Peer Review File

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Reviewer A

Very well written manuscript and highlights the need for non-invasive markers for diabetic nephropathy. The graphs, tables, and flowchart were well presented. The limitations of the study are explained well.

This manuscript highlights that this type of study needs to be reproduced in a large sample size and different ethnic group. The commercial availability of such test will hold an extreme value in prognosis of the renal lesion. The test needs to be validated by regulatory organizations before it can be commercially available.

Reply: We deeply appreciate the positive comments of the Reviewer regarding the significance of our study. We completely agree with the Reviewer that the study needs to be reproduced in a large sample size and different ethnic group, and the test should be validated by regulatory organizations for further development. We have added these valuable comments into the dissucsion section of the revised manuscript (see Page11, lines 284-286).

Reviewer B

Authors aimed to complement currently available diagnosis techniques for diabetic nephropathy. For such purpose the investigate changes in MVs concentration derived from different parts of the renal system.

Major concerns:

-Introduction. Please avoid general sentences, e.g. line 37-38. Apparently, many different aspects related to DN diagnosis are mixed-up and it is difficult to understand how and in which sense the proposed study will overcome them (heterogeneity, prevalence, "other kidney disease".

Reply: We thank the Reviewer for the valuable comment. We agree that the aspects related to DN diagnosis are complicated which makes the diangosis rather difficult in some cases. According to published studies, among diabetic patients who had kidney diseases and underwent renal biopsy, 34.5%-72.7% presented non-DN kindey injuries, of which membranous nephropathy (MN) was most frequently seen (24.1% - 32.2%), followed by minimal change disease (MCD, 6.9% -16.7%). This is why we included the proteinuric control of MN and MCD patients in the current study. We have modified our text accordingly in the Introduction of the revised manuscript (see Page 3, line 60-65).

-it is not clear which types of membrane vesicles are being isolated and the isolation procedure is not explain in detail. Authors even refer to exosomes when urine was centrifuged at 20000g. Were proteases inhibitors added prior to storage at -80ºC?

Reply: We thank the Reviewer for raising this important question and apologize for not describing the methodology clearly in the previous manuscript. We used cell-free urine

directly to quantitate microvesicles (MVs) by flowcytometry for the purpose of the study, which detected MVs of 0.2 μm to 1μm. Isolation procedure was only performed when using transmission electron microscopy to visually verify the existence of MVs in cell-free urine. Centrifugal speed of 20000g is commonly used to get MV-enriched pellets, and as pointed by the Reviewer, exosomes could also be coprecipitated. Proteases inhibitors were not added to the urine in our study. We have clarified the methodology accordingly in the revised manuscript. (see Page 5, line 120; Page 6, line 147-148).

-Defined clinical groups are confusing. For instance, the control group includes diabetics and non-diabetics while the patients group are composed by diabetics. The number of samples included in sub-groups are particularly low. Additionally, several pathologies are mixed within one group. It may be then very difficult to draw valid conclusions.

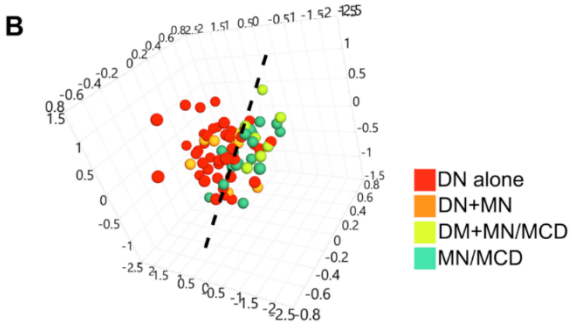
Reply: We aploplize for not clearly interpreting the clinical grouping of our study. According to published studies, about 34.5%-72.7% of renal biopsies in Diabetic patients defined non-DN injuries, among which MN and MCD took the majority. Thus our main purpose was to compare DN patients and diabetic MN/MCD patients. However, one complicated clinical situation is that some DN patients could be superimposed with other kidney injuries, mainly MN. We don't know whether urinary MVs could help differentiate DN and DN superimposed with MN, and therefore we set up these two subgroups (n=42, and 7 respectively). The other proteinuric control, the non-diabetic MN/MCD group (n=19), was included to provide more information about urinary MVs features of MN/MCD, as it has been pointed by the Reviewer, the case number of diabetic MN/MCD is limited in our cohort (n=10). Certainly the study needs to be reproduced in a large sample size multi-center study. We have dicussed about the limitation of the subgroup sample size in the discussion section of the revised manuscript (see Page11, line283-286).

-Drafting should be revised. E.g. line 152: The MV counts normalized to urine creatinine had higher ability to discern proteinuric patients than analyzed as concentration. In fact, by concentration the p value is not significant.

