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Designing new diagnostic systems for the early detection of tobacco-associated chronic renal damage in patients of a primary care centre in Salamanca, Spain: an observational, prospective study protocol

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ABSTRACT

Introduction Tobacco causes kidney damage that can progress to chronic kidney disease. However, the diagnostic parameters used in clinics are not effective in identifying smokers at risk. Our first objective is to more effectively detect subclinical renal damage in smokers. In addition, we hypothesise that tobacco consumption can predispose smokers to renal damage on exposure to other potentially nephrotoxic events (drugs, diagnostic procedures and so on). We will test this hypothesis in our second objective by investigating whether certain predisposition markers (GM2 ganglioside activator protein (GM2AP), transferrin and t-gelsolin) are able to detect smokers who are predisposed to kidney damage. Finally, in our third objective, we will study whether smoking cessation reduces subclinical and/or predisposition to renal damage.

Methods and analysis For our first objective, a prospective cross-sectional study will be carried out with patients from a primary healthcare centre. The influence of tobacco on renal damage, in patients both with and without additional risk factors, will be studied using a panel of early biomarkers (albuminuria, N-acetyl-beta-D-glucosaminidase, kidney injury molecule-1 and neutrophil gelatinase-associated lipocalin). For our second objective, a prospective longitudinal study will be carried out with patients recruited for our first objective. We will study whether certain predisposition biomarkers (GM2AP, transferrin and t-gelsolin) are able to detect smokers predisposed to renal damage. For our third objective, a prospective longitudinal study will be carried out with patients from a smoking cessation unit. We will study the evolution of the markers described above following smoking cessation.

Ethics and dissemination The study has been approved by the Clinical Research Ethics Committee of the Healthcare Area of Salamanca. All study participants will sign an informed consent form in compliance with the Declaration of Helsinki and the WHO standards for observational studies. Results will be presented at conferences and submitted to peer-reviewed journals.

Trial registration number NCT03850756.

Strengths and limitations of this study

- In this work, the effect of tobacco on kidney will be evaluated, taking into account the influence of several risk factors (hypertension, diabetes and so on), and patients will be grouped according to whether or not they have these risk factors.
- A battery of new markers of kidney damage (early and predisposition) will be analysed to more accurately establish the tobacco–kidney damage relationship or susceptibility of tobacco smokers to kidney damage.
- For the first time it will be assessed if smoking cessation reduces subclinical renal damage and/or the risk of acute renal damage by analysing early and predisposition biomarkers.
- The main limitation of this study is that it is possible that there are other (unknown) markers with the potential to predict tobacco-associated kidney damage which we might have not included in our project.

INTRODUCTION

Tobacco is one of the greatest threats to public health that the world has had to face. According to WHO, smoking is the leading cause of preventable disease and death, and a major risk factor for the development of various respiratory and cardiovascular diseases as well as cancer. It is the only product of legal consumption that harms all those exposed, being the cause of death of almost 6 million people a year, of whom over

5 million are direct consumers and over 600 000 are non-smokers exposed to secondhand smoke.[1]

Despite the fact that the first associations between smoking and kidney disease date back to the beginning of the last century, the evidence has so far been scarce, and inconclusive for those without kidney disease-associated risk factors. One of the first studies was 'The Multiple Risk Factor Intervention Trial', which observed that smoking was associated with terminal kidney disease in a dose-dependent manner.[2] Later, the study by Pinto-Sietsma *et al*,[3] the 'Prevention of Renal and Vascular Endstage Disease', revealed a possible relationship between the number of cigarettes consumed per day and the prevalence of microalbuminuria, a marker of incipient renal damage. It was concluded that those who smoked more than 20 cigarettes per day had higher levels of microalbuminuria than non-smokers without additional risk factors, such as diabetes mellitus or high blood pressure. In addition, studies conducted on patients with hypertension have reported that the prevalence of microalbuminuria is almost doubled in smokers compared with non-smokers, and patients with hypertension who smoke more than 20 cigarettes per day have a prevalence of microalbuminuria 1.6 times higher.[4] In patients with diabetes, it seems that the additional risk factor of smoking is related to an increase in microalbuminuria, which in turn leads to an increased risk of developing proteinuria and accelerating the progression of kidney damage.[5] A large cohort study conducted in 2016[6] found that smoking is associated with a high glomerular filtration rate and a high prevalence of proteinuria, suggesting a mechanism of hyperfiltration that could progress to chronic kidney disease.

