PEER REVIEW HISTORY

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ARTICLE DETAILS

TITLE (PROVISIONAL)	AN OBSERVATIONAL PROSPECTIVE STUDY PROTOCOL TO DESIGN NEW DIAGNOSTIC SYSTEMS FOR THE EARLY DETECTION OF TOBACCO-ASSOCIATED CHRONIC RENAL DAMAGE IN PATIENTS OF A PRIMARY CARE CENTER OF SALAMANCA (SPAIN)
AUTHORS	Prieto, Marta; Vicente-Vicente, Laura; Casanova, Alfredo G; Hernández-Sánchez, M Teresa; Gomez-Marcos, Manuel; Garcia-Ortiz, Luis; Morales, Ana Isabel

VERSION 1 – REVIEW

REVIEWER	Keiko Hosohata Osaka University of Pharmaceutical Sciences, Japan
REVIEW RETURNED	03-Aug-2019

GENERAL COMMENTS	This study is estimated that novel urinary biomarkers such as KIm-1 and NGAL could detect the subclinical renal damage caused by tobacco. However, information of patient's characteristics and results are missing, so results are not enough to verify this hypothesis.

REVIEWER	Gurjot Kaur School of Pharmaceutical Sciences, Shoolini University, Solan, India
REVIEW RETURNED	09-Oct-2019

GENERAL COMMENTS	I really liked the study protocol except for few minor comments listed below: 1. How do you control for the diet changes that may affect your sample population as it is already reported that some components of our diet can affect renal health? 2. In the analysis of urine samples, in the section of Proteinuria, it is not clear what 24 h stands for in the different time points mentioned before. 3. There are very few minor grammatical mistakes in the introduction.

REVIEWER	Danijela Krstic Institute of Medical Chemistry, Faculty of Medicine, University of Belgrade, Serbia
REVIEW RETURNED	13-Oct-2019

GENERAL COMMENTS	The study protocol: AN OBSERVATIONAL PROSPECTIVE STUDY PROTOCOL TO

	DESIGN NEW DIAGNOSTIC SYSTEMS FOR THE EARLY DETECTION OF TOBACCO-ASSOCIATED CHRONIC RENAL DAMAGE is clearly written and well conducted. Minor corrections: 1. Section introduction, Page 6, row 44 For the statement: "To study whether certain predisposition markers previously identified in our group (Ganglioside GM2 activator protein (GM2AP), transferrin, and t-Gelsolin).." corresponding reference(s) should be provided. 2. d should be replaced by D in the name of N-acetyl-beta-d-glucosaminidase 3. Section, Subjects of study, page 8, row 23: It is written: "A follow-up of 200 smoking patients recruited in the previous objective will be carried out, specifically 100 each from groups 1 and 3." Patients from group 1 are non-smoker? Section General variables, page 9, row 27, write biochemical parameters instead of "analytical biochemistry"

REVIEWER	Hung-Yi Chuang, MD, ScD Kaohsiung Medical University
REVIEW RETURNED	06-Nov-2019

GENERAL COMMENTS	This research protocol will investigate the kidney function damaged by tobacco uses. 1. Detecting subclinical renal damage in smokers using a panel of early biomarkers (albuminuria, NAG, KIM-1, and NGAL): they we hypothesize that tobacco consumption can predispose smokers to renal damage on exposure to other potentially nephrotoxic events, (pharmacological treatments, diagnostic procedures etc.). 2. Investigating whether certain predisposition markers (GM2AP, Transferrin, and t-Gelsolin) are able to detect smokers who are predisposed to kidney damage. 3. Studying whether smoking cessation reduces subclinical and/or predisposition to renal damage. Study design Four groups of patients: non-smoker without predisposed factors, non-smoker with predisposed factors, smoker without predisposed factors, smoker with predisposed factors. A total of 500 patients will be recruited (125 per group), which was calculated by statistical theory. For subjects 2 and 3, a longitudinal design will be proposed. The study began on March 4, 2019. The collection of samples will end in April 2022. The study will be completed in July 2022. The follow-up period will be 24 months. Questions: 1. Authors should argue the reasoning why 24 months following is enough? 2. For kidney functions, why do they pick these biomarkers? Are these markers better than glomerular filtration rate? 3. What do the biomarkers, such as ganglioside GM2 activator protein (GM2AP), transferrin, and t-Gelsolin, react or inter-react with microalbumin and the enzyme N-acetyl-beta-d-glucosaminidase (NAG), protein kidney damage molecule 1 (KIM-1), lipocalin associated with neutrophil gelatinase (NGAL)?

