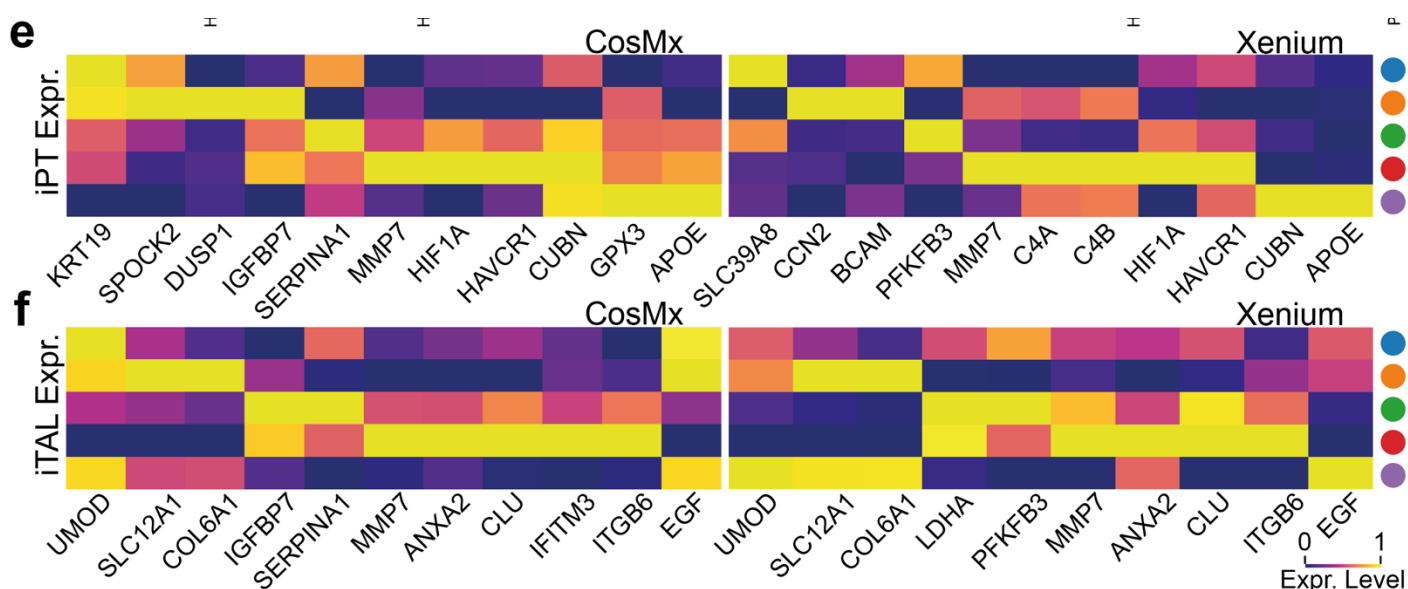


Fig. 3e: Heatmap of key differentially expressed genes of iPT center cells in MEs in the CosMx dataset (left) and Xenium dataset (right). f, Heatmap of key differentially expressed genes of iLH center cells in MEs in the CosMx dataset (left) and Xenium dataset (right). The differentially expressed (DE) genes were calculated using the Wilcoxon rank-sum test. CosMx and Xenium datasets were analyzed separately due to their differing gene panel sizes.

To mitigate these effects, we implemented a multi-layered quality control and validation framework throughout data generation and analysis. During preprocessing, we **excluded low-confidence cells**, such as those with low transcript counts or ambiguous segmentation, and **removed edge-region artifacts** that could distort spatial patterns or niche boundary definition. We also filtered out cells in the bottom 5% with the fewest spatial neighbors were excluded to ensure sufficient tissue context during niche and microenvironment analyses, minimizing edge and sparsity effects.

Recognizing the limitations of any single platform, we **integrated spatial data from two complementary high-resolution platforms—CosMx 1k and Xenium 5k—across 64 spatially profiled samples**. This fourfold expansion of spatial sampling (~4.3 million spatially resolved cells) enabled us to validate key findings across distinct technologies with different resolution profiles and gene panel sizes (1,000 vs. 5,100 genes). Despite these differences, we observed **remarkable concordance** between CosMx and Xenium datasets in both transcriptional profiles and spatial patterns. For example, **injured proximal tubule (iPT) and thick ascending limb (iLH) cells** showed consistent upregulation of **HAVCR1, MMP7, ANXA2, and ITGB6**, reinforcing the robustness of our spatially defined injury signatures (Fig. 3e-f).

The **definition of niche boundaries**—particularly in regions of high cellular density—was further supported by cross-platform validation and integration with orthogonal datasets. For instance, our characterization of a **profibrotic microenvironment**, enriched in immune and fibroblast neighbors, was not only consistent across CosMx and Xenium, but also **spatially colocalized with histological fibrosis** (as shown by Sirius Red staining, see manuscript Fig. 3n), and showed strong correlation with kidney function (eGFR; $r = -0.6$, $p < 0.001$, see manuscript Fig. 3c), reinforcing its biological and clinical relevance.

Densely packed regions posed additional challenges, especially in the context of immune cell aggregates. In particular, the **B cell-predominant immune microenvironment**, a TLS-like structure enriched in B cells, plasma cells, and CD4⁺ T cells, warranted special attention (see manuscript Fig. 5). To rigorously validate its composition and organization:

- We leveraged **Xenium 5k** to detect **CXCL13** and **CXCR5**, key markers of lymphoid tissue organization.
- We confirmed the co-expression of **TNFSF13 (BAFF)** and **TNFRSF13C (BAFF-R)**, indicating a B cell survival niche using Xenium 5k.
- We applied **imaging mass cytometry (IMC)** using a custom antibody panel (e.g., CD20, CD38, CD4, AQP1) to validate cell-cell spatial proximity at subcellular resolution.
- We further used **CosMx's ability to detect immunoglobulin isotypes (IgD, IgM, IgG, IgA)** to verify the presence of naïve and class-switched B cells.
- Finally, we integrated spatial data with a published single-cell RNA-seq B cell atlas using **scVI** and **scANVI**, which revealed a predominance of memory and naïve B cells, further corroborated by IMC staining for **CD27**, **CD11c**, and **BCL6**.

Through this multi-modal, multi-platform approach, we not only confirmed the presence of discrete spatial immune niches but also established their cellular composition and functional relevance with **transcriptional, spatial, and proteomic data**.

We fully acknowledge that **no spatial transcriptomic platform currently offers perfect positional accuracy**. However, by combining **orthogonal spatial technologies, rigorous filtering, large-scale replication, and multi-modal validation**, we have taken extensive steps to ensure that our spatial boundaries and niche definitions are both **technically sound and biologically meaningful**.

We now include this discussion in the revised manuscript to clarify our approach and highlight the measures taken to ensure robustness of spatial inference across different resolution scales.

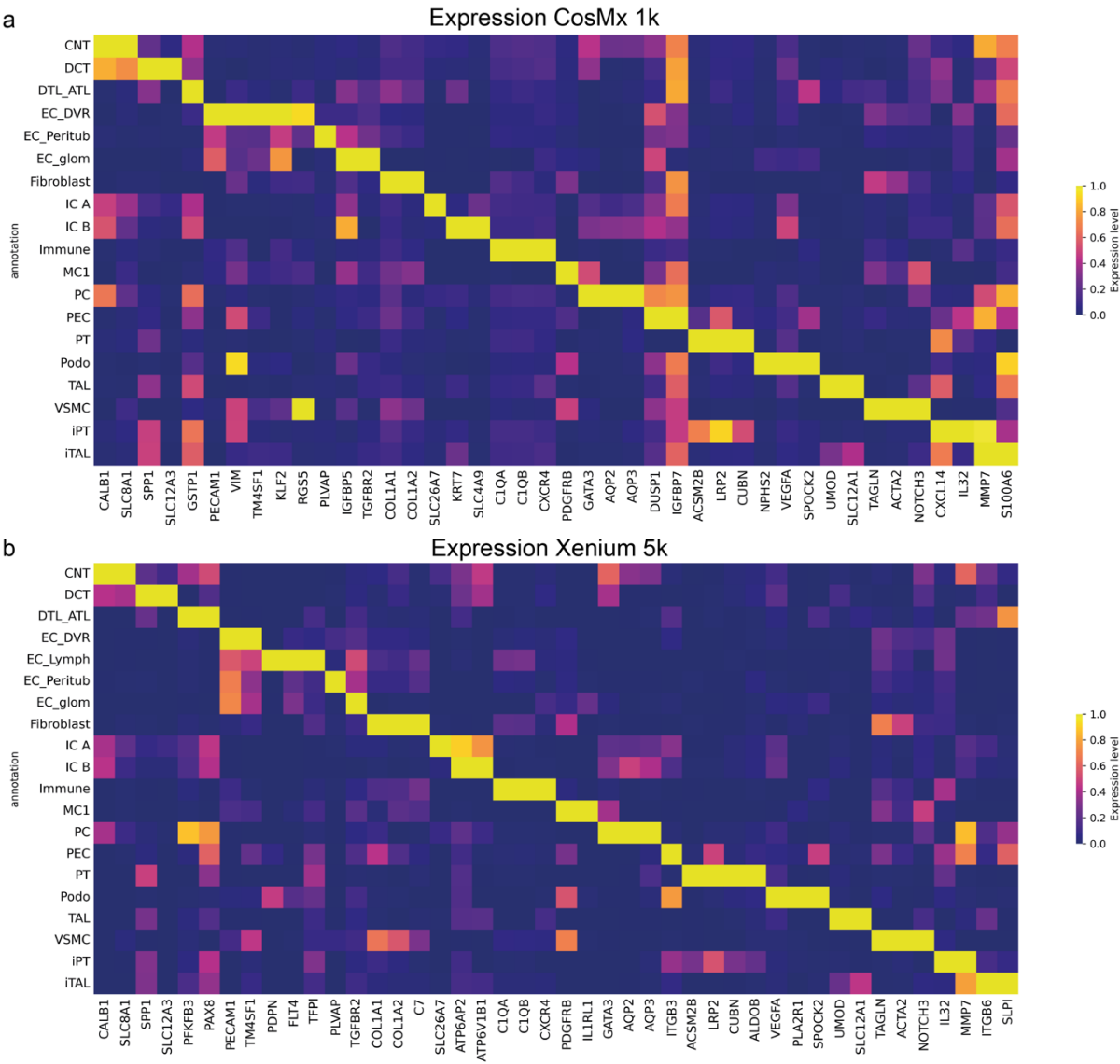
The construction of the reference space for annotation transfer warrants a more detailed explanation. Specifically, the selection criteria for anchor genes, validation of transfer accuracy, and assessment of reference space stability should be thoroughly documented. This ties back to point #1.

Response: We thank the reviewer for this important comment and fully agree that a detailed explanation of the construction and validation of the reference space used for annotation transfer is essential for transparency and reproducibility. In the revised manuscript, we have **substantially expanded both the dataset and the analysis**. Specifically, we integrated **newly generated Xenium 5k spatial transcriptomics data** and **quadrupled the number of spatially profiled samples**, leading to a unified analysis of over 5 million cells, including more than 4 million spatially resolved cells. This increased scale enabled a more rigorous construction and validation of the reference space and improved the robustness of annotation transfer across diverse samples and platforms.

Reference Space Construction: To generate a harmonized and biologically meaningful embedding across spatial and non-spatial modalities, we used **scVI**, a deep generative probabilistic model that corrects for batch effects while capturing biological variation. scVI was applied to integrate our expanded **single-nucleus RNA-sequencing (snRNA-seq) reference dataset from 150 individuals (~750,000 cells)** with the spatial transcriptomics data from both **CosMx 1k (n = 48 samples)** and **Xenium 5k (n = 16 samples)** platforms. The model used only genes shared across platforms, ensuring modality compatibility while minimizing technical bias.

Annotation Transfer Strategy: We then used **scanVI**, a semi-supervised extension of scVI, to propagate high-quality cell type labels from the snRNA-seq reference to the spatial datasets. This method avoids reliance on manually curated “anchor genes” and instead utilizes the **entire shared gene set** to model transcriptional similarity, providing a more flexible and unbiased label transfer framework that generalizes well across technologies. Labels were inferred probabilistically, allowing for confidence-aware assignments that reflect uncertainty in classification, particularly important when integrating across platforms with differing gene panels (e.g., 1,000 genes for CosMx vs. 5,100 for Xenium). After final integration of the combined datasets, UMAP and leiden clustering was calculated based on the scanVI latent space and celltypes annotated.

Validation of Transfer Accuracy: To validate annotation quality, we examined canonical marker gene expression across the inferred cell types in both CosMx and Xenium datasets. Markers such as **SLC5A2 (proximal tubule)**, **NPHS2 (podocytes)**, **CXCR4, C1QA (Immune cells)**, and **PECAM1 (endothelial cells)** were consistently enriched in their expected cell types across both platforms. These results are presented in **Extended Fig. 3 and Extended Fig. 28** and demonstrate that annotations are both platform-consistent and biologically meaningful. Furthermore, we observed high concordance in cell type frequency distributions across biological replicates and technologies, further supporting the reliability of our label transfer pipeline. Similarly,



Extended Fig. 3a: Expression of selected marker genes across annotated cell types in the CosMx dataset. For each gene, expression was averaged per cell type and scaled to a 0–1 range using MinMax normalization. The color intensity represents the relative expression level of each gene across cell types. Visualization was performed using the Seaborn heatmap function. **b:** Expression of selected marker genes across annotated cell types in the Xenium dataset. For each gene, expression was averaged per cell type and scaled to 1 as demonstrated in **Extended Fig. 36** we found no significant differences between celltype and niche annotations between the CosMx and Xenium platform when comparing samples that were assayed by both technologies.

In summary, the revised analysis incorporates a significantly expanded dataset—including **new Xenium data and additional CosMx samples**—and uses a robust probabilistic framework (scVI/scANVI) for integration and annotation transfer. The resulting cell type annotations are well-supported by canonical markers, consistent across platforms, and stable across replicates. These updates substantially improve the transparency, reproducibility, and generalizability of our cell type annotation approach. All methodological details and validation analyses are now included in the revised Methods and Supplementary Materials.

Minor Comments:

The visualisation of spatial data is informative but could be enhanced through multi-scale representations of niches, three-dimensional reconstructions where feasible, and explicit demonstration of transition zones between niches. The inclusion of validation visualisations would strengthen the presentation of results.

Response: We thank the reviewer for this thoughtful suggestion. We agree that enhanced spatial visualization is essential for interpreting tissue organization and strengthening the biological relevance of spatial transcriptomic results. In response, we have expanded and refined our data presentation across **Figures 1 through 5** to improve multi-scale visualization, incorporate additional layers of validation, and highlight transitions and spatial continuity across anatomical structures.

Multi-scale Visualization:

To facilitate interpretability across tissue scales, we now present spatial data at **three levels of resolution**:

1. **Kidney-wide representations** showing the anatomical distribution of major spatial niches across the cortex and medulla.
2. **Regional zoom-ins** to highlight the structure and transitions between adjacent niches.
3. **Cellular-level views** that illustrate niche-specific cell neighborhoods and intercellular interactions.

For example:

- **Figure 1** now includes a **side-by-side comparison of CosMx and Xenium platforms**, highlighting differences in spatial resolution and transcriptomic depth, which helps contextualize the level of detail in downstream analyses.
- **Figure 2** provides **multi-scale niche maps**, combining broad cortical-medullary coverage with **zoom-ins of niche transition zones**—e.g., between proximal tubule-dominant and immune-infiltrated regions—to illustrate spatial remodeling in disease.
- **Figures 3 and 4** focus on **tubular and immune microenvironments**, respectively, with emphasis on visualizing dense cellular niches and their local architecture. In **Figure 3**, we show side-by-side comparisons of **transcriptionally defined profibrotic microenvironments and Sirius Red-stained sections**, enabling histological validation of fibrosis-associated spatial domains.

Validation Across Modalities:

To reinforce our spatial inferences, we incorporated multiple **orthogonal validation modalities**, including:

- **Imaging mass cytometry (IMC)** to confirm the spatial proximity and composition of key cell types (e.g., CD4⁺ T cells, CD20⁺ B cells, CD38⁺ plasma cells);
- **Histological staining** (e.g., Sirius Red) to validate fibrotic zones.
- **Canonical marker expression** in both CosMx and Xenium platforms to validate transcriptional identity of spatially defined cell types.

These layers of validation are presented in **Figures 3–5**, demonstrating that our transcriptionally defined spatial niches correspond to known histological and immunological features, and are reproducible across technologies.

Clarification on 3D Reconstructions:

We appreciate the reviewer's interest in three-dimensional representations. However, we would like to clarify that our study is based on **human kidney biopsy and nephrectomy samples**, many of which were obtained through **clinical protocols**. These tissue sections are typically **limited to a thickness of ~1 mm or less**, and often represent **scarce diagnostic material**. As such, **true volumetric 3D reconstruction**—which would require serial sectioning, optical clearing, or thick tissue blocks—is not feasible in this setting due to both **technical limitations** and **constraints in sample availability**.

The reproducibility framework would benefit from the provision of a containerised analysis environment, comprehensive package versioning, and documented parameter sensitivity analyses. The inclusion of validation datasets would facilitate independent verification of the authors' findings.

Response: We thank the reviewer for this comment, we have now included the link for docker images that were used for the analysis. Specifically, a docker image for scVI/scANVI, spatial analysis, cellphoneDB, xeniumanalysis and nichecompass.

In the opinion of this reviewer, these enhancements would substantially strengthen this otherwise commendable study of kidney tissue architecture and disease progression.

Referee #2 (Remarks on code availability):

The reproducibility framework would benefit from the provision of a containerised analysis environment, comprehensive package versioning, and documented parameter sensitivity analyses. The inclusion of validation datasets would facilitate independent verification of the authors' findings.

Referee #3 (Remarks to the Author):

Summary

Dumoulin and colleagues utilize advances in spatial transcriptomic to build a single-cell atlas of the human kidney in health and chronic kidney diseases, with the focus of the latter upon diabetic kidney injury. The authors integrate spatial transcriptomic analysis with matched snRNAseq dataset and mapped the snRNAseq annotations to the spatial transcriptomic dataset, generating a high-resolution spatial map that captures the finely-tuned changes in gene expression across numerous cell types in different regions of the kidney. Using this atlas, the group defines specific parenchymal and immune cell niches via their cellular composition that are present in healthy kidneys as well as niches that are specific to disease. They go on to define the cellular microenvironments surrounding these presumably injured regions of the kidney, reporting cell types associated with disease, highlighting increased B cells, and report markers associated with pathogenesis. Most importantly, the work defines patient subgroups based on associations with niche composition, fulfilling an unmet need of classification of chronic kidney diseases that are highly heterogenous in their clinical presentation and course. Lastly, the authors combine serum and kidney proteomics to generate potential biomarkers derived from the aforementioned niches and validate these markers with prediction of disease course.

Significance

Understanding the complex cellular landscape of the kidney is crucial for the mechanistic understanding of renal physiology in health and disease. The authors provide the largest spatial single cell atlas of the kidney yet, performing analysis of large regions rather than previously-reported spot areas. Previously, the Susztak lab reported broad cellular environments within the kidney but now go on to further classify these microenvironments as distinct niches, with unique cellular composition where gene expression varies depending on specific location within the kidney, highlighting disease-specific niches. While it remains to be seen how these niches hold true in other settings of other kidney diseases, such as acute, autoimmune, and inflammatory injury, the authors make advances including the documentation of gene expression changes in the same cell type along the corticomedullary axis which reflect cell type changes to meet tissue and organ function, the integration of nuclear/cellular RNAseq data with matched spatial transcriptomic data, and the identification of unique cellular niches that are associated with disease. Furthermore, the largest conceptual advance comes from their use of the spatial niches to subdivide patients as this may bode for personalized treatment strategies. The combination of niche composition with kidney and serum proteomics to map disease states to specific cellular locations is impressive as it provides a framework in which spatial transcriptomics can be combined with proteomics (especially serum considering ease-of-sampling) and can be used to not only to identify patients with complex and heterogenous diseases into specific subgroups, but also to investigate the underlying cellular and molecular drivers of disease. While the paper falls somewhat short in providing mechanistic link underpinning what the authors describe, the paper is impactful as it provides a detailed roadmap for using these types of omics studies to stratify patients with complex/heterogeneous diseases.

Points for the authors to consider

1) While the identification of niches corresponding to “injured” cells is crucial, it would be informative to know how these injured niches (iPT and iLOH) compare to normal niches in their gene expression differences. While the supplementary tables contain what the cluster defining genes are, it should be stated in the text how the authors define iPT and PT and what the differentially expressed genes between iPT vs PT and iLOH and LOH. This is a analysis that should be stated.

Response: We thank the reviewer for this insightful comment and appreciate the opportunity to clarify our framework for defining and characterizing injury-associated spatial niches, specifically the **injured proximal tubule (iPT)** and **injured loop of Henle (iLOH)** niches. In the revised manuscript, we have now provided an in-depth explanation of how these niches were identified, how they differ from their uninjured counterparts, and how their **transcriptional signatures are conserved across analytical methods, platforms, and biological replicates**. This analysis highlights the **robustness, disease specificity, and biological relevance** of these spatial states.

Distinction Between Cell Types and Niches:

We first emphasize that our study makes a clear distinction between **cell type annotation** and **niche identification**. Cell types were defined using **scVI** and **scANVI**, integrating spatial transcriptomic data from CosMx (n = 48) and Xenium (n = 16) platforms with a comprehensive **single-nucleus RNA-seq reference dataset from 150 individuals** (~750,000 cells, Fig 1). scVI constructed a latent space capturing biological heterogeneity while correcting for batch and platform effects, and scANVI applied a semi-supervised label transfer framework to assign probabilistic cell type labels. Based on the scANVI latent space, leiden clustering was performed and celltypes annotated. These annotations were validated using canonical markers (e.g., **SLC8A1, CXCR4, PLVAP, PECAM1**) and were highly consistent across platforms (Extended Fig. 3).

Based on this, we now explicitly state what we define as IPT / iTAI cells and performed differentially expressed gene analysis (Extended Fig. 26 a-d). In **CosMx**, IPT cells with higher expression of **HAVCR1, MMP7, IGFBP7, IL32, TPM1, VCAM1, CLU, SOX4, CRYAB**, and **TNFRSF12A**, while PT cells retained expression of **SLC13A3, CUBN, LRP2, VEGFA, APOE**, and **PPARA**. In **Xenium**, IPT cells showed a highly overlapping injury profile, including **HAVCR1, IGFBP7, MMP7, IL32, ANXA3, TNC, VIM, IFITM3**, and **PIGR**, with PT cells expressing **ALDOB, AZGP1, SLC22A8, ACE**, and **ANGPTL3** (Extended Fig. 26).

In the TAL vs iLOH comparison, CosMx data revealed higher expression of **MMP7, SLPI, CLU, ANXA2, KRT19, TPM1, ITGB6**, and **TIMP1** in iLOH, with uninjured TAL cells maintaining expression of **UMOD, EGF, SLC12A1**, and **ESRRB**. Xenium again confirmed these patterns, with iLOH cells expressing **GABRP, PROM1, SOX9, ANXA2**, and **MMP7**, while TAL cells expressed **PTGER3, ATP1B1**, and **UMOD** (Extended Fig. 26).

Importantly, these transcriptional injury signatures were **highly consistent between CosMx (1,000 genes) and Xenium (5,100 genes)**, despite differences in gene panel size and detection sensitivity. Key injury markers—including **HAVCR1, MMP7, IGFBP7, TPM1, VCAM1, and SOX4** (IPT), and **MMP7, CLU, ANXA2, TPM1** (iLOH)—were robustly and reproducibly upregulated across both platforms, demonstrating strong **cross-platform reproducibility**.

In contrast, **niches were identified independently of predefined cell type labels**, using three complementary computational methods: **COVET** (Extended Fig. 13), **NicheCompass** (Extended Fig. 14), and **MiniBatch K-means** clustering of spatial neighborhoods. These methods operate at the level of **spatially organized, multicellular structures**, allowing us to capture recurrent tissue states that emerge from the spatial arrangement and interaction of diverse cell types. Each niche reflects not just a collection of cells, but a functionally and spatially coherent tissue pattern. We emphasize that **the IPT and iLOH niches are increased in abundance** in diabetic kidney disease (DKD), indicating that these are **disease-specific spatial architectures**, not mere transcriptional variants of normal cell types.

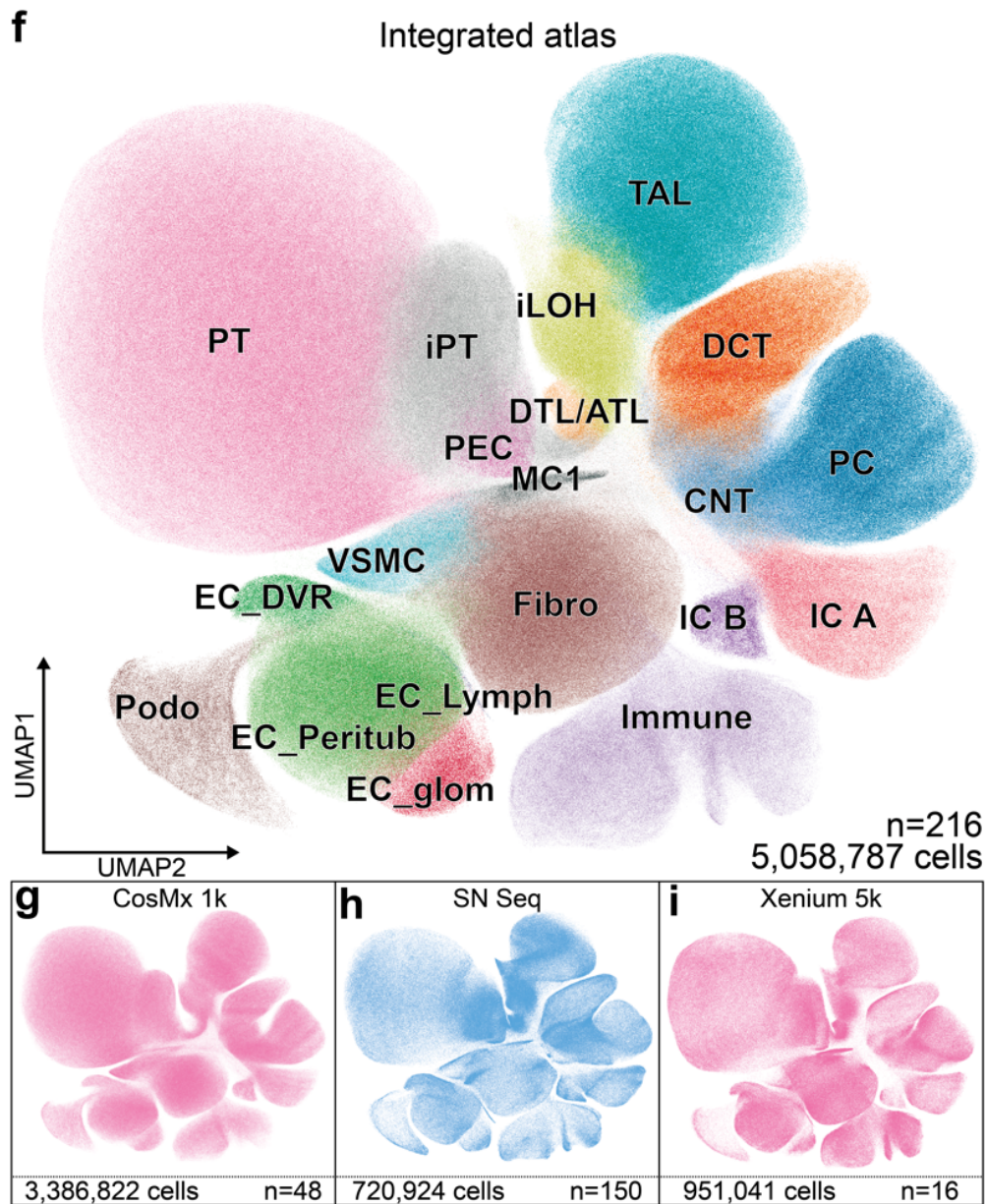
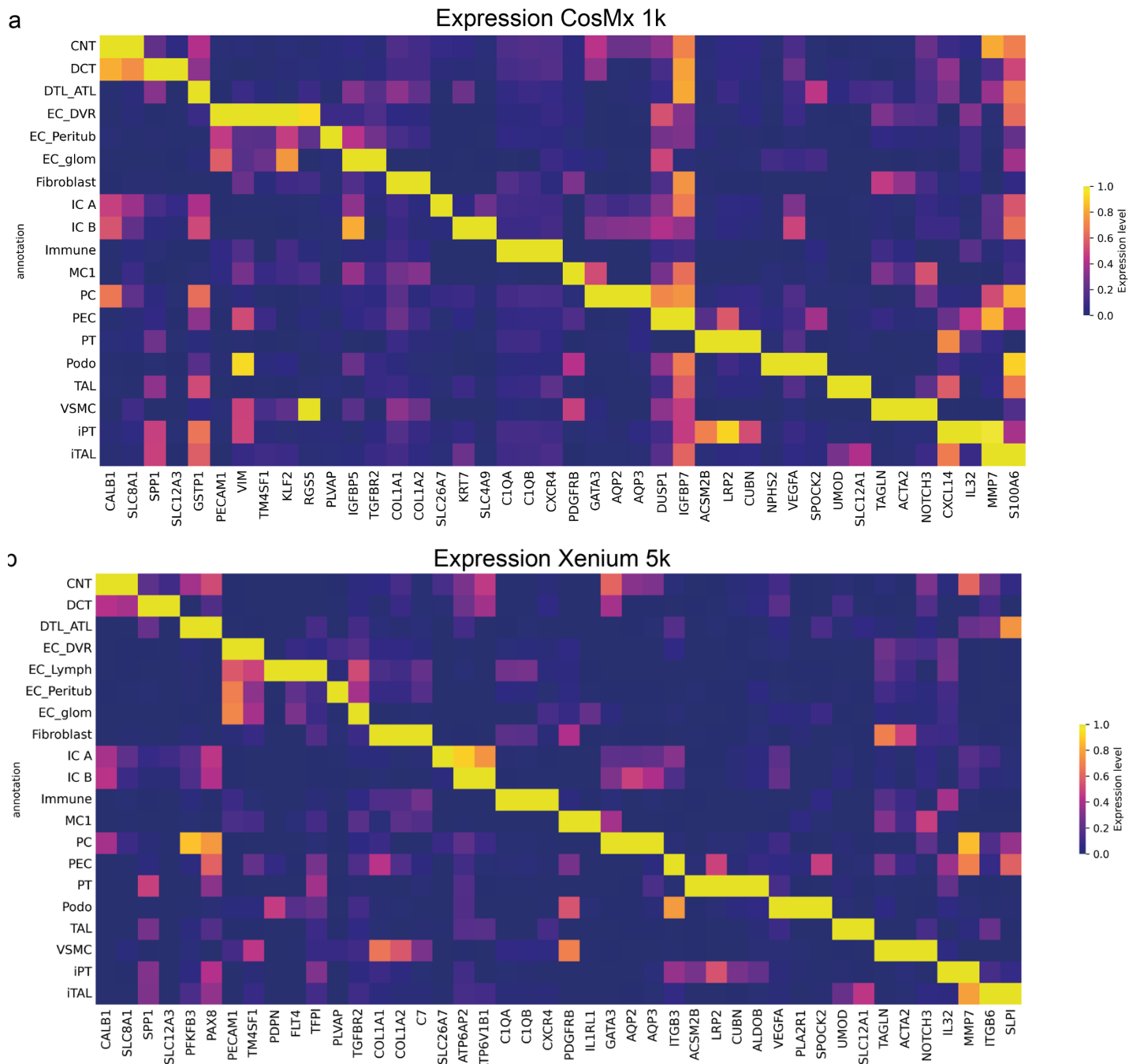


Fig. 1f: The UMAP visualization of the integrated spatial single-cell atlas. The spatial gene expression (CosMx 1k and Xenium 5k data) was integrated with dissociated single nucleus gene expression using scVI and scanVI. Each dot represents a single cell.

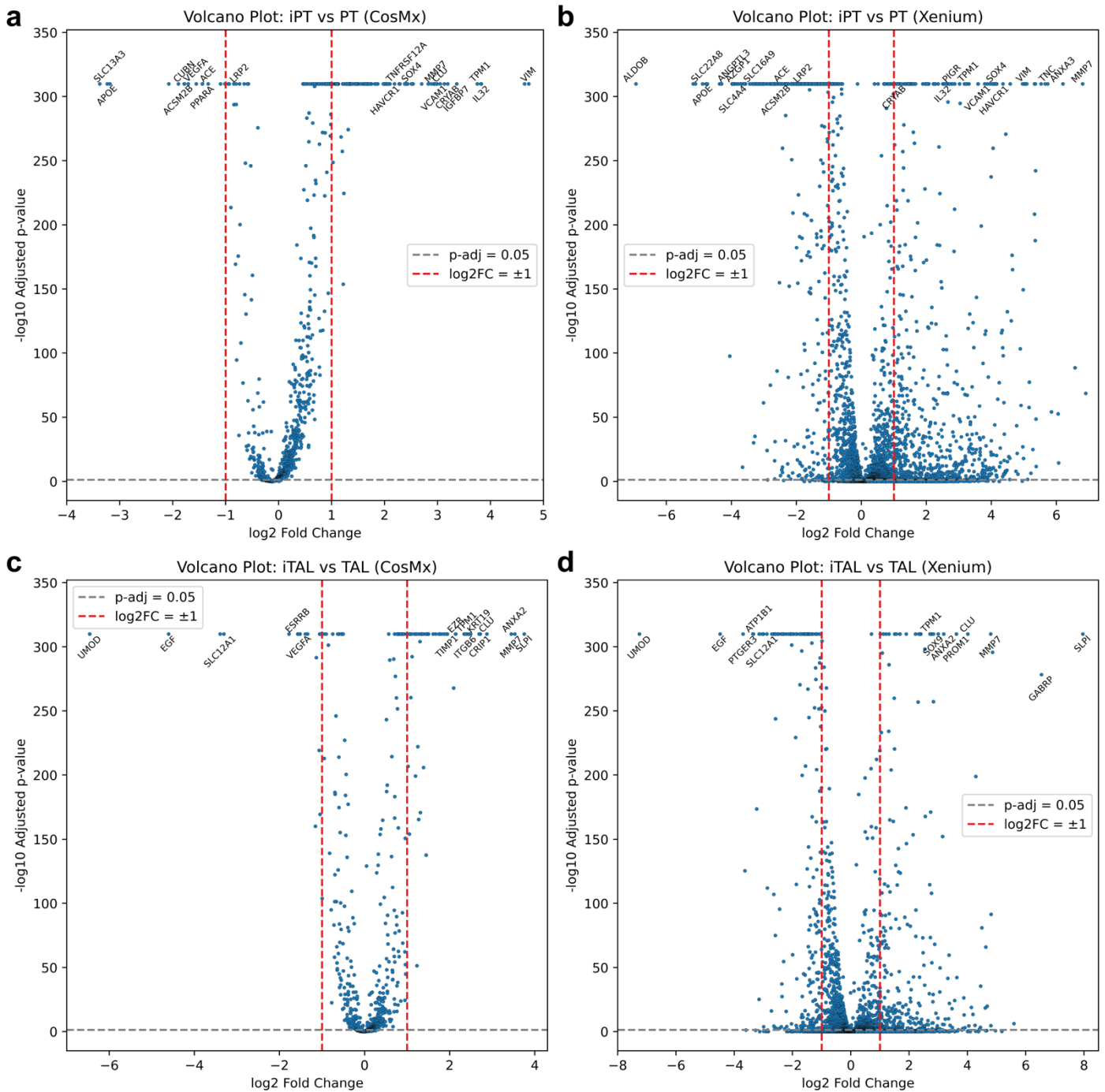
g, CosMx 1k spatial gene expression data. 48 samples and 3,386,822 cells were included in this study.

h, snRNA-seq data. 150 samples and 720,924 cells were included in this study.

The color indicates the different cell types such as: CNT, connecting tubular cells; VSMC, vascular smooth muscle cell; PC, principal cell; IC A, IC B, intercalated cells A, B; inj T, injured tubular cells; PT, proximal tubular cells; MC1, mesangial cell; LOH, thick ascending limb cells as well as DTL_ATL, distal thin limb, ascending thin limb cells; Fibro, fibroblasts; Podo, podocytes; DCT, distal convoluted tubular cells; PEC, parietal epithelial cells; EC: EC_Peritub, peritubular endothelial cells as well as EC_DVR, vasa recta endothelial cells; Immune, immune cells; EC_glom, glomerular endothelial cells.



Extended Fig. 3a: Marker gene expression of each cell population in the CosMx dataset. Mean expression of each population was calculated and scaled from 0 to 1 and depicted as a heatmap. b: Marker gene expression of each cell population in the Xenium dataset. Mean expression of each population was calculated and scaled from 0 to 1 and depicted as a heatmap.



Extended Fig. 26a: Volcano plot comparing gene expression between iPT and PT in CosMx dataset. Differential expression analysis was performed using the Wilcoxon rank-sum test between iPT and PT cells in the CosMx dataset, following normalization and log-transformation. Each point represents a gene, plotted by its log2 fold change (x-axis) and $-\log_{10}(\text{adjusted p-value})$ (y-axis). Selected biologically relevant genes are labeled. b: Volcano plot comparing gene expression between iPT and PT in Xenium dataset. Differential expression analysis was performed using the Wilcoxon rank-sum test between iPT and PT cells in the Xenium dataset, following normalization and log-transformation. Each point represents a gene, plotted by its log2 fold change (x-axis) and $-\log_{10}(\text{adjusted p-value})$ (y-axis). Selected biologically relevant genes are labeled. c: Volcano plot comparing gene expression between iLOH and TAL in CosMx dataset. Differential expression analysis was performed using the Wilcoxon rank-sum test between iLOH and TAL cells in the CosMx dataset, following normalization and log-transformation. Each point represents a gene, plotted by its log2 fold change (x-axis) and $-\log_{10}(\text{adjusted p-value})$ (y-axis). Selected biologically relevant genes are labeled. d: Volcano plot comparing gene expression between iLOH and TAL in Xenium dataset. Differential expression analysis was performed using the Wilcoxon rank-sum test between iLOH and TAL cells in the Xenium dataset, following normalization and log-transformation. Each point represents a gene, plotted by its log2 fold change (x-axis) and $-\log_{10}(\text{adjusted p-value})$ (y-axis). Selected biologically relevant genes are labeled.

2) Figure 1: Validation of spatially variable genes would be helpful, showing the differences in protein expression along the cortical and medullary sides (panel f).
 Response: We thank the reviewer for requesting additional protein-level validation. After careful consideration we concluded that we cannot generate a uniformly convincing protein map for every spatially variable gene

within the scope of this revision. To avoid presenting an incomplete or potentially misleading validation we have removed the spatially variable gene panel altogether and have withdrawn the related text.

3) Figure 2: The presence of *iPT* in control samples and diabetic samples without kidney involvement warrants further validation of the injured niches and is not mentioned in the text (panel h). The authors show fraction increases in DKD but it would also be informative to show what niches decrease in DKD (panel i).

Response: We thank the reviewer for this thoughtful and important comment. We have now revised the manuscript to better clarify how **injured niches are defined**, how they relate to kidney health and disease, and how their prevalence correlates with clinical severity. We also now discuss **niches that are decreased** in diabetic kidney disease (DKD), addressing both ends of the pathological spectrum.

First, we would like to clarify that the “control” samples used in this study were defined as kidneys with **normal estimated glomerular filtration rate (eGFR)** and **no overt histological abnormalities** on light microscopy. However, it is important to acknowledge that many of these samples are derived from **adult human nephrectomy specimens**, which may harbor **subclinical or focal injury**, particularly due to aging. In fact, the presence of low-level **HAVCR1 (KIM-1)** expression in both tissue and circulation has been shown to be a **sensitive early biomarker** of tubular injury and is predictive of future kidney function decline, even in patients with otherwise normal kidney function. **iPT and iLOH niches were absent in the rightmost samples**, which derive from **two pediatric patients with histologically pristine kidneys** and normal function (HK3565_C and Pediatric1_C, Fig. 2b). Thus, the rare detection of **injured proximal tubule (iPT)** cells or niches in some control samples may reflect **mild, subclinical injury (and/or aging)**, consistent with clinical experience and prior biomarker studies.

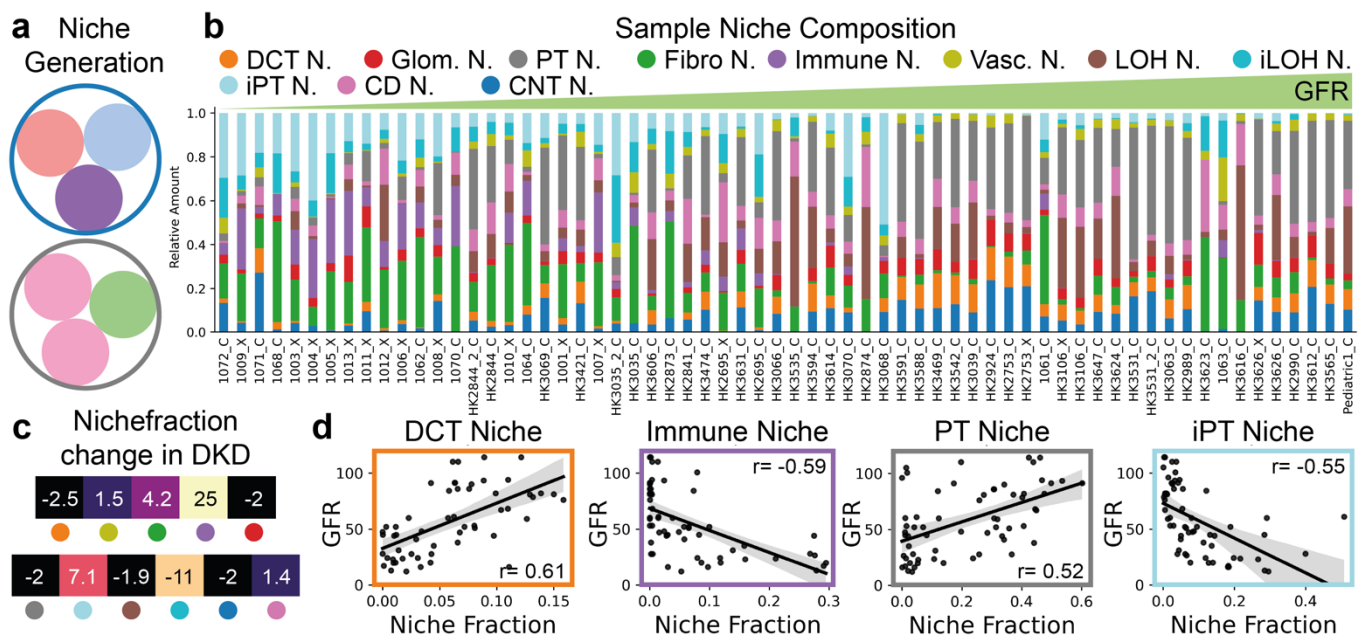


Fig 2a: Schematic of defining niches (left) and microenvironments (right). For niches, all cells were clustered into groups based on their neighborhood fraction after restricting the analysis to those with sufficient local context, removing the sparsest 5% of neighborhoods (based on total neighbor count at 80 μ m) to reduce edge effects ($n = 4,029,331$ cells). For each cell, we computed the number of neighboring cells within concentric distance shells: 0–20 μ m, 20–40 μ m, 40–60 μ m, and 60–80 μ m. This was achieved by calculating cumulative neighbor counts at 20, 40, 60, and 80 μ m, followed by subtracting counts from the smaller radius to isolate the number of neighbors in each respective shell (e.g., the 40–60 μ m shell = count at 60 μ m – count at 40 μ m). For each cell, neighbor counts were normalized by the total number of neighbors at that distance, yielding a cell-by-neighbor composition matrix that reflects the relative cellular makeup of the local environment at different spatial scales. This matrix was then scaled and subjected to principal component analysis (PCA), with the top 10 components used as input for MiniBatch K-means clustering. The number of clusters (k) was determined based on the elbow method. Niche clustering was performed on the entire dataset to define global spatial neighborhoods. b: Sample niche composition: the fraction of each niche is calculated for all cells within each sample and visualized. The samples are arranged by disease severity (GFR) from left to right. c: Change in relative niche fraction in DKD vs healthy controls. Each value represents the fold change in the average proportion of a given niche between DKD patients and healthy controls. A positive value indicates that the niche is expanded in DKD, while a negative value indicates it is more prevalent in controls. Fold changes were computed by normalizing niche frequencies within each diagnostic group, then taking the ratio of DKD to control values. d, correlation of niche fraction with eGFR using Pearson correlation. All correlations with $p < 0.001$.