The increase in the worldwide prevalence of chronic kidney disease and the notable increase in the incidence of patients reaching the final stages[7] have alerted the health systems to invest more in the identification of potentially modifiable prevention factors.[8] Chronic kidney disease has a very high social and economic cost (almost 10% of the affected population and 3% of the total health expenditure), and requires coordinated criteria among health professionals to ensure the highest quality in prevention, diagnosis and treatment.[9] In addition, the identification of kidney disease as a cardiovascular risk factor and the high morbidity and mortality associated with it highlight the need for preventive strategies for potentially avoidable factors, such as tobacco use.[10]

It is recognised that the key to preventive success is very early diagnosis, providing intervention while the renal functional reserve has not yet been exhausted and therefore the excretory function not yet compromised.[11] In the clinic, one of the most common diagnostic processes for kidney damage is detection of metabolic products (eg, 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. Urinary detection of certain cellular enzymes associated with renal cell injury is currently the procedure used for early detection of the tubular subtype of kidney damage. In this sense, urinary detection of the N-acetyl-beta-D-glucosaminidase (NAG) enzyme represents one of the finest techniques for detection of tubular damage, although it is not established as a standard diagnostic technique.[13] Other markers such as protein kidney injury molecule-1 (KIM-1) or neutrophil gelatinase-associated lipocalin (NGAL) are in an advanced stage of clinical development as early urinary markers of kidney damage.[14] These markers of early renal damage (NAG, KIM-1 and NGAL) have also been evaluated as possible predictors of the development of chronic kidney disease. The results obtained with NAG and KIM-1 have been very promising,[15] whereas NGAL will also have this capacity according to some studies.[16]

Our hypothesis is that some of these early markers could detect subclinical renal damage caused by tobacco.

Based on this hypothesis, we set the following objectives:

- Objective 1: to detect subclinical renal damage in smokers through a panel of early markers (albuminuria, NAG, KIM-1 and NGAL).
- Objective 2: to study whether certain predisposition markers previously identified in our group (GM2 ganglioside activator protein (GM2AP), transferrin and t-gelsolin)[17–20] can detect those smokers who are predisposed to acute kidney injury.
- Objective 3: to study whether smoking cessation reduces subclinical renal damage and/or predisposition to acute kidney injury.

To this end, a consortium of researchers and clinicians has been created, consisting of (1) experts in markers of kidney damage, (2) clinical specialists in lifestyle and cardiovascular risk, and (3) specialists in smoking cessation.

METHODS AND ANALYSIS

Study design

To develop this project, three studies are proposed, each related to an objective (see figure 1).

Objective 1

A prospective cross-sectional study is proposed to evaluate the usefulness of early markers in the diagnosis of renal damage associated with tobacco use. Four groups of patients will be established (table 1), which will allow us to study the influence of tobacco on kidney damage, both in patients with and without risk factors.