	4. How can the protocol provide more information than those studies before, for example, 'The Multiple Risk Factor Intervention Trial' (MRFIT), 'Prevention of Renal and Vascular Endstage Disease' (PREVEND)? 5. It is better if authors could offer their budge estimating.

REVIEWER	Zhen Wang Mayo Clinic, USA
REVIEW RETURNED	06-Nov-2019

GENERAL COMMENTS	This manuscript is a study protocol of assessing early markers for subclinical renal damage in smokers. Three subsequent studies were proposed to address three objectives. I have the following comments and suggestions. 1. Please add diagnostic and prognostic information of the proposed markers in chronic renal diseases with or without tobacco use. 2. Please describe the rationale/theatrical reasoning that these early markers would work in renal damage caused by tobacco. In other words, why the makers evaluated in chronic renal diseases in other study would work differently in patients with tobacco use. 3. Table 1, please clarify the meaning of "-". Also for "risk factors", X means patients have all of these risk factors, and "-" mean no risk factors at all? 5. Please add citation or background information about the numbers generated for sample size calculation in the sentence, "detect a minimum difference of 18 mg/g of the albumin/creatinine index between two of the four groups, considering a common standard deviation of 40 mg/g." 6. Subjects, please add more information on how patients will be sampled and recruited. Please add details on how to ensure no difference between groups on gender and age. 7. The STROBE statement is a reporting guideline, not a method guideline. Please delete the relevant sentences in the limitations.

VERSION 1 – AUTHOR RESPONSE

REVIEWERS RESPONSE
Reviewer: 1
Reviewer Name: Keiko Hosohata
Institution and Country: Osaka University of Pharmaceutical Sciences, Japan.
Please state any competing interests or state 'None declared': None.
Please leave your comments for the authors below

This study is estimated that novel urinary biomarkers such as KIm-1 and NGAL could detect the subclinical renal damage caused by tobacco. However, information of patient's characteristics and results are missing, so results are not enough to verify this hypothesis.

This manuscript includes the work protocol of the "BIOTAB" project: AN OBSERVATIONAL PROSPECTIVE STUDY PROTOCOL TO DESIGN NEW DIAGNOSTIC SYSTEMS FOR THE EARLY DETECTION OF TOBACCO-ASSOCIATED CHRONIC RENAL DAMAGE IN PATIENTS OF A PRIMARY CARE CENTER OF SALAMANCA (SPAIN). Therefore, the objective of this paper is to design a protocol to be carried out with the appropriate methodology.

In the section “METHODS AND ANALYSIS” are described the characteristics of the patients that will be recruited for the study.
It must be considered that all the results will not be available until the end of the project. After that, the findings will be published.

Reviewer: 2
Reviewer Name: Gurjot Kaur
Institution and Country: School of Pharmaceutical Sciences, Shoolini University, Solan, India Please state any competing interests or state ‘None declared’: none declared
Please leave your comments for the authors below

I really liked the study protocol except for few minor comments listed below:
1. How do you control for the diet changes that may affect your sample population as it is already reported that some components of our diet can affect renal health?

Indeed, the diet affects kidney function to patients who have previous alterations in renal function (Hershey, 2018; Riccio et al., 2015; Rysz et al., 2017). In this work, patients with impaired renal function at the recruitment time will not be included. Therefore, control of dietary changes is not considered in this protocol.

References:
Hershey, K., 2018. Renal Diet. Nurs. Clin. North Am. 53, 481–489. https://doi.org/10.1016/j.cnur.2018.05.005

Riccio, E., Di Nuzzi, A., Pisani, A., 2015. Nutritional treatment in chronic kidney disease: the concept of nephroprotection. Clin. Exp. Nephrol. 19, 161–167. https://doi.org/10.1007/s10157-014-1041-7

Rysz, J., Franczyk, B., Ciałkowska-Rysz, A., Gluba-Brzózka, A., 2017. The Effect of Diet on the Survival of Patients with Chronic Kidney Disease. Nutrients 9. https://doi.org/10.3390/nu9050495

2. In the analysis of urine samples, in the section of Proteinuria, it is not clear what 24 h stands for in the different time points mentioned before.