To assess this systematically, we substantially expanded our dataset to include **64 spatially profiled samples**, using two complementary high-resolution spatial platforms—**CosMx 1k (n=48; ~3.3 million cells)** and **Xenium 5k (n=16; ~1 million cells)**—integrated with single-nucleus RNA-seq data from 150 individuals (~750,000 cells), yielding a unified atlas of over **5 million cells**, including more than **4 million with spatial coordinates**.

Injured and uninjured proximal tubule (PT/iPT) and loop of Henle (TAL/iLOH) cells were distinguished based on clustering in the scANVI-integrated latent space and manual curation using canonical marker genes. **Uninjured PT and TAL cells** expressed **segment-specific transport and metabolic markers** (e.g., **LRP2, CUBN, PPARA** for PT; **UMOD, SLC12A1, EGF** for TAL), while **iPT and iLOH clusters** expressed **injury- and stress-response genes** including **HAVCR1, MMP7, IGFBP7, VCAM1, ANXA2, and CLU**. These injury-associated states were consistently observed across **both CosMx and Xenium platforms**, confirming **cross-platform reproducibility**.

To examine the spatial organization of these cell states, we defined **tissue-level spatial niches** using a data-driven approach based on **cell neighborhood composition**. This analysis included all >4 million high-quality cells after excluding those with insufficient local neighborhood context and removing the sparsest 5% of neighborhoods (based on total neighbor count at 80 μ m) to reduce edge effects. We computed a normalized neighbor-type vector and applied **MiniBatch K-means clustering** to identify recurrent, spatially cohesive multicellular patterns. This yielded **11 transcriptionally and anatomically distinct niches**, including both canonical structures (e.g., glomerular, vascular, homeostatic epithelial) and **injury-associated niches** such as **iPT and iLOH**.

We next assessed **sample-level niche composition** by calculating the fraction of cells belonging to each niche. These are visualized in **Fig. 2b**, where samples are ordered by eGFR. In this analysis, **iPT and iLOH niches were nearly absent in the rightmost samples**, which derive from **two pediatric patients with histologically pristine kidneys** and normal function (HK3565_C and Pediatric1_C). In contrast, **iPT and iLOH fractions increased dramatically in DKD samples**—by **7-fold and 11-fold**, respectively (Fig. 2c). This pattern strongly supports the **disease specificity** of these niches.

The reviewer also asked whether **niches decrease** in DKD—a key point that we have now addressed more explicitly in the revised text and figures. In **Fig. 2i**, we show the correlation between niche abundance and eGFR. Notably:

- The **iPT niche** was **strongly negatively correlated** with eGFR ($r = -0.55$, $p < 0.001$), consistent with its expansion in more advanced disease (Fig. 2d).
- In contrast, **homeostatic epithelial niches**, including those enriched for **proximal tubule (PT)** and **distal convoluted tubule (DCT)** cells, showed **strong positive correlations** with eGFR (PT: $r = 0.52$; DCT: $r = 0.61$; both $p < 0.001$), reflecting their **progressive depletion** in DKD (Fig. 2d).

These analyses indicate that kidney disease progression is marked not only by **the emergence of pathological spatial niches** (e.g., iPT, iLOH), but also by the **loss of normal epithelial organization**, suggesting a shift from homeostatic to maladaptive tissue states.

To further support the relevance of our niche assignments, we observed that:

- The **transcriptional signatures** of injured niches were **highly consistent across platforms** (CosMx and Xenium, Extended Fig. 26).
- The **spatial composition** of these niches was **reproducible across multiple DKD donors**.
- The niche definitions were **stable across analytical methods**, including **COVET (Extended Fig 13), NicheCompass (Extended Fig 14), and MiniBatch K-means**.

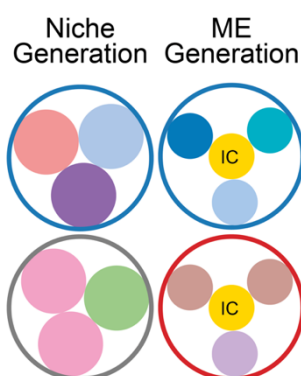
We have updated the main text to reflect these clarifications, and we thank the reviewer for prompting a deeper discussion of niche dynamics across the health–disease spectrum.

4) Figure 3: While the authors define microenvironments surrounding the injured niches, it would be interesting to see if and how microenvironments around the other niches change in DKD patients. In 3j, the authors should consider further dissection of the immune cell composition shared between iPT ME4 and iLOH ME1. What are the predominant immune cell types shared between these microenvironments?

Response: We thank the reviewer for this insightful comment. In response, we have expanded our analysis to examine the transcriptional shifts **niches** undergo in diabetic kidney disease (DKD) and have provided a detailed characterization of the **immune cell composition in the injured tubular microenvironment**.

Our analysis now leverages a **substantially expanded dataset** consisting of **64 spatially profiled human kidney samples**, spanning both healthy controls and DKD patients, profiled with **two complementary high-resolution spatial transcriptomics platforms: CosMx 1k (n=48; ~3.3 million cells) and Xenium 5k (n=16; ~1 million cells)**. These were integrated with **single-nucleus RNA-seq data from 150 individuals (~750,000 cells)** to construct a unified atlas of over **5 million cells**, including more than **4 million with spatial coordinates**. To go beyond individual cell-type analysis, we defined **spatial niches** as multicellular tissue structures based on neighborhood composition using MiniBatch K-means clustering. To ensure robust context, we included only cells with sufficient local neighborhood density, removing the sparsest 5% of neighborhoods (based on total neighbor count at 80 μm), resulting in 4,029,331 cells. This yielded 11 globally defined spatial niches, including both canonical structures (e.g., glomeruli, vasculature) and disease-associated niches such as **iPT** and **iLOH**.

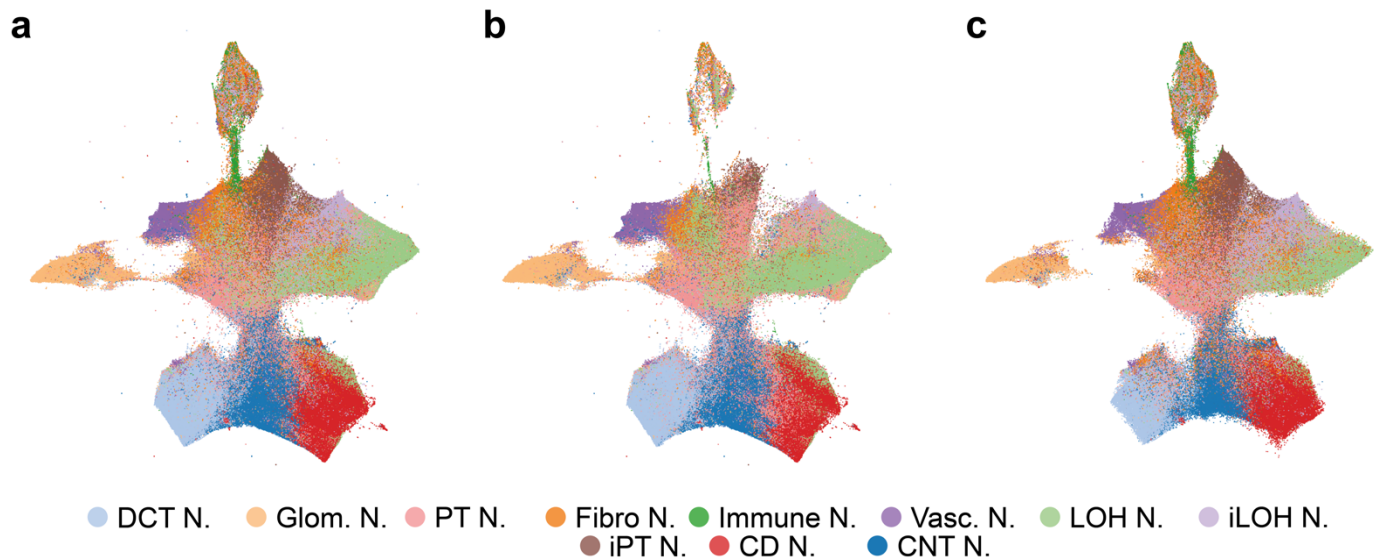
To complement this, we also defined **cell-centric microenvironments (MEs, Schematic right side)**, which reflect the immediate spatial surroundings of a specific focal cell (e.g., an injured epithelial or immune cell). For each focal cell, we calculated the normalized composition of cell types within spatial shells (20–80 μm), and clustered these local neighborhoods using MiniBatch K-means to uncover recurring spatial motifs. This dual-level analysis—niche and microenvironment—captures both tissue-scale organization and cell-specific



Schematic: Schematic of defining niches (left) and microenvironments (right). For niches, all cells were clustered based on their neighborhood fraction after restricting the analysis to those with sufficient local neighborhood density, removing the sparsest 5% of neighborhoods (based on total neighbor count at 80 μm) to reduce edge effects ($n = 4,029,331$ cells). For each cell, we computed the number of neighboring cells within concentric distance shells: 0–20 μm , 20–40 μm , 40–60 μm , and 60–80 μm . This was achieved by calculating cumulative neighbor counts at 20, 40, 60, and 80 μm , followed by subtracting counts from the smaller radius to isolate the number of neighbors in each respective shell (e.g., the 40–60 μm shell = count at 60 μm – count at 40 μm). For each cell, neighbor counts were normalized by the total number of neighbors at that distance, yielding a cell-by-neighbor composition matrix that reflects the relative cellular makeup of the local environment at different spatial scales. This matrix was then scaled and subjected to principal component analysis (PCA), with the top 10 components used as input for MiniBatch K-means clustering. The number of clusters (k) was determined based on the elbow method. Niche clustering was performed on the entire dataset to define global spatial neighborhoods. For microenvironments classification was restricted to specific subsets of cells: iPT and iLOH cells for

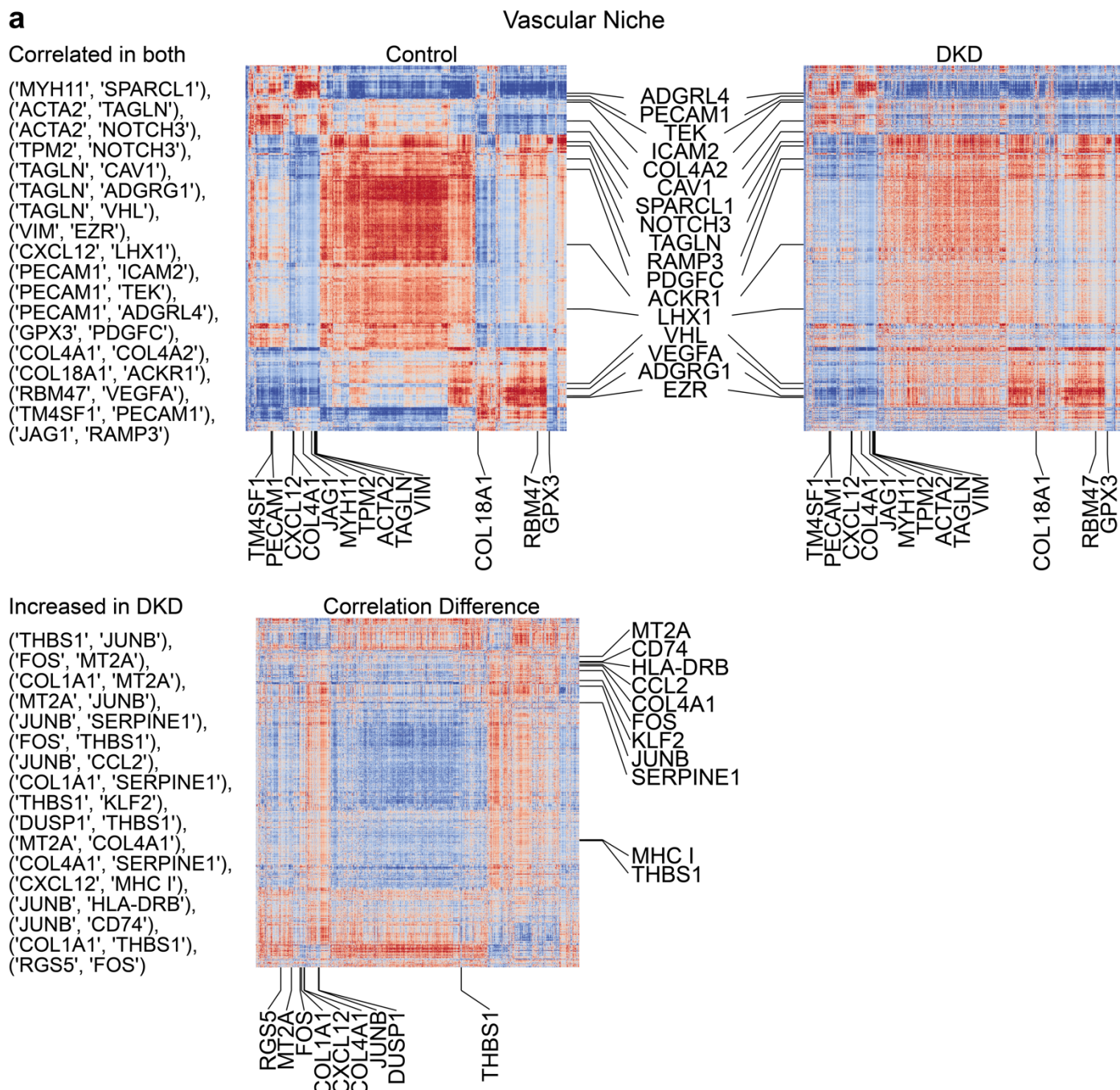
contextual cues.

As an orthogonal approach to defining cellular niches defined through MiniBatch K-means clustering, we employed the covariance environment (COVET) methodology on cells in the CosMx dataset, excluding the bottom 5% with the fewest neighbors to ensure sufficient tissue context. This approach incorporates the gene-gene covariate structure with spatial location considerations. Inputs to the COVET matrix included: (1) spatially resolved gene expression, (2) 64 highly variable genes (selected via Scanpy's `highly_variable_genes`), and (3) the number of spatial neighbors ($k = 8$). We computed an adjusted covariance matrix across the 64 genes to capture niche-specific co-expression patterns. Pairwise niche distances were then derived using Approximate Optimal Transport (AOT). Based on this distance matrix, we calculated Uniform Manifold Approximation and Projection (UMAP) for visualization after PCA which help to better examine the relationships within the COVET niche representation (Extended Fig. 13).



Extended Fig. 13: Orthogonal niche definition and visualization using COVET: We applied the recently published COVET method to define cellular niches based on spatial covariance of gene expression. Inputs to the COVET matrix included: (1) spatially resolved gene expression, (2) 64 highly variable genes (selected via Scanpy's `highly_variable_genes`), and (3) the number of spatial neighbors ($k = 8$). For each cell in the CosMx dataset (excluding the bottom 5% with the fewest neighbors to ensure sufficient tissue context), we computed an adjusted covariance matrix across the 64 genes to capture niche-specific co-expression patterns. Pairwise niche distances were then derived using Approximate Optimal Transport (AOT). Based on this distance matrix, we calculated UMAP (Uniform Manifold Approximation and Projection) for visualization after PCA which help to better examine the relationships within the COVET niche representation. Panels show UMAP embeddings of the resulting COVET adjacency matrices, with each point representing a single cell and colored by our annotation based niche annotations. **a:** UMAP for all patient samples combined. **b:** UMAP restricted to control patients. **c:** UMAP restricted to patients with diabetic kidney disease (DKD).

To address the reviewer's question and illustrate changes in niches across conditions, we analyzed spatially correlated transcripts for each niche, using Hotspot based on the niche-specific latent representations calculated by COVET analysis. This allowed us to investigate niche-specific regulatory networks: In both DKD patients and control patients, we found an overlap between spatially correlated transcripts. For example, in the vascular niche, which is shown here, we find that key vascular or mural gene pairs like MYH11-SPARCL1, ACTA2-TAGLN, ACTA2-NOTCH3, PECAM1-TEK to be correlated in both control as well as DKD patients. On the other hand, using the Hotspot analysis we were now able to highlight gene pair correlation shifts in DKD using differential correlation analysis. In the vascular niche, we found increased correlations between transcript pairs such as *COL1A1-SERPINE1*, *CXCL12-MHC I*, and *JUNB-CD74*—implicated in matrix remodeling, immune signaling, and antigen presentation—in DKD (Extended Fig. 25). In summary, using COVET and subsequently Hotspot, we could further identify conserved gene-gene correlations within all the defined niches, as well as disease-associated correlation shifts. We have performed this analysis for all niches and illustrated these across the Extended Fig. 15-25.



Extended Fig. 25: Spatially correlated transcripts in the vascular niche. Hotspot was applied to identify genes with spatially correlated expression patterns based on niche-specific latent representations calculated by COVET analysis. The heatmap displays pairwise gene-gene correlations, with stronger correlations indicated by deeper red coloring. Gene pairs conserved across both conditions are highlighted at the top. Differential correlation analysis revealed condition-specific interactions, with gene pairs significantly increased in diabetic kidney disease (DKD) highlighted (bottom).

To address the reviewer's question around the immune cell composition around the injured tubular cells, we generated a dedicated kidney immune atlas by integrating spatial transcriptomics data with single-nucleus RNA sequencing, capturing over 250,000 immune cells. To investigate how immune cells are spatially arranged in the human kidney, we clustered immune cells based on the composition of their immediate cellular neighborhoods. This analysis revealed seven distinct immune MEs enriched for specific neighboring cell types, including fibroblasts (Fig. 4f, m), injured tubular epithelial cells (Fig. 4k, r), vascular cells (Fig. 4i, p), and glomerular cells (Fig. 4j, r). Accordingly, we named these microenvironments the fibroblast-associated immune ME, injured tubular immune ME, vascular immune ME, and glomerular immune ME. In addition, we identified two immune-dense MEs. One was predominantly composed of CD4⁺ T cells, with CD8⁺ T cells and a smaller proportion of B

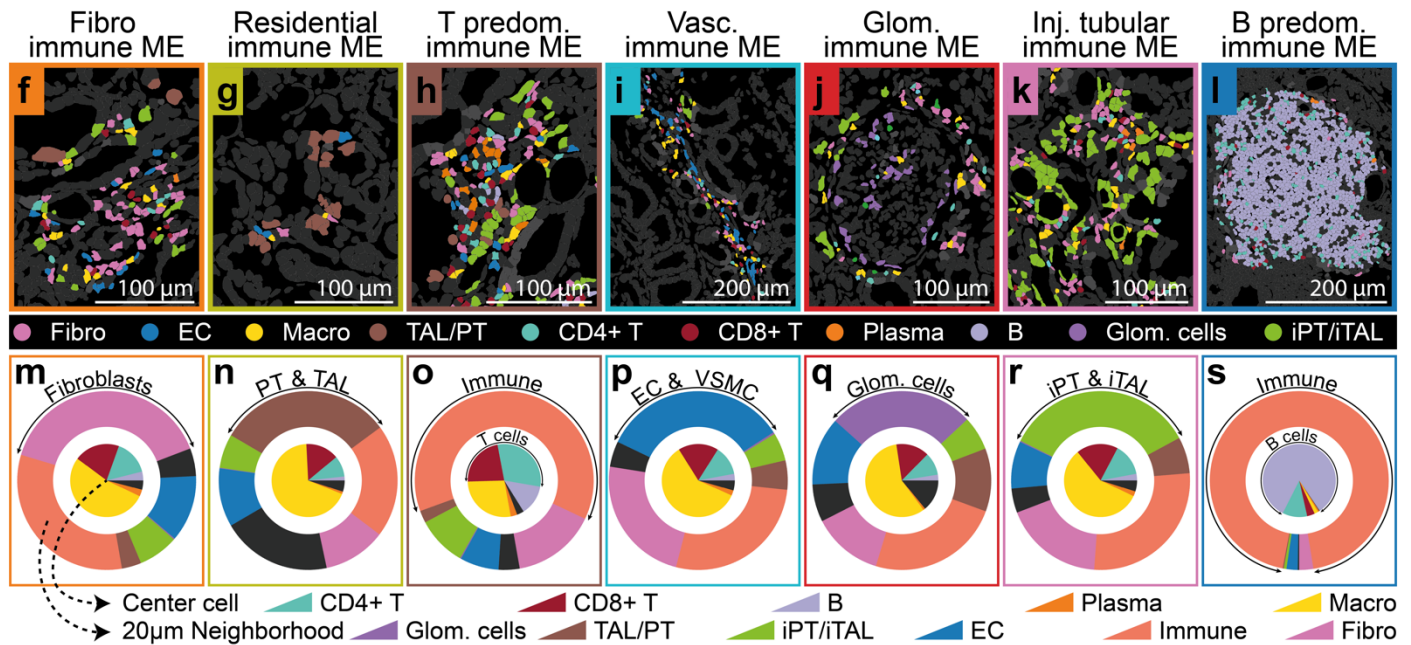


Fig. 4: Immune microenvironments: f-l representative spatial plot of the Fibro Immune, Residential immune, T predom. Immune, Vasc. Immune, Glom. Immune, Inj. Tubular immune and B predom. Immune microenvironments. m-s circular plots showing the celltype composition in the 20µm surrounding (outer circle) and the annotation of the immune cells at the center (inner circle). Celltypes are colored based on colors shown in the legend. Xenium Explorer 3 was used for the spatial plot visualization.

cells, and was termed the T cell–predominant immune ME (Fig. 4b, c). The second ME was enriched in B cells and contained CD4⁺ T cells, and was designated as the B cell–predominant immune ME.

In response to the reviewer’s question, the **injured tubular immune microenvironment (Fig. 4k) is primarily composed of macrophages**, which represent the dominant immune cell type within this ME (yellow in Fig. 4r). Additional contributions come from CD8⁺ T cells (red) and CD4⁺ T cells (green), indicating a mixed immune response centered around injured epithelial structures. This ME is defined by its spatial proximity to injured proximal tubule (iPT) and injured thick ascending limb (iTAL) cells, as illustrated in the outer ring of Fig. 4r. Given that injured tubular cells can emerge early in disease and occasionally even in control kidneys—particularly in aged individuals or following mild stress³—we also detected this ME at low frequency in control samples. This observation, along with our increased sample size, encouraged us to go beyond the initial characterization and ask whether the immune composition within this microenvironment changes with disease progression. We postulated that the immune contexture surrounding these cells evolves in the course of disease. To test this, we compared immune cell fractions within the injured tubular immune ME between control samples and kidneys from patients with diabetic kidney disease (DKD). Interestingly, we observed a significant increase in plasma cells surrounding injured tubules in DKD ($p = 0.008$), suggesting a disease-associated shift in immune cell recruitment or retention within this microenvironment.

To further probe the mechanisms underlying this enrichment, we correlated ligand–receptor interactions between iPT/iTAL cells and nearby immune populations with GFR as a marker of kidney function. This analysis revealed several key interactions:

- A decrease in ANXA1–FPR3 signaling between iTAL cells and macrophages in DKD, potentially reflecting impaired resolution of inflammation, as ANXA1–FPR interactions are known to mediate anti-inflammatory responses and macrophage homeostasis.
- An increase in TNFSF13–TNFRSF13B interactions (APRIL–TACI) between iTAL cells and plasma cells. This pathway is critical for plasma cell survival and maintenance, suggesting that the tubular epithelium in DKD may actively support plasma cell retention or expansion.
- An increase in CXCL12–CXCR4 signaling between iPT cells and plasma cells. CXCL12 is a potent chemoattractant, and its upregulation may facilitate plasma cell recruitment to the injured epithelium.

Taken together, our expanded dataset enabled us to move beyond the earlier definitions of iPT ME4 and iLOH ME1 and identify refined, recurrent injured tubular microenvironments across a larger patient cohort. Among these, we delineated a disease-associated profibrotic tubular microenvironment enriched for immune and stromal interactions, which colocalizes with regions of collagen deposition. Building on this, we leveraged our kidney immune atlas—now comprising over 250,000 cells—to investigate the spatial organization of renal inflammation in greater detail. This allowed us to define an injured tubular immune microenvironment, centered

around iPT and iTAI cells, which is predominantly enriched in macrophages and T cells. Notably, in DKD, this microenvironment shifts toward increased plasma cell infiltration. By correlating spatial ligand–receptor

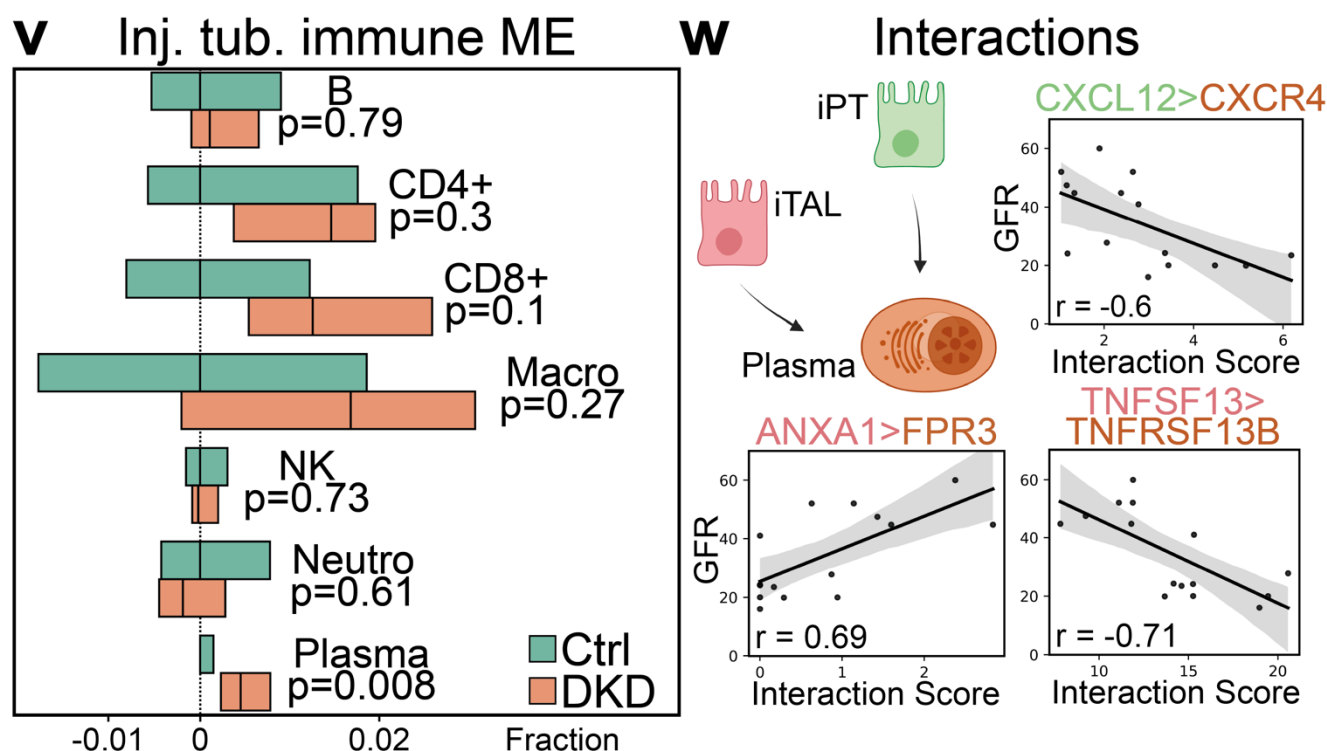


Fig 4v: Comparison of immune cell fractions within the injured tubular immune microenvironment between control and DKD samples. Shown are the relative abundances of B cells, CD4⁺ T cells, CD8⁺ T cells, macrophages, NK cells, neutrophils, and plasma cells in control (green) and DKD (red). For visualization, the median fraction of each immune cell type in control samples was calculated and subtracted from both control and DKD values, centering control medians at zero to facilitate direct comparison. Statistical comparisons were performed using Student's t-test, and p-values are indicated. w: Ligand–receptor interactions between injured proximal tubular cells, injured thick ascending limb cells and plasma cells within the injured tubular immune microenvironment were inferred using CellPhoneDB v5. The interaction scores for significantly enriched pairs were then correlated with GFR.

interactions with disease severity, we uncovered epithelial–immune signaling pathways—such as CXCL12–CXCR4 and TNFSF13–TNFRSF13B—that may support plasma cell recruitment and survival, offering mechanistic insight into how chronic inflammation and fibrosis are sustained in diabetic kidney disease.

5) Figure 4: The resident kidney immune cell microenvironment is insufficiently defined. Because these cells were not seen around injured tubular areas does not mean they are typical of the kidney. Further characterization and validation of this resident kidney immune cell microenvironment should be performed.

Response: We thank the reviewer for this important comment regarding the definition and validation of the resident kidney immune microenvironment. In the revised manuscript, we have significantly expanded our immune cell analysis to address this concern and to more rigorously define the spatial immune architecture of the human kidney across health and disease.

To accomplish this, we analyzed an **expanded spatial transcriptomics dataset of 64 human kidney samples**, incorporating **CosMx 1k** (n = 48; ~3.3 million cells) and **Xenium 5k** (n = 16; ~1 million cells), integrated with **single-nucleus RNA sequencing (snRNA-seq)** from **150 individuals (~750,000 cells)**. This yielded a **unified, multimodal atlas of over 5 million cells**, including more than **4 million spatially resolved cells**. From this integrated dataset, we generated a **kidney immune cell atlas**, capturing over **250,000 spatially**

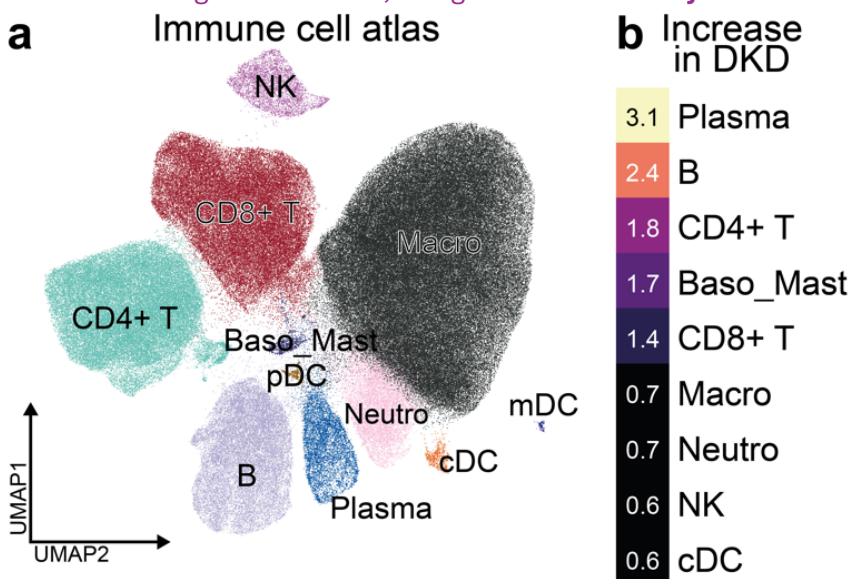


Fig 4a, Human kidney immune cell atlas. UMAP of the integrated datasets colored by cell types. Each dot represents one cell. The color represents the different cell populations: Baso/Mast, basophils and mastcells; Macro, macrophages; Neutro, neutrophils; cDC, classical dendritic cells; mDC, myeloid dendritic cells; pDC, plasmacytoid dendritic cells; B, B cells; Plasma, plasma cells; CD4+ T, CD4+ T cells; CD8+ T, CD8+ T cells; NK, natural killer cells. B, Fold change between immune cell fractions of control vs. DKD patients.

resolved immune cells (Fig. 4a). To understand their tissue localization and contextual organization, we clustered immune cells based on the **cell type composition of their immediate spatial neighborhoods**. This analysis revealed **seven robust and biologically distinct immune microenvironments (MEs, Fig. 4f-s):**

1. **Fibroblast-associated immune ME**
2. **Injured tubular immune ME**
3. **Vascular immune ME**
4. **Glomerular immune ME**
5. **T cell-predominant immune ME**
6. **B cell-predominant immune ME**
7. **Resident immune ME**

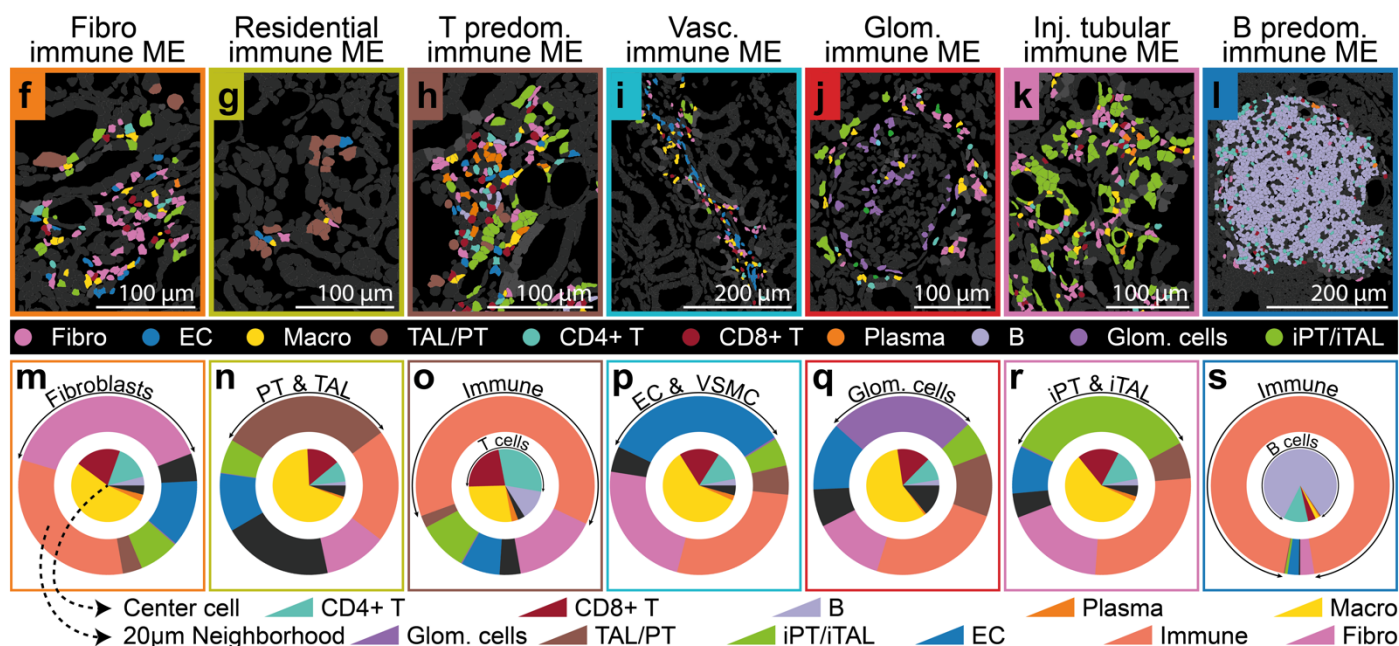


Fig 4: Immune microenvironments: f-l representative spatial plot of the Fibro Immune, Residential immune, T predom. Immune, Vasc. Immune, Glom. Immune, Inj. Tubular immune and B predom. Immune microenvironments. m-s circular plots showing the celltype composition in the 20μm surrounding (outer circle) and the annotation of the immune cells at the center (inner circle). Celltypes are colored based on colors shown in the legend. Xenium Explorer 3 was used for the spatial plot visualization.

Defining and Validating the Resident Kidney Immune Microenvironment

In response to the reviewer's specific concern about the **resident immune ME**, we agree that spatial separation from injury is not, by itself, sufficient to establish tissue residency. To this end, we implemented a **multi-layered analytical framework** to validate the identity and function of this compartment:

1. Spatial Context and Neighborhood Independence

The **resident immune ME** is defined by the **absence of spatial proximity** to injured epithelial cells, fibrotic niches, vascular structures, or dense immune aggregates. Instead, immune cells in this ME are **widely dispersed** throughout the kidney and consistently found in **non-inflamed regions**. In healthy pediatric kidneys (HK3656_C and Pediatric1_C), nearly **all immune cells localized to the resident ME** (Fig. 4e, rightmost bars), reinforcing its role as a baseline immune compartment in uninjured human kidney tissue.

2. Stability Across Disease States

While the **total number of immune cells increases in DKD** (Extended Fig 29ba, the **proportion assigned to the resident ME remains stable** across health and disease (Extended Fig 29b). This pattern is in contrast to the expansion of other immune MEs (e.g., injured tubular or fibroblast-associated MEs) in DKD, and suggests that the resident ME is **maintained independently of inflammation-driven recruitment**. Moreover, the immune cell composition of this microenvironment (mostly macrophages) was stable across disease state (Extended Fig. 29c).

3. Distinct Transcriptional Signature

We compared the gene expression profiles of **resident macrophages**, the predominant immune cell type in this ME, with those in inflammation-associated MEs.

- In **CosMx**, resident macrophages expressed higher levels of:
 - **MRC1** and **CD163**, classic markers of **tissue-resident, anti-inflammatory macrophages** (Extended Fig. 28).
 - **ZFP36**, a regulator of cytokine mRNA degradation associated with resolution of inflammation (Extended Fig. 28).

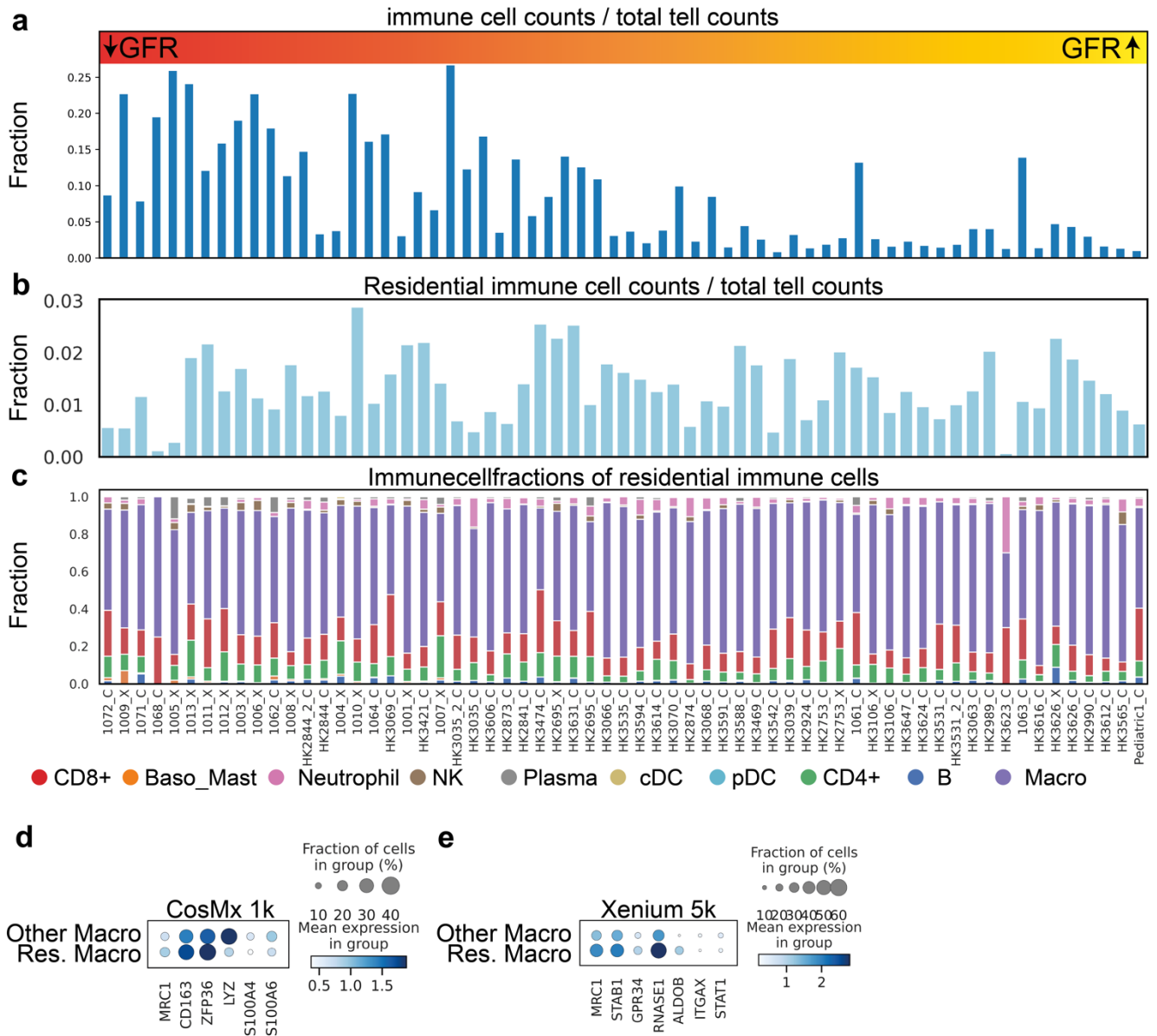
In contrast, macrophages in non-resident MEs upregulated **LYZ**, **S100A4**, and **S100A6**, markers of **monocyte-derived and inflammatory macrophage states** (Extended Fig. 28).

- In **Xenium**, resident macrophages consistently expressed **MRC1**, along with **GPR34**, **STAB1**, **ALDOB**, and **RNASE1**—genes involved in **immune regulation, scavenging, and tissue remodeling**. Non-resident macrophages upregulated **ITGAX (CD11c)** and **STAT1**, indicative of **antigen presentation and interferon signaling** (Extended Fig. 28).

These findings were consistent across both platforms and further support the **functional distinction** between resident and recruited macrophage populations.

Taken together, the **spatial independence from injury, stable representation across health and disease, broad tissue distribution, and anti-inflammatory transcriptional phenotype** of immune cells in this ME provide strong evidence that the **resident immune microenvironment represents a bona fide tissue-resident immune compartment** of the human kidney.

This is one of the first definition of residential tissue immune microenvironment, we agree that **spatial and transcriptional criteria alone cannot definitively prove residency**. Another caveat is that direct lineage tracing or in situ fate-mapping can only be performed in mice. We have therefore revised the manuscript to reflect this limitation and clarify that, while the evidence strongly supports residency, we cannot completely exclude low-level immune surveillance or quiescent infiltration.



Extended Fig. 28a: Bar plot showing the fraction of immune cells relative to all immune cells in each sample. **b:** Bar plot showing the fraction of immune cells classified as part of the residential immune microenvironment relative to all immune cells in each sample. **c:** Stacked bar plot showing the relative proportions of immune cell types within the residential immune microenvironment across all spatial transcriptomic samples (CosMx and Xenium). Each bar represents one sample, and cell type proportions were normalized by the total number of immune cells assigned to the residential microenvironment in that sample. Sample names are abbreviated in the end based on the platform they were run on (C = CosMx, X = Xenium). **d:** Visualisations of differentially expressed genes in macrophages between the residential immune microenvironment macrophages and macrophages outside of this microenvironment in the CosMx dataset and **e:** in the Xenium dataset.

In panel c, the immune cell zonation as characterized by the predominance of immune cells is insightful, yet the presence of CD8⁺ T cells in all microenvironments except the B cell microenvironment is interesting. Further interrogation of this observation may be beyond the scope of the manuscript but is worth noting. Only two patients show B cell-predominant immune microenvironments (panel d). The authors might consider not generalizing these findings. Furthermore, CD4⁺ predominant environments seem to be more consistently seen across diseased patients. The authors suggest a causal role of B cells in kidney progression but it is unclear what effects the B cells have in progressive kidney disease. Furthermore, have the authors ruled out recent viral infections? B cell TLS are seen in acute kidney injury as well as ageing so it is unclear whether the observation reported is a microenvironment unique to chronic kidney disease or kidney injury (PMID: 27699223). At some point in the future, the authors might consider examining clonal expansion within these populations. This would help our understanding the presence of B cells in these microenvironments.