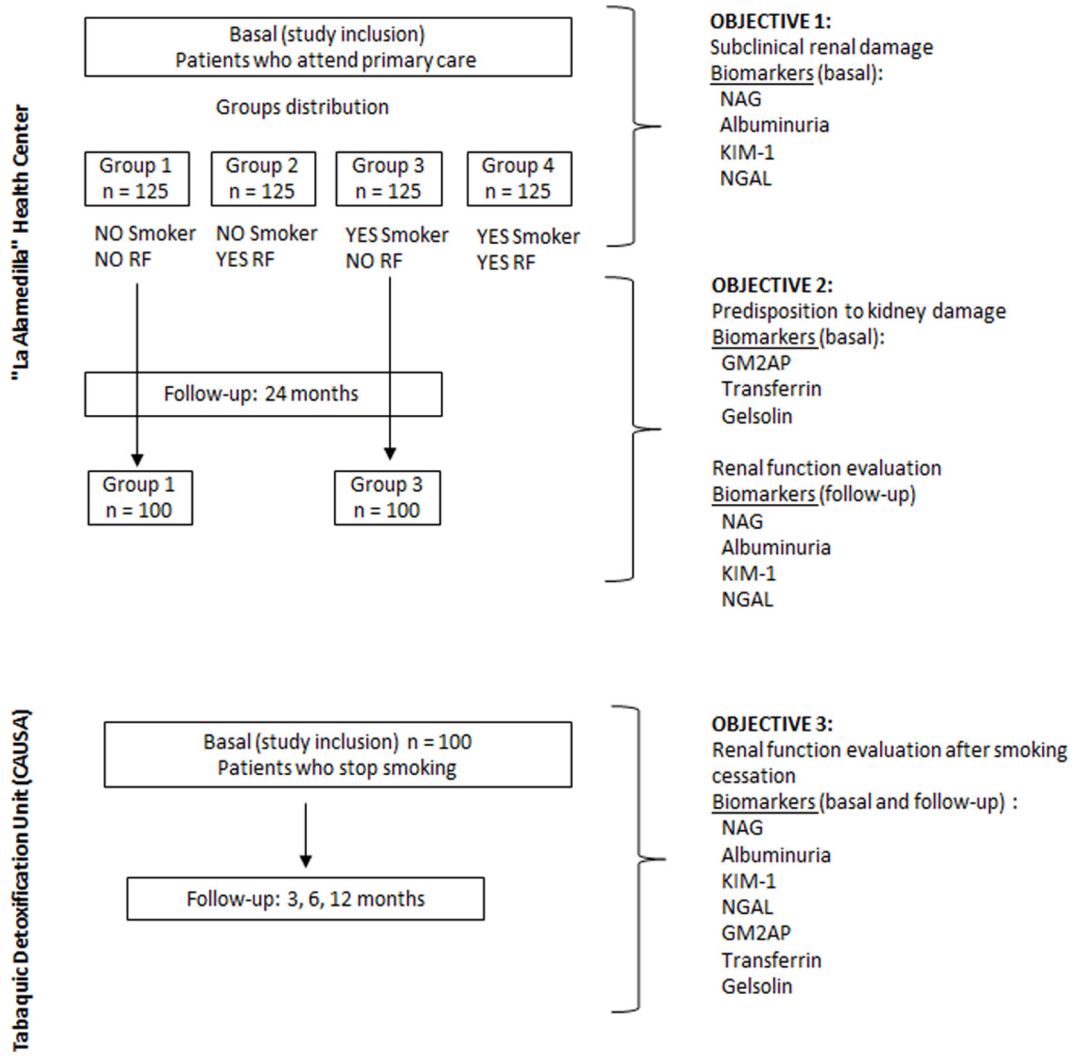

Figure 1 Study design outline. Recruitment centres, patients' distribution by groups, proposed objectives and specific biomarkers for each objective. CAUSA, University Hospital of Salamanca; GM2AP, GM2 ganglioside activator protein; KIM-1, kidney injury molecule-1; NAG, N-acetyl-beta-D-glucosaminidase; NGAL, neutrophil gelatinase-associated lipocalin; RF, risk factors.

Table 1 Groups of patients established in the study design

Group	Smoker*	Risk factors†
1		
2		X
3	X	
4	X	X

*Any person who habitually consumes tobacco at the time of sample collection or ceased smoking during the last year will be considered a smoker (see the 'Collection and processing of urine samples' section).[28]
†The following factors involved in the development of kidney damage will be considered risk factors: diabetes mellitus, hypertension and/or frequent use of non-steroidal anti-inflammatory drugs (more than 3 days a week during the 3 months prior to sampling). When a cell is marked with 'X', it is considered that the group included smokers (according to the previous definition*) or patients with at least one of the risk factors mentioned depending on the column considered.

Objective 2

A longitudinal prospective study is proposed to evaluate the usefulness of markers of predisposition to acute kidney injury in the context of the smoking patient. Patients from groups 1 and 3 (non-smokers and smokers without other risk factors that may influence predisposition, respectively) in objective 1 will be studied (see table 1).

Objective 3

A prospective longitudinal study is proposed to evaluate whether the risk of renal damage decreases with cessation of tobacco consumption. This will involve follow-up of patients from the Tabaquic Detoxification Unit of the University Hospital of Salamanca.

Sample size

The sample size has been estimated on the basis of detection of differences in the parameters analysed between the four groups defined in objective 1. As data on the variability of microalbuminuria are available from

previous studies of the population, this parameter has been used for estimation. In a previous study from our group,[21] the mean±SD of the albumin/creatinine index was 14.8±41.4 mg/g. Therefore, an 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 SD of 40 mg/g. Five subjects have been added to each group 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.

Subjects of the study

Objective 1

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 consultation until the estimated sample size is reached. During the recruitment process, we will ensure that there are no significant differences in terms of gender and age between the groups. For this purpose, during recruitment, statistical studies will be conducted to ensure that the groups are homogeneous in terms of gender and age. Thus, the subsequent recruitment can be conducted with homogeneous groups.