For greater clarity, and according to the recommendations of the reviewer, we have modified the writing of the manuscript, being the current version as follows:

“Urinary protein excretion will be evaluated by the Bradford colorimetric method.[24] It is estimated that proteinuria values in healthy patients should be 150 mg in 24 hours, or 0 - 8 mg/dL.[25]”

3. There are very few minor grammatical mistakes in the introduction.

The manuscript has been reviewed both by the authors and the translation service provided by the journal.

Reviewer: 3
Reviewer Name: Danijela Krstic
Institution and Country: Institute of Medical Chemistry, Faculty of Medicine, University of Belgrade, Serbia Please state any competing interests or state ‘None declared’: None declared
Please leave your comments for the authors below

The study protocol: AN OBSERVATIONAL PROSPECTIVE STUDY PROTOCOL TO DESIGN NEW DIAGNOSTIC SYSTEMS FOR THE EARLY DETECTION OF TOBACCO-ASSOCIATED CHRONIC RENAL DAMAGE is clearly written and well conducted.

Minor corrections:
1. Section introduction, Page 6, row 44
For the statement: "To study whether certain predisposition markers previously identified in our group (Ganglioside GM2 activator protein (GM2AP), transferrin, and t-Gelsolin).." corresponding reference(s) should be provided.

References have been added in the place indicated by the reviewer. The sentence states as it follows:
“(Objective 2) To study whether certain predisposition markers previously identified in our group (Ganglioside GM2 activator protein (GM2AP), transferrin, and t-Gelsolin), [17-20] can detect those smokers who are predisposed to acute kidney injury”.

2. d should be replaced by D in the name of N-acetyl-beta-d-glucosaminidase

“N-acetyl-beta-d-glucosaminidase” has been replaced by “N-acetyl-beta-D-glucosaminidase” throughout the entire manuscript.

3. Section, Subjects of study, page 8, row 23:
It is written: "A follow-up of 200 smoking patients recruited in the previous objective will be carried out, specifically 100 each from groups 1 and 3." Patients from group 1 are non-smoker?

The mistake has been corrected. The current sentence is as follows:
"A follow-up of 200 smoking patients recruited in the previous objective will be carried out, specifically 100 each from groups 1 and 3".

Section General variables, page 9, row 27, write biochemical parameters instead of "analytical biochemistry"

"Analytical biochemistry" has been replaced by “biochemical parameters”.

Reviewer: 4
Reviewer Name: Hung-Yi Chuang, MD, ScD
Institution and Country: Kaohsiung Medical University
Please state any competing interests or state ‘None declared’: None
Please leave your comments for the authors below

This research protocol will investigate the kidney function damaged by tobacco uses.
1. Detecting subclinical renal damage in smokers using a panel of early biomarkers (albuminuria, NAG, KIM-1, and NGAL): they we hypothesize that tobacco consumption can predispose smokers to renal damage on exposure to other potentially nephrotoxic events, (pharmacological treatments, diagnostic procedures etc.).
2. Investigating whether certain predisposition markers (GM2AP, Transferrin, and t-Gelsolin) are able to detect smokers who are predisposed to kidney damage.
3. Studying whether smoking cessation reduces subclinical and/or predisposition to renal damage.
Study design

Four groups of patients: non-smoker without predisposed factors, non-smoker with predisposed factors, smoker without predisposed factors, smoker with predisposed factors. A total of 500 patients will be recruited (125 per group), which was calculated by statistical theory.
For subjects 2 and 3, a longitudinal design will be proposed. The study began on March 4, 2019. The collection of samples will end in April 2022. The study will be completed in July 2022. The follow-up period will be 24 months.

Questions:
1. Authors should argue the reasoning why 24 months following is enough?
The follow-up of the patients is considered a proof of concept to see the evolution of the markers since each patient has an individual history of smoking in terms of time and consumption. The choice of 24 months can be a reasonable time to obtain a first data of orientation. Depending on the results obtained, the follow-up time could be extended.