We thank the reviewer for this thoughtful comment. In the revised manuscript, we have substantially expanded both the scope and resolution of our analysis. Specifically, we increased our spatial dataset fourfold, now comprising 64 spatially profiled kidney samples using two complementary high-resolution platforms—CosMx 1k (n = 48; ~3.3 million cells) and Xenium 5k (n = 16; ~1 million cells). These data were integrated with single-nucleus RNA sequencing from 150 patients (~750,000 cells), resulting in a unified atlas of over 5 million cells, including more than 4 million spatially resolved cells. Leveraging this expanded dataset, we constructed a kidney immune atlas capturing over 250,000 immune cells

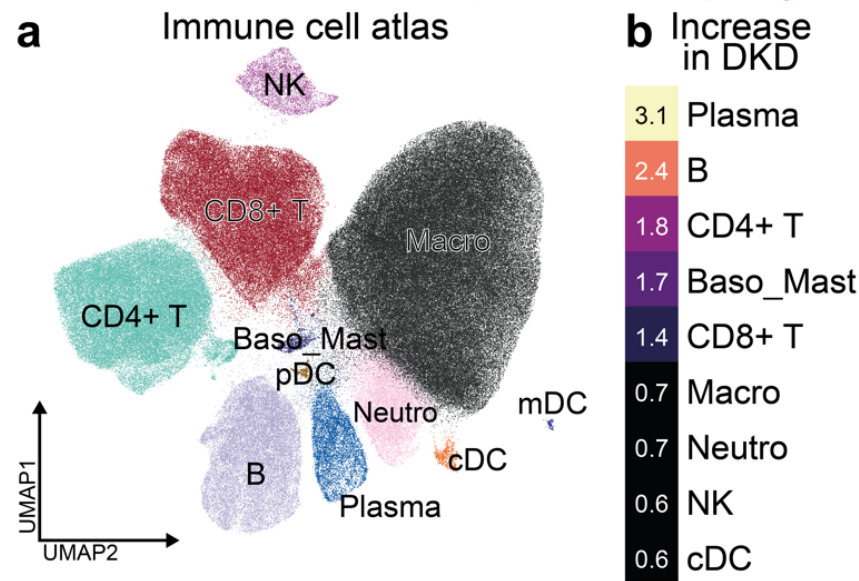


Fig 4a, Human kidney immune cell atlas. UMAP of the integrated datasets colored by cell types. Each dot represents one cell. The color represents the different cell populations: Baso/Mast, basophils and mastcells; Macro, macrophages; Neutro, neutrophils; cDC, classical dendritic cells; mDC, myeloid dendritic cells; pDC, plasmacytoid dendritic cells; B, B cells; Plasma, plasma cells; CD4+ T, CD4+ T cells; CD8+ T, CD8+ T cells; NK, natural killer cells. B, Fold change between immune cell fractions of control vs. DKD patients.

(Fig. 4a). This revealed an increase in B cells and plasma cells in kidneys from patients with diabetic kidney disease (DKD, Fig. 4b). To better understand the organization of renal inflammation in DKD, we clustered immune cells based on the composition of their immediate cellular neighborhoods, identifying seven distinct immune microenvironments (MEs). These included MEs enriched for specific neighboring cell types: fibroblasts (Fig. 4f, m), injured tubular epithelial cells (Fig. 4k, r), vascular cells (Fig. 4i, p), and glomerular cells (Fig. 4j, r),

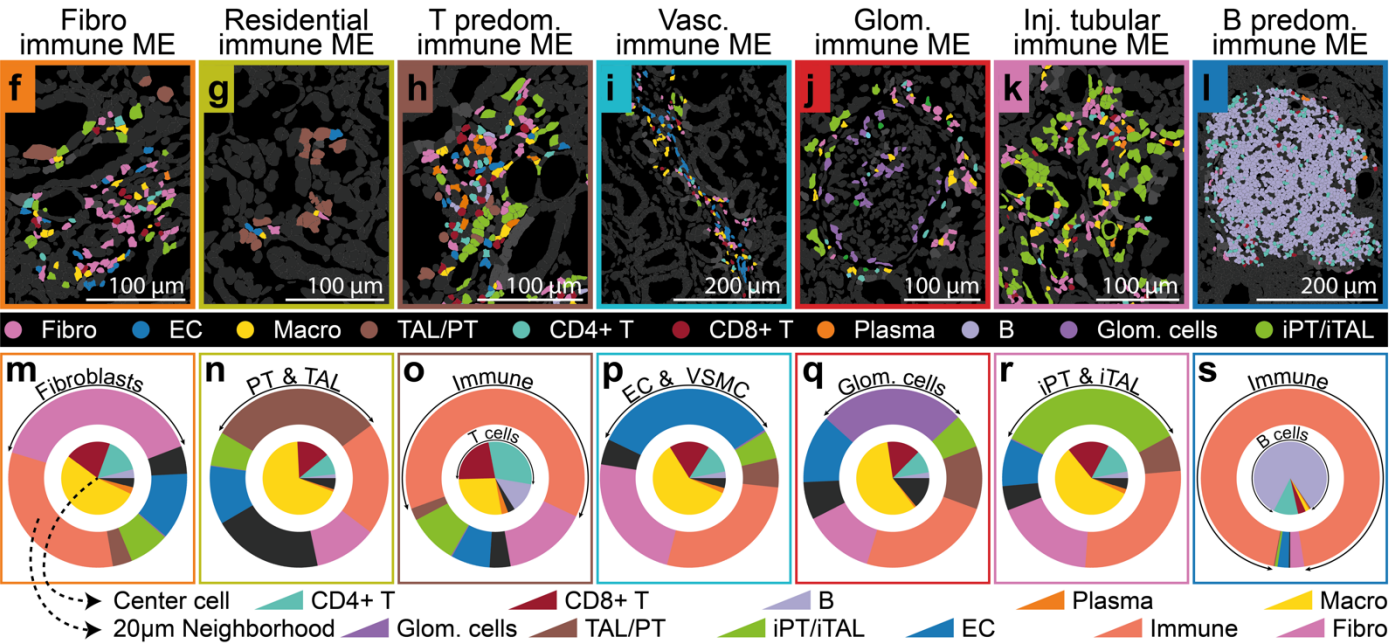


Fig 4: Immune microenvironments: f-l representative spatial plot of the Fibro Immune, Residential immune, T predom. Immune, Vasc. Immune, Glom. Immune, Inj. Tubular immune and B predom. Immune microenvironments. m-s circular plots showing the celltype composition in the 20µm surrounding (outer circle) and the annotation of the immune cells at the center (inner circle). Celltypes are colored based on colors shown in the legend. Xenium Explorer 3 was used for the spatial plot visualization.

which we termed the fibroblast-associated, injured tubular, vascular, and glomerular immune MEs, respectively. In addition, we identified two immune-dense MEs: one predominantly composed of CD4⁺ T cells

(alongside CD8⁺ T cells and B cells), termed the T cell–predominant ME (Fig. 4b, c), and another enriched in B cells with CD4⁺ T cells, designated the B cell–predominant ME. Lastly, we defined a distinct immune ME lacking proximity to injured epithelial cells, fibroblasts, glomeruli, vascular structures, or other immune cells, which we termed the residential kidney immune microenvironment (Fig. 4e, g). In the revised manuscript, we extensively characterized this compartment: The B cell rich microenvironment was reminiscent of a tertiary lymphoid structure. Notably, using the Xenium 5k assay, we detected expression

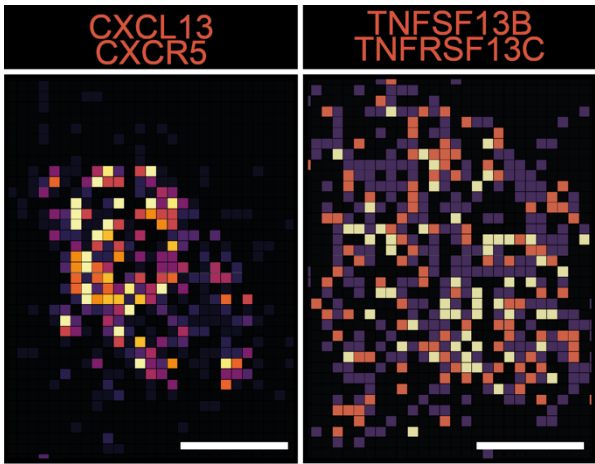


Fig 5o: Zoom into the B cell predom. Immune microenvironment shown in Fig. 4l and s. Colocalization of the expression of CXCL13 and CXCR5 (binned to 20µm) as well as TNFSF13B and TNFRSF13C. Xenium Explorer 3 was used for this visualization.

of CXCL13—a chemokine typically restricted to secondary lymphoid organs and unusual in a metabolic kidney disease like DKD—along with its receptor CXCR5 (Fig. 5o). CD4⁺ T cells within this ME expressed high levels of CXCR5 and PDCD1 (PD1), consistent with a follicular helper T cell phenotype (Extended Fig. 28g). Moreover, we found a striking colocalization between TNFSF13 (BAFF) and TNFRSF13C, further supporting a local pro-survival niche for B cells. While these findings suggest local B cell activation within a TLS-like niche, we were unable to rule out the possibility of a recent or subclinical viral infection contributing to this immune architecture. To further analyze this microenvironment, we employed multiplex protein staining using imaging mass cytometry (IMC), a technique that combines high-dimensional antibody-based detection with spatial resolution at subcellular scale. IMC uses metal-tagged antibodies and laser ablation followed by mass spectrometry to quantify protein expression across tissue sections while preserving spatial context. We applied a customized

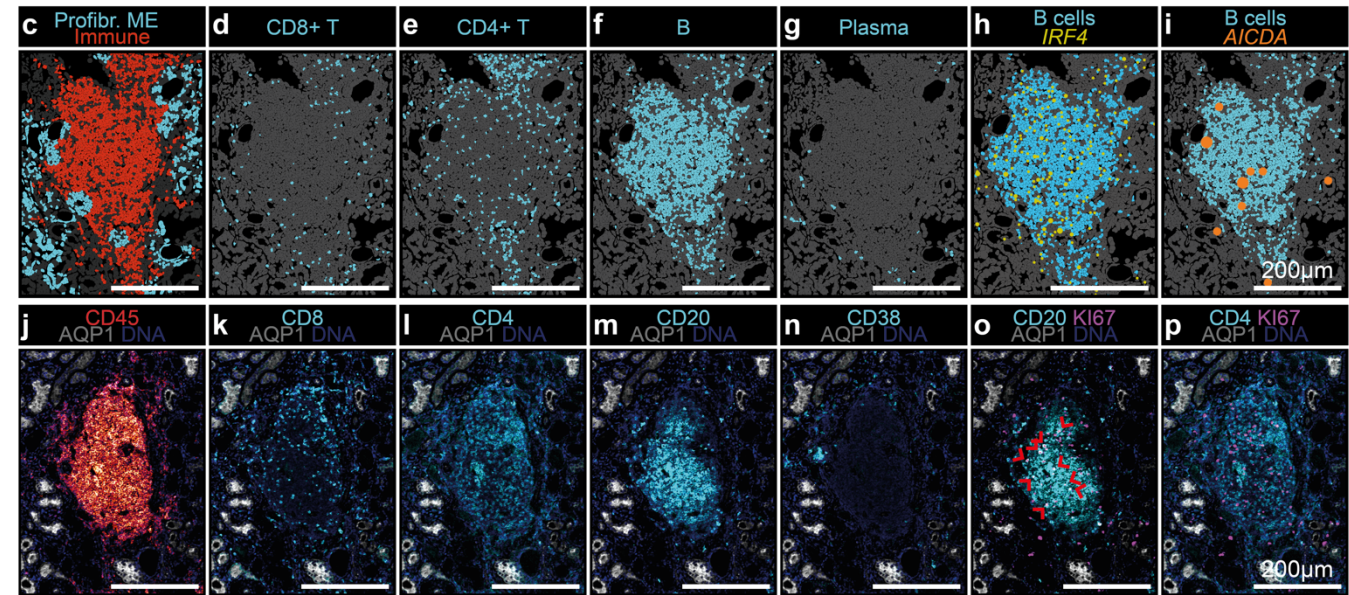


Fig 5c – i: spatial plots generated using Xenium Explorer 3 of the profibrotic tubular microenvironment and immune cells (c); immune cell types in the B cell predominant microenvironment (d-g). Expression of IRF4 and AICDA. J to p: Imaging mass cytometry using multiplex protein panel of CD45, AQP1, DNA staining, CD8, CD4, CD20, CD38, CD20, KI67.

antibody panel targeting key immune and epithelial markers, including CD4 and CD8 (to identify T cell subsets), CD20 (for B cells), CD38 (a marker of plasma cells), and CD45 (a pan-leukocyte marker, Fig. 5). Additionally, we included AQP1 to mark proximal tubular epithelium and a DNA intercalator to visualize nuclear architecture. Using this approach, we confirmed the immune cell composition of the B cell–predominant microenvironment as identified by both the CosMx and Xenium assays, including the presence of CD4⁺ T cells, CD20⁺ B cells, and CD38⁺ plasma cells in close spatial proximity.

Moreover, we integrated our Xenium-derived B and plasma cells with a previously published single-cell RNA sequencing atlas of human B cells and plasma cells. To perform this integration, we used the deep generative

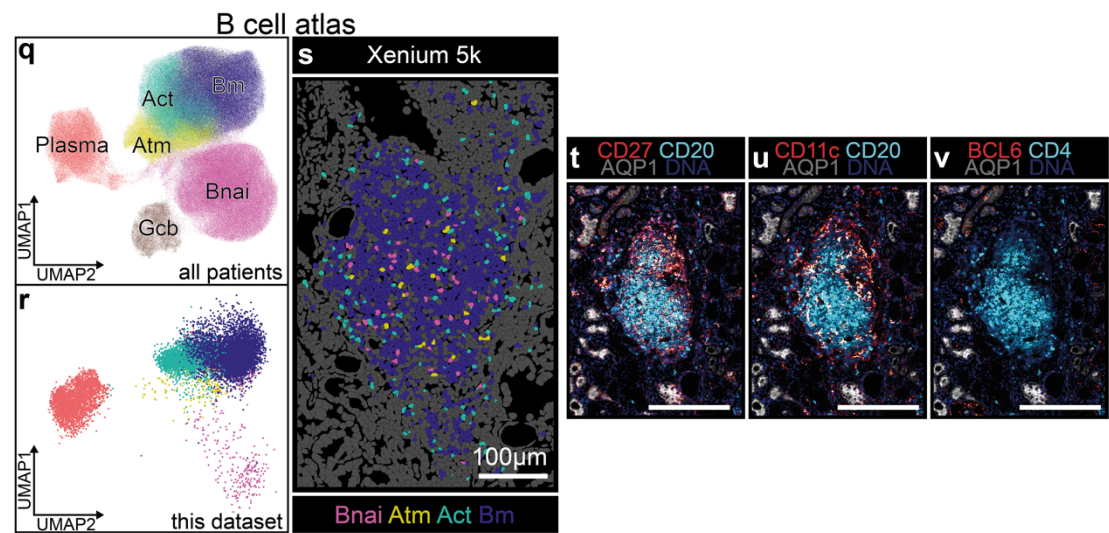


Fig 5: High-resolution spatial B and plasma cell atlas constructed by integrating our Xenium 5k spatial transcriptomics data with a publicly available single-cell RNA-sequencing reference of human B and plasma cells. Integration was performed using a two-step approach with scVI and scANVI. A four-layer scVI model (30 latent dimensions, learning rate = 0.001, early stopping with patience = 15) was trained on raw counts to create a shared latent space. scANVI was then used in a semi-supervised manner to transfer cell type annotations from the reference to the Xenium dataset. Label transfer was based on latent space proximity, followed by retraining on high-confidence annotations to refine classification. q: UMAP of the integrated datasets colored by cell types. Each dot represents one cell. The color represents the different cell populations: Plasma cells (Plasma), atypical memory B cells (Atm), activated B cells (Act), memory B cells (Bm), naïve B cells (Bnai) and germinal center B cells (GcB). r: UMAP of only Xenium cells of this study in the integrated dataset, colored by cell types. s: Spatial plots generated using Xenium Explorer 3 of the B cell subsets in the B predominant immune microenvironment. t-v: Imaging mass cytometry using multiplex protein panel of CD20, AQP1, DNA staining, CD27, CD11c, BCL6.

w ImmuneComplex Analysis

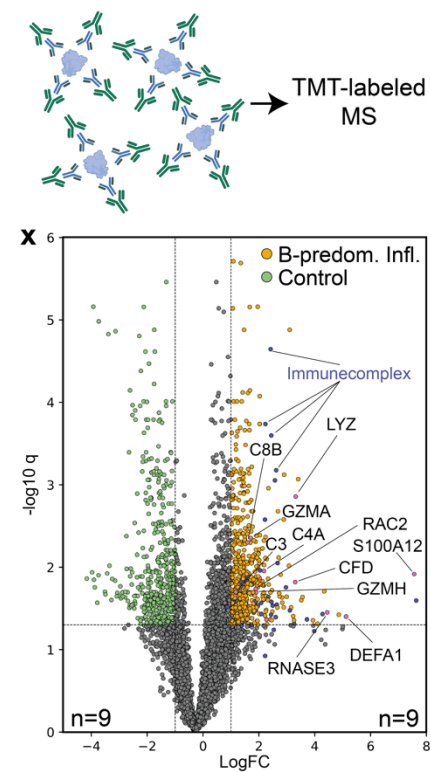


Fig. 5w: Schematic overview of the immune complex analysis. Kidney lysates from control patients and those with B cell–predominant inflammation were subjected to IgA-based immunoprecipitation to isolate antibody-bound immune complexes. x: Volcano plot showing differentially enriched proteins bound to IgA in B cell–enriched versus control kidney lysates.

models scVI and scANVI, which enable joint modeling and probabilistic label transfer across datasets with different modalities and batch effects. This approach allowed us to map spatial B and plasma cells from the kidney into transcriptionally defined B cell subsets, thereby enabling a high-resolution characterization of B cell phenotypes within the tissue context (Fig. 7q-s). We could show a high abundance of memory B cells, as well as naïve B cells and a few atypical memory b cells. Using the imaging mass cytometry and CD27, CD11c and BCL6 as markers for memory, atypical and germinal center b cells, we could corroborate these findings (Fig. 5t-v). Moreover, using Ki67 as a proliferation marker, we could identify that CD20 positive B cells proliferate, but not CD4 T cells (Fig. 5o-p). What triggers this process is unclear, however using Xenium 5k we could identify

expression of the class switch recombination enzyme *AICDA* as well as the transcription factor *IRF4* (Fig. 5h-i). This could indicate that B cells enter the tissue in these B cell aggregates via CXCL13 gradient and become plasma cells to produce antibodies. To further investigate the downstream consequences of intrarenal B cell aggregates and local immune complex formation, we identified patients with evidence of strong B cell-predominant inflammation by applying a spatial gene signature derived from the B cell-rich immune microenvironment to bulk RNA-sequencing data from the Susztaklab Biobank cohort. In these patients, we performed immunoprecipitation of IgA-containing immune complexes from paired kidney protein lysates, followed by tandem mass tag (TMT)-based quantitative mass spectrometry to characterize the protein components bound to IgA. This approach enabled us to directly profile the effector molecules associated with locally produced immune complexes in situ. In B cell-enriched samples, we observed a marked enrichment of proteins involved in immune effector functions. These included granzymes—cytotoxic enzymes released by lymphocytes that can mediate direct tissue injury—as well as multiple components of the complement cascade, such as C3, C4A, and C8B. These complement proteins are known mediators of inflammation and tissue damage when activated or deposited in the kidney.

To assess clinical relevance, we applied the **B cell-predominant ME signature** to two large bulk kidney transcriptomic cohorts:

- In the **Susztaklab Biobank (n = 843)**, the signature correlated with **increased fibrosis severity**.
- In the **TRIDENT cohort**, unsupervised clustering based on this signature identified a **B⁺ DKD subgroup (~8.4%)** that exhibited **significantly worse baseline eGFR** and **more rapid disease progression**.

We appreciate the reviewer's note regarding potential confounders such as **viral infections** or **age-related TLSs**. While patients with known viral infections were excluded, **subclinical infection cannot be definitively ruled out**.

Finally, we agree with the reviewer that **BCR clonality assessment** is a critical next step to determine whether these B cells undergo **antigen-driven expansion**, and we now highlight this as a key direction for future work.

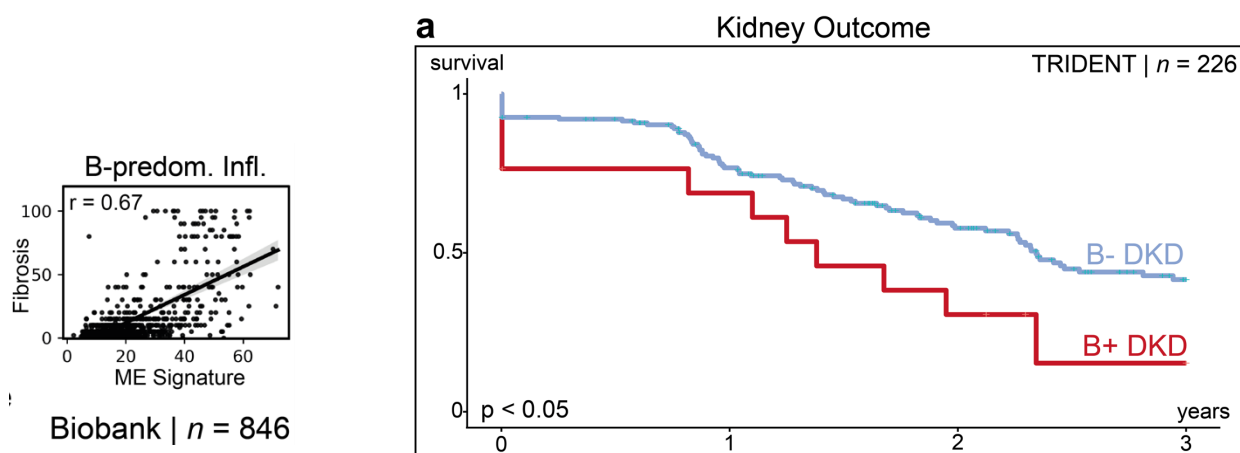


Fig. 5 left side: Correlation between a spatial gene signature derived from the B cell-predominant immune microenvironment (ME) and interstitial fibrosis in a cohort of 846 patients. Spatial signatures were identified by pseudo-bulking spatial transcriptomics data followed by differential expression analysis using DESeq2. The resulting 10-gene signature was correlated against fibrosis scores obtained from histopathological assessment of paired kidney tissue sections by an expert nephropathologist using matched bulk RNA-sequencing data using Pearson correlation ($p < 0.0001$). b: Patients were stratified based on the spatial gene signature of the B cell-predominant immune microenvironment. A 10-gene signature was derived from spatial transcriptomics data and applied to bulk RNA-sequencing profiles of kidney tissue from a large patient cohort (TRIDENT). Hierarchical clustering using Ward's method with Euclidean distance separated patients into two groups: those with low expression (B⁻) and those with high expression (B⁺) of the B ME gene signature. Kaplan-Meier survival analysis was then performed to evaluate time to a renal composite endpoint—defined as progression to dialysis, kidney transplantation, or a $\geq 40\%$ decline in baseline eGFR. The x-axis indicates time (in years), and the y-axis indicates the survival probability ($p < 0.05$).

6) Figure 5: *iPT* does not correlate with disease severity as strongly as the *iLOH* and immune niche. Does this call into question the validity of the *iPT* niche? In panel b, statistical analysis should be performed for the survival curves.

Response: We thank the reviewer for this thoughtful comment. In the revised manuscript, we have substantially expanded our dataset and refined our analytical framework to enhance the biological and clinical interpretation of spatial niches. We increased our spatial sample size fourfold, incorporating **64 human kidney samples** profiled using two complementary high-resolution platforms—**CosMx 1k** ($n = 48$; **~3.3 million cells**) and **Xenium 5k** ($n = 16$; **~1 million cells**). These were integrated with **single-nucleus RNA-sequencing data from 150 individuals (~750,000 cells)** to construct a unified atlas of over **5 million cells**, including more than **4 million spatially resolved cells**.

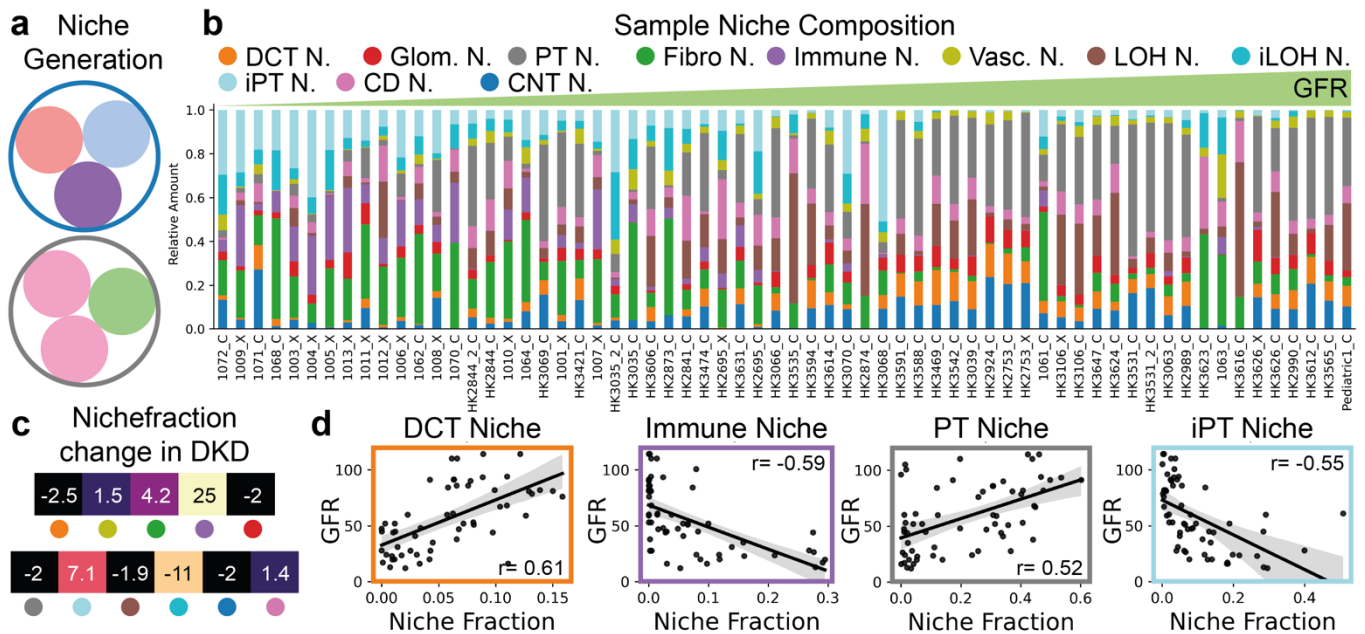
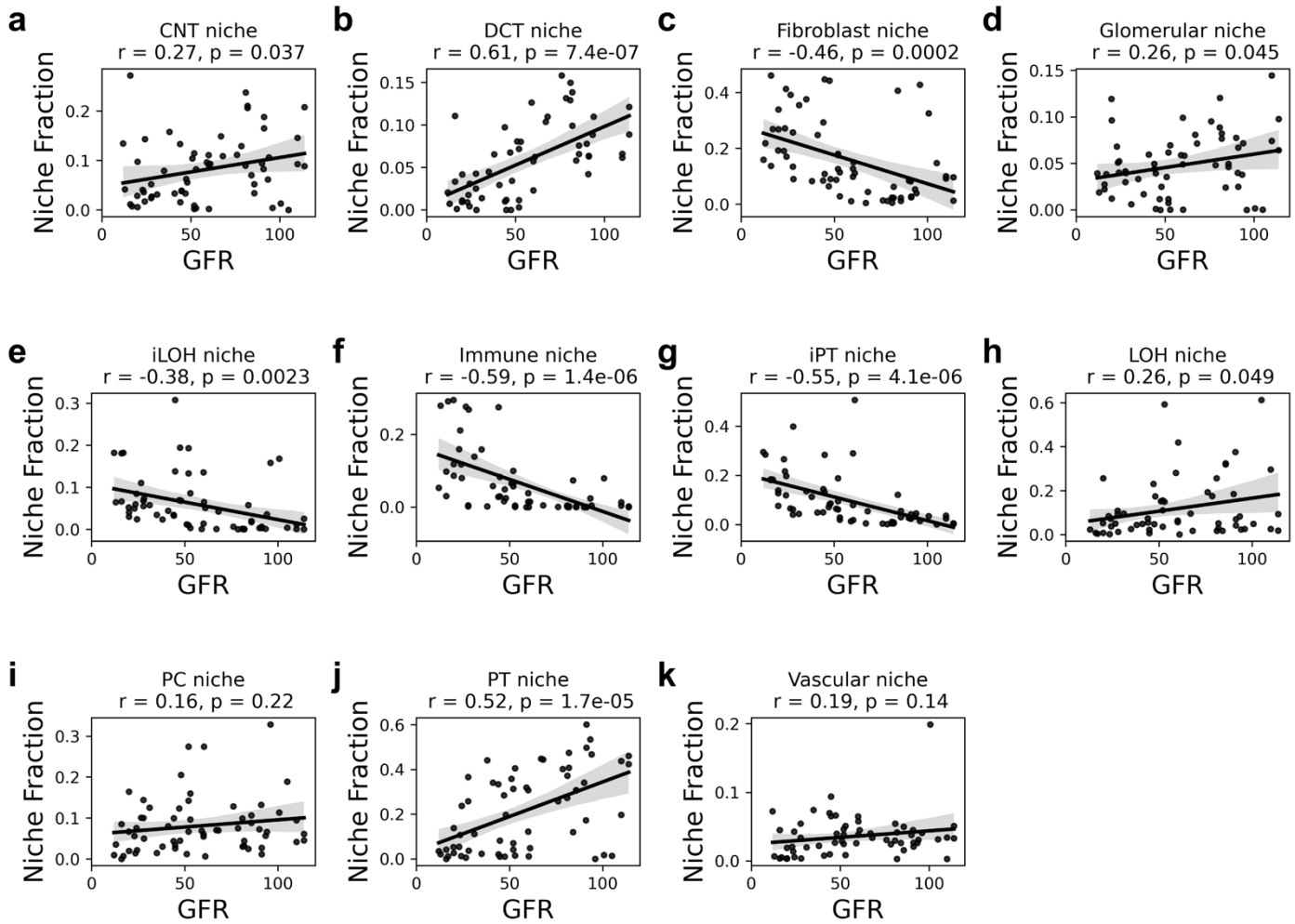


Fig 2a: Schematic of defining niches (left) and microenvironments (right). For niches, all cells were clustered into groups based on their neighborhood fraction after restricting the analysis to those with sufficient local context, removing the sparsest 5% of neighborhoods (based on total neighbor count at 80 μm) to reduce edge effects ($n = 4,029,331$ cells). For each cell, we computed the number of neighboring cells within concentric distance shells: 0–20 μm , 20–40 μm , 40–60 μm , and 60–80 μm . This was achieved by calculating cumulative neighbor counts at 20, 40, 60, and 80 μm , followed by subtracting counts from the smaller radius to isolate the number of neighbors in each respective shell (e.g., the 40–60 μm shell = count at 60 μm – count at 40 μm). For each cell, neighbor counts were normalized by the total number of neighbors at that distance, yielding a cell-by-neighbor composition matrix that reflects the relative cellular makeup of the local environment at different spatial scales. This matrix was then scaled and subjected to principal component analysis (PCA), with the top 10 components used as input for MiniBatch K-means clustering. The number of clusters (k) was determined based on the elbow method. Niche clustering was performed on the entire dataset to define global spatial neighborhoods. **b:** Sample niche composition: the fraction of each niche is calculated for all cells within each sample and visualized. The samples are arranged by disease severity (GFR) from left to right. **c:** Change in relative niche fraction in DKD vs healthy controls. Each value represents the fold change in the average proportion of a given niche between DKD patients and healthy controls. A positive value indicates that the niche is expanded in DKD, while a negative value indicates it is more prevalent in controls. Fold changes were computed by normalizing niche frequencies within each diagnostic group, then taking the ratio of DKD to control values. **d,** correlation of niche fraction with eGFR using Pearson correlation. All correlations with $p < 0.001$.

To characterize disease-associated tissue reorganization, we defined **spatial niches** based on local cell–cell neighborhood composition (Fig. 2a). Unlike microenvironments that center on a focal cell, spatial niches capture larger-scale, **emergent multicellular tissue states**. We applied a robust, data-driven framework using PCA and MiniBatch K-means clustering. This approach yielded **11 globally defined spatial niches**, including homeostatic, inflammatory, and injured epithelial compartments.

To assess clinical relevance, we computed **sample-level niche compositions** and correlated them with **estimated glomerular filtration rate (eGFR)**, a clinical measure of kidney function. As shown in **Fig. 2b** and **Fig. 2i**, both the **injured proximal tubule (iPT)** niche and the **injured loop of Henle (iLOH)** niche were:



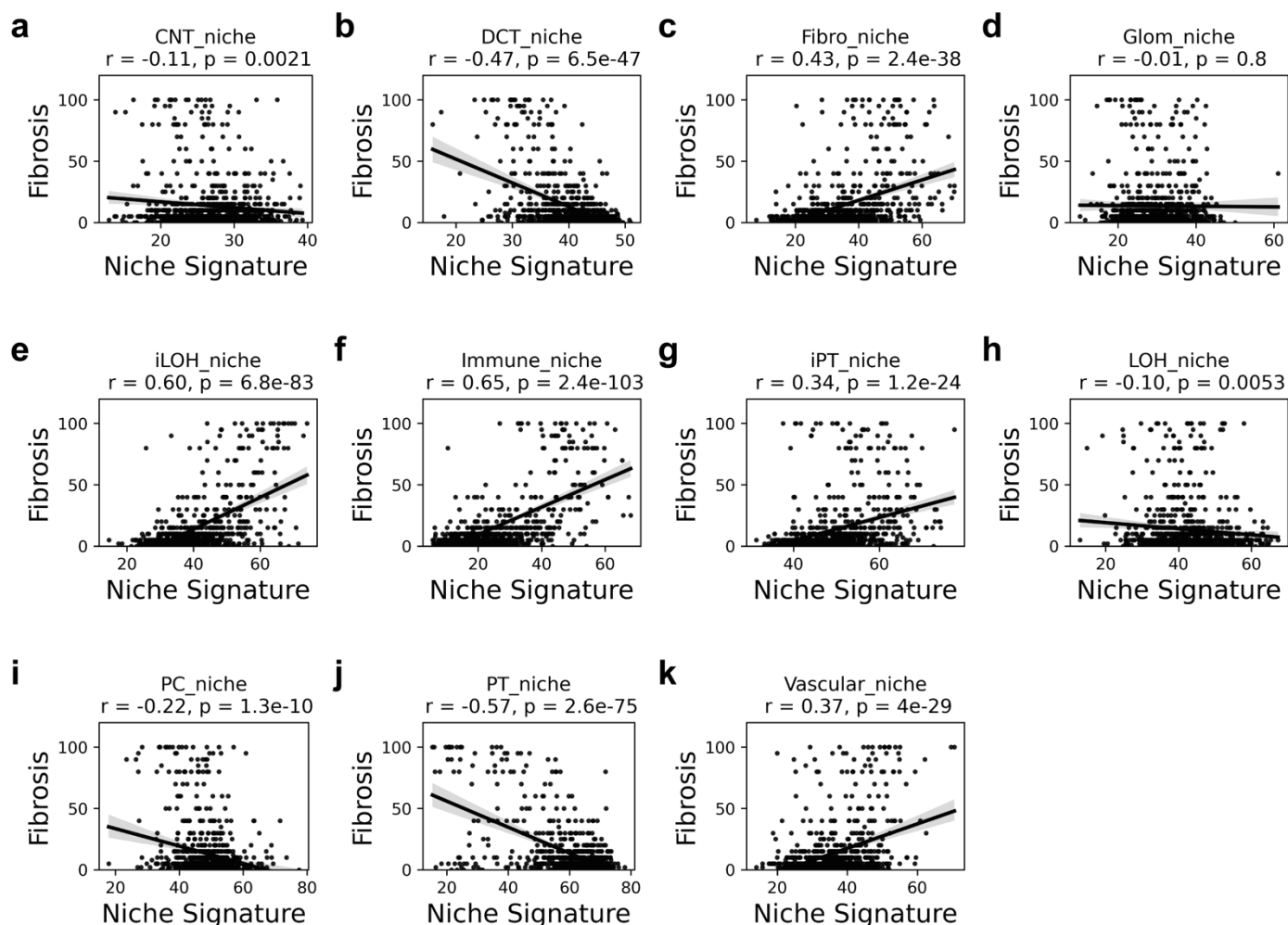
Extended Fig. 12 Correlation between glomerular filtration rate (GFR) and niche-specific cell fractions across spatial niches: Scatter plots showing Pearson correlations between estimated glomerular filtration rate (GFR) and the relative abundance of individual cell niches across all samples in the spatial transcriptomics dataset. Each plot represents a distinct spatial niche (a: CNT niche, b: DCT niche, c: Fibroblast niche, d: Glomerular niche, e: iLOH niche, f: Immune niche, g: iPT niche, h: LOH niche, i: PC niche, j: PT niche, k: Vascular niche) and shows niche fraction vs. GFR per sample. Cell fractions were calculated by normalizing the number of niche-specific cells to the total cell count within each sample. A linear regression line is overlaid, and Pearson correlation coefficients (r) and corresponding p-values are annotated.

- **Virtually absent in healthy controls**, including pediatric reference samples (Fig. 2b).
- **Significantly expanded in DKD** (7-fold increase for iPT, 11-fold for iLOH, Fig. 2c).
- And showed **strong negative correlations with eGFR** (iPT: $r = -0.55$; iLOH: $r = -0.38$; both $p < 0.01$, Fig. 2d).

These findings suggest that both iPT and iLOH niches reflect **epithelial injury programs tightly linked to disease progression**.

To explore their molecular differences, we performed differential expression and spatial signature analysis. While the **iPT niche** was defined by the expression of TNFSF10, IL32, VCAM1, HAVCR1, MMP14, CRYAB, COL27A1, TPM1, REG1A, CLCF1 the **iLOH niche** was defined by S100A2, ITGB6, PAPP2, LIF, AGR2, SLPI, IFGBP6, KRT7, ACSL4, CD44 supporting their distinct injury state. Using the spatial gene signatures for correlation with interstitial fibrosis revealed only a moderate correlation between the iPT gene signature and interstitial fibrosis ($r = -0.34$, Extended Fig. 32) in 843 patients, suggesting that while the iPT niche reflects injury, it may not directly correlate with advanced fibrotic remodeling. Fibrotic remodeling is a downstream event that may occur after transient epithelial injury has resolved, thereby weakening its association with acute injury signatures. We interpret this as biologically meaningful: injured PT cells are among the earliest responders to stress and can appear in early disease, aged tissue, or even healthy kidneys under mild stress conditions.³ Thus,

the iPT niche may mark early, and potentially reversible, epithelial injury rather than irreversible tissue remodeling. In contrast, the iLOH and immune niches showed tighter associations with chronic tissue



Extended Fig 32: The association between spatial gene signature and disease severity: Spatial gene signatures were extracted for each niche with pseudobulk counts on a sample level followed by DeSeq2. 10 genes for each spatial gene signature were used to correlate with interstitial fibrosis scored by a renal pathologist in 843 patients (Susztaklab biobank, microdissected tubule bulk sequencing).

remodeling and immune activation—hallmarks of progressive disease.

Finally, in response to the reviewer's note regarding survival analysis, we have now added **statistical analysis of the survival curves**.

Minor comments

1) Sample origin: 13 total samples: 3 control (relatively young males) vs 10 diseased: 5 with Diabetic kidney disease, 1 CKD, and 1 DM

We increased our spatial sample size fourfold, incorporating **64 human kidney samples** profiled using two complementary high-resolution platforms—**CosMx 1k (n = 48; ~3.3 million cells)** and **Xenium 5k (n = 16; ~1 million cells)**. These were integrated with **single-nucleus RNA-sequencing data from 150 individuals (~750,000 cells)** to construct a unified atlas of over **5 million cells**, including more than **4 million spatially resolved cells**. Figure 1 shows the age sex and kidney disease status information.

2) Figure 1a: Missing scale bar on PAS panel

Response: We have added a scale bar.

3) Figure 1e and 4a: It may be more helpful to place the cluster labels on each population rather than on a legend as some of the colors are difficult to distinguish from one another.

Response: We agree with the reviewer and have adjusted the legends.

4) Fig 2K, 2L: Stats missing from the plots themselves.

Response: We have added stats for the revised Figure 2.

5) Figure 3: The case is made that fibroblasts are consistently seen in close proximity to IPT and iLOH; however, the representative images shown in Figure 2f are not so convincing. PAS staining of these niches would also be beneficial.

Response: We agree with the reviewer, we have used Sirius Red staining to illustrate collagen deposition.

6) Figure 4b: The labeling scheme used here is rather confusing as similar colors are used between the outer and inner circle although they refer to different cell types.

Response: We thank the reviewer for this suggestion. In our revised manuscript, we use an alternative color scheme that we believe is easier to quickly understand.

Thank you for the opportunity to review the manuscript “Spatial Human Kidney Map Highlights B-cell driven Kidney Disease Subgroup” by Dumoulin and colleagues. This body of work applies subcellular spatial transcriptomics using the CosMx platform to profile the complex architecture of the Kidney in the context of 4 healthy kidney samples compared to tissue from 4 patients with diabetes (DM), 2 samples from a patient with chronic kidney disease (CKD) and 7 samples from 5 patients with diabetic kidney disease (DKD).

Using their analysis pipeline, the authors describe the kidney architecture spatial in reference to ssRNA seq data including spatially variable genes (figure 1). By performing “neighbourhood” analysis at a range of sizes (varying from 20-80um), niches are defined relative to central cells based on the cell type composition and local gene expression. Using this approach, the authors can characterise local microenvironments (ME) relative to structures (such as the proximal tubule and loop of Henle in) either a healthy or disease/injured state (figures 2 and 3). Based on this analysis, both the cell composition of the niche as well as the genes that are differentially expressed, and potential drivers of ligand-receptor interactions can be inferred. Based on this approach, cell types profiled at either a per sample (healthy versus disease) or local ME, outline an association with immune cell expansion/infiltration (with a particular focus on B cells in Diabetic Kidney Disease) in patients with kidney disease compared to healthy controls (Figure 4). While the findings of the study are interesting, I do have some concerns.

1. Biological insight.

The major conclusions drawn in this paper appear to confirm well-known features of diabetic renal disease rather than demonstrating novel ones. It is concluded, for example, that inflammatory changes correlate with DKD, as does an increase in mesangial cells and reduced podocytes in the glomerulus. There is a large literature on the role of inflammation on the pathogenesis of diabetic nephropathy. Similarly, the classic histological features of DKD include mesangial hypercellularity and associated glomerular (and therefore podocyte) destruction. Neither are referenced. The observation that tubular injury is associated with disease and its progression (p 13) is also well known. These observations therefore demonstrate that the technology being used in this study can replicate findings using more conventional approaches – a useful “positive control” for the approach rather than a demonstration that it can discover important new features of disease.

Response: We respectfully emphasize that this study advances well beyond previously described features. By combining a uniquely large and multimodal dataset with high-resolution spatial transcriptomics, machine learning-based analysis, and orthogonal validation strategies, we provide **novel biological insight into disease-associated tissue states, cell-cell interactions, and immune compartmentalization and the first (and even targetable) diabetic kidney disease (DKD) subtype**, that were not previously accessible using conventional histology, bulk transcriptomics, or dissociated single-cell RNA sequencing.

While we indeed confirm classical pathological features of DKD—such as mesangial expansion, podocyte loss, and inflammatory infiltration—these serve as essential **positive controls** that validate the fidelity of our spatial and analytical approaches. More importantly, we identify previously uncharacterized cell states, spatial niches, immune microenvironments, and molecular interaction networks that provide an **entirely new view of disease biology** and **directly support precision therapeutic strategies**.

1. Scale and dataset novelty

Our study integrates spatial transcriptomic data from **64 human kidney samples**, profiled using two complementary, high-resolution platforms—**CosMx 1k (n = 48; ~3.3 million cells)** and **Xenium 5k (n = 16; ~1 million cells)**—with **single-nucleus RNA-seq data from 150 individuals (~750,000 cells)**. This yields a **multi-modal, cross-platform atlas of over 5 million cells**, including **>4 million spatially resolved cells**, making it the **largest spatially resolved single-cell dataset for any human disease to date**.

We benchmark this against 23 recent high-profile *Nature* and *Nature Medicine* studies (summarized in the supplement), which include single-cell or spatial transcriptomics from human or mouse tissues. None match our study in terms of **scale, spatial resolution, sample diversity, or disease-specific focus**.