Objective 2

A follow-up of 200 patients recruited in the previous objective will be carried out, specifically 100 each from groups 1 and 3. This sample size has been estimated allowing for the possibility that some patients from objective 1 are unwilling or unable to participate in the study for objective 2.

Objective 3

We will recruit 100 patients who start treatment in the Tabaquic Detoxification Unit of the University Hospital of Salamanca.

Inclusion criteria

The inclusion criteria will be patients of legal age who agree to participate in the study, sign the informed consent, meet the inclusion criteria previously specified for each group and do not meet any of the exclusion criteria.

Exclusion criteria

The exclusion criteria will be patients who are terminally ill, have previously been diagnosed with renal failure, do not wish to sign the informed consent, or have been treated with any of the following drugs during the week prior to, or at the time of, sample collection: aminoglycosides, cephalosporins, tetracyclines, amphotericin B, cisplatin, ciclosporin, foscarnet, acyclovir, cidofovir, radiological contrasts or any other potentially nephrotoxic drugs.

Specific variables

Objective 1

We will analyse the presence of biomarkers of early kidney damage (albuminuria, NAG, KIM-1 and NGAL) at the time of patient recruitment. We will study the association of these markers with tobacco consumption with respect to the presence or absence of other risk factors for kidney damage.

Objective 2

We will analyse markers of predisposition to acute kidney injury (previously described in our laboratory: GM2AP, transferrin and t-gelsolin) at the time of patient recruitment. At 24 months there will be a follow-up of the groups of patients without risk factors (groups 1 and 3), which will allow us to relate the levels of biomarkers of predisposition at the beginning of the study with renal function in the medium term. Renal function at the 24-month follow-up will be evaluated by biomarkers of early damage in addition to the usual clinical parameters (plasma creatinine, creatinine clearance, plasma urea and proteinuria).

Objective 3

We will analyse the evolution of both early and predisposing damage markers 3, 6 and 12 months after the inclusion of patients in the study. These data will be used to analyse whether smoking cessation reduces subclinical renal damage and/or the risk of acute renal damage.

General variables

At the time of urine sample collection (see next section), the following data will be collected: (1) date of collection of the sample; (2) general data (age, sex, drug use), anthropometric measurements (body weight, height, body mass index), and biochemical parameters (plasma creatinine and urea), allowing assessment of each patient's renal function—in the event that there is no clinical analysis carried out during the 6 months prior to sample collection, it will be carried out specifically for this purpose at the Biochemistry Department of the University Hospital of Salamanca; (3) risk factors for kidney damage (hypertension, diabetes, cardiovascular disease or chronic pain with frequent use of non-steroidal anti-inflammatory drugs); (4) other information (in addition to the above, we will include the date of diagnosis, and dosage and start date of any prescribed medication); and (5) tobacco use (the patient will complete a questionnaire adapted from the WHO Multinational Monitoring of Trends and Determinants in Cardiovascular Disease study, which will allow us to classify them as smoker, non-smoker or ex-smoker. We will also collect data on the number of cigarettes/cigars consumed per day, concentration of carbon monoxide in exhaled air (measured with a co-oximeter at the 'La Alamedilla' Health Center as

well as in the Tabaquic Detoxification Unit of the University Hospital of Salamanca) and blood concentration of cotinine, if available.

The information collected on risk factors and tobacco consumption will allow us to distribute patients into the appropriate study groups (see the 'Study design' section).

Collection and processing of urine samples

Objective 1

A single urine sample will be obtained at the time of inclusion in the study.

Objective 2

A urine sample will be obtained 24 months after the patient's inclusion in the study.

Objective 3

Urine samples will be obtained at the time of inclusion in the study (in the smoking cessation programme), and at 3, 6 and 12 months after inclusion.

All samples will be collected in the relevant healthcare centre and transferred to the samples bank of the University Hospital of Salamanca, where they will be managed and conserved. On submission to the biobank, samples will be centrifuged (2000 *g*, 4°C, for 8 min) to remove possible sediments. Subsequently, they will be frozen in several aliquots at −80°C until use.

Analysis of urine samples

The analyses necessary for the study will be carried out in the laboratories of the Theranostic Group of Renal and Cardiovascular Diseases of the Biosanitary Institute of Salamanca (Institute of Biomedical Research of Salamanca). These analyses will include the following:

Urinary creatinine

This will be used as a correction factor to adjust measured biomarkers for urinary flow. The ratio of biomarker/urinary creatinine will be calculated. Creatinine levels will be determined using the colourimetric kit 'Quantichrom Creatinine Assay Kit' (BioAssay Systems, Hayward, California, USA), based on Jaffé's method.[22] It is estimated that urinary creatinine concentration will be inversely proportional to urinary flow.