2. For kidney functions, why do they pick these biomarkers? Are these markers better than glomerular filtration rate?

The markers that we are going to evaluate appear earlier than the glomerular filtration rate decline, and hence they are better for detecting early or subclinical kidney damage. Precisely our hypothesis is that these markers can detect incipient kidney damage that would never be detected by the glomerular filtration rate or plasma creatinine. This is reflected in the Introduction of the manuscript, in the following paragraph:

“It is recognized that the key to preventive success is very early diagnosis, allowing intervention while the renal functional reserve has not yet been exhausted and, therefore, the excretory function is not yet compromised.[11] In the clinic, one of the most common diagnostic tools for kidney damage is the detection of metabolic products (for example creatinine and urea), which accumulate in the blood once the renal excretory capacity begins to decrease. However, at the stage when serum urea and creatinine levels are detectably increased, more than 70% of renal function has already been lost. Thus, there is a current trend in diagnostics to identify sensitive, specific, and easily quantifiable markers that detect incipient pathophysiological events in the early stages, when the damage is less widespread.[12] These potential markers may, for example, be involved in the synthesis, activation, or inhibition of biochemical process mediators and cellular structural constituents related to processes such as apoptosis and tissue regeneration. Likewise, signs of destruction of tissues (extracellular matrix, and basal membranes) could be found in the urine, whether they are whole or degraded molecules, or remnants of organelles, cells, or tissues. The urinary detection of certain cellular enzymes associated with renal cell injury is currently the procedure for the early detection of the tubular subtype of kidney damage. In this sense, urinary detection of the enzyme N-acetyl-beta-d-glucosaminidase (NAG) represents one of the finest techniques for the detection of tubular damage, although it is not established as a standard diagnostic technique.[13] Other markers, such as protein kidney damage molecule 1 (KIM-1), or lipocalin associated with neutrophil gelatinase (NGAL), are in an advanced stage of clinical development as early urinary markers of kidney damage.[14]”

3. What do the biomarkers, such as ganglioside GM2 activator protein (GM2AP), transferrin, and t-Gelsolin, react or inter-react with microalbumin and the enzyme N-acetyl-beta-d-glucosaminidase (NAG), protein kidney damage molecule 1 (KIM-1), lipocalin associated with neutrophil gelatinase (NGAL)?

Each of these markers are independent of each other, providing different information about the damaged renal region and the altered mechanism. (Alfredo G. Casanova et al.,2020; Ferreira et al.,

2011; Quiros et al., 2010; Vicente-Vicente et al., 2015, 2013). As far as it is known, there is no relationship or interaction between them.

References:

Alfredo G. Casanova, Laura Vicente-Vicente, M. Teresa Hernández-Sánchez, Marta Prieto, M. Isabel Rihuete, Laura M. Ramis, Elvira del Barco, Juan J. Cruzb, Alberto Ortiz, Ignacio Cruz-González, Carlos Martínez-Salgado, Moisés Pescador,, Francisco J. López-Hernández, Ana I. Morales, n.d. Urinary transferrin pre-emptively identifies the risk of renal damage posed by subclinical tubular alterations. Biomedicine & Pharmacotherapy 121 (2020) 109684. https://doi.org/10.1016/j.biopha.2019.109684

Ferreira, L., Quiros, Y., Sancho-Martínez, S.M., García-Sánchez, O., Raposo, C., López-Novoa, J.M., González-Buitrago, J.M., López-Hernández, F.J., 2011. Urinary levels of regenerating islet-derived protein III β and gelsolin differentiate gentamicin from cisplatin-induced acute kidney injury in rats. Kidney Int. 79, 518–528. https://doi.org/10.1038/ki.2010.439

Quiros, Y., Ferreira, L., Sancho-Martínez, S.M., González-Buitrago, J.M., López-Novoa, J.M., López-Hernández, F.J., 2010. Sub-nephrotoxic doses of gentamicin predispose animals to developing acute kidney injury and to excrete ganglioside M2 activator protein. Kidney Int. 78, 1006–1015. https://doi.org/10.1038/ki.2010.267

Vicente-Vicente, L., Ferreira, L., González-Buitrago, J.M., López-Hernández, F.J., López-Novoa, J.M., Morales, A.I., 2013. Increased urinary excretion of albumin, hemopexin, transferrin and VDBP correlates with chronic sensitization to gentamicin nephrotoxicity in rats. Toxicology 304, 83–91. https://doi.org/10.1016/j.tox.2012.12.006