1. Barnett et al. created an organotypic atlas of human vascular cells by integrating single-cell RNA sequencing data from 19 organs across 67 donors.² In total, they analyzed ~800,000 cells (166 samples), including ~67,000 vascular cells. Of the 19 datasets, 2 were newly generated and 17 were publicly available.
2. Mathys et al. generated a single-cell transcriptomic atlas of the aged human brain by profiling six brain regions from 283 post-mortem samples across 48 individuals with and without Alzheimer's disease.³ In total, they analyzed 1.3 million cells to uncover region- and cell-type-specific alterations associated with the disease. While Mathys et al. generated an influential dissociative single-cell atlas of the aging human brain (~1.3 million cells from 48 individuals), our study not only exceeds this in total cell number and donor diversity, but crucially incorporates spatial resolution at subcellular level, preserving tissue architecture and enabling in situ analysis of cellular interactions and microenvironmental structure.
3. Wälchli et al. constructed a molecular atlas of the developing and adult human brain vasculature by performing single-cell RNA sequencing on 606,380 freshly isolated endothelial, perivascular, and tissue-derived cells from 117 samples across 68 individuals.⁴ While their study provides valuable insights into vascular development and pathology, it is based entirely on dissociative methods. In contrast, our work integrates spatial transcriptomics with single-nucleus RNA-seq to preserve tissue context and enable in situ analysis of cellular organization.
4. Matchett et al. generated a single-cell atlas of human liver regeneration by combining single-nucleus RNA sequencing (snRNA-seq) from 31 liver samples (healthy, APAP-ALF, and NAE-ALF) with spatial transcriptomics using the Visium platform on 7 samples.⁵ While this multimodal approach provides important insights into liver repair, the spatial resolution of Visium is limited and captures transcriptomes from multiple cells per spot. In contrast, our study integrates subcellular-resolution spatial transcriptomics from 64 samples (CosMx 1k, n=48; Xenium 5k, n=16) with single-nucleus RNA-seq profiles from 150 patients (~750,000 cells), enabling precise cell-level mapping and interaction analysis.
5. Lai et al. constructed a multimodal cell atlas of aging human skeletal muscle by analyzing 31 individuals (12 adult, 19 older adult) using single-nucleus and single-cell RNA sequencing (sc/snRNA-seq) and single-nucleus ATAC-seq (snATAC-seq), generating 292,423 transcriptomic profiles across 15 major cell populations.⁶ While this study provides valuable insights into age-related changes in muscle biology, the spatial dimension was inferred rather than directly measured. In contrast, our work leverages high-resolution spatial transcriptomics across 64 kidney samples (CosMx 1k, n=48; Xenium 5k, n=16) and integrates them with snRNA-seq from 150 patients (~750,000 nuclei), yielding over 5 million spatially resolved single-cell profiles.
6. Farah et al. created a spatial and transcriptional atlas of the developing human heart by combining single-cell RNA sequencing (~143,000 cells from 8 fetal hearts) with high-resolution MERFISH-based spatial transcriptomics (~250,000 cells from 3 hearts).⁷ While this approach enabled mapping of cardiac cell types and spatial organization, the MERFISH panel was limited to 238 genes. In contrast, our study focuses on spatially resolved transcriptomic profiling of human disease using higher-content technologies—CosMx (1,000 genes, n=48) and Xenium (5,100 genes, n=16).
7. Han et al. generated an epithelial cell-focused single-cell atlas of lung adenocarcinoma (LUAD) by profiling 246,102 epithelial cells from 16 early-stage LUADs and 47 matched normal lung samples using scRNA-seq.⁸ While this provided important insights into epithelial plasticity in cancer, the analysis was limited to dissociative single-cell profiling and a restricted cellular context. In contrast, our study provides spatially resolved transcriptional data from 64 samples using CosMx (1,000 genes, n=48) and Xenium (5,100 genes, n=16).
8. Yao et al. presented a whole-brain cell-type atlas of the adult mouse by integrating ~4 million high-quality scRNA-seq profiles with a spatial transcriptomics dataset of ~4.3 million cells using MERFISH.⁹ While this study delivers unmatched resolution for the mouse brain, the MERFISH profiling was limited to ~500 genes. In contrast, our study focuses on human kidney disease and provides a spatially resolved single-cell atlas of over 5 million cells using two complementary single-cell spatial transcriptomics platforms: CosMx (1,000 genes, n=48) and Xenium (5,100 genes, n=16). These were integrated with single-nucleus RNA-seq data from 150 patients (~750,000 cells).
9. Huang et al. performed combinatorial indexing-based single-cell RNA sequencing on over 1.6 million nuclei from 101 E13.5 mouse embryos representing 22 mutant and 4 wild-type genotypes.¹⁰ This whole-embryo approach provides a powerful framework for systematically assessing developmental phenotypes in mouse models. In contrast, our study focuses on human kidney disease and leverages two single-cell spatial transcriptomics platforms—CosMx (1,000 genes, n=48) and Xenium (5,100 genes, n=16)—to profile over 4 million spatially resolved human cells. These datasets were integrated with single-nucleus RNA-seq from 150 human kidney samples (~750,000 cells), resulting in a cross-technology atlas of more than 5 million cells.

10. Lake et al. applied multiple single-cell and single-nucleus RNA sequencing assays (>400,000 nuclei or cells) alongside spatial imaging technologies—Visium (22 patients) and Slide-seq2 (6 patients)—to profile healthy (45 donors) and diseased (48 patients) human kidneys, generating a valuable resource for understanding kidney injury and repair.¹¹ However, Visium offers limited spatial resolution, capturing transcriptomes at ~55 µm spots that typically include multiple cells, and Slide-seq2, while higher resolution, still does not achieve true single-cell resolution. In contrast, our study leverages two high-resolution single-cell spatial transcriptomics platforms—CosMx (1,000 genes, n=48) and Xenium (5,100 genes, n=16)—to profile over 4 million spatially resolved human kidney cells at true single-cell resolution. We further integrated these spatial data with single-nucleus RNA-seq from 150 kidney samples (~750,000 cells), generating a cross-technology reference atlas of more than 5 million cells. This makes our work to our knowledge the most comprehensive and spatially resolved single-cell resource available for any human disease to date.
11. Harnik et al. constructed a comprehensive spatial expression atlas of the adult human proximal small intestine using Visium spatial transcriptomics (8 patients), spatial proteomics, and single-molecule FISH.¹² While their study provides valuable insights, Visium lacks true single-cell resolution, with each spot covering multiple cells and thus limiting precise cell-type and spatial interaction analyses. By contrast, our study utilizes two single-cell resolution spatial transcriptomics platforms—CosMx (1,000 genes, 48 samples) and Xenium (5,100 genes, 16 samples)—across 64 kidney tissues, profiling over 4 million spatially resolved cells. When integrated with 150 single-nucleus RNA-seq profiles (~750,000 cells), this forms a cross-technology atlas of more than 5 million kidney cells, providing unmatched resolution and scale for dissecting human disease.
12. Qiu et al. applied single-cell combinatorial indexing to generate a comprehensive temporal transcriptional atlas of mouse prenatal development, profiling 12.4 million nuclei from 83 embryos across precisely staged intervals from E8 to P0.¹³ While the scale and temporal resolution of this study are impressive, the data are derived exclusively from nuclei and mouse embryos, without spatial resolution. In contrast, our study focuses on human disease and integrates single-cell spatial transcriptomics from 64 adult human kidney tissues using CosMx (1,000 genes, n=48) and Xenium (5,100 genes, n=16)—platforms that provide true single-cell resolution. Combined with 150 single-nucleus RNA-seq profiles (~750,000 cells), this dataset spans over 5 million cells, forming the most comprehensive spatially resolved human disease atlas to date.
13. Zhang et al. generated a spatially resolved single-cell atlas of the entire adult mouse brain by profiling ~10 million cells using MERFISH targeting ~1,100 genes.¹⁴ This study provides an impressive spatial transcriptomic overview at the whole-organ scale in mouse, though it remains limited to a predefined gene panel and is not focused on human disease. In contrast, our study creates a human disease-focused spatial atlas using two true single-cell spatial transcriptomics platforms—CosMx (1,000 genes, n=48) and Xenium (5,100 genes, n=16)—in combination with 150 snRNA-seq samples (~750,000 cells), totaling over 5 million human kidney cells. This integrative approach offers higher gene coverage than MERFISH and yields the most comprehensive single-cell spatial dataset yet available for any human disease.
14. Shi et al. constructed a spatial atlas of the adult mouse central nervous system by applying STARmap PLUS, an in situ sequencing method, to profile 1,022 genes across 1.09 million high-quality cells in 3D at subcellular resolution.¹⁵ While this provides remarkable spatial precision in the mouse CNS, the study is limited to a predefined gene panel and does not address human disease. In contrast, our study focuses on human pathology, integrating two true single-cell spatial transcriptomics platforms—CosMx (1,000 genes, n=48 samples) and Xenium (5,100 genes, n=16 samples)—with 150 snRNA-seq datasets (~750,000 cells), totaling over 5 million human kidney cells. This enables high-resolution spatial mapping with broader gene coverage and direct relevance to human disease, making it the largest and most comprehensive spatial single-cell resource for any human pathology to date.
15. Qian et al. generated a spatial atlas of the developing human fetal cortex by applying MERFISH with a 300-gene panel to over 18 million cells across 39 fetal brain samples, complemented by Visium (n=2) and snRNA-seq (n=3).¹⁶ While the study provides valuable insight into cortical arealization, it is limited to fetal tissue, uses a restricted gene panel, and employs only a single single-cell spatial technology. It also does not address adult human disease. In contrast, our study focuses on adult human disease—specifically diabetic kidney disease—and employs two single-cell spatial transcriptomics platforms: CosMx (1,000 genes, n=48 samples) and Xenium (5,100 genes, n=16 samples). These were integrated with snRNA-seq data from 150 samples (~750,000 cells), resulting in a dataset of over 5 million spatially resolved human cells.
16. Pei et al. constructed spatial maps of metastatic pancreatic cancer using Visium spatial transcriptomics on 49 biopsies from 13 patients¹⁷, profiling the tumor microenvironment and clonal architecture. While informative, the study is limited by the low spatial resolution of Visium. In contrast, our study focuses on diabetic kidney disease in the adult human, leveraging two high-resolution single-cell spatial transcriptomics platforms—CosMx (1,000 genes, n=48 samples) and Xenium (5,100 genes, n=16 samples)—alongside snRNA-seq data from 150 samples (~750,000 cells). This integration generated a spatial atlas of over 5 million human cells, offering superior cellular

resolution, broader gene coverage, and multimodal insight into disease-related microenvironments, establishing the most comprehensive spatial single-cell resource in human disease to date.

17. Sun et al. generated a spatially resolved single-cell brain atlas in mice, profiling 4.2 million cells across 20 ages and two rejuvenation interventions using MERFISH with a 300-gene panel.¹⁸ While this study provides valuable insights into aging, it is limited to mouse tissue, uses a restricted gene panel, and relies on a single spatial transcriptomics technology. In contrast, our study focuses on human diabetic kidney disease, combining two high-resolution single-cell spatial transcriptomics platforms—CosMx (1,000 genes; 48 samples) and Xenium (5,100 genes; 16 samples)—integrated with snRNA-seq data from 150 human samples (~750,000 nuclei). This integration yields a spatial atlas of over 5 million human cells, enabling in-depth exploration of disease-associated microenvironments at unprecedented spatial and transcriptional resolution.
18. Yayan et al. constructed a spatial human thymus cell atlas across fetal development by integrating multimodal single-cell data, spatial transcriptomics, and multiplex imaging from 29 donors.¹⁹ The spatial data was generated using the Visium platform, which provides limited spatial resolution and lacks single-cell precision. In contrast, our study focuses on human diabetic kidney disease and applies two high-resolution, single-cell spatial transcriptomics technologies—CosMx (1,000 genes; 48 samples) and Xenium (5,100 genes; 16 samples)—combined with single-nucleus RNA-seq from 150 samples (~750,000 cells). This integrated atlas of over 5 million spatially resolved human cells enables fine-scale mapping of disease-associated cellular niches and intercellular interactions.
19. To et al. created a multi-omic atlas of human embryonic skeletal development by combining paired transcriptional and epigenetic profiling of ~336,000 nuclei with spatial transcriptomics from 12 donors, focusing on joint and cranial development between 5 and 11 weeks post-conception.²⁰ Spatial transcriptomics was limited to in situ sequencing (ISS) using the 10x Genomics CARTANA platform, which targets only 150 genes, and Visium, performed on just two samples, both of which offer limited gene coverage and spatial resolution. In contrast, our study of diabetic kidney disease utilizes two high-resolution single-cell spatial transcriptomics platforms—CosMx (1,000 genes; 48 samples) and Xenium (5,100 genes; 16 samples)—integrated with single-nucleus RNA-seq from 150 patients (~750,000 cells). This results in a dataset of over 5 million spatially resolved human cells, making it the most comprehensive single-cell spatial atlas assembled for any human disease to date.
20. He et al. compiled an integrated transcriptomic cell atlas of human neural organoids by merging 36 publicly available single-cell transcriptomic datasets spanning 26 different protocols, totaling over 1.7 million cells.²¹ No new data was generated, and the atlas represents a meta-analysis of dissociated scRNA-seq data. In contrast, our study generates entirely new primary data from human kidney tissue using two single-cell spatial transcriptomics platforms—CosMx (1,000 genes; 48 samples) and Xenium (5,100 genes; 16 samples)—and integrates this with publicly available single-nucleus RNA-seq data from 150 patients (~750,000 cells). Altogether, our atlas comprises over 5 million spatially resolved human cells, providing the most comprehensive, experimentally generated single-cell spatial resource for any human disease to date.
21. Oliver et al. present a systematic integration of 25 scRNA-seq datasets from 385 samples across 189 healthy individuals, covering the entire gastrointestinal tract during development and adulthood.²² Using a standardized pipeline (scAutoQC), they assembled a healthy reference atlas of ~1.1 million dissociated single cells. In contrast, our study generates primary spatially resolved single-cell data from diseased human kidney tissue, using two distinct spatial transcriptomics platforms—CosMx (1,000 genes; 48 samples) and Xenium (5,100 genes; 16 samples)—and integrates it with single-nucleus RNA-seq from 150 patients (~750,000 cells). This results in a multi-modal atlas of over 5 million cells from more than 200 patients, offering high-resolution insight into disease-associated cellular neighborhoods and molecular states
22. Muñoz-Blat et al. employed a multi-omic approach to investigate decidualization resistance (DR) in the endometrium of women with a history of severe preeclampsia (sPE).²³ Their study combined morphological analysis with single-cell RNA sequencing (scRNA-seq) on endometrial samples from 23 patients (11 sPE, 12 controls) to characterize cellular and molecular disruptions. In contrast, our study goes beyond dissociative profiling by generating spatially resolved single-cell data from 64 human kidney tissue samples using two cutting-edge spatial transcriptomics platforms—CosMx (1,000 genes, 48 samples) and Xenium (5,100 genes, 16 samples). These datasets were integrated with single-nucleus RNA-seq profiles from 150 patients (~750,000 cells), creating a multi-modal kidney atlas of over 5 million spatially mapped cells.
23. Ounadjela et al. present a spatial multiomic atlas of the human placenta during early gestation.²⁴ They combined paired snRNA-seq and snATAC-seq, spatially resolved snATAC-seq and RNA-seq, and in situ sequencing/hybridization to map the epigenomic and transcriptomic landscape of placental development. This work was performed on tissue from eight donors at early gestational time points.

2. Complementary spatial platforms (CosMx + Xenium)

The use of two distinct spatial technologies—CosMx (1,000-gene panel) and Xenium (5,100-gene panel)—provided **cross-platform validation** of spatial patterns, niche definitions, and injury/immune states. We demonstrate **concordant transcriptional profiles** across platforms for major injured epithelial populations, immune cell types, and ligand–receptor interactions. This **platform-agnostic reproducibility** reinforces the robustness of our biological conclusions.

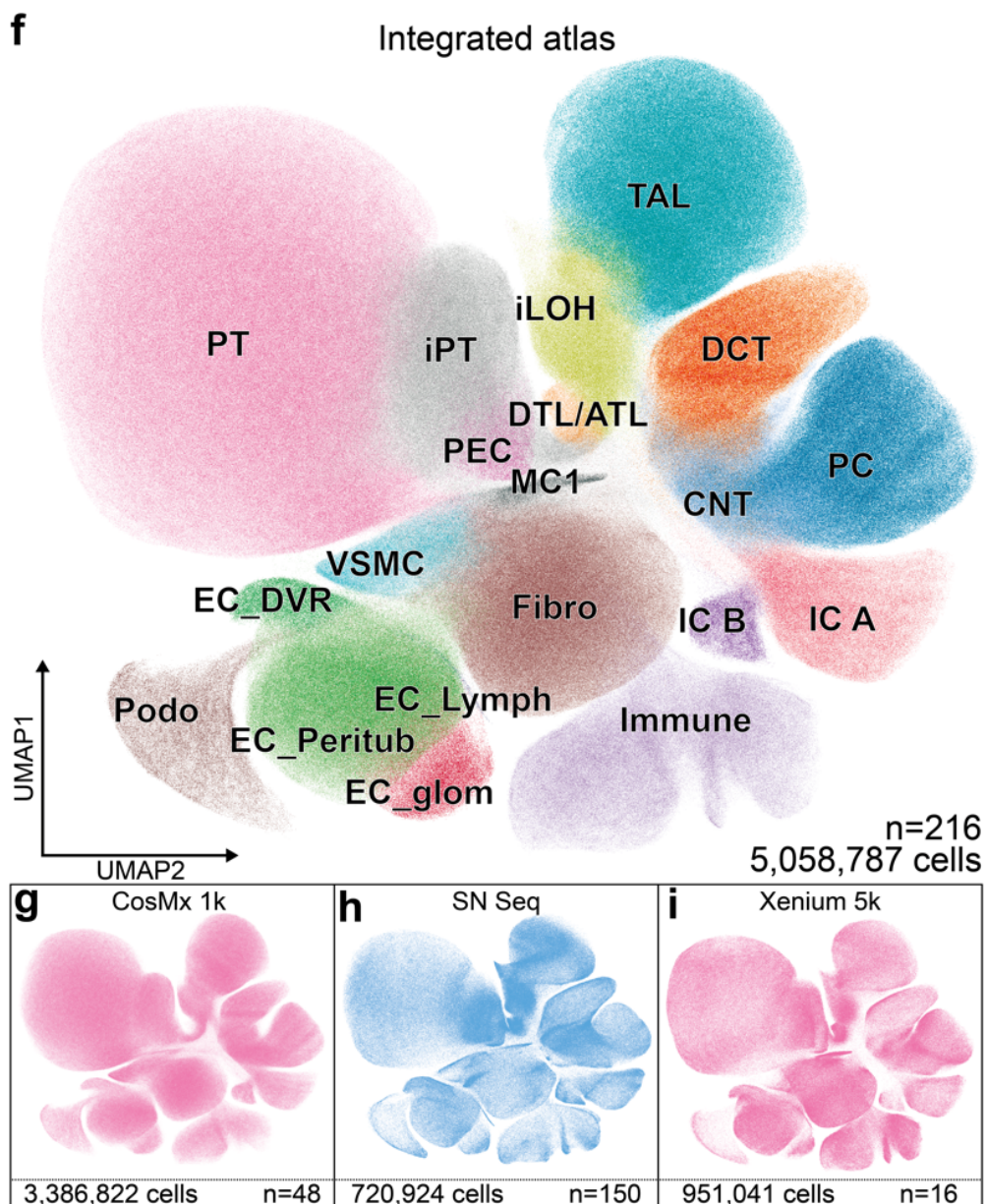


Fig. 1f: The UMAP visualization of the integrated spatial single-cell atlas. The spatial gene expression (CosMx 1k and Xenium 5k data) was integrated with dissociated single nucleus gene expression using scVI and scanVI. Each dot represents a single cell.

g, CosMx 1k spatial gene expression data. 48 samples and 3,386,822 cells were included in this study.

h, snRNA-seq data. 150 samples and 720,924 cells were included in this study.

The color indicates the different cell types such as: CNT, connecting tubular cells; VSMC, vascular smooth muscle cell; PC, principal cell; IC A, IC B, intercalated cells A, B; inj T, injured tubular cells; PT, proximal tubular cells; MC1, mesangial cell; LOH, thick ascending limb cells as well as DTL_ATL, distal thin limb, ascending thin limb cells; Fibro, fibroblasts; Podo, podocytes; DCT, distal convoluted tubular cells; PEC, parietal epithelial cells; EC: EC_Peritub, peritubular endothelial cells as well as EC_DVR, vasa recta endothelial cells; Immune, immune cells; EC_glom, glomerular endothelial cells

3. Discovery of 11 spatially defined kidney niches

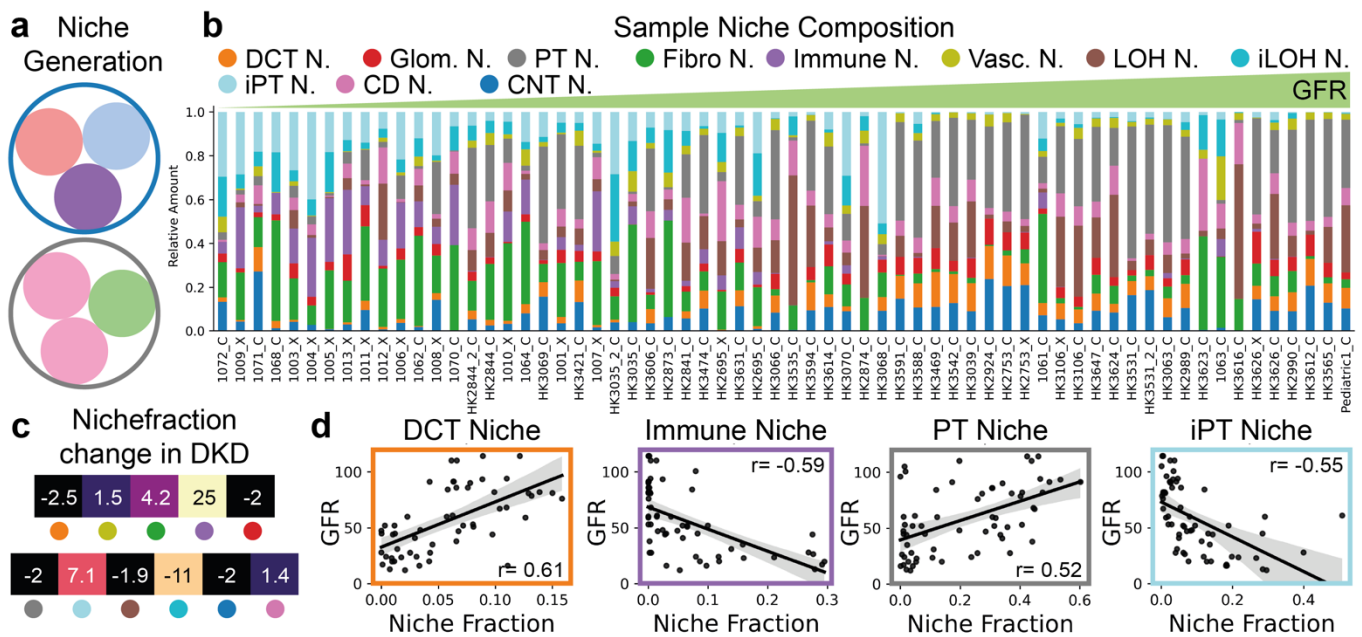


Fig 2a: Schematic of defining niches (left) and microenvironments (right). For niches, all cells were clustered into groups based on their neighborhood fraction after restricting the analysis to those with sufficient local context, removing the sparsest 5% of neighborhoods (based on total neighbor count at 80 μm) to reduce edge effects ($n = 4,029,331$ cells). For each cell, we computed the number of neighboring cells within concentric distance shells: 0–20 μm , 20–40 μm , 40–60 μm , and 60–80 μm . This was achieved by calculating cumulative neighbor counts at 20, 40, 60, and 80 μm , followed by subtracting counts from the smaller radius to isolate the number of neighbors in each respective shell (e.g., the 40–60 μm shell = count at 60 μm – count at 40 μm). For each cell, neighbor counts were normalized by the total number of neighbors at that distance, yielding a cell-by-neighbor composition matrix that reflects the relative cellular makeup of the local environment at different spatial scales. This matrix was then scaled and subjected to principal component analysis (PCA), with the top 10 components used as input for MiniBatch K-means clustering. The number of clusters (k) was determined based on the elbow method. Niche clustering was performed on the entire dataset to define global spatial neighborhoods. b: Sample niche composition: the fraction of each niche is calculated for all cells within each sample and visualized. The samples are arranged by disease severity (GFR) from left to right. c: Change in relative niche fraction in DKD vs healthy controls. Each value represents the fold change in the average proportion of a given niche between DKD patients and healthy controls. A positive value indicates that the niche is expanded in DKD, while a negative value indicates it is more prevalent in controls. Fold changes were computed by normalizing niche frequencies within each diagnostic group, then taking the ratio of DKD to control values. d, correlation of niche fraction with eGFR using Pearson correlation. All correlations with $p < 0.001$.

We define **spatial niches** as recurrent multicellular tissue structures, based on unbiased clustering of local neighborhood composition. These 11 niches reflect canonical structures (e.g., glomeruli, vasculature, Fig. 2b), homeostatic epithelial states, and **injury-associated niches** such as the **injured proximal tubule (iPT)** and **injured loop of Henle (iLOH)** niches. These injury niches are:

- **Absent in healthy pediatric controls,**
- **Increased (iPT) (iLOH) enriched in DKD,**
- And **strongly negatively correlated with kidney function** (iPT $r = -0.55$, $p < 0.001$, iLOH $r = -0.38$, $p < 0.01$).
- Using these defined spatial contexts, we identified DKD-specific gene expression changes within individual niches, reflecting altered cellular interactions. Notably, in the vascular niche, we found enrichment for pathways related to ECM remodeling, senescence, inflammatory signaling, and neovascularization (Fig. 2o).
- We analyzed spatially correlated transcripts for each niche, using Hotspot based on the niche-specific latent representations calculated by COVET analysis. This allowed us to investigate niche-specific regulatory networks: In both DKD patients and control patients, we found an overlap between spatially correlated transcripts. For example, in the vascular niche, which is shown here, we find that key vascular or mural gene pairs like MYH11-SPARCL1, ACTA2-TAGLN, ACTA2-NOTCH3, PECAM1-TEK to be correlated in both control as well as DKD patients. On the other hand, using the Hotspot analysis we were now able to highlight gene pair correlation shifts in DKD using differential correlation analysis. In the vascular niche, we found increased correlations between transcript pairs such as COL1A1-SERPINE1, CXCL12-MHC I, and JUNB-CD74—implicated in matrix remodeling, immune signaling, and antigen presentation—in DKD (Extended Fig. 25). In summary, using COVET and subsequently Hotspot,

we could further identify conserved gene-gene correlations within all the defined niches, as well as disease-associated correlation shifts. We have performed this analysis for all niches and illustrated these across the Extended Fig. 15-25.

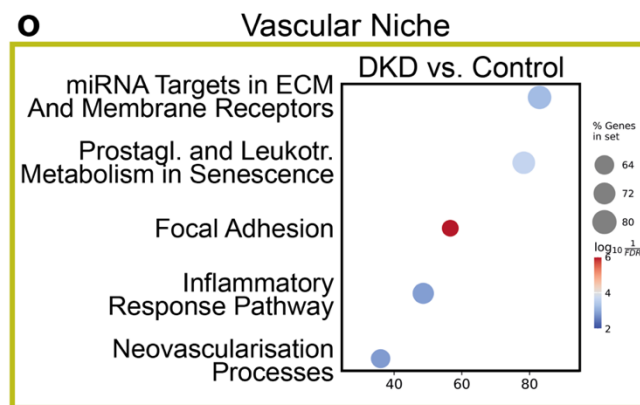
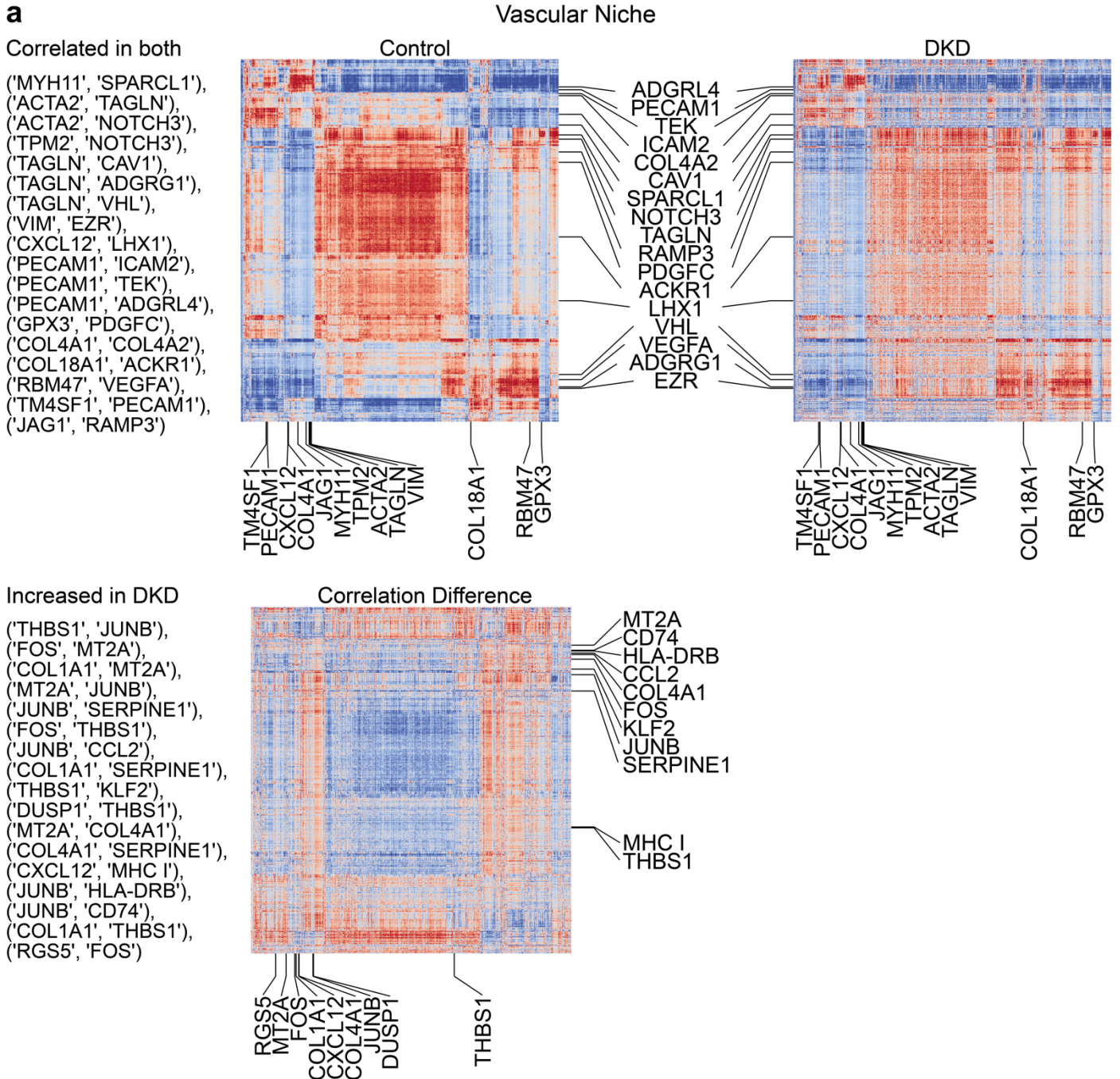


Fig. 2o: Differential gene expression analysis was performed between control and DKD samples specifically within the vascular niche, using the Wilcoxon rank-sum test on imputed gene expression data. Gene set enrichment analysis was subsequently conducted using GSEApY with the Wikipathways 2021 database. Displayed pathways reflect significantly enriched gene sets with an adjusted p-value < 0.01.

These findings extend beyond prior knowledge by showing that epithelial injury in DKD manifests as **spatially organized, disease-specific tissue units**, rather than diffuse or isolated events.



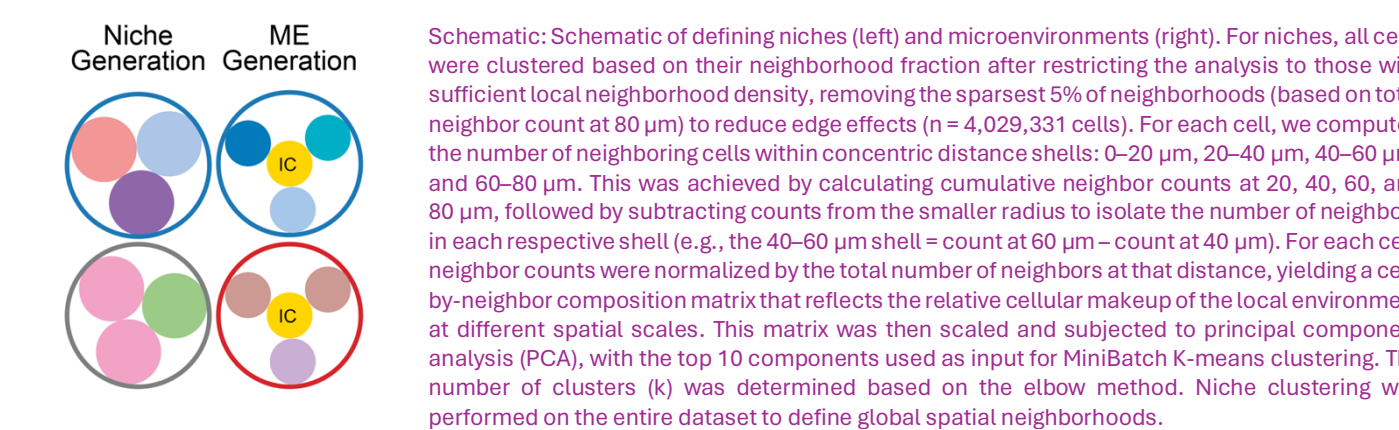
Extended Fig. 25: Spatially correlated transcripts in the vascular niche. Hotspot was applied to identify genes with spatially correlated expression patterns based on niche-specific latent representations calculated by COVET analysis. The heatmap displays pairwise gene-gene correlations, with stronger correlations indicated by deeper red coloring. Gene pairs conserved across both conditions are highlighted at the top. Differential correlation analysis revealed condition-specific interactions, with gene pairs significantly increased in diabetic kidney disease (DKD) highlighted (bottom).

4. Cell-centric microenvironments

In addition to tissue-scale niches, we defined **microenvironments (MEs)** centered on individual injured epithelial cells or immune cells (Schematic). Cells change their gene expression profiles based on the neighboring cells due to cell-cell interactions, such a macrophage next to different genes when is next to injured cells compared to a healthy cell. These local interaction neighborhoods capture **context-dependent remodeling** of the microenvironment, such as:

- The **profibrotic ME**, characterized by proximity to immune cells and fibroblasts and spatially overlapping with **collagen-rich fibrotic areas** (validated by Sirius Red staining);
- Shifts in local signaling pathways, such as increased **EGFR and PDGFR signaling**, linked to matrix deposition.

This dual perspective—tissue-centric niches and cell-centric MEs—offers a novel lens to study disease progression and the evolution of pathological tissue architecture.



5. Construction of a spatial immune atlas

We generated a **kidney immune cell atlas comprising >250,000 immune cells**, spatially resolved across 64 patients. Using neighborhood-based clustering, we identified **seven distinct immune microenvironments**, each enriched for specific neighbor types (Fig. 4f-s):

1.

Fibroblast-associated immune ME
2.

Injured tubular immune ME
3.

Vascular immune ME
4.

Glomerular immune ME
5.

T cell–predominant immune ME
6.

B cell–predominant immune ME
7.

Resident immune ME

These MEs were stable across samples and platforms and revealed **structured immune–epithelial interactions** not previously mapped in human kidney disease. Notably, the **resident immune ME** was enriched for **anti-inflammatory, tissue-resident macrophages** and maintained stable abundance across health and disease—suggesting a role in immune homeostasis.

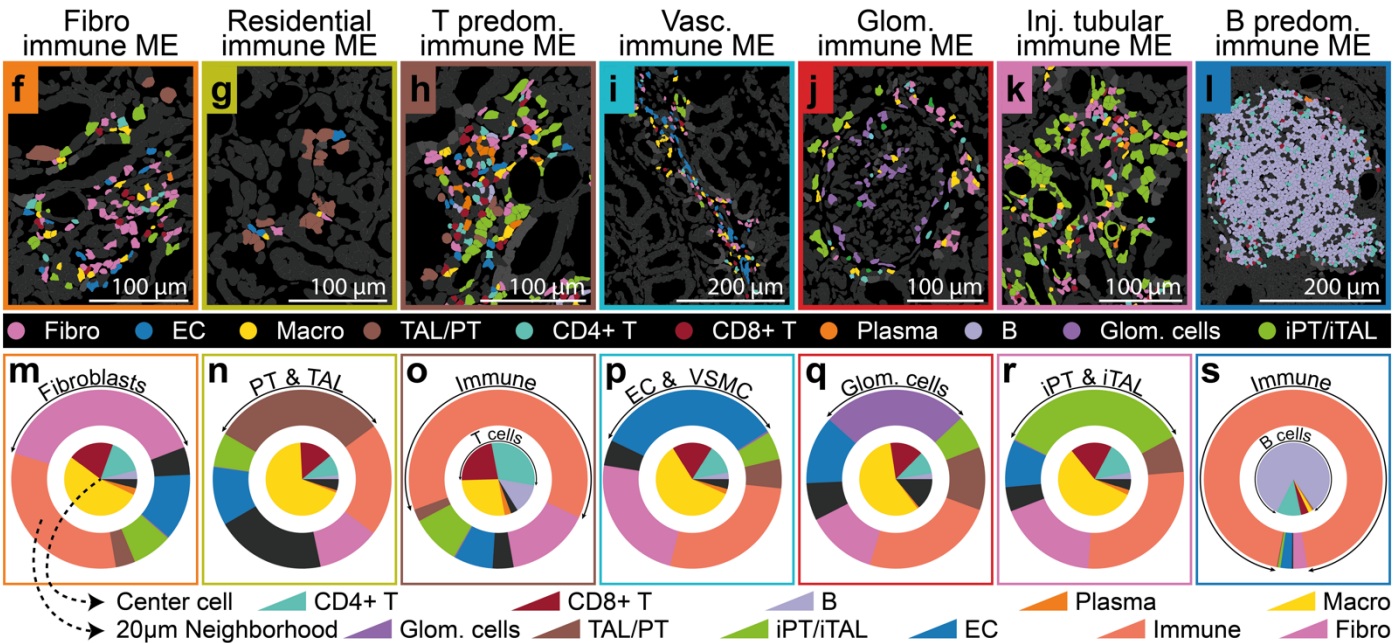


Fig 4: Immune microenvironments: f-l representative spatial plot of the Fibro Immune, Residential immune, T predom. Immune, Vasc. Immune, Glom. Immune, Inj. Tubular immune and B predom. Immune microenvironments. m-s circular plots showing the celltype composition in the 20μm surrounding (outer circle) and the annotation of the immune cells at the center (inner circle). Celltypes are colored based on colors shown in the legend. Xenium Explorer 3 was used for the spatial plot visualization.

6. Discovery of disease severity–associated cell–cell interactions

By correlating **ligand–receptor interaction strength** with **clinical metrics (eGFR)**, we identify the first **disease severity–dependent, spatially anchored interaction networks** in human DKD (Fig. 2p–r). Examples include:

- **IL6–IL6R** and **PLAU–PLAUR** (glomerular endothelial cells to podocytes),

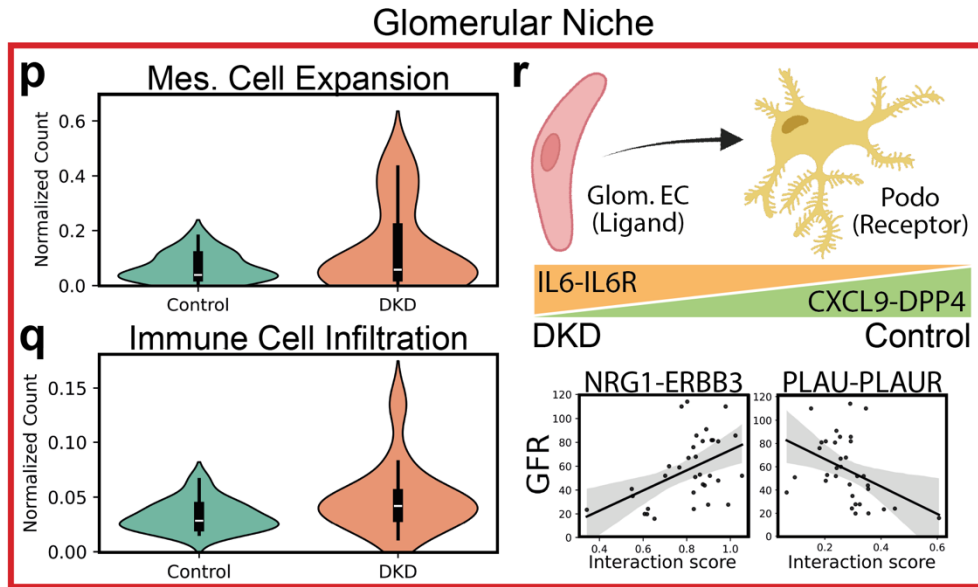


Fig. 2p: analysis of the glomerular niche: The fraction of mesangial cells in the glomerular was calculated and compared between control and DKD patients ($p < 0.05$). q: The fraction of immune cells in the glomerular was calculated and compared between control and DKD patients ($p < 0.05$). r: Ligand–receptor interactions originating from glomerular endothelial cells and targeting podocytes were inferred using CellPhoneDB v5, with microenvironments defined at the sample level. Analyses were performed using imputed gene expression to maximize sensitivity. Only statistically significant ligand–receptor pairs were retained for downstream analysis. Interaction scores were then correlated with estimated glomerular filtration rate (eGFR) across samples.

7. Mapping immune–epithelial interactions

We show that inflammation in DKD is **not globally increased**, but **spatially organized** around injured tubules, vasculature, and glomeruli (Fig. 4t–w). Notable interactions include:

- **CXCL12–CXCR4** and **TNFSF13–TNFRSF13B (APRIL–TACI)** signaling between injured epithelium and plasma cells;
- **FN1–ITGA4B7** between mesangial cells and macrophages, negatively correlated with eGFR;
- **BMP4–BMPR2** between podocytes and CD8⁺ T cells, positively correlated with eGFR.

This work creates the first **spatially informed immune signaling network** for DKD, with direct implications for understanding and treating tissue-specific inflammation.

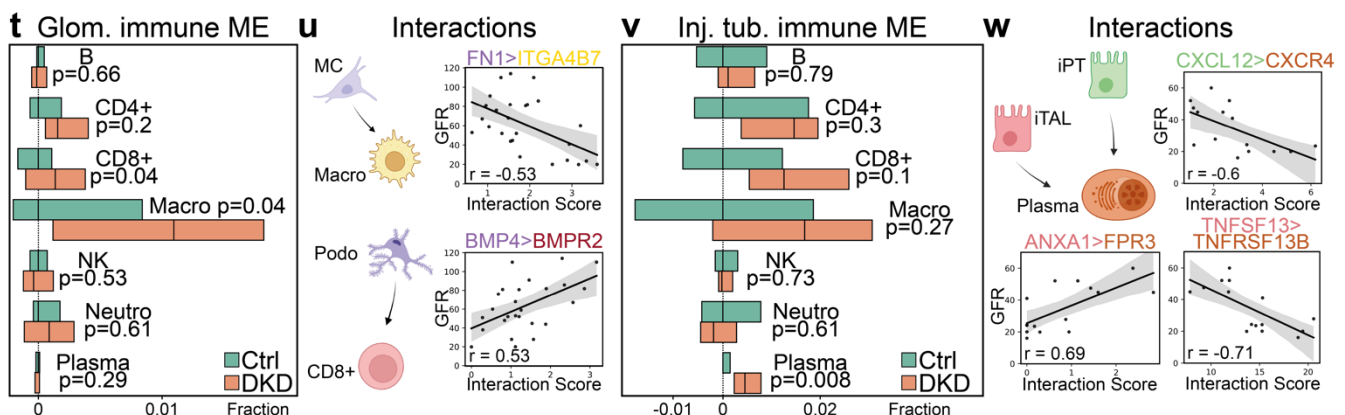


Fig 4v: Comparison of immune cell fractions within the injured tubular immune microenvironment between control and DKD samples. Shown are the relative abundances of B cells, CD4⁺ T cells, CD8⁺ T cells, macrophages, NK cells, neutrophils, and plasma cells in control (green) and DKD (red). For visualization, the median fraction of each immune cell type in control samples was calculated and subtracted from both control and DKD values, centering control medians at zero to facilitate direct comparison. Statistical comparisons were performed using Student's t-test, and p-values are indicated. w: Ligand–receptor interactions between injured proximal tubular cells, injured thick ascending limb cells and plasma cells within the injured tubular immune microenvironment were inferred using CellPhoneDB v5. The interaction scores for significantly enriched pairs were then correlated with GFR.

8. Spatial and molecular definition of fibrotic remodeling

We precisely localize **fibrosis-associated epithelial and stromal states** and define ligand–receptor axes that correlate with **COL1A1 expression and Sirius Red staining (Fig. 3o)**, including:

- **JAG2–NOTCH, PDGFB–PDGFRB, and EGFR signaling**, all spatially restricted to fibrotic niches.

These spatially restricted interactions support novel therapeutic targeting strategies and provide insight into how **early epithelial injury transitions to irreversible fibrosis**.