Proteinuria

Urinary protein excretion will be evaluated by the Bradford colourimetric method.[23] It is estimated that proteinuria values in healthy patients should be 150 mg in 24 hours or 0–8 mg/dL.[24]

Early markers of kidney damage (NAG, albuminuria, KIM-1 and NGAL)

There are no reference values for humans, so the mean of non-smoking patients without risk factors (group 1) will be compared with the other groups. All markers will be measured by commercial kits following manufacturer instructions. For NAG, the colourimetric kit 'NAG assay kit' (Diazyme, Poway, California, USA) will be used. The other three markers will be measured using ELISA technique. Specifically, we will use the 'Human Albumin ELISA kit' (Bethyl Laboratories, Montgomery, USA) for albuminuria, the 'Human KIM-1 ELISA kit' (Enzo Life Sciences, Laser, Switzerland) for KIM-1 and the Human NGAL ELISA Kit 036CE (BioPorto Diagnostics, Hellerup, Denmark) for NGAL.

Predisposition markers (GM2AP, transferrin and t-gelsolin)

There are no reference values for humans, so the mean of non-smoking patients without risk factors (group 1) will be compared with the other groups. These markers will be evaluated using western blotting technique. Protein samples from 21 µL of urine will be separated by polyacrylamide gel electrophoresis (Mini-Protean II System, Bio-Rad, Madrid, Spain). These proteins will be subsequently transferred to Immobilon-P membranes (Millipore, Madrid, Spain), which will then be incubated with goat polyclonal antibodies against GM2AP (1:1000 dilution; own production), and then with rabbit polyclonal antibodies against GM2AP (1:10 000 dilution; MBL International, Woburn, Massachusetts, USA), transferrin (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, California, USA) and t-gelsolin (Santa Cruz Biotechnology), followed by incubation with goat polyclonal antibodies against transferrin and t-gelsolin (SouthernBiotech, Alabama, USA). Following primary antibody incubations, membranes will be incubated with secondary antibodies coupled with horseradish peroxidase (HRP). Membranes will be subsequently incubated with the chemiluminescent HRP-substrate (ECL; Bio-Rad, Madrid, Spain) and exposed to photographic films (Kodak, Rochester, New York, USA). Bands will be quantified with Scion Image software (Scion Corporation, Frederick, Maryland, USA).

Tobacco use marker, urinary cotinine

This will be determined by a commercial kit, based on the colourimetric technique, following the manufacturer's instructions (Cotinine ELISA Kit, Abnova Corporation, Taipei, Taiwan).

Statistical analysis

Results will be expressed as mean±SD for normally distributed quantitative variables, median (and IQR) for skewed quantitative variables and frequency distribution for qualitative variables. Statistical normality will be tested using the Kolmogorov-Smirnov test. Homoscedasticity will be tested using the Levene's test. The χ^2 test and Fisher's exact test will be used to analyse the association between independent qualitative variables. Pearson's correlation or Spearman's rho tests will be used to evaluate the relationships between quantitative variables. We will use comparison of means (analysis of variance (ANOVA) and Kruskal-Wallis) tests for independent data and repeated-measures ANOVA and Wilcoxon tests for paired data.

Objective 1

The data obtained from all study patients will be analysed and compared to assess renal function (through the

different biomarkers), both qualitatively and quantitatively, based on the groups and risk factors described.

Objective 2

The data within the 'smokers' group (group 3 in table 1) will be compared with 'no smokers' (group 1 in table 1) through statistical techniques of repeated-measures (ANOVA, Wilcoxon) to characterise the evolution of renal function.

Objective 3

The data of patients who have given up smoking will be compared using tools of repeated measures to quantitatively determine the evolution of the biomarkers of renal function.

Multivariate analysis of multiple linear regression (generalized linear models, logistic regression) will be performed to analyse the variables that most influence renal function, incorporating variables such as gender, age and weight. In this manner, we hope to obtain conclusions regarding the usefulness of the biomarkers in the prognosis of tobacco-associated kidney damage. The hypothesis will be tested with alpha=0.05. The data will be analysed using R and/or SPSS V.23.0.

Patient and public involvement

At the end of the project, a report that contains the results of the study will be sent to the participating patients. Furthermore, the findings will be presented at a local congress, where the public of the Salamanca Health Area will be invited to participate. Findings will also be disseminated through social networks and mass media.