Vicente-Vicente, L., Sánchez-Juanes, F., García-Sánchez, O., Blanco-Gozalo, V., Pescador, M., Sevilla, M.A., González-Buitrago, J.M., López-Hernández, F.J., López-Novoa, J.M., Morales, A.I., 2015. Sub-nephrotoxic cisplatin sensitizes rats to acute renal failure and increases urinary excretion of fumarylacetoacetase. Toxicol. Lett. 234, 99–109. https://doi.org/10.1016/j.toxlet.2014.11.033

4. How can the protocol provide more information than those studies before, for example, 'The Multiple Risk Factor Intervention Trial' (MRFIT), 'Prevention of Renal and Vascular Endstage Disease' (PREVEND)?

The MRFIT trial (Multiple Risk Factor Intervention Trial) is a randomized, multicenter study designed to study the effects of controlling blood pressure, serum cholesterol and smoking levels on the incidence of ischemic coronary disease (Whelton et al., 1996). Our study aims to see the incidence on renal function.
On the other hand, the Prevention of Renal and Vascular End Stage Disease (PREVEND) study attempts to establish the natural course of albuminuria and its relationship with renal and cardiovascular pathology, in a large representative cohort of the general population (Pinto-Sietsma et al., 2000). Our study aims to find markers of early kidney damage and predisposition in smoking patients.

References:
Pinto-Sietsma, S.J., Mulder, J., Janssen, W.M., Hillege, H.L., de Zeeuw, D., de Jong, P.E., 2000. Smoking is related to albuminuria and abnormal renal function in nondiabetic persons. Ann. Intern. Med. 133, 585–591. https://doi.org/10.7326/0003-4819-133-8-200010170-00008

Whelton, P.K., Perneger, T.V., He, J., Klag, M.J., 1996. The role of blood pressure as a risk factor for renal disease: a review of the epidemiologic evidence. J Hum Hypertens 10, 683–689.

5. It is better if authors could offer their budge estimating.

This study was funded by the Spanish Ministry of Science Innovation and Universities (MICINN) and the Carlos III Health Institute/European Regional Development Fund (ERDF) (grant number: PI17/01979) with 105,270 €.

Reviewer: 5
Reviewer Name: Zhen Wang
Institution and Country: Mayo Clinic, USA
Please state any competing interests or state ‘None declared’: None declared.
Please leave your comments for the authors below

This manuscript is a study protocol of assessing early markers for subclinical renal damage in smokers. Three subsequent studies were proposed to address three objectives. I have the following comments and suggestions.

1. Please add diagnostic and prognostic information of the proposed markers in chronic renal diseases with or without tobacco use.
Following their recommendations, we have added information regarding the diagnosis and prognosis of biomarkers in chronic kidney disease. The paragraph inserted, in the introduction section, is as follows:

“These markers of early renal damage (NAG; KIM-1 and NGAL), have also been evaluated as possible predictors in the development of chronic kidney disease. The results obtained with NAG and KIM-1 have been very promising, [15] whereas NGAL would also have this capacity according to some studies. [16]”

On the other hand, as far as we know, there are no published data regarding the use of these markers to the diagnosis and prognosis of chronic kidney disesase caused by tobacco. Therefore, our study will be a pioneer in that regard.

2. Please describe the rationale/theatrical reasoning that these early markers would work in renal damage caused by tobacco. In other words, why the makers evaluated in chronic renal diseases in other study would work differently in patients with tobacco use.

Our hypothesis is that tobacco, due to the mechanisms involved in its toxic effect, such as oxidative stress, inflammation, increased blood pressure, intrarenal vasoconstriction or hypoxia (Orth and Hallan, 2008) could produce subclinical renal damage. The markers that detect subclinical injury may be related, for example, to the synthesis, activation or inhibition of mediators of biochemical processes, or to the generation of molecules from cellular constituents as a result of processes such as apoptosis or tissue regeneration (Bonventre et al., 2010; Zhou et al., 2008).