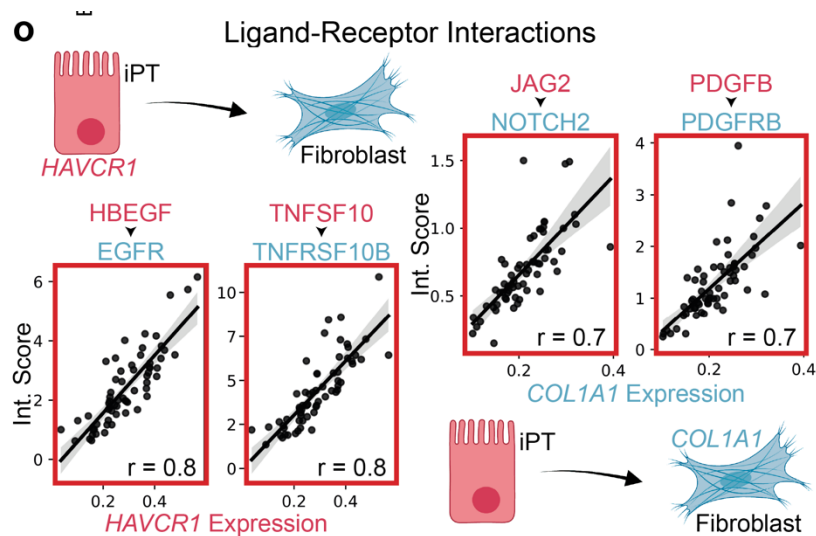


Fig. 3o: Left: Ligand–receptor interactions between injured proximal tubular cells and fibroblasts within the profibrotic microenvironment were inferred using CellPhoneDB v5. The interaction scores for significantly enriched pairs were then correlated with HAVCR1 expression in proximal tubular cells. Right: The same interaction scores were correlated with COL1A1 expression in fibroblasts. Each dot represents one sample.

9. Identification and subtyping of intrarenal B cell clusters

We identified a **B cell–predominant immune ME** resembling **tertiary lymphoid structures (TLSs)** in a **subset of DKD patients**. This ME is characterized by:

- CXCL13–CXCR5 expression,
- BAFF–BAFF-R signaling,
- CD20⁺ B cell proliferation (Ki67⁺),
- Class-switch recombination (AICDA, IRF4),
- And formation of plasma cells (CD38⁺), confirmed by **IMC and transcriptomic profiling (Fig. 5)**.

Using scVI/scANVI, we integrated these spatial B cells with a reference B cell atlas and identified **naïve, memory, atypical memory, and germinal center–like B cell subsets** in situ. To our knowledge, this represents the **first subtyping of intrarenal B cells in DKD using spatial methods**.

10. Therapeutic targetability of immune niches

Proteomic analysis of IgA immune complexes revealed enrichment of **complement components (C3, C4A, C8B)** and **granzymes** in B cell–rich kidneys, linking this immune niche to **antibody-mediated tissue injury**. Based on this, we propose **three repurposable therapeutic strategies**:

1. **B cell depletion** (e.g., rituximab, ofatumumab),
2. **BAFF/APRIL inhibition** (e.g., belimumab),
3. **Complement inhibition** (e.g., iptacopan), all with known efficacy in immune kidney diseases.

11. Development of a biomarker for B⁺ DKD

We generated a spatial gene signature from the B cell–predominant ME and applied it to **two independent cohorts** (Susztaklab Biobank, n = 843; TRIDENT), identifying a **B⁺ DKD subgroup (~8.4%)** with:

- **Faster progression to ESKD**, confirmed by Kaplan–Meier survival analysis and multivariable Cox modeling.

This provides the foundation for **biomarker-based patient stratification** in future trials.

Conclusion

In sum, while we reproduce classical DKD features as internal validation, the true strength of this study lies in its **novel biological insight**. We describe **entirely new spatial tissue units, microenvironmental**

reprogramming, targetable molecular pathways, and disease-associated immune niches—all revealed by integrating high-resolution spatial transcriptomics with machine learning and orthogonal validation. We have revised the manuscript to better reflect these contributions and thank the reviewer for encouraging us to more clearly articulate the **biological novelty and translational significance** of this work.

The description of a microenvironment in which B cells predominate is interesting, and a more detailed analysis of it would be of interest. The finding that there is an increase in immune infiltrate including B cells in diabetic kidney disease has been reported previously, and a correlate with infiltrate and disease progression is not unexpected. In the absence of applying CosMx to refine B cell subtypes via gene signatures at single cell resolution (outside of inflammatory pathways consistent with DKD), it is difficult to determine the additional value of this analysis compared to existing studies. What subset of B cells are present, what are their isotypes, are they associated with other features of tertiary lymphoid structures etc.. This is a potentially interesting finding that needs to be described in more detail, validated, and correlated with disease in a larger cohort. That a “large number” of renal function/disease associated genes identified in a GWAS are expressed in B cells is inconclusive. Have the authors compared the expression of GWAS-implicated genes across all kidney-resident immune cells to determine whether B cells uniquely express these genes at higher levels?

We thank the reviewer for this insightful comment and the opportunity to clarify both the novelty and added value of our findings. We appreciate that B cell infiltration in diabetic kidney disease (DKD) has been noted in previous studies, particularly in dissociative single-cell datasets and bulk transcriptomic analyses. However, we respectfully submit that **the biological significance of intrarenal B cells in DKD—and their spatial architecture, functional phenotypes, and relationship to disease progression—has not been characterized or validated until now.**

Previous small studies have reported **B cell increases**, but these findings were not consistent across datasets, often limited by small sample sizes, originated from bulk tissue analysis, lack of spatial context, and minimal phenotyping. Most critically, **the association between B cell infiltration and kidney disease progression has not been robustly demonstrated or clinically stratified at the level of spatially organized microenvironments or molecular phenotypes.** Indeed, we show that only 8% of cases are from the B+ DKD subtype (which is a bit contrast to prior papers). We therefore respectfully disagree with the assertion that this relationship is well established. Our work fills this gap by providing an integrated, spatially resolved, and clinically anchored analysis of B cell biology in DKD.

To address the reviewer’s concerns in detail, we leverage our expanded dataset including 64 samples, to construct a **spatial immune atlas of >250,000 immune cells (Fig. 4a).**

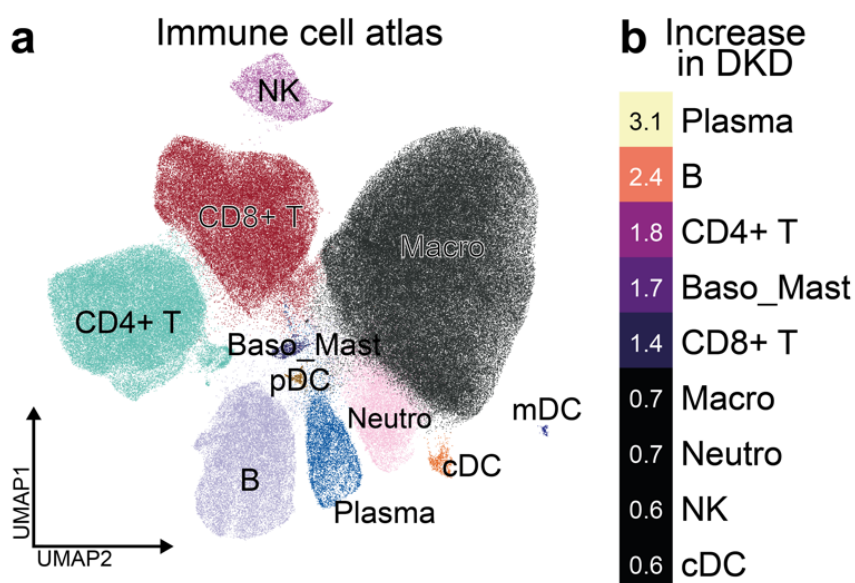


Fig 4a, Human kidney immune cell atlas. UMAP of the integrated datasets colored by cell types. Each dot represents one cell. The color represents the different cell populations: Baso/Mast, basophils and mastcells; Macro, macrophages; Neutro, neutrophils; cDC, classical dendritic cells; mDC, myeloid dendritic cells; pDC, plasmacytoid dendritic cells; B, B cells; Plasma, plasma cells; CD4+ T, CD4+ T cells; CD8+ T, CD8+ T cells; NK, natural killer cells. B, Fold change between immune cell fractions of control vs. DKD patients.

Through this framework, we identify immune microenvironments and identify a B cell-predominant immune microenvironment in a subset of DKD patients. Here B cells reside next to follicular T helper cells, as evidenced by the expression of CXCR5 and PDCD1 specifically in this immune microenvironment in CD4+ T cells (Extended

Fig. 29g). Moreover, we found expression of CXCL13 and CXCR5 as well as TNFSF13B and TNFRSF13C in this microenvironment further **supporting the tertiary lymphoid structure formation (Fig. 5o).**

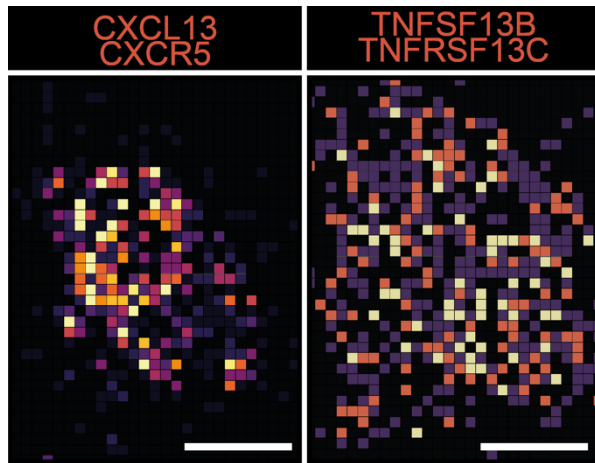


Fig 5: Zoom into the B cell predominant immune microenvironment shown in Fig. 4l and s. Colocalization of the expression of CXCL13 and CXCR5 (binned to 20 μ m) as well as TNFSF13B and TNFRSF13C. Xenium Explorer 3 was used for this visualization.

Analyzing B cell subsets: using scVI/scANVI, we integrated our spatial Xenium-derived B and plasma cells with a reference atlas of human B cell states. This revealed: Memory B cells, Naïve B cells and Atypical memory B cells. This was confirmed by imaging mass cytometry with a multiplex protein staining, using CD27 as memory B cell marker, BCL6 as germinal center marker and CD11c for atypical memory B cells (Fig. 5q-v).

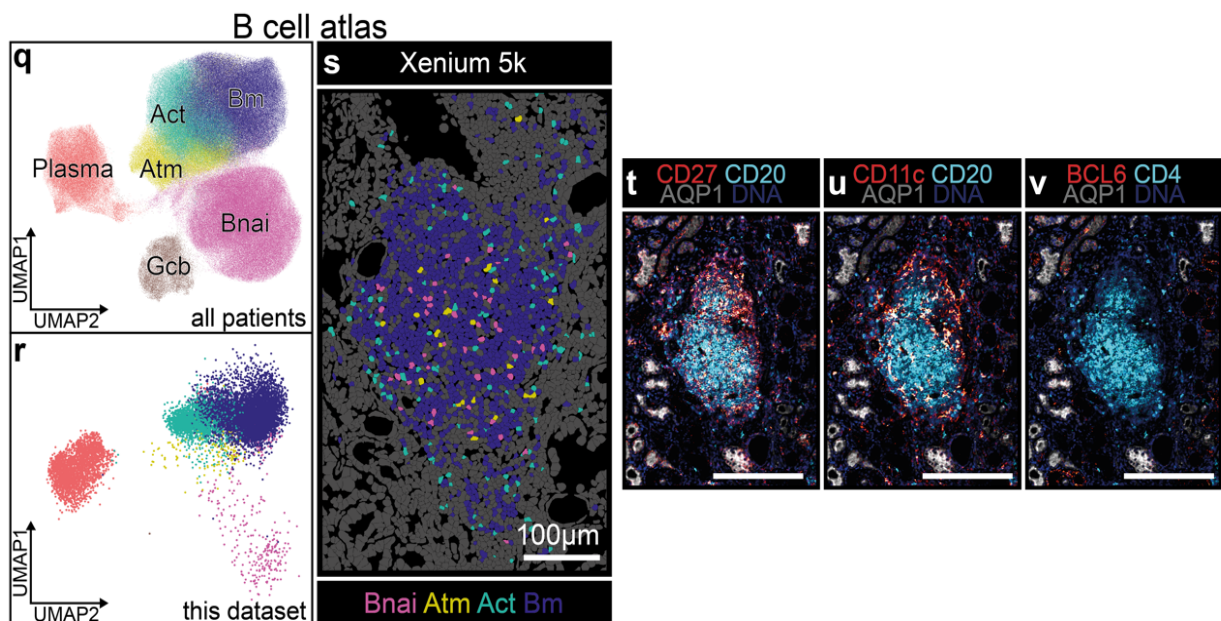
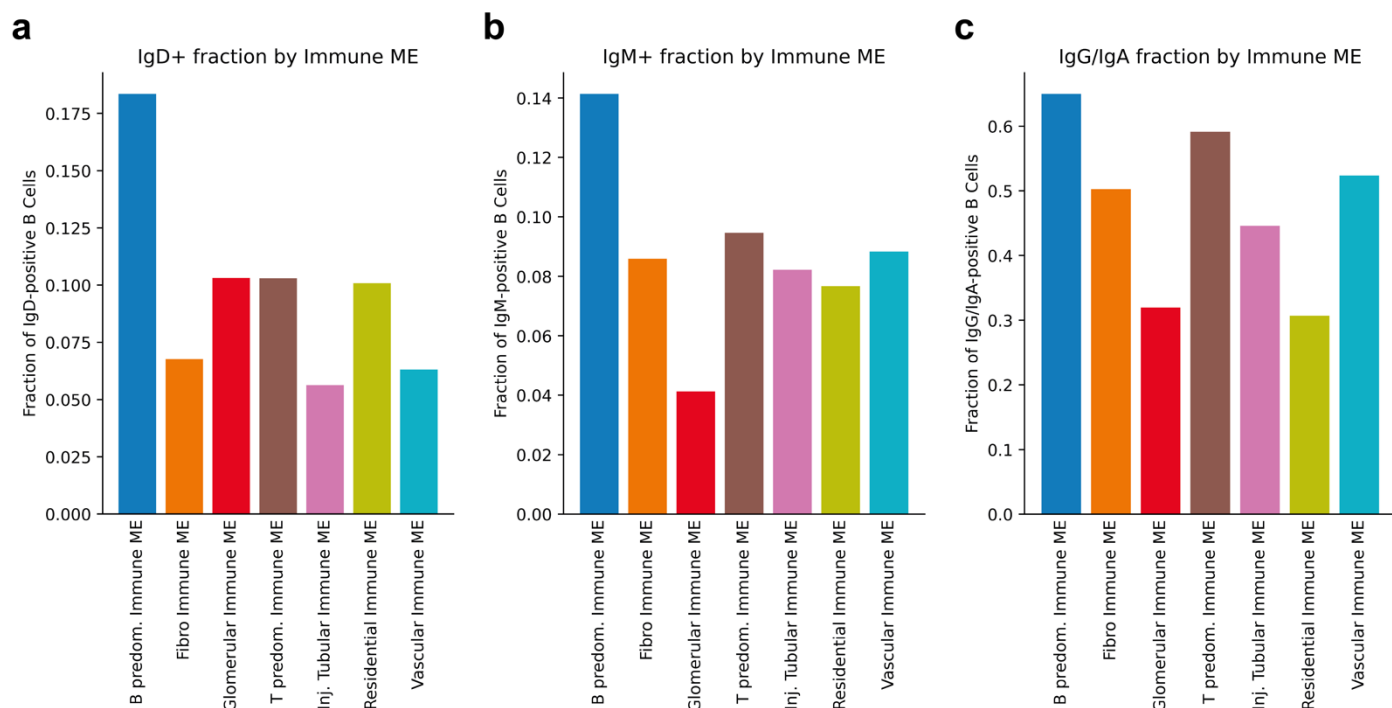


Fig 5: High-resolution spatial B and plasma cell atlas constructed by integrating our Xenium 5k spatial transcriptomics data with a publicly available single-cell RNA-sequencing reference of human B and plasma cells. Integration was performed using a two-step approach with scVI and scANVI. A four-layer scVI model (30 latent dimensions, learning rate = 0.001, early stopping with patience = 15) was trained on raw counts to create a shared latent space. scANVI was then used in a semi-supervised manner to transfer cell type annotations from the reference to the Xenium dataset. Label transfer was based on latent space proximity, followed by retraining on high-confidence annotations to refine classification. q: UMAP of the integrated datasets colored by cell types. Each dot represents one cell. The color represents the different cell populations: Plasma cells (Plasma), atypical memory B cells (Atm), activated B cells (Act), memory B cells (Bm), naïve B cells (Bnai) and germinal center B cells (Gcb). r: UMAP of only Xenium cells of this study in the integrated dataset, colored by cell types. s: Spatial plots generated using Xenium Explorer 3 of the B cell subsets in the B predominant immune microenvironment. t-v: Imaging mass cytometry using multiplex protein panel of CD20, AQP1, DNA staining, CD27, CD11c, BCL6.

Analyzing B cell isotypes: We used the CosMx 1k platform to classify B cells into four categories—IgD⁺, IgM⁺, IgA/G⁺, or negative—based on normalized and scaled expression of *IGHD*, *IGHM*, *IGHA1*, *IGHG1*, and *IGHG2*, assigning each cell to the dominant isotype expressed. Extended Fig. 31a shows the Fraction of IgD⁺ B cells across immune MEs. The B cell-predominant immune ME showed a significantly higher proportion of IgD⁺ B cells compared to all other MEs (Mann-Whitney U test, $p = 2.38 \times 10^{-11}$). B shows the fraction of IgM⁺ B cells across immune MEs. A significantly higher proportion of IgM⁺ B cells was also observed in the B cell-predominant immune ME (Mann-Whitney U test, $p = 1.06 \times 10^{-4}$). c: Fraction of IgA/G⁺ B cells across immune MEs. No

significant difference was observed between the B cell–predominant ME and other immune MEs. To characterize B cell isotype distribution across immune microenvironments (MEs), we classified B cells into four categories—IgD⁺, IgM⁺, IgA/G⁺, or negative—based on the dominant normalized and scaled expression *IGHD*, *IGHM*, *IGHA1*, *IGHG1*, and *IGHG2*. We observed that the B cell–predominant immune ME contained a significantly higher fraction of IgD⁺ B cells compared to all other immune MEs (Mann–Whitney U test, $p = 2.38 \times 10^{-11}$). Similarly, IgM⁺ B cells were also significantly enriched in the B cell–predominant ME ($p = 1.06 \times 10^{-4}$). In contrast, no significant differences were observed in the distribution of IgA/G⁺ B cells between the B cell–predominant and



Extended Fig. 31: To investigate antibody isotype distribution within spatial immune microenvironments (MEs), B cells were classified into four categories—IgD⁺, IgM⁺, IgA/G⁺, or negative—based on normalized and scaled expression of *IGHD*, *IGHM*, *IGHA1*, *IGHG1*, and *IGHG2*, assigning each cell to the dominant isotype expressed. a: Fraction of IgD⁺ B cells across immune MEs. The B cell-predominant immune ME showed a significantly higher proportion of IgD⁺ B cells compared to all other MEs (Mann-Whitney U test, $p = 2.38 \times 10^{-11}$). b: Fraction of IgM⁺ B cells across immune MEs. A significantly higher proportion of IgM⁺ B cells was also observed in the B cell-predominant immune ME (Mann-Whitney U test, $p = 1.06 \times 10^{-4}$). c: Fraction of IgA/G⁺ B cells across immune MEs. No significant difference was observed between the B cell-predominant ME and other immune MEs.

other immune MEs. These findings indicate that the B cell-predominant ME is enriched for early-stage or naive B cells, while class-switched B cells are more broadly distributed across immune microenvironments.

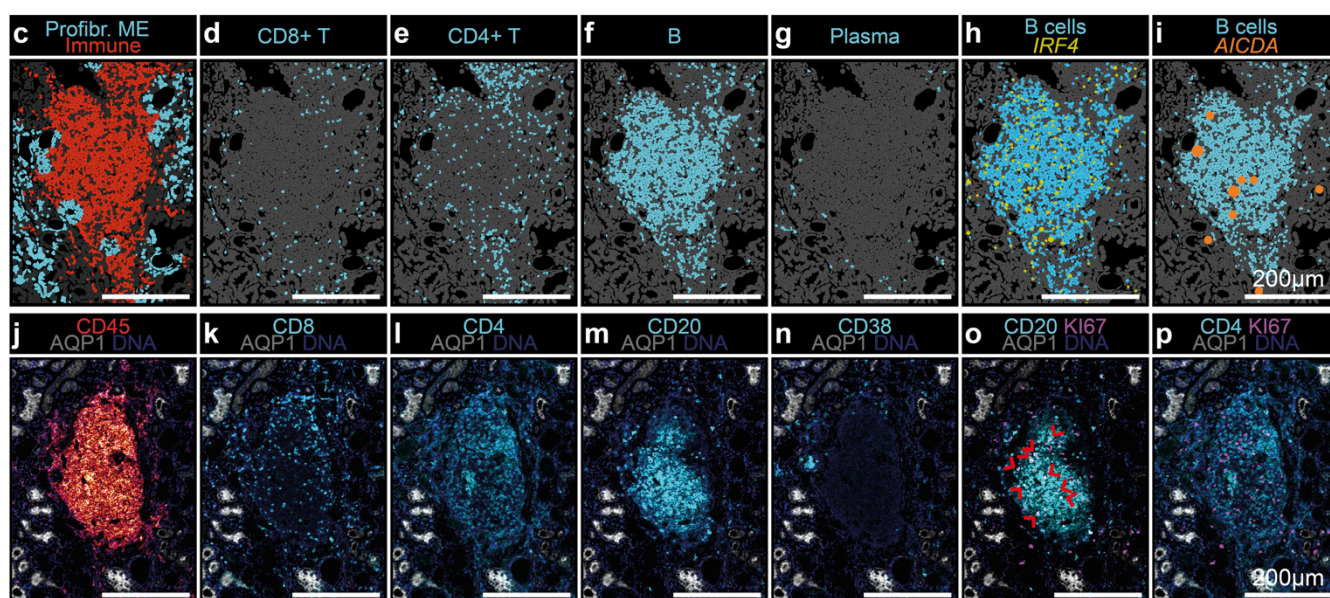


Fig 5c – i: spatial plots generated using Xenium Explorer 3 of the profibrotic tubular microenvironment and immune cells (c); immune cell types in the B cell predominant microenvironment (d-g). Expression of *IRF4* and *AICDA*. J to p: Imaging mass cytometry using multiplex protein panel of CD45, AQP1, DNA staining, CD8, CD4, CD20, CD38, CD20, KI67.

Moreover, B cells within this microenvironment expressed **AICDA**, a key enzyme mediating class-switch recombination, and **IRF4**, a transcription factor essential for plasma cell differentiation. Imaging mass cytometry further revealed that these B cells were **Ki67-positive**, indicating active proliferation. Together, these findings suggest that B cells in this niche undergo activation, class switching, and differentiation toward antibody-producing cells, features not shown previously in DKD (Fig. 5).

Correlation with disease severity and progression

We derived a **spatial gene signature** from the B cell–predominant ME and applied it to:

- The **Susztaklab Biobank (n = 843)**: Strong negative correlation with **interstitial fibrosis**;
- The **TRIDENT DKD cohort**: Patients with a high B cell signature (**B⁺ DKD, ~8.4%**) had:
 - **Lower baseline eGFR**,
 - **Faster progression to end-stage kidney disease**, validated by **Kaplan–Meier survival analysis**.

These analyses provide **robust evidence of clinical relevance**, distinguishing our findings from prior reports of generalized immune infiltration.

Comparison of GWAS gene expression across immune subsets

We appreciate the reviewer’s skepticism regarding our claim that a "large number" of kidney disease–associated GWAS genes are expressed in B cells. To clarify:

- Our **recent GWAS of kidney function in >2.2 million individuals** (Liu et al. Science 2025) identified **1,026 loci** and **1,060 candidate genes**.
- In this study, using snRNA sequencing data we could map the expression of **45 GWAS-prioritized genes to B cells**.

While we agree that expression alone does not imply causality, this analysis **adds weight to the hypothesis that B cells may play a genetically mediated role in DKD pathogenesis**, especially when considered alongside spatial, transcriptional, and proteomic evidence.

Therapeutic implications and clinical translation

Our findings identify three **repurposable therapeutic strategies** for patients with B⁺ DKD:

1. **B cell–targeted therapies** (e.g., rituximab, ofatumumab),
2. **BAFF/APRIL inhibition** (e.g., belimumab),
3. **Complement blockade** (e.g., iptacopan).

We further propose that **CD19 CAR T cell therapy**—currently being trialed in autoimmune disease—could overcome the **tissue-penetration limitations of anti-CD20 monoclonals**. Preliminary retrospective analysis in a **lymphoma cohort (n = 64, eGFR < 60)** treated with CD19 CAR T cells showed **statistically significant improvements in kidney function** at 2 months post-treatment (Extended Fig. 34). While not definitive, this finding supports the **biological plausibility and therapeutic potential** of targeting intrarenal B cells in DKD.

Conclusion:

Our work provides a detailed, spatially resolved, and clinically validated characterization of a **B cell–predominant immune microenvironment in DKD**. Using CosMx, Xenium, and IMC, our analysis adds substantial value beyond prior studies and provides a compelling rationale for **precision immunotherapy trials in B⁺ DKD**. We have updated the manuscript to incorporate these clarifications and thank the reviewer for the opportunity to strengthen our work.

2. Sample size

The techniques and analyses used appear appropriate. One concern, however, is the very low sample number, with only five individuals with DKD being compared with four healthy control samples. It is also not always clear which diseases are being compared with healthy. The initial analysis appears to include all three disease subcohorts together with the healthy one. It is not clear if the replicate samples (that expand the DKD cohort from 5 to 7, for example) are treated as independent values during statistical analysis – this would not seem appropriate and given the cohort sizes their exclusion might make a difference to conclusions.

Response: We thank the reviewer for this important comment. In response, we have substantially expanded our dataset, increasing the number of spatially profiled samples fourfold to a total of 64, using two complementary high-resolution platforms—**CosMx 1k** (n = 48; ~3.3 million cells) and **Xenium 5k** (n = 16; ~1 million cells). These spatial datasets were integrated with **single-nucleus RNA-sequencing data from 150 patients** (~750,000 cells), resulting in a unified atlas comprising **over 5 million cells**, including more than **4 million spatially resolved profiles**. To the best of our knowledge, this constitutes the **largest and most comprehensive spatially resolved single-cell atlas of any human disease to date**. The revised dataset includes **15 control samples** and **29 from patients with diabetic kidney disease (DKD)**.

In the revised manuscript, we leverage this expanded dataset to comprehensively characterize **spatial kidney niches**, which we define as spatially and transcriptionally distinct tissue units composed of characteristic cell types, their organization, and interactions (e.g., glomeruli, vasculature, or fibrotic zones). Across all 64 samples, we identified **11 distinct spatial niches**. When comparing niche fractions between control and DKD samples, we observed a **7-fold increase in the iPT niche**, an **11-fold increase in the iLOH niche**, and a **25-fold increase in the immune niche** (Fig. 2c). These comparisons were performed using a **subset of samples comprising only Control and DKD cases**.

We also assessed the clinical significance of spatial niches by correlating **niche abundance with kidney function (eGFR)**. These correlations were performed using **all samples** in the dataset. Using the defined niche framework, we identified DKD-specific gene expression changes within individual niches, reflecting altered cell–cell interactions. For example, in the vascular niche, we found **enrichment of pathways related to extracellular matrix remodeling, senescence, inflammatory signaling, and neovascularization**. We analyzed spatially correlated transcripts for each niche, using Hotspot. This allowed us to investigate niche-specific regulatory networks as well as differentially correlated transcripts across all niches (Extended Fig. 15-25).

We further observed a marked increase in **mesangial and immune cell fractions within the glomerular niche** in DKD samples (Fig. 2p–q), consistent with established pathological features. Importantly, using **spatially resolved ligand–receptor inference via CellPhoneDB**, we identified disease-associated signaling between **glomerular endothelial cells and podocytes**, including increased inflammatory pathways. This ligand–receptor analysis was **limited to Control vs. DKD samples**.

To further refine the spatial characterization of injured tubules and immune cells in DKD, we defined **cell-centric microenvironments (MEs)** (Fig. 11, right). For each focal cell, we assessed neighboring cell compositions within concentric spatial shells (20–80 μm), normalized the profiles, and clustered them using **MiniBatch K-means** to identify recurrent local interaction patterns. We focused particularly on **injured proximal tubule (iPT)** and **thick ascending limb (iTAL)** cells, and identified **five distinct microenvironments**, including a **profibrotic ME** enriched in **immune and fibroblast neighbors**. This ME was strongly enriched in DKD (comparison limited to Control vs. DKD) and showed a **robust negative correlation with GFR** ($r = -0.6$, $p < 0.001$, Fig. 3c), indicating its clinical relevance. This correlation was performed using **all samples**.

Despite differences in gene panel size between **CosMx (1,000 genes)** and **Xenium (5,100 genes)**, we observed **highly consistent transcriptional signatures across platforms**. Gene expression analyses were performed on all samples and separately for each platform to account for differences in gene coverage and technical characteristics.

To validate the spatial identity of the **profibrotic ME**, we performed **Sirius Red staining** on adjacent tissue sections from the same patient. This ME strongly overlapped with **regions of collagen deposition**, confirming its spatial alignment with histologically confirmed fibrosis. In our revised analysis, we also examined **ligand–receptor interactions between iPT cells and fibroblasts** within the profibrotic ME and correlated these interactions with injury markers (e.g., HAVCR1), using **all samples**.

We also constructed a **dedicated kidney immune atlas** by integrating spatial transcriptomic and snRNA-seq data, capturing **over 250,000 immune cells** from all 64 samples. In this atlas, we observed a marked **increase in B cells and plasma cells in DKD samples** (comparison limited to Control vs. DKD). To examine the organization of immune cells in situ, we clustered immune cells from **all samples** based on their spatial neighborhoods. This revealed **seven distinct immune MEs**, enriched for specific neighboring cell types (e.g., fibroblasts, injured tubules, vasculature, glomeruli). We defined these as **fibroblast-associated, injured tubular, vascular, glomerular, T cell–predominant, and B cell–predominant** immune MEs.

To further investigate **immune–epithelial spatial interactions** in DKD, we analyzed samples from both control and DKD patients. In glomerular regions, we observed **increased intraglomerular macrophages and CD8⁺ T cells** in DKD, along with **altered ligand–receptor signaling** that correlated with kidney function. Similarly, in the **injured tubular microenvironment**, we found **significant enrichment of plasma cells** and DKD-specific increases in pro-survival and chemotactic signals such as **TNFSF13–TNFRSF13B and CXCL12–CXCR4** (Fig. 4t–w).

We acknowledge that a **small number of samples were profiled more than once**, either across platforms (e.g., CosMx and Xenium) or as technical replicates. These replicates were treated as **independent data points**, reflecting distinct experiments with different measurement noise, cell counts, and field-of-view selection. Given the overall **large sample size (n = 64)** and **low proportion of repeated samples**, we believe this approach is appropriate and does not materially affect the conclusions. We have now clarified in the figure legends which samples were used in each comparison.

Moreover, we prioritized **external validation** of our findings in four large independent cohorts:

1. The **CD19 CAR T cell lymphoma cohort (n = 45)** with impaired kidney function,
2. The **TRIDENT DKD cohort (n = 226)** with longitudinal outcomes,
3. The **Susztaklab Biobank (n = 843)** with paired kidney tissue and clinical data,
4. The **UK Biobank** (3,309 individuals with Diabetes mellitus) with plasma proteomics and eGFR measurements.

This external validation further strengthens the robustness and clinical generalizability of our findings.

3. Comparison with previous work.

Many findings in the study are incremental in reference to a similar study recently published (July 2024) by the same authors in *Nature Genetics* (<https://doi.org/10.1038/s41588-024-01802-x>). In this study a similar sized cohort (including an overlap of samples analysed in this submitted manuscript) are profiled spatially using Visium genome sequencing at 50um resolution. Using the same custom gene panel in this study, CosMx was previously performed on two samples (1 Healthy, 1 disease) to train methodology to resolve, or infer, cell types at single cell resolution. Therefore, while only 2 samples are analysed at subcellular resolution in the previously published manuscript (compared to 16 here), there is significant overlap in the kidney structures that can be identified, and possibly meta-analysis that could have been performed to reach the same conclusions. For example, expression of immune signatures (such as B cells) that are not reported in the previous manuscript could be analysed from this data without increased resolution as opposed to the fibrotic niche/microenvironment. Thus, while this manuscript does report 14 more samples with increased resolution, there is significant overlap between the two studies in design, methodology and patient cohorts.

Nature Genetics covering more patient samples and a greater number of cells (though these were not included in this study).

We thank the reviewer for this thoughtful comment and the opportunity to clarify the differences between our current study and our previous publication in *Nature Genetics*. While both studies investigate the human kidney, they differ substantially in **resolution, scope, methodology, and key findings**.

The *Nature Genetics* study featured broad **dissociated single-cell/nucleus profiling** (338,565 sc/snRNA-seq and snATAC-seq cells from 65 patients) and incorporated **spot-level spatial transcriptomics** using the 10X Visium platform on 14 samples. Each Visium spot (~55–100 µm) captures transcripts from multiple cells, limiting resolution. Although that study included CosMx data from two samples, mostly to verify cell types and gene expression. The spatial profiling was neither the central focus nor performed at the scale or resolution of the current work, such as we could not compare cases and controls in our prior paper.

In contrast, the current study employs two high (single cell)-resolution spatial platforms—CosMx (n = 48, ~3.3 million cells) and Xenium (n = 16, ~1 million cells)—together profiling over 4 million spatially resolved cells across 64 samples. These data were integrated with single-nucleus RNA sequencing from 150 patients (~750,000 cells), yielding an atlas of over 5 million cells. To our knowledge, this represents the most comprehensive spatially resolved single-cell resource available for any human disease.

Key distinctions include:

- **Resolution:** The previous study used spot-level Visium data; here, we achieve true single-cell and subcellular resolution, enabling fine-grained neighborhood and interaction analyses.
- **Analytical focus:** The prior work spatially validated cell types. This study centers on identifying disease-associated spatial niches, immune microenvironments, and cell–cell signaling circuits—analyses only feasible with single-cell spatial resolution.

- **Methodological innovation:** We employed advanced computational methods, including 3 complementary niche and neighborhood analysis, ligand–receptor inference, and immune microenvironment mapping, tailored to high-resolution spatial data.
- **Sample size:** Given only 2 samples were analyzed previously using the CosMx platform, while 64 samples analyzed using complementary approaches CosMx/Xenium.
- **New biological insights:** The current study reveals B cell–driven microenvironments associated with progressive diabetic kidney disease, defines immune–epithelial cross-talk, and identifies actionable signaling pathways, none of which were addressed in the earlier publication.

To ensure the broader relevance of our findings, we validated key spatial signatures across four independent, large-scale cohorts—including the TRIDENT study, Susztaklab Biobank, UK Biobank, and a CD19 CAR T cell lymphoma cohort with impaired kidney function. The consistency of associations across these datasets further underscores the novelty and translational potential of our study. In summary our previous work was mostly about defining cell types, our current work identifies an important and targetable disease subtype that could even be identified based on blood biomarkers. We will ensure these distinctions are clearly stated in the revised manuscript to avoid confusion and to accurately reflect the unique contributions of this high-resolution spatial atlas.

4. Literature

Little attempt is made to compare the findings of this study to the literature (incl ref 15). There is, for example, a literature on the association of diabetic nephropathy with inflammation and immune dysfunction. Comparisons should be made between the niches described and conventional histological features that correlate with them (e.g. comment on mesangial hypercellularity above). Some previous single cell and spatial studies of kidney disease are dismissed out of hand (see refs 15 and 21-26 in the discussion – mouse studies might well be very relevant) or not discussed in detail (e.g. Lake BB Nature 2023). These studies need to be compared to the present one in more detail to avoid the inadvertent implication of novelty.

Response: We thank the reviewer for this important comment and the opportunity to clarify how our work compares to and extends existing literature. We agree that inflammation and immune dysfunction have been broadly associated with diabetic kidney disease (DKD), and we acknowledge that previous single-cell and spatial studies, including Lake et al. (Nature 2023) and others cited in references 15 and 21–26, have made important contributions to our understanding of kidney pathology. However, we respectfully emphasize that our study goes well beyond previous observations. By combining a uniquely large and multimodal dataset with true single-cell spatial resolution, machine learning–based analysis, and orthogonal validation strategies, we provide novel biological insight into disease-associated tissue states, cellular interactions, and immune compartmentalization in DKD—insights that were not previously accessible using conventional histology, bulk transcriptomics, or dissociated single-cell RNA sequencing.

In our **revised manuscript, we have expanded the dataset fourfold to include 64** spatially profiled human kidney samples, using two complementary high-resolution platforms—CosMx 1k (n = 48; ~3.3 million cells) and Xenium 5k (n = 16; ~1 million cells). These spatial data were integrated with single-nucleus RNA sequencing from 150 patients (~750,000 cells) to build a unified multimodal atlas of over 5 million cells, including more than 4 million spatially resolved profiles. This represents, to the best of our knowledge, the largest and most comprehensive single-cell spatial atlas of any human disease to date.

Using this dataset, **we provide a detailed characterization of eleven computationally identified spatially defined kidney niches, and identify significant remodeling of niche composition in DKD.** For example, we observe a seven-fold increase in the iPT niche and an eleven-fold increase in the iLOH niche (Fig. 3c). These niche-level changes are not only statistically significant but are also tightly correlated with kidney function: the iPT niche exhibits a strong negative correlation with eGFR ($r = -0.55$, $p < 0.001$), while homeostatic epithelial niches, such as the PT ($r = 0.52$, $p < 0.001$) and DCT ($r = 0.61$, $p < 0.001$), are positively correlated with kidney function and are among the most depleted in DKD. Additionally, we observe a 25-fold increase in the immune niche in DKD, with strong correlation to disease severity ($r = -0.59$, $p < 0.001$). Within the glomerular niche, we detect a marked expansion of mesangial and immune cells in DKD samples (Fig. 2p–q), consistent with historical histological descriptions such as those by Kimmelstiel and Wilson, and later summarized by Avraham et al., who described glomerular hypercellularity, nodular sclerosis, and matrix expansion in DKD. Our spatial analysis

builds directly upon these observations by assigning transcriptional identity to these cell populations and mapping their niche-level organization at single-cell resolution.

We have also **directly compared our results to prior large-scale studies** such as the KPMP Atlas. The Lake et al. study combined dissociated single-cell and single-nucleus RNA-seq with spatial profiling using Visium and Slide-seq2. While this work provided valuable insights into kidney injury and repair, the spatial resolution of Visium (55–100 μm per spot) and Slide-seq2 do not permit true single-cell mapping or precise definition of local cellular neighborhoods. In contrast, our CosMx and Xenium platforms achieve true single-cell spatial resolution and higher gene coverage (up to 5,100 genes), enabling us to define cell-type-specific injury programs (e.g., iLOH) and their spatially organized microenvironments. For example, we identified five distinct injury-associated microenvironments centered on IPT and iTAL cells, including a profibrotic ME characterized by proximity to fibroblasts and immune cells, enriched for MMP7, ANXA2, CLU, ITGB6, and IFITM3—transcriptional signatures that are both spatially localized and strongly associated with fibrosis and clinical decline.

We agree with the reviewer that **mouse studies remain informative**, and we have expanded our discussion to acknowledge work such as Polonsky et al., who applied seqFISH to investigate kidney responses to ischemia–reperfusion injury in mice. While they identified early tertiary lymphoid structure–like aggregates lacking B cells, our data show that in human DKD, mature B cell–predominant immune microenvironments can form with hallmark features of tertiary lymphoid structures. Using Xenium, we detected spatially restricted expression of CXCL13 and its receptor CXCR5, and confirmed the presence of follicular helper T cells. Imaging mass cytometry (IMC) further validated the presence and proximity of CD20⁺ B cells and CD4⁺ T cells. This was complemented by spatial transcriptomic detection of AICDA and IRF4, indicating local class-switch recombination and plasma cell differentiation.

To further define **B cell phenotypes**, we integrated Xenium-derived B and plasma cells with a published **single-cell B cell atlas using scVI/scANVI**. This revealed transcriptionally defined subtypes including memory, naïve, and atypical memory B cells. We validated these in situ using IMC markers (CD27, CD11c, BCL6) and demonstrated local proliferation with Ki67. Using CosMx, we were also able to directly detect IgD, IgM, IgG, and IgA isotypes at single-cell resolution, further defining the antibody landscape of the B cell–predominant niche.

In patients with a **high spatial B cell signature**, we performed **IgA immune complex pulldown followed by TMT-labeled proteomics**, identifying local enrichment of complement proteins (C3, C4A, C8B) and granzymes, consistent with antibody-mediated tissue injury. These data support a functional role for these structures in disease pathology, consistent with prior murine studies by Cippa et al., who showed that B cell–driven responses can emerge in the kidney even in the absence of foreign antigen.

Importantly, we used our **B cell spatial signature to stratify patients in two independent cohorts: the SusztaLab Biobank (n = 843) and TRIDENT (n = 226)**. In both, patients with high B cell signature (B⁺ DKD) had and more rapid progression to end-stage kidney disease, with survival differences confirmed by Kaplan–Meier and multivariable Cox analysis. Thus, our study not only maps the composition and structure of these immune niches, but also links them to clinical trajectory and identifies them as potential therapeutic targets.

In summary, while we replicate known features of DKD, we believe the biological insight and spatial resolution offered by **this study rewrites the landscape of DKD**. Our work reveals novel spatial niches, disease-associated microenvironments, new molecular signaling axes, and previously uncharacterized immune structures that are linked to patient outcomes and amenable to therapeutic targeting. We have revised the manuscript to better contextualize our findings within the existing literature and thank the reviewer for prompting this important clarification.

5. Other issues

a) The term "kidney disease " needs to be used with care. It is not possible to generalise to all kidney disease from this small study focused on diabetic nephropathy, and yet this is implied on occasion, including in the title. Care should also be taken to avoid implying causation where association is described – again one reading of the title would imply this (another would imply that DKD is B cell-driven).

Response: We thank the reviewer for emphasizing the importance of terminological precision and caution in interpreting association versus causation. We fully agree that findings from a focused study on diabetic kidney disease (DKD) should not be generalized to all kidney diseases, and that our language must reflect association rather than implying causality.

To address these concerns, we have implemented the following targeted changes:

1) Title revision for specificity and neutrality:

Original title: "Spatial human kidney map reveals a B cell driven disease state"

Revised title: " Spatial Single Cell Atlas of Diabetic Kidney Disease Reveals a B Cell-Rich Disease Subgroup"

→This change narrows the scope explicitly to DKD and replaces the term "driven" with "rich" to avoid implying a causal relationship.

2) Consistent terminology throughout the manuscript

We carefully reviewed the entire text and replaced generic references to "kidney disease" with "diabetic kidney disease (DKD)" or "the DKD cohort," wherever appropriate.

The more general term "kidney disease" is now only used when referring to prior literature that addresses broader renal conditions.

3) Clarification of association vs. causation

We revised statements that previously suggested causality. For example, phrases like "B cells drive progression" and "B cell mediated remodeling" have been reworded to "B cell rich microenvironments are associated with progression" and "B cell associated remodeling."

We hope that these revisions make clear that our study provides descriptive and associative insights into B cell-enriched immune environments in DKD, without asserting broader generalizations or causative conclusions. We appreciate the reviewer's input, which helped strengthen the clarity and precision of our manuscript.

b) The clinical cohorts are not well described. Disease course and stage, past and current treatment and ethnicity could all impact on the data collected – but this information is not provided or not discussed. How is DKD, DM and CKD defined? How is variable renal function taken into account? How are the repeat specimens related to each other (some seem contemporaneous - as GFR is the same for each - others not)? Such clinical detail is important in a disease-focused study, especially one with small patient numbers.

Response: We have substantially expanded our dataset, increasing the number of spatially profiled samples fourfold to a **total of 64, analyzed** using two complementary high-resolution platforms: CosMx 1k (n = 48; ~3.3 million cells) and Xenium 5k (n = 16; ~1 million cells). These spatial datasets were integrated with single-nucleus RNA sequencing data from 150 patients (~750,000 cells), resulting in a unified multimodal atlas of over 5 million cells, including more than 4 million with spatial resolution.

The revised dataset includes spatial data from 15 control samples and 29 samples from patients with diabetic kidney disease (DKD). Patient demographics are now reported in detail: 26% were female, the mean age was 57.3 years, and 13 samples were from Black patients. Estimated GFR was evenly distributed across the DKD cohort (see Fig. 1d), and we have now included this panel in the main figure in response to the reviewer's request (Fig. 1 c-e).