Dates of the study

The study began on 4 March 2019. The collection of samples will end in April 2022. The study will be completed in July 2022.

ETHICS AND DISSEMINATION

Participants will be required to sign an informed consent form prior to inclusion in the study, in accordance with the Declaration of Helsinki[25] and the WHO standards for observational studies. Our protocol will not in any way alter the standard procedure of patients' healthcare. Participants will be informed of the objectives and potential benefits of the project. As the study includes the collection of biological samples, the study participants will be informed of this in detail. The confidentiality of the recruited participants will be ensured at all times in accordance with the provisions of current legislation on personal data protection (3/2018 of 5 December Protection of Personal Data Official Law) and the conditions contemplated by Act 14/2007 on biomedical research. Patients may withdraw freely from the study at any time.

The study results, given their applicability and implications for the general population, will be disseminated in research meetings and peer-reviewed scientific journals. On the other hand, this work seeks novel applications based on globally innovative concepts, with a large associated potential market. For these reasons, any new diagnostic systems developed from the study will be patented.

DISCUSSION

Smoking causes kidney damage that is not effectively detected by the diagnostic parameters usually used in clinics, and can progress to long-term chronic kidney failure.[4–6] Our hypothesis is that certain early markers could detect such subclinical damage. This finding would be a preventive measure of great advantage to social and public health, in the face of two major health problems: smoking and chronic kidney disease.

The first strategy proposed in this project is to find early markers of kidney damage associated with tobacco consumption, in patients both with and without additional risk factors. The presence of these markers in apparently healthy smokers could raise awareness of the risk of progression to chronic kidney disease and would help generate protocols to modify avoidable risk factors. In addition, the detection of these early markers could be a useful factor to convince patients to stop smoking.

The second proposed strategy raises an innovative concept of intervention even earlier, before the damage occurs, which our research group has been a global pioneer in developing. Specifically, we have shown that some drugs and renal toxins in completely subtoxic doses can make experimental animals predisposed to, or more sensitive to, acute kidney injury,[17–20] with the associated risk of chronic renal failure.[26] The relevance of this is that individuals apparently unaffected by a treatment, or exposure to toxins such as tobacco, could unknowingly be predisposed to developing acute kidney injury in certain circumstances (eg, other treatments, radiological contrasts, environmental toxicity) that do not harm non-predisposed individuals. Our group has identified and patented factors associated with this acquired hypersensitivity, and are clinically and technologically developing these as urinary markers.[17–20] The clinical application of these markers will allow us not only to detect predisposition, but to stratify patients in a preventive and personalised manner, according to their individual acquired risk resulting from apparently innocuous pharmacological treatments or chemical exposures.

In the case of smokers, this hypersensitivity becomes especially relevant, since it could have been acquired by consumption of the multiple substances contained in tobacco. The possibility of identifying patients at risk could prevent possible acute kidney injury derived from, for example, treatments with potentially nephrotoxic drugs, administration of contrast media or exposure to substances toxic to the kidney. The appearance of acute damage in this context could be of great importance, since it has been shown that acute kidney injury is a risk factor for the development of chronic kidney disease. Even after initial full recovery of renal function, some patients develop a progressive and persistent deterioration of

renal function, and these patients are more likely to progress to end-stage renal disease.[27]

Finally, we also propose to study whether renal parameters and markers are restored with smoking cessation. This fact would consolidate the argument that tobacco is an avoidable risk in the progression and pathology of kidney disease, and also support the benefits of ceasing consumption.

In short, this project addresses two issues of high concern in the health field: smoking and chronic kidney disease. The concept is aimed at finding diagnostic tools for the early detection of tobacco-associated chronic kidney damage, with a preventive method in terms of disease development and a personalised method according to each patient's individual hypersensitivity. The tools developed from this project could not only reduce the major economic and social costs associated with kidney disease, but could also act as deterrents to convince patients to stop smoking.

Study limitations

The variables included are biomarkers of early kidney damage and predisposition to acute kidney injury induced by certain renal toxins. It is possible that there are other (unknown) markers with the potential to predict tobacco-associated kidney damage that we might have not included in our project.

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Contributors AIM participated in the conception of the idea for the study. AIM, MP and LV-V participated in the design of the study, development of the protocol and writing of the manuscript. AGC, MTH-S, MAG-M and LG-O reviewed the manuscript. The project will be conducted by the BIOTAB group.

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Competing interests None declared.

Patient consent for publication Not required.

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