References:
Orth, S.R., Hallan, S.I., 2008. Smoking: a risk factor for progression of chronic kidney disease and for cardiovascular morbidity and mortality in renal patients--absence of evidence or evidence of absence? Clin J Am Soc Nephrol 3, 226–236. https://doi.org/10.2215/CJN.03740907

Bonventre, J.V., Vaidya, V.S., Schmouder, R., Feig, P., Dieterle, F., 2010. Next-generation biomarkers for detecting kidney toxicity. Nat. Biotechnol. 28, 436–440. https://doi.org/10.1038/nbt0510-436

Zhou, Y., Vaidya, V.S., Brown, R.P., Zhang, J., Rosenzweig, B.A., Thompson, K.L., Miller, T.J., Bonventre, J.V., Goering, P.L., 2008. Comparison of kidney injury molecule-1 and other nephrotoxicity biomarkers in urine and kidney following acute exposure to gentamicin, mercury, and chromium. Toxicol. Sci. 101, 159–170. https://doi.org/10.1093/toxsci/kfm260

3. Table 1, please clarify the meaning of "-". Also for "risk factors", X means patients have all of these risk factors, and "-" mean no risk factors at all?

To make the table clearer, the symbol "-" has been removed. In addition, the meaning of "X" has been added at the bottom of the table.

4. Please add citation or background information about the numbers generated for sample size calculation in the sentence, "detect a minimum difference of 18 mg/g of the albumin/creatinine index between two of the four groups, considering a common standard deviation of 40 mg/g."

In a previous study from our group (Gomez-Marcos et al., 2016), the mean and the standard deviation (SD) of the albumin/creatinine index was 14.8 ± 41.4 mg/g (data not published). We have used this data to estimate the sample size, considering a SD of 40 mg/g

We have modified the paragraph about sample size estimation, being the current as follows. (Pag, line):

"The sample size has been estimated on the basis of detection of differences in the parameters analyzed between the four groups defined in objective 1. As information on the variability of microalbuminuria is available from previous studies of the population, this parameter has been used for the estimation. In a previous study from our group,[22] the mean and the standard deviation (SD) of the albumin/creatinine index was 14.8 ± 41.4 mg/g. therefore, a SD of 40 mg/g has been considered to estimate the sample size. Accepting an alpha risk of 0.05 and a beta risk of 0.2 in a bilateral contrast, 120 subjects in each group are required to detect a minimum difference of 18 mg/g of the albumin/creatinine index between two of the four groups, considering a common standard deviation of 40 mg/g. Each group has been increased by 5 subjects to account for possible losses due to technical issues, meaning that a total of 500 subjects will be recruited (125 per group). Using the same criterion, the number of patients needed for objective 3 has been estimated as 100".

Reference:

Gomez-Marcos, M.A., Martinez-Salgado, C., Gonzalez-Sarmiento, R., Hernandez-Rivas, J.M., Sanchez-Fernandez, P.L., Recio-Rodriguez, J.I., Rodriguez-Sanchez, E., García-Ortiz, L., 2016. Association between different risk factors and vascular accelerated ageing (EVA study): study protocol for a cross-sectional, descriptive observational study. BMJ Open 6, e011031. https://doi.org/10.1136/bmjopen-2016-011031

5. Subjects, please add more information on how patients will be sampled and recruited. Please add details on how to ensure no difference between groups on gender and age.

Information regarding the recruitment of patients has been added to clarify this point. Specifically, the modified paragraph is as follows:

“Patients of legal age from the "La Alamedilla" Health Center of Salamanca (Spain) will be included. A total of 500 patients will be recruited (125 per group) at the general practitioner consult until reaching the estimated sample size. During the recruitment process we will ensure that there are no significant differences in terms of gender and age between the groups. To that purpose, while the recruitment is done, statistical studies will be conducted to ensure that the groups are homogeneous in terms of gender and age. Thus, the subsequent recruitment can be directed to have homogeneous groups”.

7. The STROBE statement is a reporting guideline, not a method guideline. Please delete the relevant sentences in the limitations.

Everything related to the STROBE statement in the “Study Limitations” section has been removed

VERSION 2 – REVIEW

REVIEWER	Hung-Yi Chuang, MD, ScD Kaohsiung Medical University, Taiwan
REVIEW RETURNED	10-Dec-2019

GENERAL COMMENTS	I have no more comments.