Every patient in the Trident cohort had pathologically confirmed diabetic nephropathy (by gold standard RPS classification of GBM measurement). The Trident study did not exclude subjects with additional diagnosis such as acute kidney injury, hypertensive disease or other GN, which was rare. These samples were retained for completeness and potential future cross-disease comparisons but were not included in the DKD group. To ensure clinical specificity, samples from patients with diabetes or hypertension who had preserved kidney function (eGFR > 60 mL/min/1.73m²) were not classified as DKD. Instead, these were labeled as "DM" or "HTN" to reflect underlying comorbidities without overt kidney disease. A small number of samples were profiled twice,

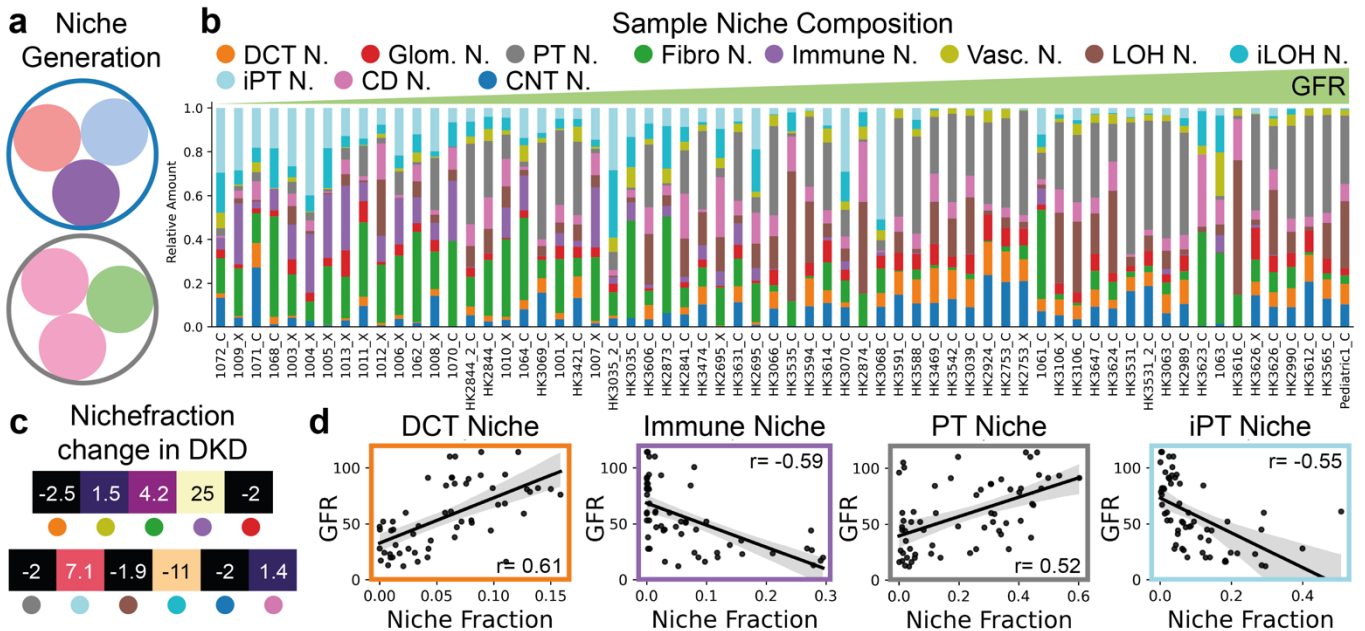
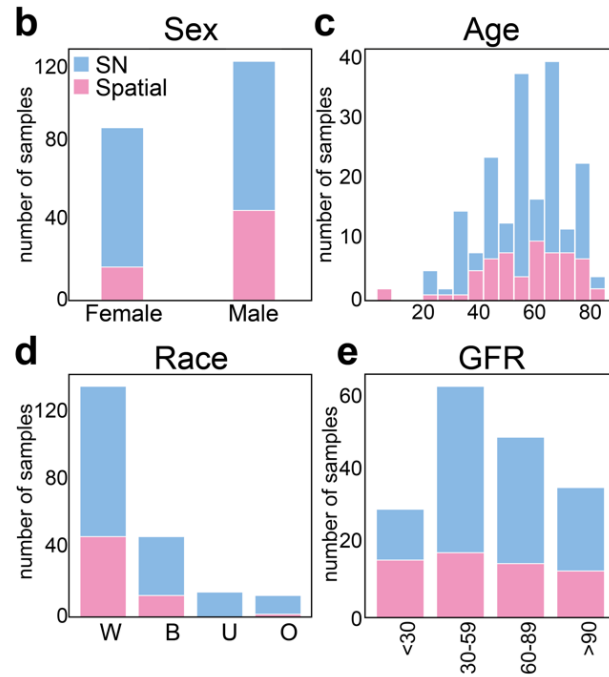


Fig 2a: Schematic of defining niches (left) and microenvironments (right). For niches, all cells were clustered into groups based on their neighborhood fraction after restricting the analysis to those with sufficient local context, removing the sparsest 5% of neighborhoods (based on total neighbor count at 80 μ m) to reduce edge effects ($n = 4,029,331$ cells). For each cell, we computed the number of neighboring cells within concentric distance shells: 0–20 μ m, 20–40 μ m, 40–60 μ m, and 60–80 μ m. This was achieved by calculating cumulative neighbor counts at 20, 40, 60, and 80 μ m, followed by subtracting counts from the smaller radius to isolate the number of neighbors in each respective shell (e.g., the 40–60 μ m shell = count at 60 μ m – count at 40 μ m). For each cell, neighbor counts were normalized by the total number of neighbors at that distance, yielding a cell-by-neighbor composition matrix that reflects the relative cellular makeup of the local environment at different spatial scales. This matrix was then scaled and subjected to principal component analysis (PCA), with the top 10 components used as input for MiniBatch K-means clustering. The number of clusters (k) was determined based on the elbow method. Niche clustering was performed on the entire dataset to define global spatial neighborhoods. **b:** Sample niche composition: the fraction of each niche is calculated for all cells within each sample and visualized. The samples are arranged by disease severity (GFR) from left to right. **c:** Change in relative niche fraction in DKD vs healthy controls. Each value represents the fold change in the average proportion of a given niche between DKD patients and healthy controls. A positive value indicates that the niche is expanded in DKD, while a negative value indicates it is more prevalent in controls. Fold changes were computed by normalizing niche frequencies within each diagnostic group, then taking the ratio of DKD to control values. **d:** correlation of niche fraction with eGFR using Pearson correlation. All correlations with $p < 0.001$.

either on different platforms or as technical replicates on the same platform. We treated each profile as an independent datapoint, given that these represent distinct experiments and measurements. Considering the large overall sample size, we found this approach is justified and does not disproportionately influence the results.

In the revised version of the manuscript, we made a concerted effort to incorporate renal function as a key clinical variable throughout our analyses: We provide a comprehensive characterization of 11 spatially defined kidney niches and identify significant remodeling in diabetic kidney disease (DKD). With the expanded cohort, we explicitly incorporated renal function by correlating niche abundance with eGFR (Fig. 2d). This revealed strong associations between spatial architecture and kidney function. For instance, the injured proximal tubule (IPT) and thick ascending limb (iLOH) niches were markedly expanded in DKD (7- and 11-fold, respectively; Fig. 2c) and negatively correlated with eGFR (IPT: $r = -0.55$, $p < 0.001$). In contrast, homeostatic epithelial niches (e.g., PT: $r = 0.52$; DCT: $r = 0.61$) showed positive correlations and were depleted in disease. The immune niche also showed a strong negative correlation ($r = -0.59$), underscoring global architectural shifts with declining function. To further contextualize these changes, we analyzed glomerular composition and found increased mesangial and immune cell fractions in DKD.

Ligand–receptor analysis **uncovered disease-associated interactions**, including elevated IL6–IL6R signaling between endothelial cells and podocytes. Correlating interaction strength with eGFR revealed functionally relevant pathways: NRG1–ERBB3 was positively associated with eGFR, whereas CXCL9–DPP4 and PLA2G2B–PLA2G2B were negatively correlated, suggesting roles in progression. By incorporating spatial context, we defined cell states based on both transcriptome and local neighborhood, identifying five distinct microenvironments (MEs) around injured tubules. Among these, a profibrotic ME—enriched in immune and fibroblast neighbors—was strongly associated with DKD and negatively correlated with eGFR ($r = -0.6$). Ligand–receptor correlations with markers of injury (HAVCR1) and fibrosis (COL1A1) implicated EGFR, Notch, and PDGF signaling in epithelial–stromal crosstalk (Fig. 3o). Similarly, with the help of an expanded immune cell atlas, we defined immune microenvironments. We explicitly incorporate variable renal function into the analysis of the glomerular and injured tubular immune microenvironment: In glomeruli, DKD samples showed increased infiltration by macrophages and CD8⁺ T cells. Correlation with eGFR highlighted FN1–ITGA4B7 (mesangial–macrophage) as a potential profibrotic signal, while BMP4–BMP2 (podocyte–CD8⁺ T cell) may represent protective signaling. Finally, interactions between IPT/iTAL cells and plasma cells revealed disease-associated patterns, including reduced ANXA1–FPR3 signaling (anti-inflammatory), and increased TNFSF13–TNFRSF13B and CXCL12–CXCR4 signaling, suggesting a supportive niche for sustained immune cell retention in DKD (Fig. 4t–w).

In summary, we have **expanded the cohort** and provided **detailed clinical annotation**, including patient demographics, disease stage, and comorbidities. Importantly, **variable renal function (eGFR) is now explicitly integrated into our spatial and molecular analyses**, allowing us to assess how changes in tissue architecture and cell–cell interactions relate to kidney function across disease severity.

c) The study analysed spatiotemporal gene expression patterns along the corticomedullary axis, linking these patterns to physiological roles such as water resorption, calcium handling, and bicarbonate transport. The authors make a number of points regarding spatial gene expression (Figure 1f) all of which are inferred based on computational prediction. Have any of these been experimentally validated? Later injured proximal tubule and loop of Henle disease-specific niches suggested a role for fibrosis-related markers and signalling pathways in disease progression. Have any of the computationally predicted receptor-ligand interactions been functionally validated?

Response: We thank the reviewer for this important comment and the opportunity to clarify the scope of our experimental validation and the biological insight derived from our spatial transcriptomic analysis. We agree that certain physiological functions of the kidney—such as water reabsorption, ion transport, and inflammation—are well established. However, we respectfully emphasize that the goal of this study was not to redefine basic physiology, but to provide a high-resolution, multimodal, and spatially resolved framework for understanding how disease alters tissue architecture, cellular interactions, and immune landscapes in the human kidney.

Our study was designed as a comprehensive human tissue atlas aimed at generating **testable clinical hypotheses**, rather than a mechanistic dissection of individual pathways. The latter would require animal model studies, which unfortunately fail to recapitulate human DKD. In keeping with this objective, and in response to the reviewer’s concern, we have removed the analysis of corticomedullary spatial gradients in physiological gene expression (Fig. 1f) from the revised manuscript, as it relied on computational inference and did not directly impact our core conclusions.

To address the reviewer’s question more fully regarding experimental validation, we have substantially expanded the dataset, quadrupling the number of spatially profiled samples to include **64 human kidney tissues** analyzed using two complementary high-resolution spatial platforms—**CosMx 1k (n = 48)** and **Xenium**

5k (n = 16). These datasets were integrated with **single-nucleus RNA sequencing from 150 individuals**, yielding a multimodal atlas of **over 5 million cells**, including more than **4 million spatially resolved profiles**. The breadth and resolution of this dataset enabled detailed characterization of 11 spatially defined kidney niches and revealed significant remodeling of tissue architecture in diabetic kidney disease (Fig. 2a-d).

For example, we observed a 7-fold increase in the iPT niche and an 11-fold increase in the iLOH niche in DKD (Fig. 2c). These injury-associated niches showed strong negative correlations with kidney function (eGFR), while homeostatic niches—such as PT and DCT—were depleted in disease and positively correlated with function. This correlation-based validation was performed across all samples and is shown in Extended Fig. 12.

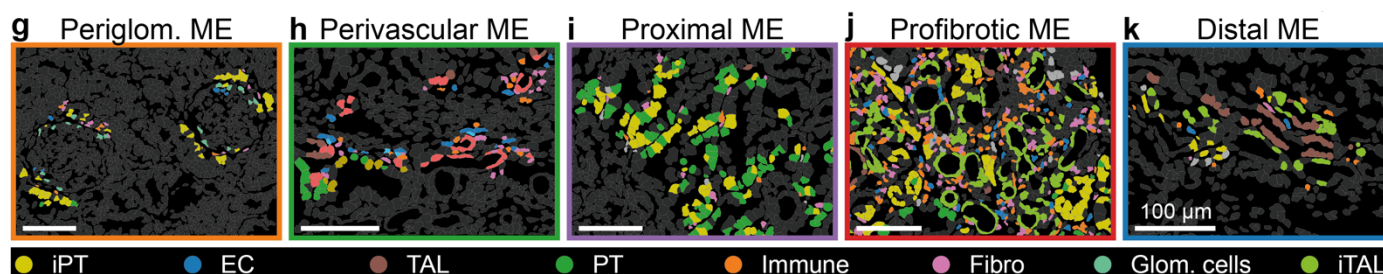


Fig. 3g-k: Representative image of the periglomerular ME. h, Representative image of the perivascular ME. i, Representative image of the proximal ME. j, Representative image of the profibrotic ME. k, Representative image of the distal ME. Spatial plots were generated using Xenium Explorer 3. Celltypes are indicated by their color. iPT: injured PT cells, EC: endothelial cells, including peritubular and distal vasa recta endothelial cells, TAL: thick ascending loop cells, PT: proximal tubular cells, Immune: Immune cells, Fibro: Fibroblast cells, Glom. cells: Glomerular cells, including parietal epithelial cells, podocytes and glomerular endothelial cells. iTAL: injured thick ascending loop cells.

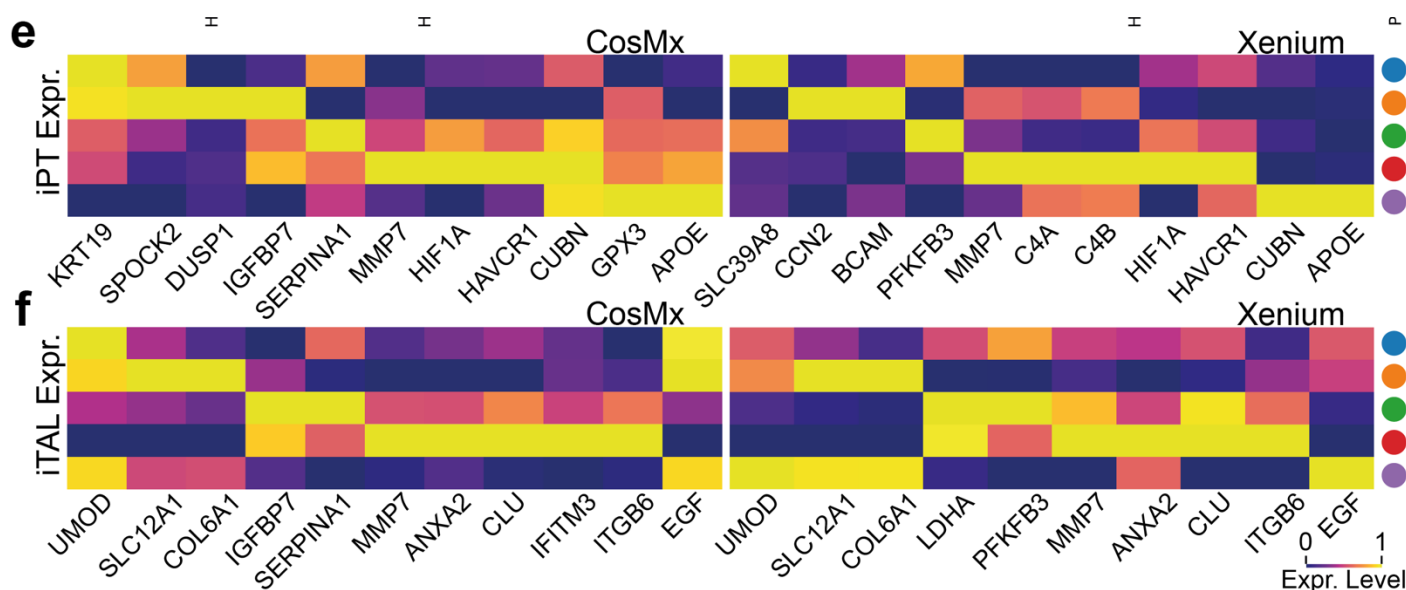


Fig. 3e: Heatmap of key differentially expressed genes of iPT center cells in MEs in the CosMx dataset (left) and Xenium dataset (right). f, Heatmap of key differentially expressed genes of iLOH center cells in MEs in the CosMx dataset (left) and Xenium dataset (right). The differentially expressed (DE) genes were calculated using the Wilcoxon rank-sum test. CosMx and Xenium datasets were analyzed separately due to their differing gene panel sizes.

To further refine our understanding of spatial pathology in DKD, we introduced a complementary analysis based on **cell-centric microenvironments (MEs)**, which describe the local neighborhood of a given focal cell. Using this approach, we identified five injury-associated microenvironments, including a **profibrotic ME** enriched in immune and fibroblast neighbors (Fig. 3g-k). This ME was strongly associated with declining kidney function ($r = -0.6$, $p < 0.001$) and was confirmed to spatially overlap with **Sirius Red-positive regions** marking collagen deposition. Thus, this transcriptionally and spatially defined ME maps onto active fibrotic remodeling (Fig. 3n).

To explore **cell-cell signaling within the profibrotic ME**, we used ligand–receptor inference and correlated interaction strength with markers of tubular injury (e.g., HAVCR1) and fibrosis (e.g., COL1A1). We identified HBEGF–EGFR and TNFSF10–TNFRSF10B as enriched ligand–receptor pairs linking injured proximal tubules to fibroblast activation (Fig. 3o). These interactions were further supported by correlation with bulk RNA-seq data from an independent cohort (Extended Fig. 27 and 30). Additionally, we identified JAG2–NOTCH and PDGFB–

PDGFRB as key pathways associated with matrix production, consistent with prior literature. Importantly, these predictions were validated using two independent transcriptomic datasets and matched histological staining.

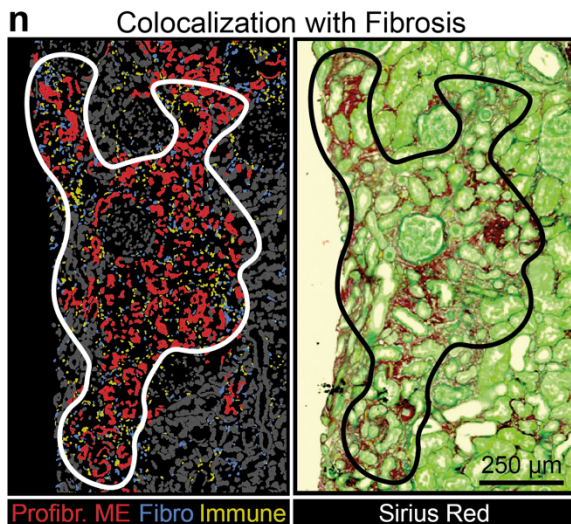


Fig. 3n: Left side: Spatial plot of injured epithelial cells that belong to the profibrotic microenvironment (red) together with Fibroblasts (blue) and immune cells (yellow). Other cells are depicted in grey. Spatial plot was

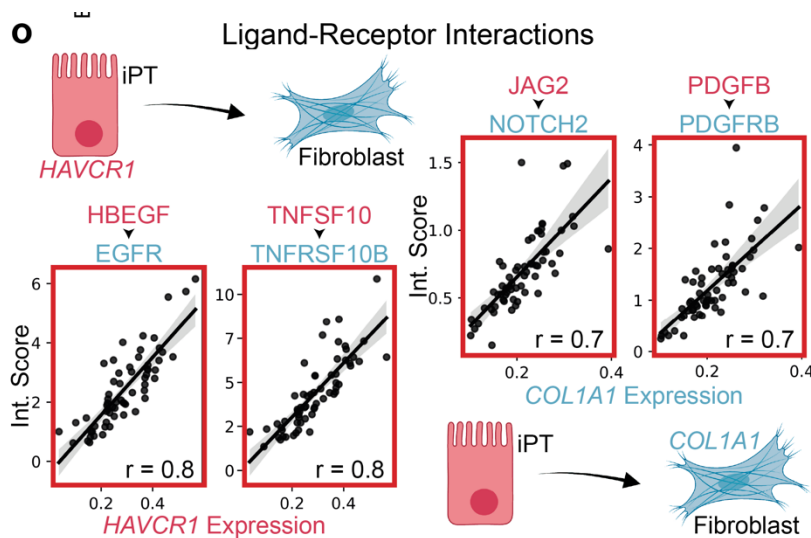


Fig. 3o: Left: Ligand-receptor interactions between injured proximal tubular cells and fibroblasts within the profibrotic microenvironment were inferred using CellPhoneDB v5. The interaction scores for

To investigate immune–epithelial crosstalk, we constructed a **dedicated immune atlas** comprising **over 250,000 immune cells** (Fig. 4a). We identified **seven spatially distinct immune microenvironments**, including fibroblast-associated, injured tubular, vascular, glomerular, T cell–predominant, and B cell–predominant compartments (Fig. 4f–s). Within the **injured tubular immune ME**, we found a significant enrichment of **plasma cells** in DKD samples. Spatial ligand–receptor analysis revealed DKD-associated increases in **TNFSF13–TNFRSF13B (APRIL–TACI)** and **CXCL12–CXCR4**, suggesting that injured epithelium supports plasma cell recruitment and survival. These interactions were corroborated by correlation with plasma cell markers (e.g., MZB1) in bulk RNA-seq from the Susztaklab Biobank.

We then focused on the **B cell–predominant ME**, which was present in a subset of DKD patients and showed hallmark features of a **tertiary lymphoid structure (TLS)**. Using **Xenium 5k**, we detected spatial expression of **CXCL13 and CXCR5**, a follicular T–B axis typically restricted to secondary lymphoid organs. CD4⁺ T cells within this niche expressed CXCR5, consistent with a **follicular helper T cell phenotype**, while **TNFSF13 and TNFRSF13C colocalization** supported a local **B cell survival niche**.

To validate these findings at the protein level, we employed **imaging mass cytometry (IMC)**, using a custom panel of antibodies for CD4, CD8, CD20, CD38, CD45, and AQP1. IMC confirmed the spatial proximity of CD4⁺ T cells and CD20⁺ B cells (Fig. 5a–m). To further resolve B cell states, we integrated our Xenium-derived B and plasma cells with a reference atlas using scVI and scANVI, identifying memory, naïve, and atypical memory B cells (Fig. 5p–u). IMC with markers CD27, CD11c, and BCL6 corroborated these designations. We also used CosMx to directly detect immunoglobulin isotypes (IgD, IgM, IgG, IgA), confirming the presence of class-

switched and naïve B cells within the TLS-like niche. Notably, Ki67 staining revealed proliferating CD20⁺ B cells, and transcriptomic data showed **AICDA and IRF4 expression**, indicating **class switch recombination and plasma cell maturation in situ** (Fig. 5f, g).

To assess functional consequences, we performed **IgA immunoprecipitation and TMT-labeled mass spectrometry** on tissue samples from patients with strong B cell spatial signatures. This revealed immune complex-associated proteins such as granzymes and complement components (C3, C4A, C8B), implicating antibody-driven injury (Fig. 5x). This extends prior findings in mouse models and provides the first multi-modal, spatially anchored evidence for a mature, functional B cell-rich niche in human DKD.

Finally, to explore therapeutic implications, we applied the B cell-predominant ME signature to the SusztaLab Biobank and TRIDENT cohorts. In both, we identified a **B⁺ DKD subgroup (~8.4%)** associated with **greater fibrosis, lower eGFR, and faster progression to end-stage kidney disease** (Fig. 5v). These findings support therapeutic targeting of this compartment using existing agents (e.g., rituximab, belimumab, iptacopan), and raise the possibility of future trials evaluating **tissue-penetrating B cell-directed therapies**, such as **CD19 CAR T cells**, which we show may improve kidney function in patients outside classical autoimmune contexts (Extended Fig. 34).

In conclusion, we provide **multi-modal orthogonal validation**—including spatial transcriptomics, protein imaging, mass spectrometry, and bulk RNA-seq correlation—across multiple platforms and independent cohorts. Functional validation of ligand-receptor interactions remains extremely challenging as mouse models or organoids do not recapitulate human DKD. Our goal remains to generate high-confidence, biologically interpretable maps of cell-cell signaling and spatial architecture in DKD, forming a foundation for mechanistic follow-up studies and precision therapeutic strategies. We have revised the manuscript to reflect these clarifications and thank the reviewer for encouraging a deeper discussion of validation and prior literature.

d) The analysis offers a snapshot of kidney niches, that lacks temporal dynamics to understand how niches evolve over time or under disease progression. A small number of the FFPE samples analysed come from the same individual. Were these taken at distinct times and have they been assessed for temporal changes in niche composition that may be associated with disease?

Response: We thank the reviewer for this insightful comment. We agree that understanding how kidney microenvironments may evolve over time in diabetic kidney disease (DKD) would be invaluable. However, kidney biopsy is rarely performed in patients with DKD, which is an important practical limitation to obtaining longitudinal spatial data. There are 2 aspects of repeated biopsies, the first one is reproducibility. In our current dataset, the few repeated specimens derive from adjacent sections of the same formalin-fixed, paraffin-embedded (FFPE) tissue block. In addition, we have analyzed tissue samples using both the CosMx and the Xenium methods, indicating that the identified niches are robust. The second aspect is looking at changes as the disease progress. This could be achieved by repeated biopsies or a large number of samples obtained from patients with different stage of kidney disease. At first, we opted for the second approach. This is the first human kidney spatial transcriptomics information at scale and future studies shall address limitations of repeated biopsies and multiple other issues.

Given these constraints, we instead leveraged our expanded cross-sectional dataset to infer potential disease trajectories. In the revised manuscript, we substantially increased the number of spatially profiled samples fourfold to 64, using two complementary high-resolution platforms—CosMx 1k (n = 48; ~3.3 million cells) and Xenium 5k (n = 16; ~1 million cells). These spatial datasets were integrated with single-nucleus RNA sequencing data from 150 individuals (~750,000 cells), generating a unified multimodal atlas comprising over 5 million cells, including more than 4 million spatially resolved cells. With this large and heterogeneous dataset, we comprehensively mapped the cellular and spatial architecture of the human kidney and defined 11 distinct spatial niches. We observed substantial remodeling of these niches in DKD, particularly in the injured proximal tubule (iPT) and injured thick ascending limb (iLOH) compartments. These two niches were expanded 7-fold and 11-fold, respectively, in DKD compared to controls (Fig. 3c). Importantly, we assessed the clinical relevance of these changes by correlating niche abundance with kidney function, measured by estimated glomerular filtration rate (eGFR). This revealed robust and consistent associations: the iPT niche negatively correlated with eGFR ($r = -0.55$, $p < 0.001$), suggesting that its expansion reflects advancing disease severity. Similarly, the

immune niche also showed a strong negative correlation with eGFR ($r = -0.59$, $p < 0.001$), consistent with growing immune infiltration as function declines. In contrast, homeostatic epithelial niches—such as those enriched for proximal tubule (PT, $r = 0.52$, $p < 0.001$) and distal convoluted tubule (DCT, $r = 0.61$, $p < 0.001$) cells—were progressively depleted in more advanced disease. Together, these cross-sectional patterns suggest ordered and coordinated shifts in spatial architecture as kidney function deteriorates, providing a valuable proxy for temporal progression. While such correlations cannot establish causality or capture individual patient trajectories, they offer strong biological plausibility for sequential niche remodeling during DKD progression.

In summary, longitudinal analysis was not the aim of the current project. Our study provides a high-resolution cross-sectional snapshot of DKD microenvironments across a broad spectrum of disease severity and the identification of a specific disease subtype which is associated not only with more severe outcome but also potentially targetable by available B-cell targeted therapies. Future studies can address issues around repeated biopsies.

e) The authors then went on to generate a spatially resolved kidney immune cell atlas using seven (1x single cell (sc) RNA-Seq, 5x snRNA-Seq and 1x CosMx) datasets with 143 patients and 165,161 kidney immune cells. This approach is not without its pitfalls. Differences in experimental conditions, platforms (snRNA-seq, scRNA-seq, CosMx), and sample preparation methods could introduce batch effects that distort biological signals. The use of three and five-layer neural network models in SCVI/scANVI with 30 latent dimensions may overfit or underfit depending on the dataset size and complexity. Fixed parameters (e.g., 3,000 highly variable genes) might not be optimal for all datasets, potentially excluding important genes or introducing bias. Data quality across sources (e.g., KPMP and CosMx datasets) might vary, and lower-quality datasets could disproportionately influence integration outcomes.

Response: We thank the reviewer for this thoughtful and important comment. We appreciate the opportunity to elaborate on our data integration approach and address the potential pitfalls associated with integrating datasets across different technologies and sources.

We fully agree that integrating data from multiple platforms—including snRNA-seq, scRNA-seq, and CosMx spatial transcriptomics—requires careful attention to batch effects, sample-specific variability, and differences in data quality. To mitigate these challenges, we employed **scVI** and **scANVI**, probabilistic deep generative models specifically designed for large-scale, cross-platform single-cell data integration. These models are now widely regarded as among the most effective methods for complex multi-modal integration tasks, due to their scalability, flexibility, and demonstrated performance in minimizing batch effects while preserving biological structure (as benchmarked in Luecken et al., *Nat Methods* 2022).

In our revised analysis, we used **~750,000 single-nucleus RNA-seq profiles from 150 individuals**—drawn from three independent and diverse sources: the KPMP consortium, the Susztak Lab, and the Humphreys Lab. This reference framework was designed to ensure broad biological coverage and minimize sampling bias. To address potential variability across sources and technologies, we implemented a **multi-layered correction strategy**. Specifically, we modeled:

- **Platform identity** (CosMx, Xenium, SNseq) as the primary batch variable,
- **Sample identity** and **data source** (KPMP vs. Susztak Lab vs. Humphreys Lab) as additional categorical covariates,
- And included **continuous covariates** such as total RNA counts and mitochondrial gene content.

This modeling framework allowed us to account for both platform-level and sample-level technical variation, while retaining subtle biological variation such as immune activation and disease-specific transcriptional programs. We also applied **early stopping (patience = 15 epochs)** during training to prevent model overfitting.

To ensure robustness and transparency, we **benchmarked our integration pipeline** against several commonly used methods (Scanorama, Harmony, Liger, unintegrated) using the framework proposed by Luecken et al. This included randomly downsampling 10% of the CosMx and SNseq datasets and evaluating performance using a combination of batch mixing and biological conservation metrics. **scANVI consistently outperformed other methods** in aggregate score (Extended Fig. 1), achieving superior batch correction and preservation of meaningful transcriptional identity.

Method	Bio conservation					Batch correction					Aggregate score		
	Isolated labels	KMeans NMI	KMeans ARI	Silhouette label	cLISI	Silhouette batch	iLISI	KBET	Graph connectivity	PCR comparison	Batch correction	Bio conservation	Total
scANVI	0.77	0.89	0.63	0.76	1.00	0.89	0.02	0.51	0.99	0.99	0.68	0.81	0.76
scVI	0.59	0.72	0.43	0.59	1.00	0.91	0.03	0.51	0.96	1.00	0.68	0.66	0.67
LIGER	0.55	0.57	0.31	0.55	1.00	0.94	0.08	0.67	0.77	0.99	0.69	0.59	0.63
Harmony	0.53	0.63	0.37	0.54	0.99	0.84	0.23	0.47	0.58	1.00	0.62	0.61	0.62
Scanorama	0.53	0.63	0.36	0.54	1.00	0.93	0.04	0.23	0.66	0.88	0.55	0.61	0.59
Unintegrated	0.53	0.60	0.35	0.54	1.00	0.76	0.00	0.06	0.63	0.00	0.29	0.60	0.48

Extended Fig. 1: Benchmarking dataset integration: Methods are sorted by overall score. Overall score is a weighted mean of the batch correction score and the bio conservation score, which in turn are a mean of the individual metrics within the category. Metrics have previously been described (Luecken et al.). Briefly, these metrics were grouped into two broad categories: conservation of biological variance (Bio conservation) and removal of batch effects. All scores are normalized between 0 and 1, where higher values indicate better performance.

We also agree that parameter tuning, such as the number of latent dimensions or selection of highly variable genes, can influence integration outcomes. For our main analysis, we used **3,000 highly variable genes** and **30 latent dimensions**, parameters widely used and validated across recent benchmarking studies. We further evaluated the effect of these choices on embedding stability and found that the results were robust to moderate variation in gene selection and dimensionality (data not shown, available upon request).

To maintain internal consistency and reduce noise, we excluded the scRNA-seq dataset from the integration in the revised version of the immune cell atlas and used only single-nucleus RNA-seq and spatial transcriptomics (CosMx and Xenium). We also ensured that only high-quality cells were included. The QC for all samples used in this revised work are summarized in Extended Fig. 5-11. After integration, we projected all cells into a shared latent space and performed unsupervised Leiden clustering to define immune and parenchymal cell populations. This provided a unified cellular taxonomy across platforms, enabling spatial mapping and functional analysis at high resolution.

In summary, we agree that multi-platform integration requires careful handling. We addressed these challenges through the use of state-of-the-art generative models (scVI/scANVI), rigorous batch correction, careful parameter selection, and benchmarking against alternative methods. We believe this strategy ensures high biological fidelity while minimizing technical confounding, and we hope this clarification is helpful. We have updated the manuscript to reflect these points more clearly.

Finally, as the authors describe cell dissociation artifacts can lead to selective loss of fragile cell types which might distort cell type representation. How have the authors addressed these potential concerns?

We thank the reviewer for raising this point. We agree that cell dissociation artifacts—particularly the selective loss of fragile or rare cell types—are a well-recognized concern in dissociative single-cell and single-nucleus RNA-seq studies. Indeed, several reports, including Humphreys et al. (JASN), have documented reduced recovery of certain immune subsets in single-nucleus RNA-seq compared to single-cell RNA-seq.

However, we would like to clarify that our primary analysis is based on **spatial transcriptomics**, using **two high-resolution, imaging-based platforms—CosMx and Xenium—which do not rely on tissue dissociation**. As such, these spatial approaches are **inherently immune to dissociation-related biases** and preserve both fragile and morphologically embedded cell types, including immune cells, epithelial cells, and stromal populations, within their native tissue architecture.

The single-nucleus RNA-seq data in our study (~750,000 cells from 150 individuals) were not used to compare cell fractions across modalities, but rather to:

1. Serve as a **reference for cell type annotation** and benchmarking;

2. Enable **probabilistic label transfer** into the spatial datasets via scVI/scANVI; and
3. Impute gene-level information beyond the targeted gene panels used in spatial assays.

We intentionally avoided direct comparisons of cell-type proportions between spatial and dissociative platforms, recognizing the inherent modality-specific biases. Instead, we focused on characterizing **cell identity, transcriptional state, and spatial organization** within intact tissue, leveraging the strengths of each data type in a complementary fashion.

Moreover, the consistency of our results across both **CosMx (1,000 genes)** and **Xenium (5,100 genes)** platforms—including spatial niche composition, gene expression signatures, and ligand–receptor interactions—further supports the robustness of our findings and suggests minimal distortion from cell-type dropout.

In summary, while dissociation artifacts are a valid concern for single-cell and single-nucleus transcriptomic approaches, our study design—centered on high-resolution spatial platforms—largely avoids these limitations. We hope this clarification is helpful and have added a note to the manuscript to explicitly make this point.

f) Finally, the authors explored the relationship between the cellular niches they identified and kidney disease severity. The iLOH ME1 niche was found to be strongly associated with interstitial fibrosis (Figure 5a) and clustering of patients from the TRIDENT study using this spatial gene signature defined three groups of patients, iLOH-, iLOH+ and iLOH+ B+. These three groups differ in baseline kidney function with eGFR being 49.5, 33 and 19 cc/min/1.72m² in the three groups, respectively. Unsurprisingly, patients with the lowest baseline eGFR, and by definition most severe disease, reached the renal composite endpoint fastest (Figure 5b). Do any of the other spatial gene signatures stratify patients in a manner that correlates with renal outcome but doesn't simply reflect baseline disease severity?

Response: We thank the reviewer for this thoughtful and important question. We appreciate the concern regarding the relationship between baseline kidney function and outcome, and the opportunity to clarify the purpose of our stratification approach and the broader goals of the study.

We would like to emphasize that the **primary objective of this work was not to identify general prognostic markers of disease severity or progression**, but rather to use high-resolution spatial transcriptomics to identify **mechanistically defined, discriminatory biomarkers** that distinguish molecular subtypes within diabetic kidney disease (DKD) with potential **clinical translation and therapeutic opportunity**.

DKD remains a **heterogeneous disease** but is currently **diagnosed and treated as a single entity**, based on non-specific features such as glomerular basement membrane thickening and albuminuria. To date, **no molecular subtypes of DKD have been defined or clinically adopted**, and thus **no precision treatment strategies exist**. Our study addresses this gap by using spatial profiling to identify a **previously unrecognized subgroup of DKD characterized by intrarenal B cell-predominant inflammation**—a rare but clinically meaningful subset with distinct biology and potential therapeutic implications. Because this B⁺ DKD subgroup is uncommon, it is critical to identify **discriminatory biomarkers** that can stratify patients for potential therapy, ideally through **non-invasive, blood-based assays**. Therefore, the goal of our stratification was not to recapitulate eGFR-based risk prediction, but rather to define and validate a **mechanistically grounded disease subtype** with actionable molecular features.

To support this aim, we expanded our dataset to include **64 spatially profiled human kidney samples** using **two complementary high-resolution spatial transcriptomics platforms (CosMx 1k and Xenium 5k)**, integrated with **single-nucleus RNA-seq data from 150 individuals**, creating a multimodal atlas of **over 5 million cells**. Using this atlas, we identified **11 distinct spatial tissue niches** and found substantial remodeling in DKD. For example, the iPT and iLOH niches were 7- and 11-fold enriched in DKD, respectively, and strongly negatively correlated with kidney function. Homeostatic epithelial niches (PT and DCT) were depleted and positively correlated with eGFR, consistent with disease-associated architectural shifts.

We further defined seven spatial **immune microenvironments (MEs)**, including fibroblast-associated, injured tubular, vascular, glomerular, T cell–predominant, and B cell–predominant compartments. The **B cell–predominant ME**, which resembles a **tertiary lymphoid structure (TLS)**, was identified only in a subset of DKD patients. Using **Xenium 5k**, we detected **CXCL13–CXCR5 signaling** and **co-localization of BAFF and BAFF-R**, while CD4⁺ T cells expressed high levels of CXCR5, consistent with a **follicular helper T cell phenotype**. These features were validated using **imaging mass cytometry (IMC)**, which confirmed spatial proximity of CD4⁺ T cells, CD20⁺ B cells, and CD38⁺ plasma cells.

We used scVI/scANVI to integrate Xenium-derived B and plasma cells with a reference single-cell B cell atlas, revealing **memory, naïve, and atypical memory B cells**. These findings were confirmed by IMC using CD27, CD11c, and BCL6 staining. CosMx also enabled direct detection of immunoglobulin isotypes (IgD, IgM, IgG, IgA), supporting **in situ class switching**. Ki67 staining demonstrated that CD20⁺ B cells were proliferative, and AICDA and IRF4 expression suggested **ongoing maturation into plasma cells**.

To understand the downstream consequences of this local immune activation, we performed **IgA immunoprecipitation followed by TMT mass spectrometry**, identifying **granzymes and complement components (C3, C4A, C8B)** in immune complexes—implicating antibody-mediated injury.

To test whether this spatial phenotype corresponded to a **clinically meaningful disease subtype**, we applied the B cell ME signature to two independent cohorts. In the **Susztaklab Biobank (n = 843)**, the signature correlated with **increased fibrosis**. In the **TRIDENT cohort (n = 226)**, we identified a **B⁺ DKD subgroup (~8.4%)** with significantly **lower baseline eGFR and more rapid progression** to a composite renal endpoint. While these patients had more advanced disease at baseline, our objective was not to show that spatial signatures outperform eGFR, but rather to **demonstrate that spatially resolved immune phenotypes identify mechanistically distinct disease subtypes** with unique molecular features and therapeutic relevance.

Indeed, spatial gene signatures derived from other immune and injury microenvironments—such as the profibrotic ME or iPT—were detectable in patients with only moderate eGFR impairment and may serve in the future as early indicators of disease subtype before overt clinical decline. These analyses suggest that spatial features **complement, rather than duplicate**, existing clinical metrics, and may be particularly useful for stratifying intermediate-risk patients or identifying those likely to respond to targeted therapies.

Finally, our study offers a direct path to **precision therapeutics**. The B⁺ DKD subtype is characterized by spatially organized B cell–driven inflammation and antibody production, and aligns with established therapeutic targets:

- **B cell–depleting agents** (e.g., rituximab, ofatumumab),
- **BAFF/APRIL pathway inhibitors** (e.g., belimumab),
- **Complement-targeting therapies** (e.g., iptacopan), all of which are FDA-approved for other immune-mediated kidney diseases and could be repurposed.

In summary, our goal was not to generate prognostic models based on spatial data alone, but to define and validate **discriminatory, mechanistically meaningful tissue subtypes in DKD**. The B cell–predominant immune niche represents such a subtype—rare but reproducible, spatially and transcriptionally distinct, linked to clinical outcomes, and amenable to targeted therapy. We thank the reviewer for the opportunity to clarify this important distinction and have updated the manuscript accordingly.

g) Kidney and blood proteome analyses identified 394 significantly altered proteins in kidney tissue and 217 altered proteins in blood samples from patients with the iLOH⁺ gene signature. An overlap of 111 proteins was observed (Figure 5e), suggesting these proteins may serve as biomarkers of tubular damage and kidney disease risk. Comparing the degree of overlap based on p value thresholds will result in an underestimation of the degree of overlap due to differences in the number of proteins measured and relative sample sizes. What is the correlation between effect sizes for proteins measured in both studies? This is likely to produce a better indication of the degree of overlap between the two modalities.

Response: We thank the reviewer for the thoughtful suggestion regarding the interpretation of proteomic overlap and the utility of comparing effect sizes. In the original version of the manuscript, we examined overlap between proteins significantly altered in kidney tissue and those altered in plasma within the iLOH⁺ group. We agree that comparing based solely on p-value thresholds may underestimate biological overlap due to differences in sample size, measurement platforms, and variance structure. Evaluating effect size correlations across tissue and plasma indeed represents a more informative approach for cross-modal validation.

However, in the revised manuscript, we refined our strategy to focus not on the iLOH⁺ subgroup, but rather on the **B cell–predominant immune microenvironment**, which emerged as a **more mechanistically defined and clinically actionable DKD subtype**. Our primary goal shifted from identifying generic markers of tubular injury to defining discriminatory biomarkers that reflect this rare but prognostically important immune phenotype.