Reply: We thank the Reviewer for the advise. We have modified the result description accordingly (See Page 7, line 182-183).

-PCA is not clear and it is difficult to interpret.

Reply: Thanks for the comment. We used PCA to provide a visual information about the potential difference in urinary EVs between DN patients and proteinuric controls. And the difference was further validated by ROC curve analysis. In order to better interpret the PCA result, we outlined the proposed divisions (see Figure 3B).



-Please clarify the usefulness of Figure 3E.

Reply: Thanks for the question. We used Figure 3E to give an overview of the relationships between urinary MVs of different kidney cell origin and various renal pathological lesions. It was made based on the results of Spearman correlation analysis. We have included the correlation results in Supplementary Table S4 of the revised manuscript.

Supplementary Table S4. Correlations between urinary microvesicles and pathological features in diabetic nephropathy

	Glomerular sclerosis		Ischemic sclerosis		Mesangial expansion		Tubular injury score		Chronic tubulointerstitial score		Interstitial inflammation score		Vascular score	
	r	*P*	r	*P*	r	*P*	r	*P*	r	*P*	r	*P*	r	*P*
Podocyte MVs														
nephrin$^+$	0.151	0.300	0.109	0.462	0.278	0.053	0.098	0.501	-0.059	0.687	-0.109	0.456	0.217	0.135
podocin$^+$	0.036	0.808	0.118	0.430	0.209	0.153	0.203	0.165	-0.141	0.340	-0.202	0.169	0.225	0.124
nephrin$^+$ podocin+	0.122	0.404	0.088	0.554	0.270	0.061	0.067	0.646	-0.075	0.609	-0.132	0.365	0.166	0.254
Proximal tubular MVs														
AQP1$^+$	-0.058	0.694	0.161	0.276	0.277	0.054	0.458	**<0.001**	-0.070	0.635	-0.140	0.337	0.251	0.082
megalin$^+$	0.088	0.549	0.065	0.659	0.404	**0.004**	0.352	**0.013**	-0.031	0.834	-0.096	0.512	0.442	**<0.001**
AQP1+ megalin$^+$	-0.134	0.360	-0.020	0.893	0.354	**0.013**	0.349	**0.014**	-0.008	0.959	0.033	0.821	0.325	**0.023**
Endothelial MVs														
CD31$^+$	0.065	0.669	-0.063	0.679	0.306	**0.039**	0.503	**<0.001**	0.100	0.507	0.022	0.884	0.349	**0.017**
CD144$^+$	0.072	0.635	0.020	0.899	0.385	**0.008**	0.446	**0.002**	0.110	0.466	-0.005	0.975	0.473	**<0.001**
CD31$^+$ CD144$^+$	0.121	0.421	0.046	0.763	0.388	**0.008**	0.481	**<0.001**	0.138	0.361	0.039	0.797	0.391	**0.007**

DN, diabetic nephropathy; AQP1, Aquaporin 1; MVs microvesicles. *P* values < 0.05 are shown in bold.

-Results, first paragraph and table 1. p value in the way it is presented may not be clarifying. Please explain existing differences between DN and proteinuric controls in alpha1-MG, NAG and serum creatinine. Indeed, there are differences between them.

Reply: We thank the Reviewer for raising this important question and apologize for not clearly presenting the *P* value in our previous manuscript. The *P* values listed in Table 1 are from ANOVA analysis showing whether there is any difference among the four groups of patients. We also performed t test to investigate the potential difference between any of the two groups of the patients. Those having statistical significance are marked with * and annotated in the footnotes of Table 1. We have interpreted the analysis methodology in the

footnotes of Table 1, and made a clearer presentation in the first paragraph of the result section(see Page 7 line 171-172).

As it is shown in Table 1, there was no significant difference in the levels of urinary alpha1-MG and NAG, among the four groups of patients (by ANOVA after log transformation for normal distribution) or between any of the two groups (by t test with a Bonferroni posttest). As pointed by the Reviewer, the limited case number of subgroups could diminish the statistical power. We therefore combined the two DN subgroups, and the two proteinuric subgroups respectively. By this way, urinary alpha1-MG was found to be significantly higher in the total DN patients compared with the total proteinuric controls (23.8(15.8, 69.5) vs 47.3(19.1, 111.0), $P = 0.049$). Urinary NAG remained no significant difference. Serum creatinine was higher in DN patients compared to proteinuric controls. We have added the above data and linked it to the more advanced tubular injury in DN patients in the result and discusssion sections of the revised manuscript (Page 7, line 177-178; Page 10, line 252).