In the revised manuscript, we developed spatial gene expression signatures for **the B cell–predominant immune microenvironment, that can identify the B-cell subtype by examining bulk tissue samples** (which is more clinically translatable than single cell spatial analysis). These signatures were derived independently and directly from our spatial transcriptomic dataset. To construct spatial niche signatures, we employed a pseudobulk approach by aggregating gene expression across all cells assigned to a given niche, on a per-sample basis. We then performed differential expression analysis between niche types using the DESeq2 package to identify genes specifically enriched in each spatial microenvironment. For downstream biomarker discovery, we applied these spatial signatures to a bulk RNA sequencing dataset of the TRIDENT cohort of patients with diabetic kidney disease (n = 226). We stratified patients based on expression of the B cell–predominant immune ME signature. To identify candidate biomarkers specific to this B cell–enriched disease state, we conducted differential expression analysis. This analysis, performed using the Limma package, revealed a set of candidate tissue biomarkers specifically elevated in the B cell–associated subset, representing potential biomarkers of immune-driven disease activity. Here, we highlight some of these tissue biomarkers (Fig. 5y). These biomarkers

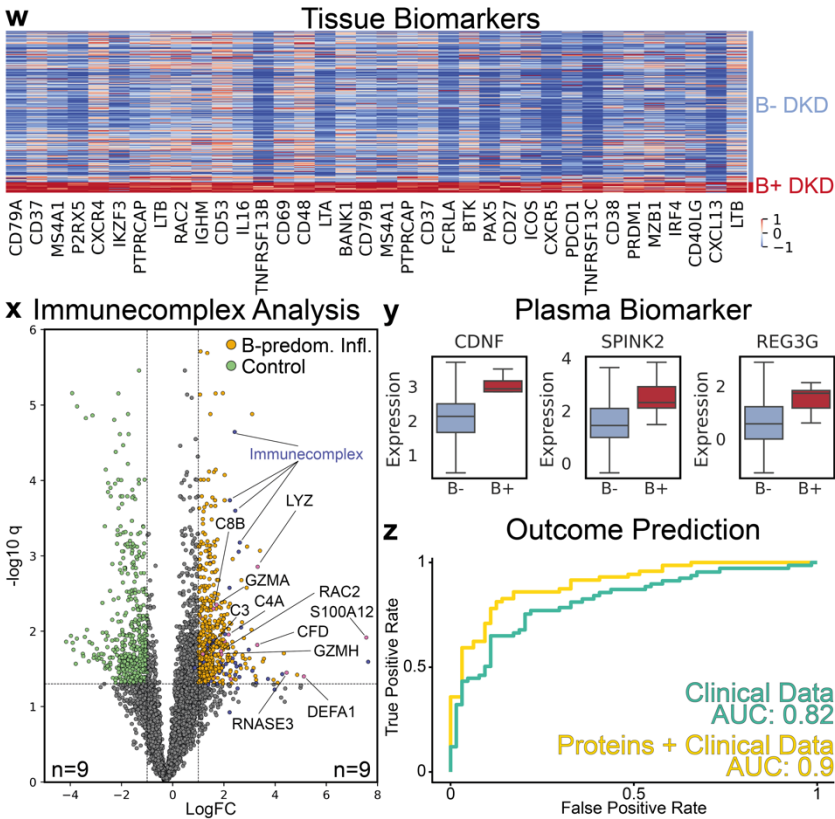


Fig. 5w: To identify candidate protein biomarkers associated with disease-relevant microenvironments, we first derived 10-gene spatial signatures for the B cell–predominant immune microenvironment using high-resolution spatial transcriptomics. These signatures were applied to bulk RNA-sequencing data from the TRIDENT Cohort to stratify patients (n=223) based on spatial microenvironment enrichment. Differential expression analysis was then performed using bulk RNAseq and the Limma R package. Depicted here are a subset of the significant DEGs.

x: Using specific antibodies against IgA, pulldown from 8 controls and 8 patients with B cell predominant inflammation from the Susztaklab Biobank was performed and analyzed using TMT-labeled mass spectrometry. Volcano plot of proteins bound to immune complexes in B-predom. Inflammation vs. Control.

y: Paired plasma proteomic from patients with B+ and B- DKD in the TRIDENT cohort was used to train an elastic-net logistic regression with the Synthetic Minority Oversampling Technique (SMOTE). To address class imbalance, we trained a classifier optimized via 5-fold cross-validation. Depicted are 3 proteins of that model.

z: Proteins with non-zero coefficients in the final model were then incorporated into a Cox proportional hazards model assessing kidney survival (death, dialysis, or $\geq 40\%$ eGFR decline). This protein-based model significantly improved risk prediction beyond clinical features alone, as evidenced by a higher mean C-statistic across 1,000 bootstrap resamples.

include: CD19, CD79A, PAX5, FCRLA, BTK: Classical markers of B cells, indicating increased B cell infiltration or expansion. CXCL13, LTA, LTB: Chemokines central to the formation of tertiary lymphoid structures (TLSs), guiding B and T cell recruitment. IL16: A cytokine involved in immune cell chemoattraction and activation. MZB1, CD38, PRDM1: Marker associated with antibody-secreting plasma cells, supporting local immunoglobulin production. TNFRSF13B (TACI): A BAFF/APRIL receptor crucial for B cell survival and class-switch recombination, highlighting sustained B cell activity within the tissue. Together, these markers indicate the presence of an active, locally organized B cell response with potential implications for immune complex-mediated injury. CXCR5, PDCD1, CD40LG: Canonical markers of T follicular helper (Tfh) cells. CXCR5 enables migration of Tfh cells into B cell follicles and CD40LG encodes CD40L, a key costimulatory molecule required for B cell activation, class-switching, and germinal center formation (Fig. 5w).

To investigate the functional consequences of B cell–predominant inflammation, we performed immune complex analysis by isolating IgA-bound proteins from kidney tissue of 8 controls and 8 patients with a B cell–rich gene signature from the Susztaklab Biobank, using IgA-specific pulldown followed by tandem mass tag (TMT)-labeled mass spectrometry (Fig. 5x). This approach enabled us to identify proteins enriched in immune complexes from B cell–inflamed samples, revealing the presence of effector molecules such as granzymes and components of the complement cascade, which may contribute to local tissue injury.

To find plasma biomarkers that could help discriminate these two patient populations, we used paired plasma proteomic from patients with B+ and B- DKD in the TRIDENT cohort to train an elastic-net logistic regression with the Synthetic Minority Oversampling Technique (SMOTE). To address class imbalance, we trained a classifier optimized via 5-fold cross-validation. The final model included 14 proteins, of which some are depicted in Fig. 5y. Moreover, we then incorporated proteins with non-zero coefficients into a Cox proportional hazards model assessing kidney survival (death, dialysis, or $\geq 40\%$ eGFR decline). This protein-based model significantly improved risk prediction beyond clinical features alone, as evidenced by a higher mean C-statistic across 1,000 bootstrap resamples (Fig. 5z). For external validation of the B+DKD protein set, we analyzed data from 3,309 UK Biobank participants with a diagnosis of diabetes mellitus and complete clinical information. In this independent cohort, the B+DKD protein set, when combined with known clinical predictors (Kidney Failure Risk Equation; KFRE), achieved an AUC of 0.7047, exceeding the performance of the clinical model (KFRE; AUC = 0.6286), thereby supporting its potential utility for risk stratification beyond established clinical predictors (Extended Fig. 38a). Moreover, quartile-based β -coefficients from the Cox model showed that individuals above the highest quartile (Q5) of the B+DKD protein set had a markedly increased risk of progression, exceeding that predicted by the KFRE model (Extended Fig. 38b). This threshold may therefore help to identify a high-risk subgroup, that could be amenable to B cell–directed interventions.

While we agree that effect size correlation between tissue and plasma measurements would add nuance to biomarker discovery, in this revision we **shifted the analysis toward biomarker identification for a rare DKD subtype characterized by B-cell enrichment**, rather than general tubular injury. Given the heterogeneity in measurement platforms (TMT vs. SomaScan vs. Olink) and the clinical and biological rarity of the B⁺ DKD group, direct cross-modal correlation of effect sizes was not feasible within this current study design. We agree that future studies, especially those with **paired tissue and plasma proteomic data**, should explicitly test this and further validate the translational value of niche-derived biomarkers.

In summary, we have focused on a **mechanistically grounded, spatially localized disease subtype**—the B⁺ DKD group—and linked it to both **tissue-level immune processes** and **circulating biomarkers**, across independent platforms and cohorts. While cross-modal effect size correlation remains an important future direction, we believe our current strategy appropriately prioritizes biological interpretability and clinical relevance. We have clarified these points in the revised Results and Discussion sections.

Comment: h) Differentially expressed proteins found to be common to both modalities showed a strong positive correlation with clinical outcome, measured as either a kidney-specific or composite outcome. The hazard ratios (HR) for both outcomes appear identical (Figure 5f) suggesting that the association with combined outcome is driven predominantly by the association with kidney outcome. Do any of the protein biomarkers show an association with death alone? In addition, the HR observed for all patients are broadly similar to those seen for the patient groups with the lowest eGFRs. Are similar HR seen for the patients that don't have hypertension, diabetes or chronic kidney disease and who are likely to have preserved eGFR? Finally, can a sparse multivariate

predictive model be developed from this collection of potential biomarkers that might have utility in predicting kidney disease outcome in an unseen cohort of patients?

Response: Thank you for this suggestion, however **we shifted the focus of the analysis the revised manuscript, we developed spatial gene expression signatures specific to the B cell–predominant immune microenvironment.** Understanding and clinical identification of this disease subgroup could offer new precision therapeutics for patients with DKD. These signatures were independently derived directly from our spatial transcriptomic dataset. To construct the spatial niche signatures, we applied a pseudobulk strategy by aggregating gene expression across all cells assigned to a given niche on a per-sample basis. We then performed differential expression analysis between niche types using the DESeq2 package to identify genes selectively enriched in each spatial microenvironment.

For downstream biomarker discovery, we applied these spatial signatures to **bulk RNA-sequencing data** from the TRIDENT cohort of patients with diabetic kidney disease (n = 226). Patients were stratified based on expression of the B cell–predominant immune ME signature. To identify candidate biomarkers associated with this B cell–enriched disease state, we conducted differential expression analysis using the Limma package. This analysis revealed a set of tissue biomarkers significantly elevated in the B cell–associated subset, representing potential indicators of immune-driven disease activity (Fig. 14y).

Highlighted biomarkers include:

- **CD19, CD79A, PAX5, FCRLA, BTK** – canonical B cell markers indicating increased infiltration or expansion;
- **CXCL13, LTA, LTB** – chemokines essential for tertiary lymphoid structure (TLS) formation, promoting B and T cell recruitment;
- **IL16** – a cytokine mediating immune cell chemoattraction and activation;
- **MZB1, CD38, PRDM1** – markers of antibody-secreting plasma cells, supporting local immunoglobulin production;
- **TNFRSF13B (TACI)** – a BAFF/APRIL receptor critical for B cell survival and class-switch recombination, indicative of sustained B cell activity;
- **CXCR5, PDCD1, CD40LG** – classical markers of T follicular helper (Tfh) cells, reflecting support for B cell activation, class switching, and germinal center formation.

Together, these markers reflect an active, locally organized B cell response with potential implications for immune complex–mediated tissue injury.

To examine the functional consequences of B cell–predominant inflammation, we performed immune complex analysis by isolating IgA-bound proteins from kidney tissue of 8 controls and 8 patients with a B cell–rich gene signature from the SusztaLab Biobank. Using IgA-specific pulldown followed by tandem mass tag (TMT)–labeled mass spectrometry (Fig. 5x), we identified proteins enriched in immune complexes from B cell–inflamed samples. These included effector molecules such as granzymes and components of the complement cascade, suggesting a potential mechanism for local tissue damage.

In response to the reviewer’s request, we **also sought circulating biomarkers capable of distinguishing B+ from B– DKD.** We used paired plasma proteomic data from the TRIDENT cohort to train an elastic-net logistic regression model, incorporating the Synthetic Minority Oversampling Technique (SMOTE) to address class imbalance. Model performance was optimized via 5-fold cross-validation. The final classifier included 14 proteins, a subset of which is shown in Fig. 5y. We then incorporated the non-zero coefficient proteins into a Cox proportional hazards model to assess kidney survival, defined as death, dialysis, or $\geq 40\%$ eGFR decline. This protein-based model significantly improved risk prediction beyond clinical features alone, as evidenced by a higher mean C-statistic across 1,000 bootstrap resamples (Fig. 5z). For external validation of the B+DKD protein set, we analyzed data from 3,309 UK Biobank participants with a diagnosis of diabetes mellitus and complete clinical information. In this independent cohort, the B+DKD protein set, when combined with known clinical predictors (Kidney Failure Risk Equation; KFRE), achieved an AUC of 0.7047, exceeding the performance of the clinical model (KFRE; AUC = 0.6286), thereby supporting its potential utility for risk stratification beyond established clinical predictors (Extended Fig. 38a). Moreover, quartile-based β -coefficients from the Cox model showed that individuals above the highest quartile (Q5) of the B+DKD protein set had a markedly increased risk of progression, exceeding that predicted by the KFRE model (Extended Fig. 38b). This threshold may therefore help to identify a high-risk subgroup, that could be amenable to B cell–directed interventions.

Regarding the reviewer's question on hazard ratios and additional endpoints: While we acknowledge the relevance of modeling mortality and other outcomes, our current analysis was **not designed as a general prognostic modeling effort**. Rather, the intent was to map **spatially resolved disease states** and define **mechanism-based biomarkers** tied to a **rare, but actionable, immune subtype** of DKD. We did not attempt to develop a multivariate sparse predictive model across all subgroups or assess death-specific hazard ratios. These are important future directions but fall outside the scope of the present work.

In summary, the **goal of this study was not to find biomarkers of general disease severity**, but rather to **identify a mechanistically distinct, B cell-enriched DKD subtype** that had not previously been defined. Given the rarity of this group, a **blood-based discriminatory biomarker panel** is essential for future trial design and clinical targeting. We agree that future studies should include **sparse multivariate models, stratification by comorbidities**, and **specific mortality analyses** to further expand the utility of these findings. However, we believe the **strength of this study lies in its mechanistic clarity and spatial precision**, and have clarified these points in the revised manuscript.

Referee expertise:

Referee #1: kidney, sc omics

Referee #3: B cells

Referee #4: immunogenetics

Referees' comments:

Referee #1 (Remarks to the Author):

The study has substantially improved by clarifying previous issues, adding 64 new samples and clearly explaining the computational methods. The niche concept has been nicely validated using three independent methods. I thank the authors for their excellent work on revising the manuscript. Overall, my remaining concern is with the spatial relevance of the gene-gene correlations, spatial relevance of the LRs reported from cellphoneDB cell-cell communication analyses and multiple hypothesis correction of the p-values reported in most sections of the paper.

Extended Figure 15 to Figure 25 shows spatially correlated genes in different niches and a comparative differential correlation analysis then identifies gene correlations increased in disease (DKD) over controls. This is a really interesting way to find unique and common gene-gene patterns. The niches are found in a spatially constrained manner. The question now is: are any of these gene-gene correlations obtained convey any spatial relationship? Can these gene-gene correlations be used to identify long and short-range interactions?

Response: Yes, the reported gene-gene correlations convey spatial information. Niches in our workflow are defined in a spatially constrained manner and COVET learns a niche-specific latent representation that captures the gene-gene covariance structure across the index cell and its k nearest spatial neighbors ($k = 8$). Hotspot is then applied to these embeddings. Because the embeddings summarize local spatial neighborhoods, the resulting interaction scores reflect spatially localized co-expression: high-scoring gene pairs consistently co-vary within similar local niches.

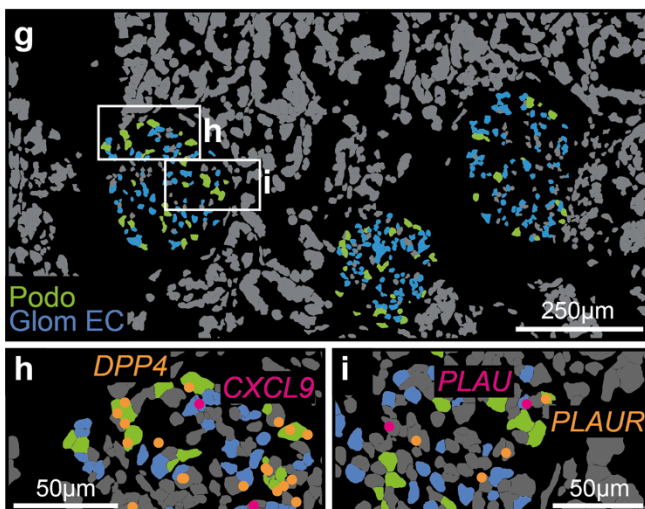
With respect to interaction range, the current analysis is specifically designed to detect short-range relationships. The use of 8-nearest-neighbor spatial neighborhoods is an intentional constraint and limits inference to local interactions. Long-range interactions are therefore outside the scope of the present study.

In addition to the above point in line 246-251, are these correlations reported after adjusting for multiple hypothesis correction?

Response: Yes, all gene-gene correlations from hotspots reported in the paper were identified using Hotspot's local-correlation test with Benjamini-Hochberg FDR control. Specifically, within each niche we computed pairwise local-correlation statistics and retained only gene pairs passing $q < 0.05$.

Figure 2r - are these ligand-receptors spatially constrained? It would be interesting to show some close distance proximity of these LR pairs and their corresponding cell types.

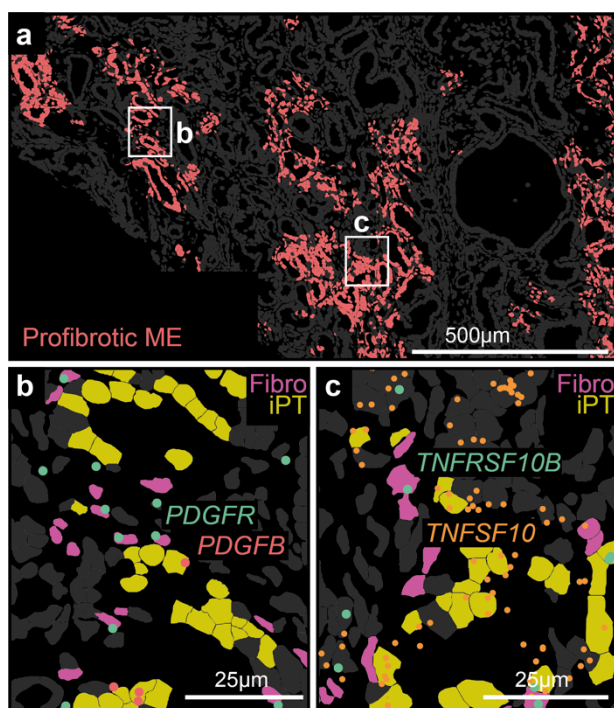
Response: We thank the reviewer for this question and have added an additional supplementary figure to illustrate the spatial colocalization of glomerular endothelial podocyte crosstalk.



Extended Data Fig. 6g, Glomerular niche with podocytes highlighted in green and glomerular endothelial cells (Glom EC) highlighted in blue. Scale bar, 250 μ m. h-i, Podocytes and Glom are pseudocolored as indicated; corresponding transcripts are overlaid in matching colors. Scale bar, 50 μ m.

Figure 3o shows really robust correlation with a nice narrow confidence interval. However, what is not clear is - given that these are findings reported from the spatial data, are these also spatially co-localised or in close proximity?

Response: We thank the reviewer for this question and have therefore added an additional supplementary figure to illustrate the spatial colocalization of *PDGFB* and *PDGFR* as well as *TNFRSF10B* and *TNFSF10*.



Extended Data Fig 8a, Injured tubular profibrotic microenvironment (red), as in Fig. 3. Scale bar, 500 μ m. b–c, Fibroblasts and injured proximal tubule (iPT) cells are pseudocolored as indicated; corresponding transcripts are overlaid in matching colors. Scale bar, 25 μ m.

Figure 4u and 4w, I am concerned about the statistical relevance after controlling for multiple hypothesis correction.

Response: We appreciate the reviewer's attention to statistical considerations. Because ligand–receptor analyses evaluate a very large search space, stringent multiple testing correction at this exploratory stage would substantially inflate type II error and obscure biologically meaningful relationships. We therefore consider this analysis hypothesis-generating rather than confirmatory.

To increase rigor, we validated the top-ranked ligand–receptor pairs in an independent bulk tubulointerstitial RNA-seq dataset of 843 human kidney samples, where these pairs showed strong positive correlations with XBP1, a plasma cell marker (Extended Fig. 10e–n). This cross-platform validation supports their biological relevance.

Line 478 “organized B cell–predominant microenvironment that case be revealed by spatial transcriptomics.” is not clear. Do the authors want to say “can” instead of “case”

Response: We thank the reviewer for this remark and have changed the text accordingly.

Referee #3 (Remarks to the Author):

Dumoulin, et al., Spatial single-cell atlas of diabetic kidney disease reveals a B-cell-rich disease subgroup

Summary: The authors strengthen their findings by quadrupling the sample size, combining two different spatial transcriptomic platforms, and identifying eleven distinct and reproducible spatial niches that can be generalized across humans, not heretofore done at this scale and resolution. Using unbiased discovery, they identified distinct spatial niches, shared across both patients and healthy controls, that were orthogonally validated with different computational methods and inked to changes in eGFR. They go on to define injured tubular and immune cell microenvironments according to their putative roles in driving disease pathogenesis, by defining the nature of their interactions with surrounding cells. Lastly, they identify a B cell-rich microenvironment that identified a patient subgroup more likely to progress to renal failure. In toto, the work details a largesspatial atlas at the single-cell resolution for a human disease, with clinically-relevant findings for DKD that sheds light on potential therapeutic targets.

The authors have addressed concerns raised at time of original review, as detailed below.

An additional point: while the authors' demonstration that cell therapy mediated B-cell depletion in lymphoma leads to (quite modest) improvement in kidney function(extended figure 34), this observation does not support their contention that kidney-infiltrating B cells are associated with poor outcome in DKD. There are multiple possible explanations why renal function improves following such therapy, for example, improvement in overall organismal physiology. Moreover, since control kidneys (of the type found in lymphoma, given that such patients lacked intrinsic evidence of kidney disease) lacked significant B-cell infiltration, it is an overreach to use such modest data to validate a central thesis of the paper, which is otherwise supported.

Response: We thank the reviewer for this thoughtful comment. We agree that the lymphoma dataset provides only indirect and limited support and that alternative explanations cannot be excluded. To avoid overstating this observation, we have removed this supplementary figure and all associated text from the manuscript.

In response to original review:

1) While the identification of niches corresponding to "injured" cells is crucial, it would be informative to know how these injured niches (iPT and iLOH) compare to normal niches in their gene expression differences. While the supplementary tables contain what the cluster defining genes are, it should be stated in the text how the authors define iPT and PT and what the differentially expressed genes between iPT vs PT and iLOH and LOH. This is a analysis that should be stated.

The authors sufficiently address this point by defining the transcriptomic differences between healthy and injured niches, demonstrating that key markers of damage were upregulated across both niches and across platforms. Furthermore, the authors provide a comprehensive and robust analysis that supports disease-specific spatial niches present in DKD.

2) Figure 1: Validation of spatially variable genes would be helpful, showing the differences in protein expression along the cortical and medullary sides (panel f).

Response: We thank the reviewer for requesting additional protein-level validation. After careful consideration we concluded that we cannot generate a uniformly convincing protein map for every spatially variable gene within the scope of this revision. To avoid presenting an incomplete or potentially misleading validation we have removed the spatially variable gene panel altogether and have withdrawn the related text.

While removing the data entirely sidesteps the question, validating the cell types that comprise the individual niches with protein staining (1-2 markers per stain) would suffice but understandable that not every spatially variable gene could be validated with protein confirmation.

3) Figure 2: The presence of iPT in control samples and diabetic samples without kidney involvement warrants further validation of the injured niches and is not mentioned in the text (panel h). The authors show fraction increases in DKD but it would also be informative to show what niches decrease in DKD (panel i). The authors provide a reasonable biological explanation for the presence of certain "injury" niches in control samples and highlight a finding that may explain subclinical injury / aging. Furthermore, the authors added the requested analysis and demonstrate a shift from homeostatic to damaged tissue states.

Response: We thank the reviewer. We have strengthened the description in the Results to explicitly note the biological plausibility of low-level injury signatures in controls and aging-related changes, and we highlight both increases and decreases in niche proportions in DKD.

4) Figure 3: While the authors define microenvironments surrounding the injured niches, it would be interesting to see if and how microenvironments around the other niches change in DKD patients. In 3j, the authors should consider further dissection of the immune cell composition shared between iPT ME4 and iLOH ME1. What are the predominant immune cell types shared between these microenvironments?

The authors sufficiently address these points by examining niche-specific regulatory networks and demonstrating changes in gene-gene correlations in DKD. Specifically, they highlight that changes that are

associated with disease and perform this analysis on all eleven niches. The authors also go further by generating a kidney immune atlas to identify seven distinct immune microenvironments and their cellular composition. Furthermore, they perform extensive analysis including ligand-receptor interaction analysis to probe how nearby immune cells are interacting with parenchymal cells.

Response: We thank the reviewer for recognizing these expansions. We have further clarified in the text which immune populations are shared between IPT ME4 and iLOH ME1, and we explicitly list the predominant immune cell types contributing to these microenvironments.

5) Figure 4: The resident kidney immune cell microenvironment is insufficiently defined. Because these cells were not seen around injured tubular areas does not mean they are typical of the kidney. Further characterization and validation of this resident kidney immune cell microenvironment should be performed. Also, in panel c, the immune cell zonation as characterized by the predominance of immune cells is insightful, yet the presence of CD8⁺ T cells in all microenvironments except the B cell microenvironment is interesting. Further interrogation of this observation may be beyond the scope of the manuscript but is worth noting. Only two patients show B cell-predominant immune microenvironments (panel d). The authors might consider not generalizing these findings. Furthermore, CD4⁺ predominant environments seem to be more consistently seen across diseased patients. The authors suggest a causal role of B cells in kidney progression but it is unclear what effects the B cells have in progressive kidney disease. Furthermore, have the authors ruled out recent viral infections? B cell TLS are seen in acute kidney injury as well as ageing so it is unclear whether the observation reported is a microenvironment unique to chronic kidney disease or kidney injury (PMID: 27699223). At some point in the future, the authors might consider examining clonal expansion within these populations. This would help our understanding the presence of B cells in these microenvironments.

The authors address these comments by defining residential immune microenvironment based on spatial contexts, stability across health and disease, and distinct transcriptomic signature. They adequately define the residential macrophage phenotype and show how they are different from disease-driven recruited macrophages. The expanded dataset also supports the idea that the cell microenvironment is enriched in DKD. And application of the B cell microenvironment signature showed that the signature correlated with fibrosis severity, worse eGFR, and more rapid disease progression. Furthermore, the authors define the TLS-like structures present in these DKD patients, validated with protein staining and highlight a key direction for future work by interrogating these structures and the antigen specificity of the lymphocytes that comprise them. While the authors could not rule out subclinical infection, patients with known viral infections were excluded. Lastly, the authors generalize their findings with validation with external cohorts, including examining how B cell depletion could improve kidney outcomes.

Response: We thank the reviewer for these insights. We now explicitly define the resident kidney immune microenvironment based on spatial context, transcriptional identity, and stability in health and DKD, and we clarify that the B cell-predominant microenvironment is present only in a subset of DKD patients. We also explicitly state that patients with known viral infections were excluded, although subclinical infection cannot be entirely ruled out. We agree that clonal expansion analysis will be highly informative and highlight this as a future direction for understanding the antigen specificity and functional roles of B cell TLS-like structures.

6) Figure 5: IPT does not correlate with disease severity as strongly as the iLOH and immune niche. Does this call into question the validity of the IPT niche? In panel b, statistical analysis should be performed for the survival curves.

Using the expanded dataset, the authors demonstrate the validity of the IPT and iLOH niches by the drastic and statistically-supported expansion in disease. Furthermore, the authors provide a biological explanation for the dichotomy in the correlation with IPT and iLOH and fibrosis with the former being associated with acute injury rather than fibrosis. Importantly, they posit an interventional window prior to irreversible tissue scarring that could, in theory, be detected by the presence of IPT.

All minor comments addressed, except please note one additional comment: line 564, should read “positive correlation” as extended figure 33I demonstrates.

Response: We thank the reviewer. The expanded dataset confirms the biological and statistical validity of both the IPT and iLOH niches, and we have strengthened the text to explain the distinct temporal and biological roles of the two injury states. As requested, we now include statistical testing for the survival curves in Figure 5b. Moreover, we have changed the phrasing in line 564.

In total, the authors have their submission and increased the scientific robustness of their study, and have improved the quality and impact of the manuscript. While the mechanistic link between the presence of B cell-TLS-like niches and disease progression remains unclear, the authors do show that local antibodies bind immune activation components, an interesting future direction that should be explored to understand how this directly causes tissue damage.

Referee #4 (Remarks to the Author):

Thank you for the opportunity to review the revised manuscript “Spatial Single-Cell Atlas of Diabetic Kidney Disease Reveals a B-Cell-Rich Disease Subgroup” by Dumoulin and colleagues.

The revised manuscript presents a substantial advance over the authors' previous publication in Nature Genetics, offering significant enhancements in resolution, methodological innovation and biological insight into diabetic kidney disease (DKD). The authors have transitioned from spot-level spatial transcriptomics to single-cell resolution, generating one of the largest datasets of its kind. This increase in resolution allows for a more precise exploration of cellular interactions and microenvironments, providing significantly more detail and enabling analyses that were previously unattainable. This single-cell precision contrasts sharply with the broader, less detailed scope of the previous work, offering a more granular understanding of the kidney's cellular architecture.

The methodology in the analysis of these complex datasets represents a significant improvement from the previous version. At the practical level, the introduction of a new framework for defining kidney tissue organization based on spatial niches marks a shift from traditional histological classifications. This novel approach enhances our understanding of kidney tissue architecture and pathology, offering insights into complex cellular interactions and their implications for disease mechanisms. At the methodological level, new analysis includes clarification of analysis pipelines implemented, QC/data and some issues that are now resolved using two complementary datasets (CosMx and Xenium). I also note that the authors have toned down claims regarding biomarkers which are appreciated. Combined, these innovations set the current study apart from the prior work, which largely focused on cell type validation without delving into niche-specific disease mechanisms or interactions. Finally, these datasets will be a valuable resource for the community it will lead to further advances in the field. Biologically, the discovery of a B-cell–predominant immune microenvironment adjacent to the injured loop of Henle introduces a novel aspect to DKD pathogenesis, suggesting potential new targets for therapeutic intervention. Additionally, the stratification of DKD patients based on spatial niches provides a pioneering approach to defining molecularly distinct patient subtypes, a critical step towards personalised medicine. This stratification and its implications for precision diagnostics and treatment were not explored in the earlier study, highlighting the current work's unique contributions to the field. In summary, the revised manuscript not only advances the technological and analytical capabilities in spatial transcriptomics but also delivers new biological insights with clinical implications. These advances present a leap beyond incremental progress, positioning the study as a significant contribution to the understanding and potential treatment of DKD.

Below I have detailed comments and clarifications for the manuscript that should be addressed.

For the uninitiated, the overlap of transcripts covered between the xenium and CosMx datasets will be unclear. On line 111, this overlap should be defined for the readers. Furthermore, specifically what aspects of the kidney cell subsets are covered and omitted by both methodologies should be defined. In addition, matched control and DKD samples should be show in figure 1 illustrating representative data both platforms. Putting this information in the context of the Kidney and disease context are important and could be summarized briefly by broad analysis of pathways and cell types covered by the two approaches.

Response: Samples were profiled using the CosMx Nanostring platform, profiling 1,000 transcripts (n = 48), and the Xenium 10X platform profiling 5,100 transcripts (n = 16), with an overlap of 656 transcripts that were profiled by both technologies.” These 656 overlapping genes include all major cell-type markers necessary to define kidney parenchymal, endothelial, stromal, and immune populations across both platforms. To help readers understand which genes are present in each platform, we now explicitly annotate platform-specific genes in Figure 3i,l.

We have also updated Figure 1 to include representative control and DKD samples from both Xenium and CosMx, allowing readers to directly compare outputs from the two platforms.

Furthermore, we performed additional comparative analyses using donors profiled on both Xenium and CosMx (n = 3). For each sample and platform, we computed the fraction of cells per cell type, reshaped these measurements into paired vectors, and compared CosMx vs Xenium using the two-sided Wilcoxon signed-rank test. We did not observe significant differences in cell-type frequency estimates between platforms (Supplementary Fig. 10a,b).

We performed the same paired analysis on niche assignments and likewise observed no significant differences (Supplementary Fig. 10c,d).

To further support transparency, we now provide a table listing all platform-exclusive genes in Supplementary Table 28.

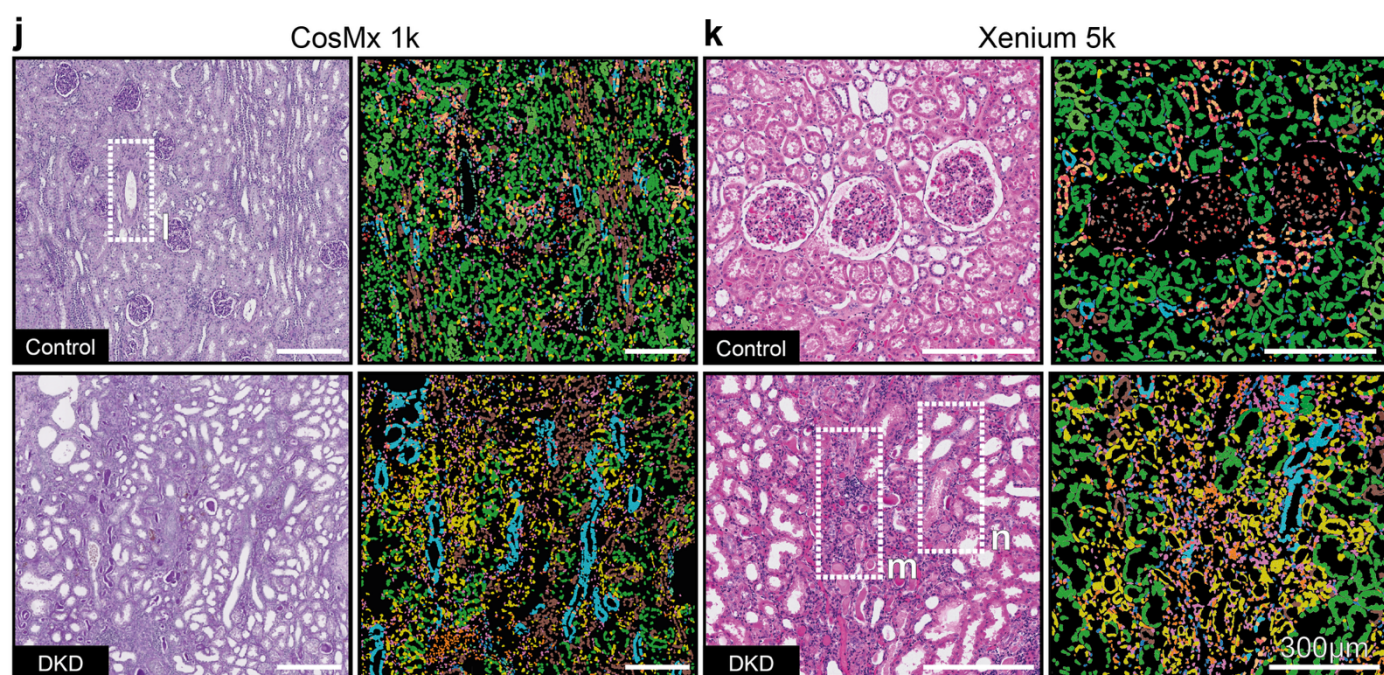
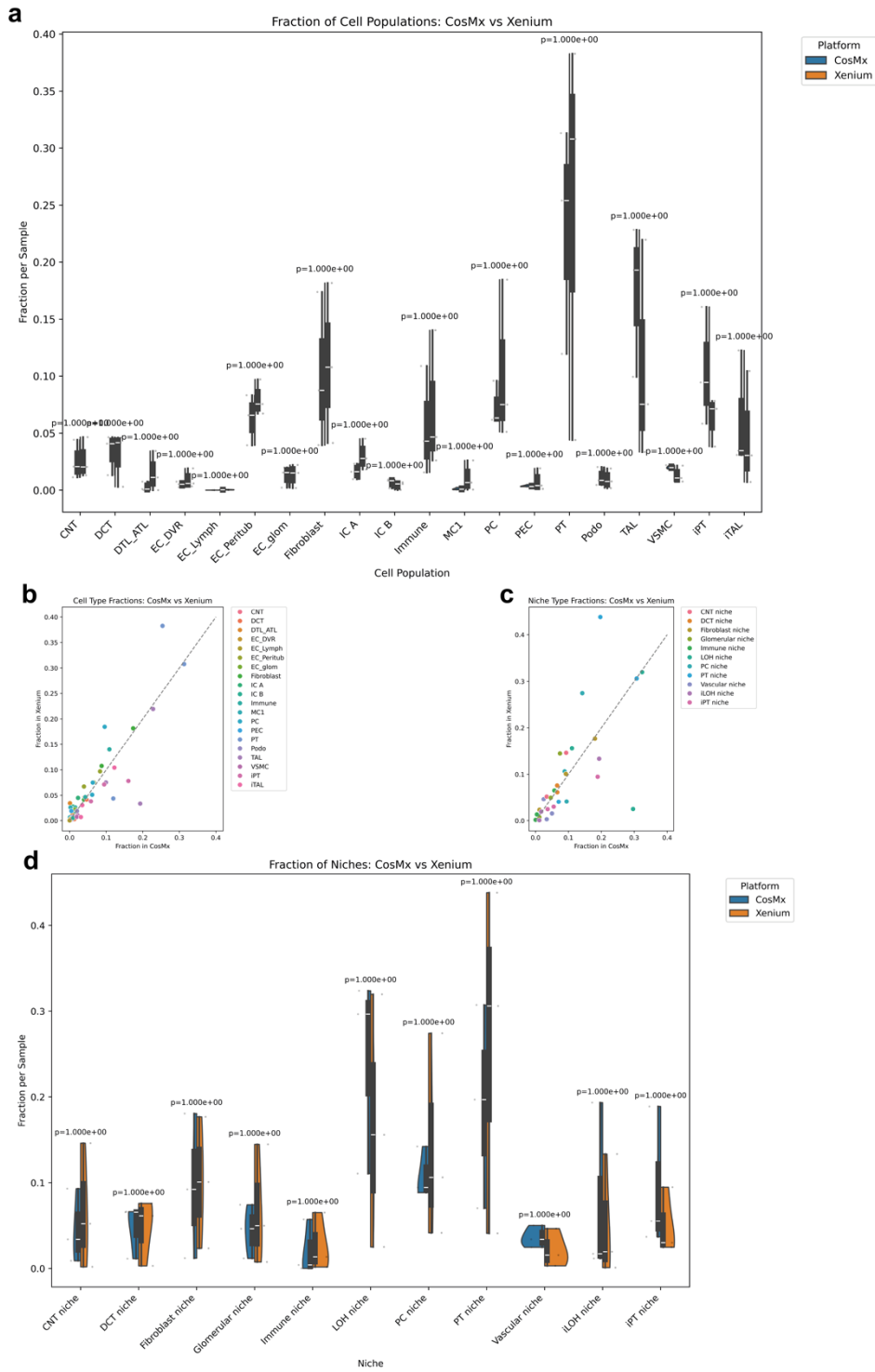


Fig 1j, PAS staining and CosMx 1k cell-type maps of a healthy pediatric kidney (top) and a DKD kidney (bottom). Cells are shown by spatial location with segmentation masks (Nanostring pipeline). Scale bar: 300 μ m.

k, HE staining and Xenium 5k cell-type maps of a healthy kidney (top) and a DKD kidney (bottom). Cells are shown by spatial location with their segmentation masks (10x Genomics Xenium Explorer 3.2.0). Scale bar: 300 μ m.



Extended Fig. 36: To compare cell population representation across spatial transcriptomics platforms, we selected kidney samples profiled by both CosMx and Xenium (HK2695, HK3626, HK3106). Cells from these samples were subset from the full dataset, and the cell type annotation was retained. Each cell was labeled by its corresponding platform and patient identifier. For each sample and technology, we computed the fraction of cells assigned to each cell type by dividing cell type counts by the total number of cells per sample and platform. The resulting fractions were reshaped into a paired format to enable direct comparison between CosMx and Xenium measurements per cell type and donor. Statistical comparisons were performed using the two-sided Wilcoxon signed-rank test for each cell type. Fractions were visualized using split violin plots with overlaid strip plots to show paired values across platforms. Adjusted p-values were annotated for each cell population comparison. c, d: To compare niche representation across spatial transcriptomics platforms, we selected kidney samples profiled by both CosMx and Xenium (HK2695, HK3626, HK3106). Cells from these samples were subset from the full dataset, and the cell type annotation (annotation_updated) was retained. Each cell was labeled by its corresponding platform and patient identifier. For each sample and technology, we computed the fraction of cells assigned to each cell type by dividing cell type counts by the total number of cells per sample and platform. The resulting fractions were reshaped into a paired format to enable direct comparison between CosMx and Xenium measurements per cell type and donor. Statistical comparisons were performed using the two-sided Wilcoxon signed-rank test for each cell type. Fractions were visualized using split violin plots with overlaid strip plots to show paired values across platforms. Adjusted p-values were annotated for each cell population comparison.

In line with this issue, there are instances where the findings of the two data sets do, and do not agree, for example, for example line 311: “The proximal tubular microenvironment, enriched for PT and DCT neighbors (Fig. 3i, l), featured iPT cells expressing CUBN, APOE, and GPX3 (CosMx only), and iLOH cells expressing EGF (both datasets), SLC12A1, and COL6A1 (Xenium), all consistent with a relatively healthier tubular state.”. The lack of correlation in data sets is not discussed thoroughly enough for the reader to critically understand the differences in these data sets. Therefore, some analysis of these differences should be provided in the extended data. As Xenium 5K is wide coverage with lower sensitivity, is the issues that these genes drop out and hence custom probes sets with lower coverage but more sensitivity would resolve this? Furthermore, how is this issue resolved when the situation occurs in the opposite direction (present in CosMx but not Xenium 5k).

Response: We agree with the reviewer that this is an important issue. Recent comparative work (e.g., Lermi et al., 2025) assessed Xenium (human 289-gene lung panel plus 50 add-ons) against the CosMx 1k universal panel and concluded that CosMx generally exhibits higher sensitivity and dynamic range—detecting more transcripts and subtle changes—whereas Xenium provides greater specificity and robustness. Our study, however, was not designed to benchmark probe performance between platforms. Instead, we addressed cross-platform variability by integrating datasets with scVI/scANVI, probabilistic deep generative models well suited for large-scale, multi-modal single-cell integration and widely benchmarked for minimizing batch effects while preserving biological structure (Luecken et al., Nat Methods, 2022). In our latent modeling, we treated platform identity (CosMx, Xenium, snRNA-seq) as the primary batch factor, incorporated sample identity and data source (KPMP, Susztak Lab, Humphreys Lab) as additional categorical covariates, and included continuous covariates such as total RNA counts and mitochondrial content. This multi-layer correction explicitly accounts for platform-level and sample-level technical effects, allowing us to focus on conserved biological signals rather than assay-specific probe behavior.

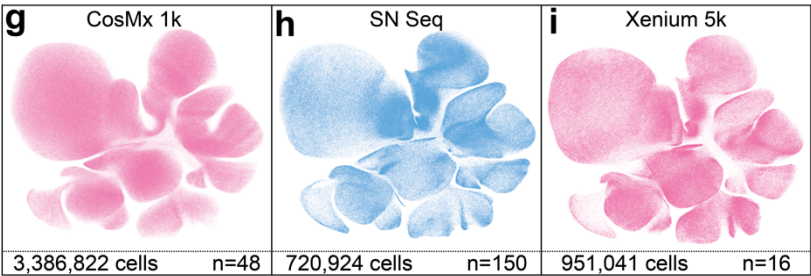


Fig 1g, CosMx 1k spatial gene expression data. 48 samples and 3,386,822 cells were included in this study.
h, snRNA-seq data. 150 samples and 720,924 cells were included in this study.
i, Xenium 5k spatial gene expression data. 16 samples and 951,041 cells were included in this study.

As shown in Fig. 1g–i, the technologies align well in the integrated space, enabling joint cell-type annotation and downstream analyses. Because cell-type calls are comparatively robust to probe-content differences, We performed an additional paired comparison using samples profiled on both Xenium and CosMx (n = 3). For each donor and platform, we computed the fraction of cells per cell type (cell-type counts divided by the total cells for that donor-platform), reshaped these fractions into paired measurements, and compared CosMx vs. Xenium with two-sided Wilcoxon signed-rank tests per cell type. We observed no statistical differences between platforms (Supplementary Fig. 10a, b). We applied the same paired analysis to kidney niche assignments and likewise found no significant differences (Supplementary Fig. 10c, d). This concordance indicates that platform-specific probe differences do not materially bias cell-type or niche composition estimates in our data, supporting the robustness and generalizability of downstream biological conclusions. Between the CosMx 1k platform and the Xenium 5k platform 656 genes overlapped. To help the reader in understanding which gene was profiled in which platform we indicated this in the Figure 3 i, l.

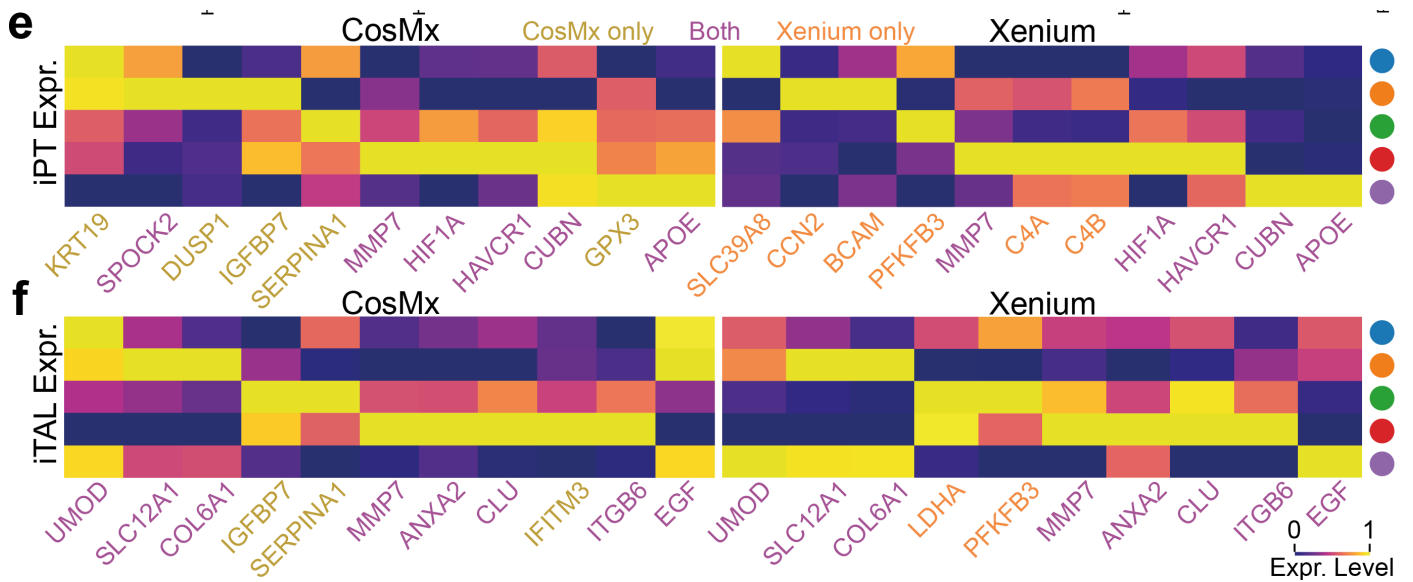


Fig 3e, Heatmap of key differentially expressed genes of iPT center cells in microenvironments in the CosMx dataset (left) and Xenium dataset (right). Color of gene name indicates whether gene was profiled only in CosMx, only in Xenium or in both assays. **f**, Heatmap of key differentially expressed genes of iLOH center cells in microenvironments in the CosMx dataset (left) and Xenium dataset (right). Color of gene name indicates whether gene was profiled only in CosMx, only in Xenium or in both assays.

Figure 3: The analysis presented figure 3 is central to the identification of different environments within the kidney. To me there are portions of this discussion of data where it is currently unclear whether the identification of niches/microenvironments is performed at the cell level, via binned transcripts within different sizes of analysis (20, 40, 80u,) or a combination of both. This is important as identification of different environments with highly altered cell numbers compared between two samples (i.e. inflamed v non-inflamed; fibrotic v non-fibrotic and so on or ranked by eGFR) will generate distinct gene expression data when analyzed at the local environment level. Thus, these data do not always reflect a change in cell state, and instead cell composition. Thus, these interpretations need to be clarified.

Response: We agree with the reviewer and have improved the phrasing. The gene expression comparisons provided in Fig. 3e and f are at the center cell level. It now reads in the manuscript as follows (lines 760ff): “Using neighborhood composition, we identified five distinct injured tubular microenvironments, each defined by unique neighboring cell types and transcriptional states (Fig. 3g–i, Supplementary Tables 12–15, Extended Fig. 7a–e). The proximal tubular microenvironment, enriched for PT and DCT neighbors, showed relatively healthy iPT and iLOH center cells expressing CUBN, APOE, GPX3, EGF, and COL6A1. The distal microenvironment, enriched for TAL, DTL/ATL, PC, CNT, and IC neighbors, featured UMOD+/EGF+ iLOH and KRT19+/SLC39A8+ iPT center cells. The periglomerular microenvironment, adjacent to glomerular cells, showed SPOCK2+/DUSP1+/IGFBP7+ iPT and CCN2+/BCAM+ iLOH cells at the center, suggesting early stress and matrix remodeling. The perivascular microenvironment, enriched for EC_DVR and VSMC neighbors, displayed SERPINA1+ and PFKFB3+ iPT/iLOH center cells, consistent with hypoxia-driven metabolic reprogramming. Finally, the profibrotic microenvironment, rich in immune and fibroblast neighbors, featured MMP7+/HAVCR1+/HIF1A+ iPT and MMP7+/ANXA2+/CLU+/ITGB6+ iLOH center cells, indicating proinflammatory and profibrotic activation consistent with prior reports.”

The Figure 3m is derived from comparison of the aggregated microenvironments. We have clarified this accordingly (line 777ff): “The profibrotic microenvironment was markedly expanded in DKD (Fig. 3d) and negatively correlated with eGFR ($r = -0.6$, $p < 0.001$; Fig. 3c). Differential expression analysis of aggregated microenvironment profiles...”.

Therefore, in Fig. 3e and f we analyze the cell states of the center cell, whereas in Fig. 3m analyze expression profile of the entire microenvironment. Comparison of the aggregated expressions of these microenvironments represents a valuable analysis as it compares spatially defined, biologically coherent units that are invisible to standard bulk RNA-seq and therefore provides complementary information to the center-cell analyses.

Figure 4: I think there is an error in the figure legend as the text does not match with the figure. Figure 4c should be annotated as it is unclear what tissue state this reflects. It also means linking the data to interpretations by the authors, for example: “We observed striking differences in the spatial distribution of immune cell types. While macrophages and plasma cells were broadly dispersed, CD4+ and CD8+ T cells

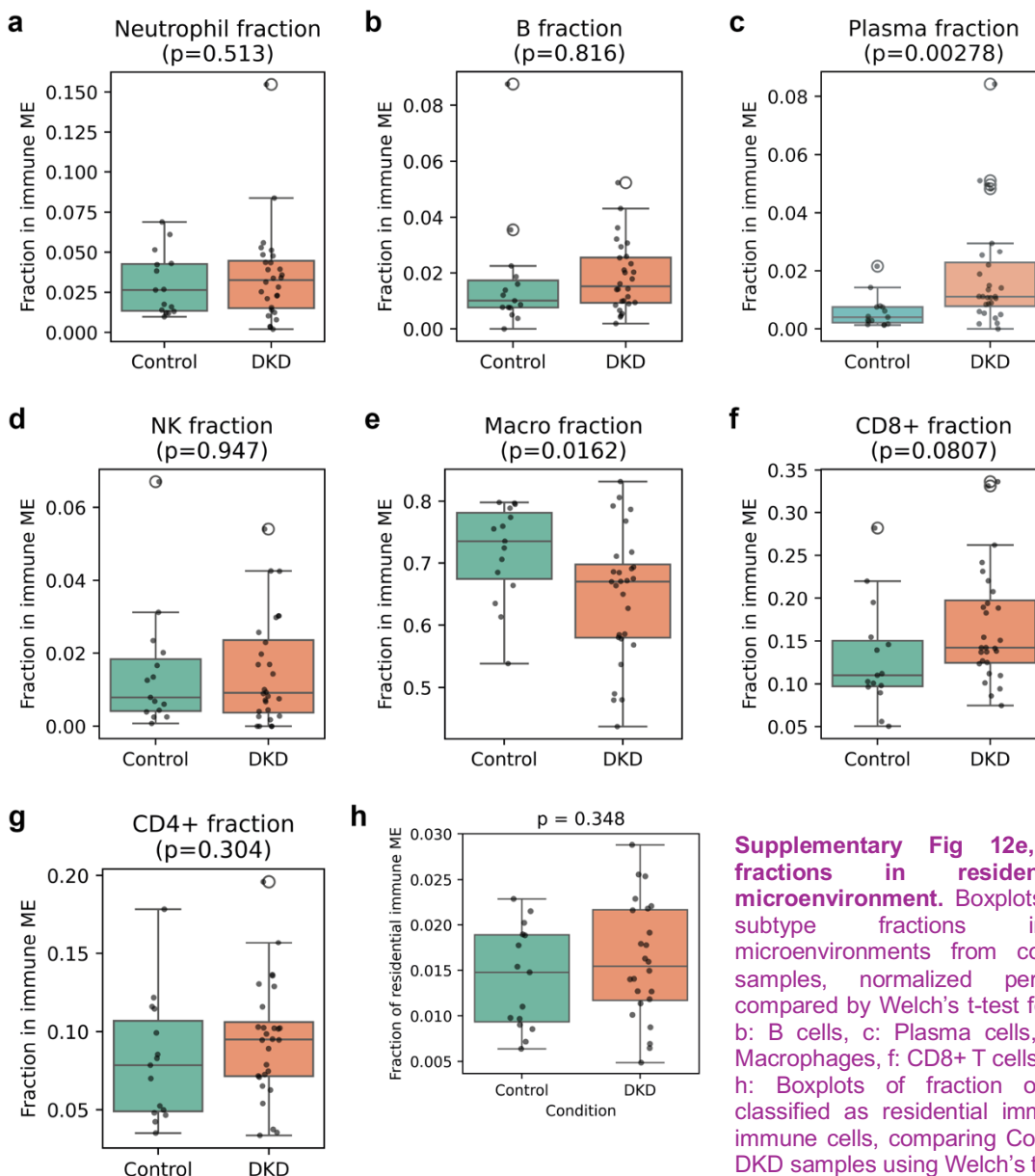
displayed non-random patterns of localization, with B cells showing the most spatially restricted distribution (Fig. 4c).” is difficult.

Response: We thank the author for this remark and have annotated the figure 4c accordingly. The respective text now reads (line 968ff): “Spatial mapping in DKD revealed that while macrophages and plasma cells were broadly distributed, CD4⁺/CD8⁺ T cells and especially B cells showed localized clustering; B cells also had the highest proportion of immune neighbors, indicating enrichment within dense immune microenvironments (Fig. 4c, Extended Fig. 10c).”

We have also updated the text of the figure legend.

The statement: “consistent with this, in two healthy pediatric kidney samples (Fig. 4e, rightmost bars, HK3656_C and Pediatric1_C), the vast majority of immune cells localized to the residential immune microenvironment. Moreover, this compartment remained proportionally stable across healthy and DKD samples (Extended Fig. 29a), and its cellular composition was largely preserved despite broader immune shifts in disease (Extended Fig. 29b).” is difficult to justify in the absence of statistical tests. These should be performed and provided to validate these statements.

Response: We appreciate the reviewer’s concern and have included additional statistical tests. Specifically, we compared the fraction of each immune cell population within the residential immune microenvironment between Control and DKD samples. We then performed a t test for each immune cell type. We did not find statistically significant differences between the two groups except for plasma cells, that were found more often in DKD (Supplementary Fig. 12a-g). Similarly, we compared the proportion of immune cells assigned to the residential immune microenvironment between Control and DKD samples. Here, we did not observe significant differences (Supplementary Fig. 12h).



Supplementary Fig 12e, Immune cell fractions in residential immune microenvironment. Boxplots of immune cell subtype fractions in residential microenvironments from control and DKD samples, normalized per sample and compared by Welch’s t-test for a: Neutrophils, b: B cells, c: Plasma cells, d: NK cells, e: Macrophages, f: CD8⁺ T cells, g: CD4⁺ T cells. h: Boxplots of fraction of immune cells classified as residential immune cells of all immune cells, comparing Control samples vs. DKD samples using Welch’s t-test.

I do not necessarily agree with the statement on line 436: “Unbiased analysis revealed a marked immune cell enrichment within the glomerular niche.” Only two (macrophages and CD8) of the analysed populations reached statistical significance, and both these populations only just reached the 0.05 cutoff. These statements should be toned down.

Response: We thank the author for his comment. We have rephrased the statement. It now reads: “Unbiased analysis revealed increased immune infiltration within the glomerular niche in DKD (Fig. 2q).”

Density plots such as those shown in Figure 5o can be misleading. Can the authors replace these data with Xenium explorer raw transcript counts overlayed on H&E.

Response: We thank the reviewer for this comment and have replaced Figure 5o with an overlay of raw transcript counts with HE.

o Spatial Colocalization

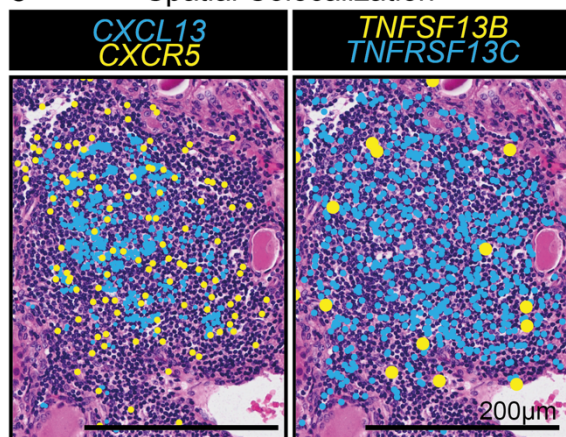


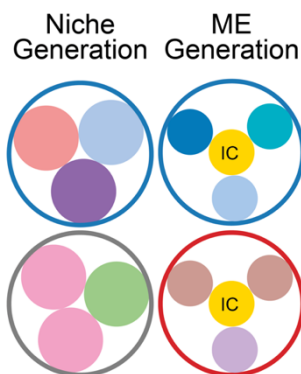
Fig 5o: Xenium spatial plot of transcript expression of CXCL13/CXCR5 and TNFSF13B/TNFRSF13C overlayed with H&E staining. Each dot represents expression of a transcript. Plots were generated by 10x Genomics Xenium Explorer 3.2.0. Scale bar, 200 µm.

MINOR

In scientific writing niche and microenvironment are often used interchangeably. This section of the niche/microenvironment analysis terminology in the manuscript is quite rigid in its current form. I am in no way suggesting a re-write of any of this, but would strongly suggest toning down the language and instead propose the authors make a clear statement at the beginning of their analysis along the lines of: “For our studies, we have performed analysis at the niche and microenvironment level. Herein, we define a niche as X and a microenvironment as Y” I think a minor clarification along these lines is important and a simple resolution that will avoid semantics in the field going forward.

Response: We thank the reviewer for this thoughtful comment. We agree that “niche” and “microenvironment” are often used interchangeably, but we believe it is important to distinguish them. In our manuscript, a kidney niche denotes a recurring, multicellular neighborhood—a reproducible spatial pattern of cell types and organization observed across samples. By contrast, a microenvironment is cell-centric: it is defined around a specific cell type and captures how its immediate spatial context relates to that cell’s gene-expression and signaling state.

We have changed the phrasing to accommodate for the reviewer’s concern (line 419f): We introduce the kidney niche terminology at the beginning of the second paragraph of the result section: “While key structures are well characterized, we hypothesized that spatial transcriptomics could reveal additional patterns of cellular organization—termed “niches” throughout this study.”



Schematic: Schematic of defining niches (left) and microenvironments (right). For niches, all cells were clustered based on their neighborhood fraction after restricting the analysis to those with sufficient local neighborhood density, removing the sparsest 5% of neighborhoods (based on total neighbor count at 80 µm) to reduce edge effects (n = 4,029,331 cells). For each cell, we computed the number of neighboring cells within concentric distance shells: 0–20 µm, 20–40 µm, 40–60 µm, and 60–80 µm. This was achieved by calculating cumulative neighbor counts at 20, 40, 60, and 80 µm, followed by subtracting counts from the smaller radius to isolate the number of neighbors in each respective shell (e.g., the 40–60 µm shell = count at 60 µm – count at 40 µm). For each cell, neighbor counts were normalized by the total number of neighbors at that distance, yielding a cell-by-neighbor composition matrix that reflects the relative cellular makeup of the local environment at different spatial scales. This matrix was then scaled and subjected to principal component analysis (PCA), with the top 10 components used as input for MiniBatch K-means clustering. The number of clusters (k) was determined based on the elbow method. Niche clustering was performed on the entire dataset to define global spatial neighborhoods.

We introduce microenvironments at the beginning of the third paragraph of the result section and make a clear distinction to niches: “Beyond identifying spatial niches, spatial transcriptomics captures how a cell’s state and gene expression vary with its immediate surroundings—termed “microenvironments” here (Fig. 3a). Unlike multicellular niches, microenvironments reflect local context-dependent changes within a specific cell type. We focused on injured tubular (iPT, iLOH) and immune cell microenvironments to understand how local interactions contribute to disease progression.” Additionally, to aid the reviewer in this distinction we include schematics of how we define niche vs. microenvironment in Fig. 2a and 3a.

The manuscript does need a thorough re-read to minor issues with grammar and spelling, mostly plurals. Some examples are shown below but there are others.

Response: We thank the reviewer for this comment and have grammar and spell checked the manuscript.

Figure 2C is difficult to interpret in its current state. I understand it is using the annotation from Figure 2b, but with the wide range of colours/niches it requires a significant amount of cross referencing. A more simple annotated Bar graph could be easier to interpret.

Response: We thank the reviewer for the helpful comment. We have updated the labeling in Fig. 2b and reordered Figs. 2c and 2n so that the color order in Figs. 2c, 2e, and 2n now exactly matches Fig. 2b. This alignment should simplify interpretation and reduce the need for cross-referencing.

Line 311 – “the state and gene expression of a given cell type change depending on its immediate surroundings; we call this microenvironment (Fig. 3a)” is not grammatically correct. Potentially: “Herein we refer to this as a microenvironment”

Response: We thank the reviewer for this comment. We have rephrased the sentence accordingly, it now reads: “Beyond identifying spatially organized niches, spatial transcriptomics reveals how a cell type’s state and gene expression vary with its immediate surroundings, a concept we refer to as the microenvironment (Fig. 3a).”

Line 316: “We focused on the injured tubular epithelial cells (iPT and iLOH) and immune cells microenvironments, as both are highly dynamic in disease in hope of understanding how local change contribute to disease progression.” Is grammatically incorrect. Furthermore, what do the authors mean by highly dynamic? spatially, temporally? With disease?

Response: We thank the reviewer for this comment. We have rephrased the sentence accordingly. It now states: “We focused on injured tubular (iPT, iLOH) and immune cell microenvironments to understand how local interactions contribute to disease progression. “

Line 426: “..... immune microenvironment was characterized by” Should be immune microenvironments

Response: We thank the reviewer for this comment and have corrected the phrasing.

Remarks on code availability: No additional comments.

Referee #5 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Referee #6 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Referees' comments:

Referee #1 (Remarks to the Author):

The authors have largely addressed my questions, and I appreciate their responses and the additional work provided. The revision has greatly improved the manuscript. However, the issue concerning the spatial relevance of the identified CCIs has not been satisfactorily resolved. For example, in the authors' rebuttal (Figure 3o), the expression pattern of the DPP4 transcript appears to extend beyond cell boundaries. Furthermore, in the PLAUI and PLAUR gene plots (right), most of the displayed transcripts do not fall within the blue (glomerular EC) or green (podocyte) regions. Again, in figures 8b and 8c. Most TNFSF10 transcripts are outside the cells of interest. This could point to an issue with segmentation and QC of xenium data. Xuanyuan et al. (<https://www.nature.com/articles/s41467-025-62599-9>) mention re-segmenting xenium data using Baysor. Could the authors comment on the quality measures taken to optimize segmentation?

Response: We thank the reviewer for their careful and thoughtful evaluation of the revised manuscript and for noting that the revision has substantially improved the work. We fully agree that careful consideration of segmentation quality is essential for the correct interpretation of spatial transcriptomic data, and we welcome the opportunity to explain in more detail how the observed transcript distributions arise from the characteristics of the technology and analysis pipeline used, and why they do not reflect a segmentation or quality control concern.

Below, we address the reviewer's question in a structured manner. First, we clarify an important conceptual point regarding how transcript coordinates and cell boundaries are represented in subcellular spatial transcriptomics platforms such as Xenium. Second, we describe the specific Xenium Prime 5k segmentation workflow used in this study and how it differs from earlier Xenium implementations that motivated the use of Baysor in prior work. Third, we discuss considerations and trade-offs associated with Baysor-based resegmentation and why this approach is not ideal. Finally, we provide quantitative quality control benchmarks demonstrating that our data are comparable in quality to publicly available Xenium Prime reference datasets.

Interpretation of transcript localization in subcellular spatial transcriptomics data: A key aspect underlying the reviewer's concern is the understandable expectation that transcripts corresponding to a given cell should visually fall within the two-dimensional polygon defining that cell's segmentation mask. While this intuition is very reasonable, it does not strictly apply to Xenium or other subcellular-resolution spatial transcriptomics platforms, and deviations from this expectation do not, on their own, indicate a segmentation issue.

In Xenium, individual RNA molecules are detected as diffraction-limited fluorescent spots and assigned precise spatial coordinates that reflect their physical location within the tissue section. These coordinates represent the position of the RNA molecule itself, rather than an inferred cell identity. Cell segmentation, in contrast, produces two-dimensional polygons that approximate whole-cell boundaries as projected onto a single tissue plane. Importantly, transcript coordinates are not constrained to lie strictly within these polygons, nor are they reassigned to cell interiors after segmentation. This design choice reflects a deliberately conservative approach aimed at minimizing false-positive transcript-to-cell assignments. Several biological and technical factors therefore contribute to the presence of transcripts that appear outside cell masks, even when segmentation is accurate: **Three-dimensional cellular structure projected onto a two-dimensional plane:**

Cells are three-dimensional structures, whereas Xenium data are generated from thin tissue sections. As a result, transcripts originating from the cytoplasm or membrane of a cell whose nucleus is captured in the plane of section may project outside the corresponding two-dimensional polygon inferred from nuclear and interior staining. This effect is particularly relevant for elongated or polarized cell types, such as endothelial cells and podocytes. **Subcellular and membrane-associated localization of transcripts:** Many of the genes highlighted by the reviewer (for example, DPP4, PLAUI, PLAUR, and TNFSF10) encode membrane-associated, secreted, or extracellularly acting proteins. Transcripts encoding such proteins are often enriched near the cell periphery, at apical or basolateral membranes, or within cellular processes that extend beyond the nuclear-defined core of the cell. In thin sections, these transcripts can therefore legitimately fall outside conservative cell boundaries defined by segmentation. **Conservative segmentation to avoid over-assignment:** The Xenium Prime segmentation pipeline prioritizes specificity over

sensitivity in transcript assignment. As a consequence, cell masks may conservatively under-represent the full cytoplasmic extent of a cell, particularly in densely packed tissues such as the glomerulus. Transcripts near cell borders or within fine cellular extensions may therefore appear outside the annotated mask. **Unassigned or extracellular transcripts:** A subset of detected transcripts is expected to remain unassigned to any cell due to partial RNA degradation, sectioning effects, or true extracellular RNA. The presence of such transcripts is a recognized feature of high-resolution spatial transcriptomic data and does not imply segmentation failure.

For these reasons, visual inspection alone—particularly inspection based on whether individual transcripts fall strictly within cell polygons—is not a reliable indicator of segmentation quality. These considerations apply broadly across subcellular spatial transcriptomics platforms, including Xenium and CosMx.

Differences between early Xenium workflows and the Xenium Prime 5k pipeline used here:

The reviewer references a recent study by Xuanyuan et al.¹, in which the authors resegmented Xenium data using Baysor. We agree that Baysor was a reasonable and well-justified choice in that specific context. However, the need for Baysor in that study arose from limitations of earlier Xenium workflows that do not apply to the Xenium Prime 5k chemistry and analysis pipeline used in the present work.

In the cited study, the authors used an earlier Xenium implementation based on a relatively small custom gene panel (300 genes) together with a nuclear stain. At that time, the 10x Genomics pipeline generated nuclear masks and inferred whole-cell boundaries by expanding each nucleus into the surrounding space. These inferred boundaries were flattened into two-dimensional masks and used for transcript assignment and downstream analyses. Because this nucleus-expansion-based approach provided only an approximate estimate of true cell boundaries and did not incorporate membrane or cytoplasmic information, post hoc resegmentation with Baysor was a reasonable strategy to improve cell delineation.

By contrast, the Xenium Prime 5k workflow used in this study employs a fundamentally different segmentation strategy. Specifically, it integrates multimodal information from nuclear staining, interior RNA signal, and membrane-associated markers to infer whole-cell boundaries directly. This approach is conceptually similar to segmentation strategies used by CosMx and represents a substantial advance over earlier nucleus-expansion-based methods.

Multimodal hierarchical segmentation in Xenium Prime 5k: In the Xenium Prime 5k pipeline, segmentation proceeds hierarchically and is prioritized on a per-cell basis, using the most informative available signals: **Boundary stain-based segmentation:** When membrane staining is available, cell boundaries are inferred directly from cell-surface markers, such as ATP1A1 and E-cadherin for epithelial cells and CD45 for immune cells. This provides the closest approximation to true biological cell membranes and enables accurate identification of multinucleated or anucleate cells, with overlapping nuclei assigned accordingly. **Interior RNA-based segmentation:** For cells lacking clear boundary staining, segmentation is guided by interior RNA signal, particularly the 18S rRNA stain, in combination with DAPI. This step uses a combination of deep learning-based inference and controlled nuclear expansion to approximate the cytoplasmic extent of the cell. In densely packed tissues, this can yield irregular but biologically plausible outlines. **Fallback nuclear expansion:** Only for cells lacking both boundary and interior signals does the pipeline revert to limited nuclear expansion, extending each nucleus up to a maximum of 5 μm or until it encounters a neighboring cell boundary.

This hierarchical approach ensures that segmentation is driven by the most biologically informative signal available for each cell, rather than by a single global rule. Importantly, the pipeline does not aim to maximize transcript capture within each cell at the expense of boundary accuracy.

In our dataset, the majority of cells were segmented using the interior RNA-based workflow, reflecting tissue-specific staining characteristics of the kidney. This pattern falls well within the range observed in publicly available Xenium Prime demonstration datasets and does not suggest a technical limitation.

Considerations regarding Baysor-based resegmentation: We fully recognize the value of Baysor in specific settings, particularly for datasets generated using earlier segmentation strategies or with limited staining information. At the same time, both the original Baysor developers and subsequent benchmarking studies^{2,3} have highlighted important trade-offs associated with its use. Baysor infers cell boundaries by jointly modeling transcriptional composition and spatial proximity and can perform segmentation using transcript distributions alone. While this approach can reduce apparent spurious co-expression by enforcing parsimonious expression profiles, it often results in a twofold increase in the number of segmented cells^{2,3} even after filtering out low-coverage cells.² There is limited evidence that these additional cells consistently correspond to bona fide biological cells, and the increase in cell number appears to reflect algorithmic optimization rather than biological structure.² In the context of Xenium Prime data, which already incorporate membrane and interior RNA information, applying Baysor carries a risk of introducing over-segmentation. For analyses focused on spatial relationships and CCIs, such effects could complicate interpretation by fragmenting biologically coherent cells and inflating apparent interaction counts. For these reasons, and in accordance with current best-practice recommendations from 10x Genomics, we did not apply Baysor resegmentation to our data.

Quantitative quality control metrics and benchmarking: To further address the reviewer's concern regarding data quality, we performed extensive quality control analyses and benchmarked our dataset against publicly available Xenium Prime 5k demonstration datasets provided by 10x Genomics.

Across the 16 samples included in this study, approximately 83 percent of detected transcripts were classified as high quality based on a Phred score of at least 20, indicating a high level of confidence in correct transcript decoding (Figure 1). To contextualize this value, we examined eight publicly

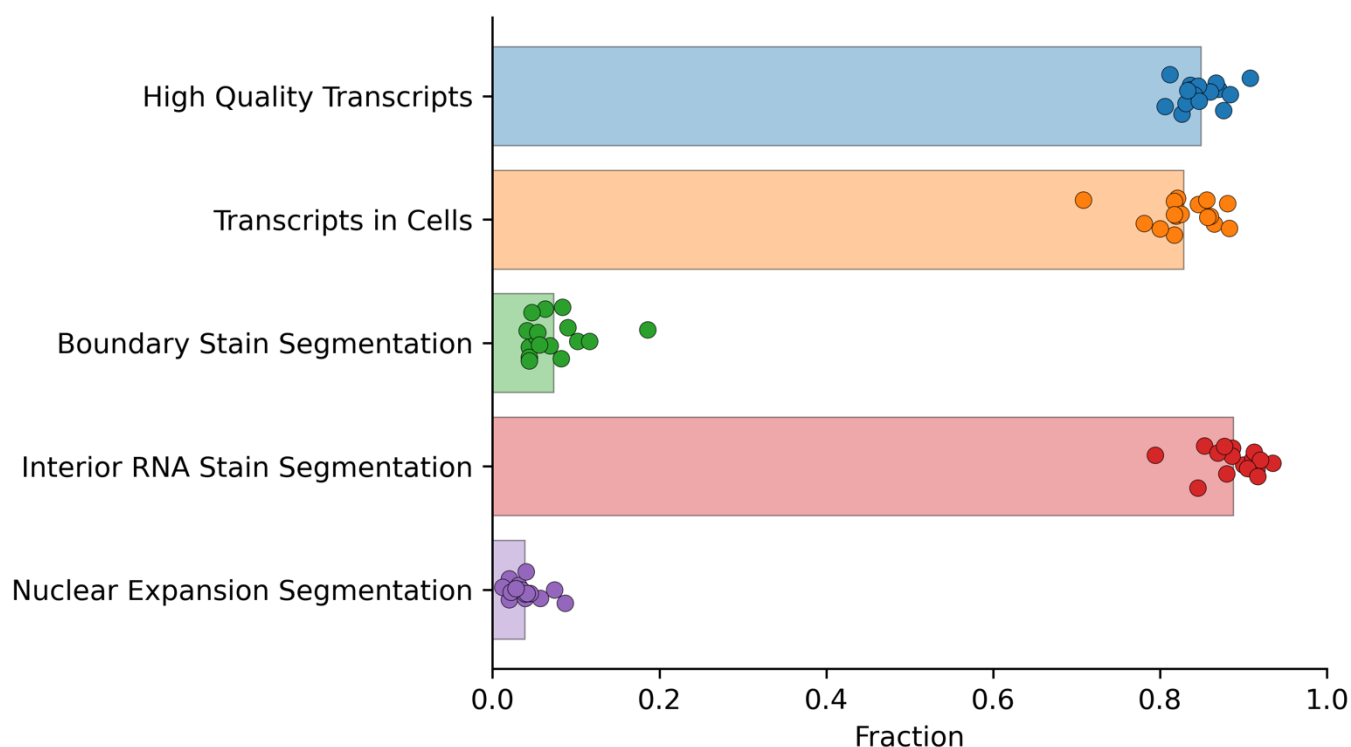


Figure 1: Quality Control of 10X Xenium datasets presented in this study.

available Xenium Prime 5k datasets spanning a range of tissue types, including ovarian cancer, lung adenocarcinoma, breast cancer, cervical cancer, melanoma, prostate carcinoma, and reactive lymph node. The proportion of high-quality transcripts in our dataset closely matched the mean values observed in these reference datasets (Figure 2).

Similarly, the fraction of transcripts assigned to segmented cells in our data was comparable to that observed in the demonstration datasets. While there was variability in the relative contribution of

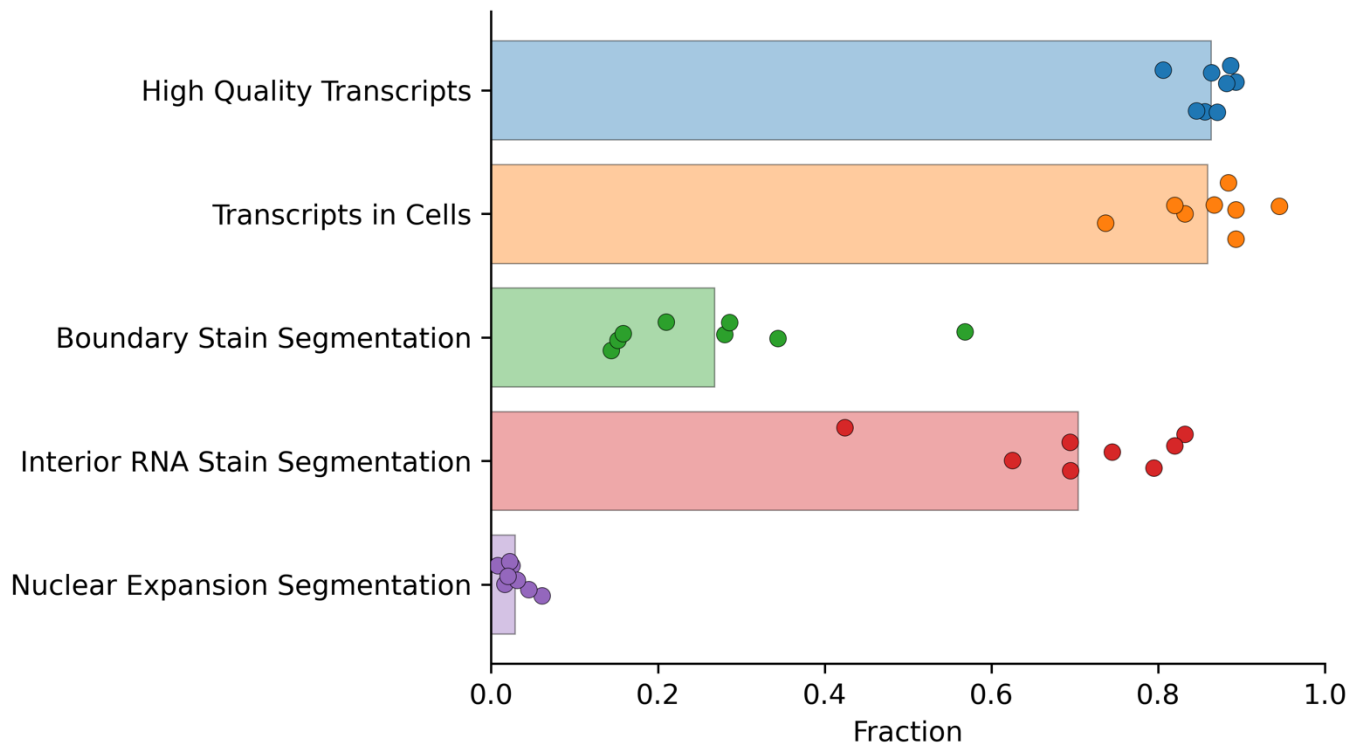


Figure 2: Quality Control of 10X Xenium datasets that are available on the 10X genomics website (<https://www.10xgenomics.com/datasets>). We subsetting to human datasets profiled on the Xenium platform with Prime chemistry yielding 8 datasets: 2 ovarian cancer, a lung adenocarcinoma, a breast cancer, cervical cancer, skin melanoma, prostate carcinoma, reactive lymph node dataset.

boundary stain–based versus interior RNA–based segmentation across datasets, such variability is expected given tissue-specific differences in morphology and staining performance (Figure 1, 2). Taken together, these comparisons indicate that our data meet established benchmarks for Xenium Prime 5k experiments and do not show evidence of systematic segmentation failure or compromised quality.

In summary, the apparent extension of transcripts beyond cell boundaries in our Xenium plots reflects a well-understood and expected feature of subcellular spatial transcriptomics data and does not indicate a failure of segmentation or quality control. The Xenium Prime 5k multimodal segmentation pipeline used here represents a substantial advance over earlier nucleus-expansion–based workflows and removes the need for Baysor resegmentation in this context. Our data meet established quality benchmarks and align with current best practices adopted in multiple recent high-impact studies:

- Lermi et al. “Comparison of imaging based single-cell resolution spatial transcriptomics profiling platforms using formalin-fixed paraffin-embedded tumor samples” *Nature Communications* 2025
- Steele et al. “A single-cell and spatial genomics atlas of human skin fibroblasts reveals shared disease-related fibroblast subtypes across tissues” *Nature Immunology* 2025
- Aminu et al. “CoCo-ST detects global and local biological structures in spatial transcriptomics datasets” *Nature Cell Biology* 2025
- Liu et al. “Conserved spatial subtypes and cellular neighborhoods of cancer-associated fibroblasts revealed by single-cell spatial multi-omics” *Cancer Cell* 2025
- Blampey et al. “Novae: a graph-based foundation model for spatial transcriptomics data” *Nature methods* 2025
- Ren et al. “Systematic benchmarking of high-throughput subcellular spatial transcriptomics platforms across human tumors” *Nature communications* 2025

We hope that this expanded explanation addresses the reviewer’s concern and clarifies the rationale underlying our analytical choices.

In particular, in light of the colocalisation, it would be good to have more information on how various gene-gene co-localisation associations are quantified.

Response: We thank the reviewer for this question. To quantify gene–gene colocalization, we used a graph-based local correlation approach closely following Hotspot and applied it to niche-specific latent representations derived from the COVET analysis. First, we constructed a cell–cell similarity graph in which each node represents a cell and edges connect cells that are close to each other in the COVET latent space. We then quantified gene–gene colocalization by asking whether two genes tend to be expressed in the same local neighborhoods of this graph.

For each pair of genes, we computed a local correlation score that measures how often the two genes are expressed together in neighboring cells within the similarity graph. To do this, gene expression values were first standardized so that they are comparable across cells. We then evaluated whether high expression of one gene in a given cell tends to coincide with high expression of the second gene in nearby cells, with closer neighbors contributing more strongly to the score. In this way, the statistic captures whether two genes show coordinated expression within the same local regions of the latent niche space. Importantly, this approach captures local co-expression within shared neighborhoods rather than global correlation across all cells. Finally, the resulting scores were converted into Z-scores by comparison to a gene-wise null model, and these Z-scores are reported in the gene–gene colocalization heatmaps.

We have adjusted the method section accordingly, it now reads:

For analysis of spatially correlated transcripts, we used Hotspot⁴ for each niche, based on the niche-specific latent representations calculated by COVET analysis. Specifically, we first construct a cell–cell similarity graph in which nodes represent cells and edges encode similarity in the COVET latent space. Gene–gene colocalization is then quantified using a local correlation statistic that evaluates whether two genes tend to be expressed in the same regions of this similarity graph. For a pair of genes x and y , the local correlation is defined as:

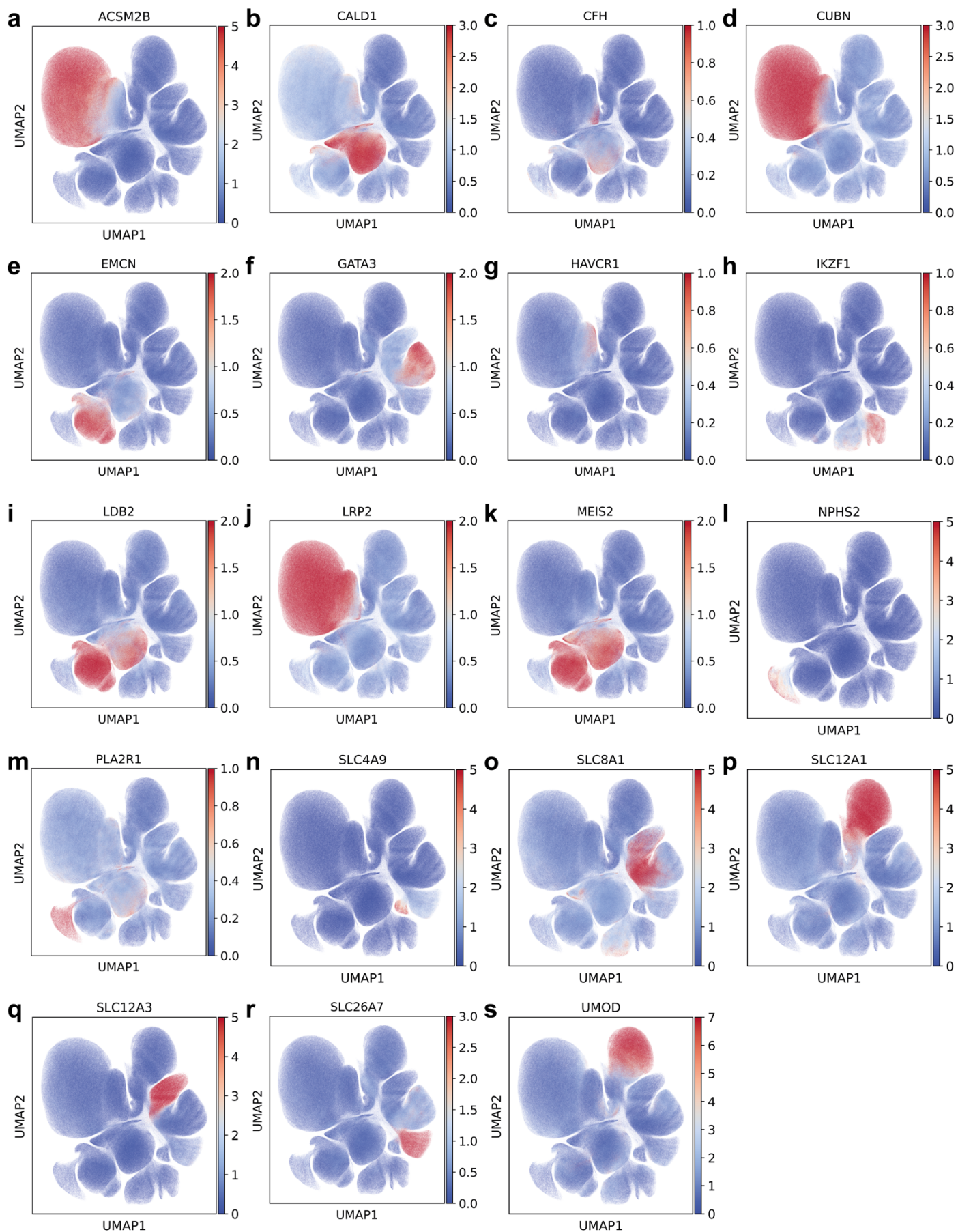
$$H_{xy} = \sum_{(i,j) \in E} w_{ij} (\tilde{x}_i \tilde{y}_j + \tilde{y}_i \tilde{x}_j),$$

where $\tilde{x}_i$ and $\tilde{y}_i$ denote standardized expression values of genes x and y in cell i , respectively, and the sum is taken over all neighboring cell pairs (i, j) in the similarity graph with edge weights w_{ij} .

The difference in gene–gene correlations between DKD and control was calculated by subtracting the control matrix from the DKD matrix. To normalize values across datasets, each correlation matrix (control, DKD, and the difference matrix) was scaled to the range $[-1, 1]$ using min–max scaling. Hierarchical clustering was applied to the scaled control matrix to define a consistent gene ordering, which was subsequently used to reorder all matrices for visualization. Clustered heatmaps were generated using Seaborn’s `clustermap` and `heatmap` functions, with selected gene pairs highlighted to visualize conserved and differential co-regulation.

Finally, can the authors add how the gene imputation is carried out?

Response: We thank the reviewer for this question. As outlined in the Methods, we used the final scANVI latent space of the integrated dataset for gene imputation. For each spatial cell, distances to its 15 nearest snRNA-seq neighbors were computed by constructing a k-nearest neighbor graph based on Euclidean distance in the latent space. The expression of 3,000 highly variable genes from the snRNA-seq dataset was then inferred for each spatial cell by averaging the corresponding gene expression values of its nearest neighbors, with contributions weighted by the squared inverse of their latent-space distances, such that closer neighbors contributed more strongly. We found that the resulting imputed expression patterns aligned well with known cell identities, as canonical marker genes localized to their expected populations (Supplementary Fig. 9, Supplementary Table 5).



Supplementary Fig. 9: Imputed gene expression of the spatial transcriptomics data. UMAP visualization showing the expression of ACSM2B (a), CALD1 (b), CFH (c), CUBN (d), EMCN (e), GATA3 (f), HAVCR1 (g), IKZF1 (h), LDB2 (i), LRP2 (j), MEIS2 (k), NPHS2 (l), PLA2R1 (m), SLC4A9 (n), SLC8A1 (o), SLC12A1 (p), SLC12A3 (q), SLC26A7 (r), UMOD (s) across all cells in the dataset. Color intensity represents imputed gene expression levels.

1. Xuanyuan, Q. *et al.* Multimodal spatial transcriptomic characterization of mouse kidney injury and repair. *Nat Commun* **16**, 7567 (2025).
2. Jones, D. C. *et al.* Cell simulation as cell segmentation. *Nat Methods* **22**, 1331–1342 (2025).
3. Petukhov, V. *et al.* Cell segmentation in imaging-based spatial transcriptomics. *Nat Biotechnol* **40**, 345–354 (2022).
4. DeTomaso, D. & Yosef, N. Hotspot identifies informative gene modules across modalities of single-cell genomics. *ce/s* **12**, 446-456.e9 (2021).

Referee #4 (Remarks to the Author):

Thank you for the opportunity to re-review this manuscript.

I am happy that the author have addressed all my comments and I think the manuscript is significantly better and will be a valuable contribution to the field.

Response: We thank the reviewer for their careful and thoughtful evaluation of the revised manuscript and for noting that it will be a valuable contribution to the field.

I just wanted to raise that there are a few very technical statistical issues raised by reviewer #1 that are beyond my skillset and I cannot add any comments around them so you will need to consult with them.

Apologies again on the delay in my review.

Referee #5 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.

Referee #5 (Remarks on code availability):

N/A

Referee #6 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports.