UNIVERSIDAD DE EXTREMADURA

TESIS DOCTORAL

INFLUENCIA DE LA VARIABILIDAD GENÉTICA EN LA VÍA DE LA CICLOOXIGENASA SOBRE LA FUNCIÓN CARDIORRENAL DE PACIENTES CON NEFROPATÍA VASCULAR

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PROGRAMA DE DOCTORADO EN BIOMARCADORES DE SALUD Y ESTADOS PATOLÓGICOS

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Esta tesis cuenta con la autorización del director/a y coodirector/a de la misma y de la Comisión Académica del programa. Dichas autorizaciones constan en el Servicio de la Escuela Internacional de Doctorado de la Universidad de Extremadura.

2024

A mi abuelo Ángel

"The scientist is free, and must be free to ask any question,
to doubt any assertion, to seek for any evidence, to correct any errors."

Julius Robert Oppenheimer

The results of this PhD thesis have been published in the following scientific publications:

1.- Genetic variants in PGE2 receptors modulate the risk of nephrosclerosis and clinical outcomes in these patients

Luz María González; Nicolás Roberto Robles; Sonia Mota-Zamorano; José Manuel Valdivielso; Juan López-Gómez; Guillermo Gervasini

Journal of Personalized Medicine 2021; 11, 772, ISSN 2075-4426

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2.- Tag-SNPs in Phospholipase-Related Genes Modify the Susceptibility to Nephrosclerosis and its Associated Cardiovascular Risk.

LM González; NR Robles; S Mota-Zamorano; JC Arévalo; JM Valdivielso; J López-Gómez; G Gervasini.

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3.- Influence of variability in the cyclooxygenase pathway on cardiovascular outcomes of nephrosclerosis patients

Luz M González, Nicolás R Robles, Sonia Mota-Zamorano, José M Valdivielso, Laura González-Rodríguez, Juan López-Gómez, Guillermo Gervasini

Scientific Reports 2023; 13:1253, ISSN 2045-2322

Impact index: 4.6, Q2

Other related publications in the field of nephrology not included in this volume

1. Polymorphisms in glucose homeostasis genes are associated with cardiovascular and renal parameters in patients with diabetic nephropathy
Mota-Zamorano, S; González, LM; Robles, NR; Valdivielso, JM; Arévalo-Lorido, JC; López-Gómez, J; Gervasini, G
Annals of Medicine, 2022 October; 54 (1), 3038-3050
Impact index: 5.348, Q2

2. Body Fat Distribution, Adipocytokines Levels and Variability in Associated Genes and Kidney Transplant Outcomes. Body Fat Distribution, Adipocytokines Levels and Variability in Associated Genes and Kidney Transplant Outcomes.
García-Pino, G; Luna, E; Blanco, L; Tormo, MA; Mota-Zamorano, S; González, LM; Azevedo, L; Robles, NR; Gervasini, G
Progress in Transplantation, 2022 June; 32(2):112-119
Impact index: 1.065, Q4

3. A Custom Target Next-Generation Sequencing 70-Gene Panel and Replication Study to Identify Genetic Markers of Diabetic Kidney Disease
Mota-Zamorano, S; González, LM; Robles, NR; Valdivielso, JM; Cancho, B; López-Gómez, J; Gervasini, G
Gene, 2021 December; 12 (12), 1992
Impact index: 3.9, Q2

4. Genetics variants in the epoxygenase pathway of arachidonic metabolism are associated with eicosanoids levels and the risk of diabetic nephropathy
Mota-Zamorano, S; Robles, NR; González, LM; Valdivielso, JM; López-Gómez, J; Cancho, B; García-Pino, G; Gervasini, G
Journal of Clinical Medicine 2021; 10(17), 3980
Impact index: 4.241, Q1

5. Plasma and urinary concentrations of arachidonic acid-derived eicosanoids are associated with diabetic kidney disease
Mota-Zamorano, S; Robles, NR; López, J; Cancho, B; González, LM; García-Pino, Guadalupe; Navarro, ML; Gervasini, G
EXCLI Journal, 2021 March; 20, 698
Impact index: 4.068, Q2

6. Effect of leptin concentrations and leptin receptor gene polymorphisms on the outcome of renal transplantation
García-Pino, G; Luna, E; Mota-Zamorano, S; González, LM; Tormo, MA; Gervasini, G
Archives of Medical Science, Epub ahead of print
Impact index: 3.318, Q2

7. Combined donor-recipient genotypes of leptin receptor and adiponectin gene polymorphisms affect the incidence of complications after renal transplantation
Mota-Zamorano, S; Luna, E; García-Pino, G; González, LM; Guillermo, G
Molecular Genetics and Metabolism Reports 2020 September; 25, 100648
Impact index: 2.797, Q3

8. Polymorphisms in vasoactive eicosanoid genes of kidney donors affect biopsy scores and clinical outcomes in renal transplantation
Mota-Zamorano, S; González, LM; Luna, E; Fernández, JJ; Gómez, A; Nieto-Fernández, A; Robles, NR; Guillermo Gervasini
PloS one 2019 October; 14(10)
Impact index: 2.740, Q2

Abbreviations

ADMA	Asymmetric Dimethylarginine
AUC	Area under the curve
BMI	Body Mass Index
BTP	Beta-trace protein
ccIMT	Common Carotid Intima-media Thickness
CKD	Chronic Kidney Disease
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration equation
COX	Cycloxigenase
CV	Cardiovascular
EP	Prostaglandin E receptors
ESKD	End-Stage Kidney Disease
HDL	High-density lipoproteins
K^+	Potassium
KIM-1	Kidney Injury Molecule-1
L-FABP	Liver-type Fatty Acid-binding Protein
MDRD	Modification of Diet in Renal Disease equation
Na^+	Sodium
NAG	N-acetil-D-glucosaminidasa
NGAL	Neutrophil Gelatinase-associated Lipocalin
NSAID	Non-steroidal Anti-inflammatory Drugs
OR	Odd ratio
PG	Prostaglandin
PGE2	Prostaglandin E2
ROC	Receiver Operating Curve
RRT	Renal Replacement Therapy
RAAS	Renin-Angiotensin-Aldosterone System
SNP	Single Nucleotide Polymorphism
UMOD	Uromodulina

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Resumen

La prostaglandina E2 (PGE2) es un importante agente mediador de la lesión renal. Las diferencias genéticas en la vía de la PGE2 pueden influir en el modo en que el organismo produce, metaboliza e interactúa con la PGE2. Esto tiene efectos potenciales sobre las condiciones patológicas. Nuestro objetivo era determinar la variabilidad genética en los genes que codifican la vía de la PGE2 y estudiar las asociaciones con el riesgo de nefroesclerosis y los resultados clínicos. Identificamos 220 tag-SNPs que capturan la variabilidad global en los genes implicados (*PLA2G4, PLAG7, SCARB1, PTGS1, PTGS2, PTGES, PTGES2, PTGES3, PTGER1, PTGER2, PTGER3* y *PTGER4*) que conducen a la síntesis y acción de la prostaglandina E2 (PGE2), y se examinaron en 1209 pacientes con nefroesclerosis y controles. El efecto de estas variantes se evaluó mediante análisis de regresión multivariante.

En relación con el riesgo de desarrollar la enfermedad, rs10846744 y rs838880 en *SCARB1* mostraron odds ratio (OR)=0,66 (0,51-0,87), p=0,003 y 1,48 (1,11-1,96), p=0,007. Además, *PLA2G4A* y *PLA2G7* contenían varios SNP asociados con medidas de aterosclerosis, tales como, el grosor íntima-media de la carótida común (ccIMT), la presencia de placas, número de placas detectadas y progresión del ccIMT a los 2 años (p-valores significativos que oscilan entre 0,0004 y 0,0005). entre 0,0004 y 0,047). Dos SNP (rs11790782 y rs2241270) en *PTGES* estaban asociaron con una mayor presión arterial sistólica y diastólica [portadores frente a no portadores OR=5,23 (1,87-9,93), p=0,03 y 5,9 (1,87-9,93), p=0,004]. *PTGS1*(COX1) rs10306194 se asoció asociado con una mayor progresión del ccIMT [OR 1,90 (1,07-3,36), p=0,029], presencia de placa carotídea [OR 1,79 (1,06-3,01), p=0,026] y gravedad de la aterosclerosis (p=0,041). La región proximal del gen *PTGER3* se señaló como relevante para la eGFR (los valores de p para los SNP identificados oscilaron entre 0,0003 y 0,038). Dos SNP consecutivos en *PTGER3*, rs2284362 y rs2284363, disminuyeron significativamente la presión sistólica (p=0,005 y p=0,0005), diastólica (p=0,039 y p=0,005) y la presión del pulso (p=0,038 y 0,014).

Se realizó un seguimiento de los pacientes durante una mediana de 47 meses (7-54) para evaluar el riesgo cardiovascular (CV). El análisis de las curvas ROC mostró que la adición de *PTGS2*rs4648268 y *PTGES3*rs2958155 y rs11300958, a un modelo predictivo de eventos CV que contenía factores de riesgo clásicos, mejoraba significativamente su capacidad predictiva (el valor AUC aumentó del 78,6% al 87,4%, p=0,4%). 87.4%, p=0.0003). Este aumento siguió siendo significativo tras corregir por pruebas múltiples.

La identificación de variantes genéticas en la vía de la PGE2 puede ser útil para controlar la evolución clínica, sobre todo CV, de los pacientes con nefroesclerosis.

Summary

Prostaglandin E2 (PGE2) is a major actor mediating renal injury. Genetic differences in the PGE2 pathway may influence how the body produces, metabolises and interacts with PGE2. This has potential effects on pathological conditions. We aimed to determine genetic variability in the genes coding for PGE2 pathway and study associations with nephrosclerosis risk and clinical outcomes. We identified 220 tag-SNPs capturing global variability in the genes involved (*PLA2G4*, *PLAG7*, *SCARB1*, *PTGS1*, *PTGS2*, *PTGES*, *PTGES2*, *PTGES3*, *PTGER1*, *PTGER2*, *PTGER3* and *PTGER4*) leading to prostaglandin E2 (PGE2) synthesis and actions, and examined 1209 nephrosclerosis patients and controls. The effect of these variants was evaluated by multivariate regression analyses.

Related to the risk of developing the disease, rs10846744 and rs838880 in SCARB1 showed significant odds ratios (OR)=0.66 (0.51-0.87), p=0.003 and 1.48 (1.11-1.96), p=0.007. In addition, *PLA2G4A* and *PLA2G7* harboured several SNPs associated with measures of atherosclerosis, namely common carotid intima-media thickness (ccIMT), presence of plaques, number of plaques detected and ccIMT progression at 2 years (significant p-values ranging from 0.0004 to 0.047). Two SNPs (rs11790782 and rs2241270) in *PTGES* were associated with higher systolic and diastolic blood pressure [carriers versus non-carriers OR= 5.23 (1.87-9.93), p=0.03 and 5.9 (1.87-9.93), p=0.004]. *PTGS1*(COX1) rs10306194 was associated with greater progression of ccIMT [OR 1.90 (1.07-3.36), p=0.029], presence of carotid plaque [OR 1.79 (1.06-3.01), p=0.026] and severity of atherosclerosis (p=0.041). A proximal region of the *PTGER3* gene was marked as relevant for eGFR (p-values for identified SNPs ranged from 0.0003 to 0.038). Two consecutive *PTGER3* SNPs, rs2284362 and rs2284363, significantly decreased systolic (p=0.005 and p=0.0005), diastolic (p=0.039 and p=0.005) and pulse pressure (p=0.038 and 0.014) values.

Patients were followed for a median of 47 months (7-54) to assess cardiovascular (CV) risk. The analysis of ROC curves showed that the addition of *PTGS2* rs4648268 and *PTGES3* rs2958155 and rs11300958, to a predictive model of CV events containing classical risk factors, significantly improved its predicting ability (AUC value increased from 78.6% to 87.4%, p=0.0003). This increase remained significant after correcting for multiple testing.

The determination of genetic variants in the PGE2 pathway may be useful to manage the clinical evolution, particularly CV-related, of patients with nephrosclerosis.

Introduction

2. Introduction

In this introduction, the fundamental aspects of nephrosclerosis, the importance of the cyclooxygenase pathway in the disease and suggestive data on the relevance of genetic variability in this metabolic route will be presented. All are collected in the three scientific articles included in this doctoral thesis, studying each part of the pathway separately but always in the same population and methodology.

2.1. Chronic kidney disease

Chronic Kidney Disease (CKD) is a global health problem, currently affecting approximately 10% of the population, equivalent to around 850 million people. It is expected to be the fifth leading cause of death in the world by 2040 (Foreman et al., 2018). This high prevalence reflects the impact of risk factors such as diabetes, hypertension, obesity, and an aging population. It is characterized by the gradual and irreversible loss of kidney function over more than three months leading to the accumulation of toxins and waste products in the body (Stevens & Levin, 2013).

Demographic differences related to race, age, and sex can influence the occurrence of CKD. Certain racial and ethnic groups may have a higher susceptibility to CKD due to genetic factors or disparities in healthcare access. Age is another significant factor, as the risk of developing CKD tends to increase with age (Campbell et al., 2021). Even if age is a factor to take into account when talking about the incidence of CKD, this age is also distributed differently among the different diagnoses of kidney disease (Figure 1), where we can observe the older population, those over 75 years of age, have a higher percentage of renal vascular disease. These findings highlight the importance of early diagnosis, which would improve the health of these patients in advance, reducing the healthcare costs of the derived diseases. In terms of sex, CKD prevalence may vary between males and females, with some studies indicating a slightly higher prevalence among males (García et al., 2022; Kao et al., 2022; Mayne et al., 2023).

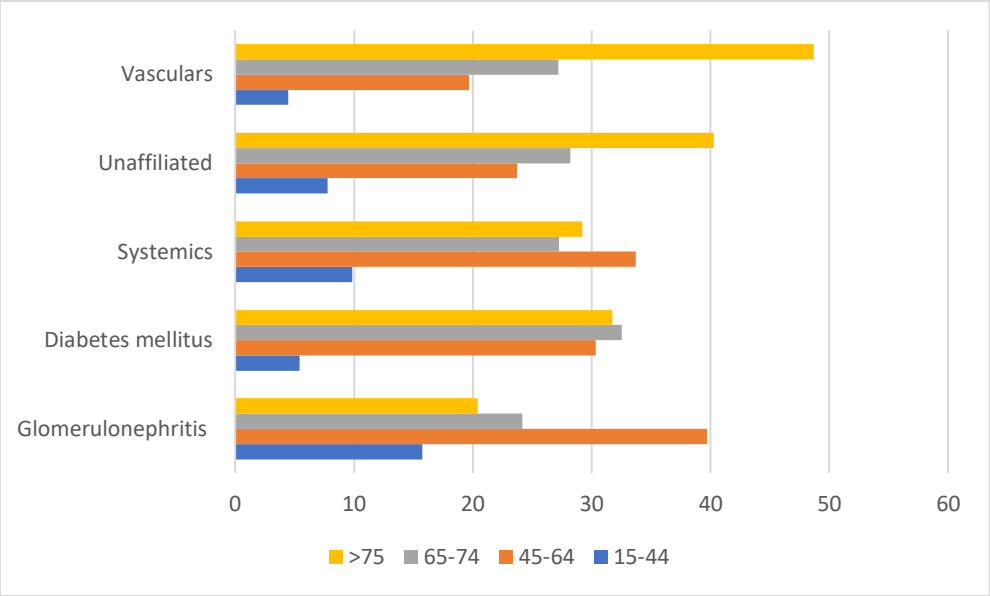

Figure 1. Ages differences into distribution of chronic kidney disease from the Spanish Society of Nephrology

In 2022, the Spanish Society of Nephrology produced an updated compilation document with incidence and prevalence data associated with renal pathology in Spain (S.E.N., 2022). First, incidence refers to the number of new cases of disease in a population in a certain period of time. The evolution of incidence in Spain in recent years has increased the need for renal replacement therapy (RRT) from 121.1 persons per million population (ppm) to 141.4 ppm (Figure 2a). During the 2020 health emergency there was a decrease. Examining the incidence by age group we observe that patients older than 75 years have higher rates of RRT followed closely by the next lower age group. This incidence is also differentiated within the Spanish national context, where we observe Catalonia and the Canary Islands are the communities with 172.4 ppm and 171.4 ppm respectively. Extremadura, our region, shows an incidence of 141 ppm, close to the national average incidence of 141.1 ppm (Figure 2b).

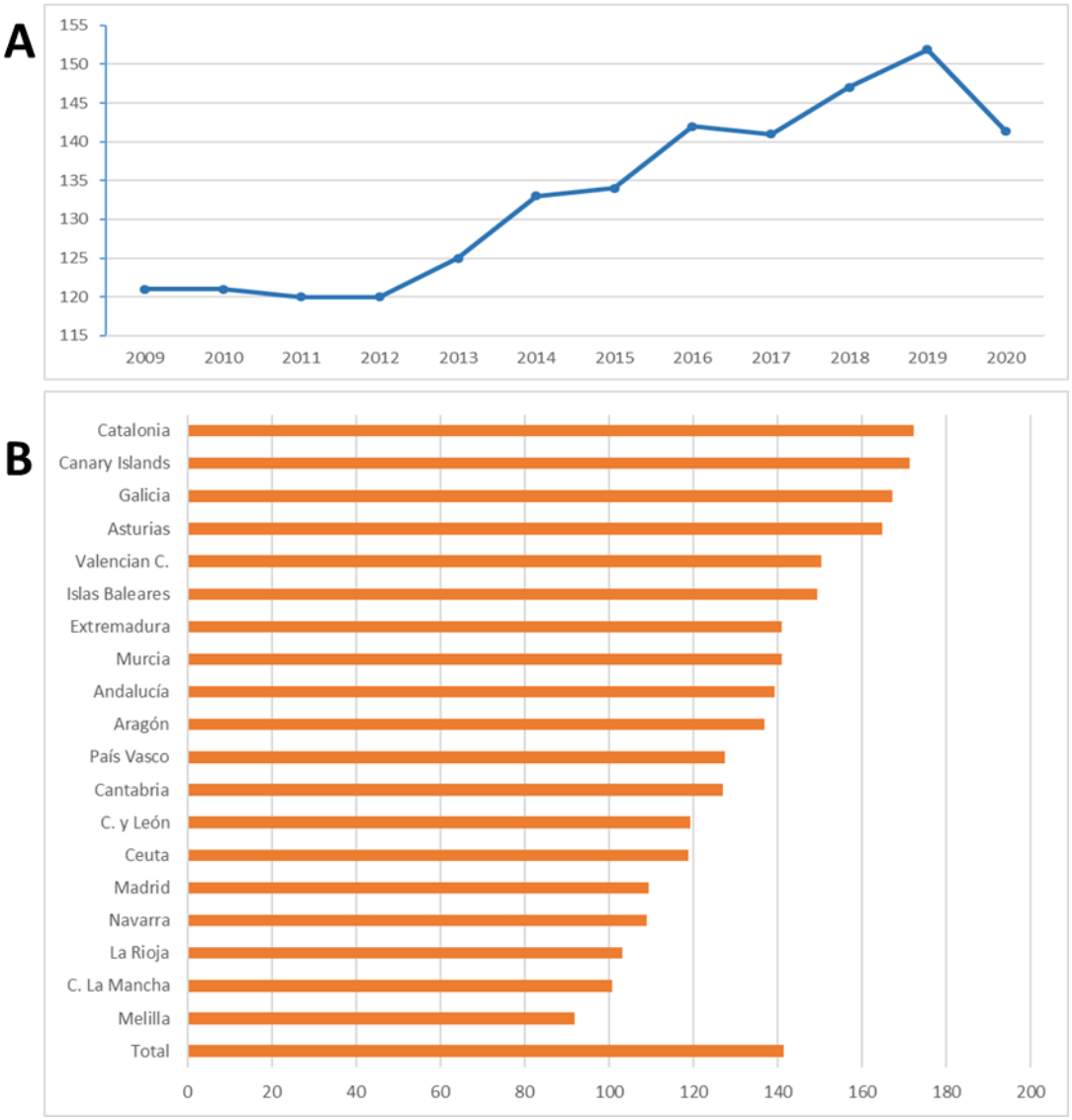

Figure 2. Evolution of incidence in Spain per year (A) and per Spanish regions (B).

We can also examine the importance of CKD in Spain based on the prevalence of CKD. Prevalence is the proportion of the population affected by a specific disease at a certain moment in time, including both new diagnoses and patients who have been treated for the disease for a long time. An increase of almost 30% is observed in the last decade in Spain, with 1363 ppm required any of the RRT modalities (Figure 3a). A higher number of the population with RRT is concentrated in patients over 75 years of age, again close behind patients in the next age range (64-75 years). In terms of prevalence at national context, the Valencian Community is in first place with 1614 ppm, leaving Catalonia in third place with 1555 ppm. Extremadura is in tenth place with a prevalence of 1300 ppm, slightly below the national average of 1363 ppm (Figure 3b).

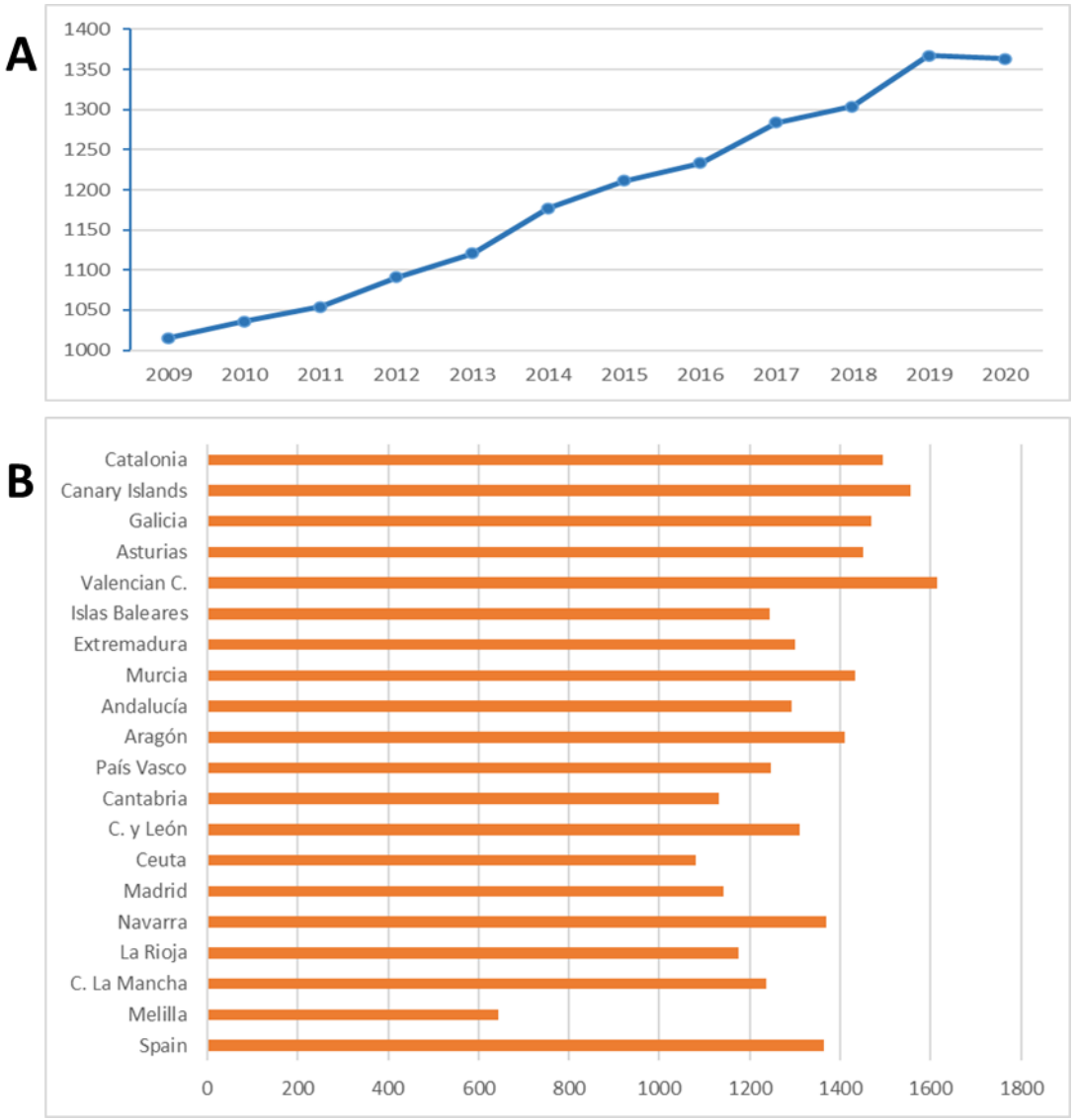

Figure 3. Evolution of prevalence of chronic kidney disease in Spain per year (A) and per Spanish regions (B)

CKD is typically classified into five stages based on the glomerular filtration rate (GFR), which measures how effectively the kidneys filter waste from the blood. In the early stages (Stages 1 and 2), the kidney damage may be present, but the symptoms are usually mild or absent. As the disease progresses to Stages 3, 4, and 5, the symptoms become more pronounced, including fatigue, swelling in the legs or ankles, changes in urine output, and difficulty concentrating (Table 1). In the most severe stage (Stage 5), also known as end-stage kidney disease (ESKD), the kidneys have lost nearly all their function, requiring dialysis or a kidney transplant to sustain life.

Table 1. Current chronic kidney disease nomenclature used by Kidney Disease Improving Global Outcomes (KDIGO) ('KDIGO Guideline', 2022)

Prognosis of CKD by GFR and albuminuria categories: KDIGO 2012				Persistent albuminuria categories Description and range		
				A1	A2	A3
				Normal to mildly increased	Moderately increased	Severely increased
				< 30 mg/g < 3 mg/mmol	30–300 mg/g 3–30 mg/mmol	> 300 mg/g > 30 mg/mmol
GFR categories (ml/min/1.73 m²) Description and range	G1	Normal or high	≥ 90			
	G2	Mildly decreased	60–89			
	G3a	Mildly to moderately decreased	45–59			
	G3b	Moderately to severely decreased	30–44			
	G4	Severely decreased	15–29			
	G5	Kidney failure	< 15			

Green, low risk (if no other marker of kidney disease, no CKD); Yellow, moderately increased risk; Orange, high risk; Red, very high risk. GFR; glomerular filtration rate

One of the challenges with CKD is that it often progresses slowly and may not cause noticeable symptoms in the early stages. As a result, many individuals may be unaware that they have CKD until it reaches more advanced stages. Common risk factors for CKD include diabetes, high blood pressure, obesity, smoking, and a family history of kidney disease (Figure 4). Certain ethnic groups, such as African Americans, Hispanics, and Native Americans, have a higher prevalence of CKD (Adedinsewo et al., 2022). Additionally, renal health can also affect a patient's condition (Lateef, 2022), leading to depression, anxiety and cognitive impairment. The economic burden of CKD can be considerable, with costs related to medications, dialysis treatments, hospitalisations and, potentially, a kidney transplant.

The diagnosis of CKD involves disease progression to the point of requiring RRT, which may be solved by renal transplantation or dialysis. However, the main burden of CKD is accelerated ageing and premature death, associated with increased cardiovascular (CV) risk (Ortiz et al., 2022). Patients undergoing any RRT have a 10-100 times higher annual mortality compared to their age group, making CV disease the leading cause of death in RRT patients. The associated CV diseases include coronary heart disease, stroke, peripheral arterial disease

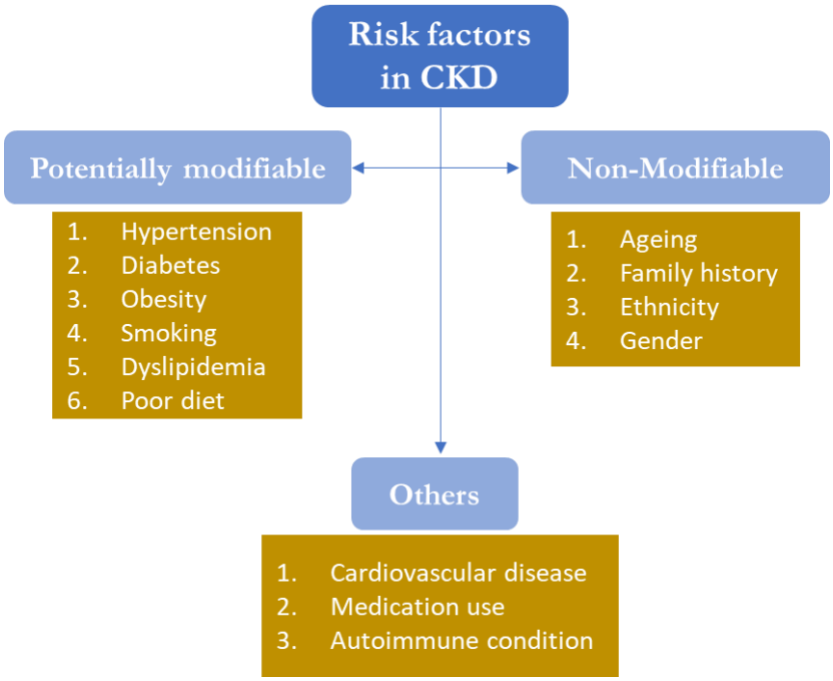

Figure 4. Common risk factors for chronic kidney disease (CKD)

and heart failure. This impact of CV diseases on CKD reflects the involvement of common pathophysiological mechanisms due in part to the high number of risk factors shared by both diseases (Matsushita et al., 2022; Ortiz, et al., 2022).

2.1.1. Nephrosclerosis

Nephrosclerosis or vascular nephropathy is a disease that refers to kidney disorders associated with vascular pathology. This chronic, progressive disease can lead to impaired kidney function and, in severe cases, kidney insufficiency.

Prolonged high blood pressure increases the pressure on the arterioles in the kidneys, causing damage to the walls of the blood vessels. Over time, thickening and narrowing of the arterioles occurs, reducing blood flow to the kidneys and affecting kidney function (Marín et al., 2002). This mechanism eventually affects haemodynamic (blood volume and pressure), electrolyte and acid-base balance. All of this is associated with an increased risk of CV disease (Zoccali, 2006). The constriction of renal arterioles and the consequent reduction in blood flow may contribute to the development of hypertension, exacerbating the progression of both diseases. This interaction between nephrosclerosis and CV disease highlights the need for early detection, prevention and appropriate treatment of nephrosclerosis to mitigate its adverse effects on renal and CV health.

In addition to hypertension, other factors may contribute to the development of nephrosclerosis. These include diabetes, high cholesterol levels, smoking, obesity and ageing. These risk factors may further increase the risk of arterial damage and progression of nephrosclerosis.

The initial stage of nephrosclerosis is often deceptive and may occur over a long period of time. Although progression of kidney damage is usually slower than in patients with diabetic nephropathy, the high prevalence of the disease, the large inter-individual variability in its progression and probably an unfavourable genetic profile (González et al., 2010) result in a proportion of cases where a vicious circle occurs: renal failure worsens CV risk and this, in turn, increases disease progression to ESKD (Robles et al., 2010).

There are no diagnostic methods to detect which patients will progress poorly and no specific therapies have been developed to improve the currently prescribed renal preventive and cardioprotective treatments [renin-angiotensin-aldosterone system blockers (RAAS), lipid-lowering agents or antiplatelet agents]. Therefore, new biomarkers, including those of genetic nature, are needed to complement classical clinical markers that can both guide individual prognosis and support the development of specific treatments targeting the etiopathogenic mechanisms of the disease, the nature of which has not been well defined to date (González et al., 2010).

2.1.1.1. Pathogenesis of Nephrosclerosis

The pathogenesis of nephrosclerosis involves a vicious cycle of hypertension, endothelial dysfunction, glomerular damage, inflammation, and fibrosis that lead to the progressive damage and scarring of the renal structures (Meyrier, 2015). Understanding these mechanisms is crucial for developing effective strategies to prevent and manage nephrosclerosis and its complications.

High blood pressure is a major risk factor and initiator of nephrosclerosis (Hill, 2008). Prolonged elevation in blood pressure causes increased strain on the small blood vessels (arterioles) within the kidneys. The sustained pressure damages the endothelial cells lining the arterioles, leading to endothelial dysfunction. Hypertension may be primary (with no specific cause, 85% of cases) or secondary (with an identified cause). Renal blood flow gradually decreases as diastolic blood pressure increases and arteriolar sclerosis begins to develop. The glomerular filtration rate remains normal until late in the disease; as a

consequence, the filtration fraction increases. Coronary, cerebral and muscular blood flow are maintained except in the presence of severe atherosclerosis in these vascular sites.

In nephrosclerosis, endothelial dysfunction occurs due to chronic hypertension. It impairs the normal regulation of blood flow and the release of vasoactive substances, such as nitric oxide (Carlström, 2021) (Figure 5). This contributes to constriction and remodelling of arterioles. In addition, ageing is associated with a reduction in nitric oxide, and this lower level contributes to salt sensitivity [i.e., lower salt intake increases blood pressure more compared to younger people (Fujiwara et al., 2000). Reduced nitric oxide due to stiff arteries is related to salt-sensitive hypertension, which is an inordinate increase of > 10 to 20 mmHg in systolic blood pressure after a large sodium load. If the kidneys do not produce adequate amounts of vasodilators, blood pressure may increase. Consequently, endothelial dysfunction significantly affects blood pressure (Roumeliotis et al., 2020).

Constriction of the arterioles decreases the blood supply to the glomeruli, the functional units of the kidneys responsible for filtration (Freitas et al., 2022). Reduced blood flow (ischaemia) to the glomeruli causes glomerular damage, which can disrupt the filtration process and impair kidney function. In many patients with hypertension, sodium transport across the cell wall is abnormal due to dysfunction or inhibition of the sodium-potassium pump (Na^+, K^+-ATPase) (Palmer et al., 2019) or increased permeability to sodium ions. The result is an elevation of the intracellular sodium concentration, which makes the cell more sensitive to sympathetic stimulation. Calcium follows sodium, so intracellular calcium accumulation may be responsible for the increased sensitivity.

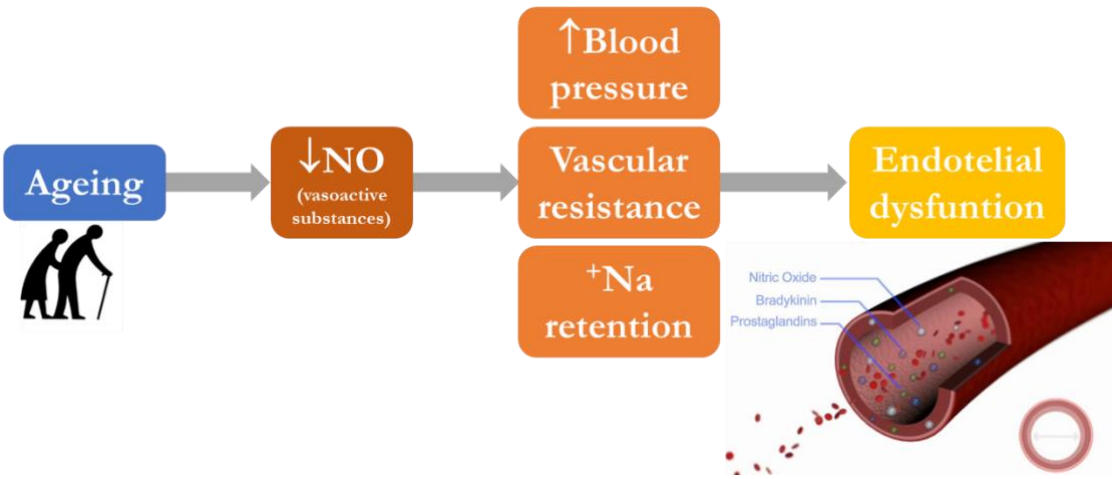

Figure 5. Blood flow regulation by vasoactive substances (NO, nitric oxide)

Reduced blood flow and pressure in the kidneys activates RAAS (Figure 6), a hormonal system involved in the regulation of blood pressure and fluid balance. Activation of the RAAS results in the production of angiotensin II, a potent vasoconstrictor which further constricts arterioles, increasing kidney damage (Park et al., 2000).

Blood volume and, consequently, blood pressure are both controlled by the renin-angiotensin-aldosterone system (Hsu et al., 2021). The juxtaglomerular apparatus produces the enzyme renin, which catalyses the conversion of angiotensinogen to angiotensin I. This inactive substance is degraded primarily in the lungs, but also in the kidneys and brain, by the angiotensin-converting enzyme (ACE). It is then converted to angiotensin II, a potent vasoconstrictor that also stimulates autonomic centres in the brain to raise sympathetic tone and release aldosterone and vasopressin. Blood pressure rises as a result of sodium and water retention caused by aldosterone and vasopressin (Tiwari et al., 2006). As hypokalaemia (3.5 mEq/L [3.5 mmol/L]) increases vasoconstriction by blocking potassium channels, aldosterone also stimulates potassium excretion. Although it has significantly less pressor activity than angiotensin II, angiotensin III, this is a circulatory hormone that stimulates aldosterone release in a similar way. Drugs that inhibit ACE do not entirely stop the production of angiotensin II because kinase enzymes also convert angiotensin I to angiotensin II.

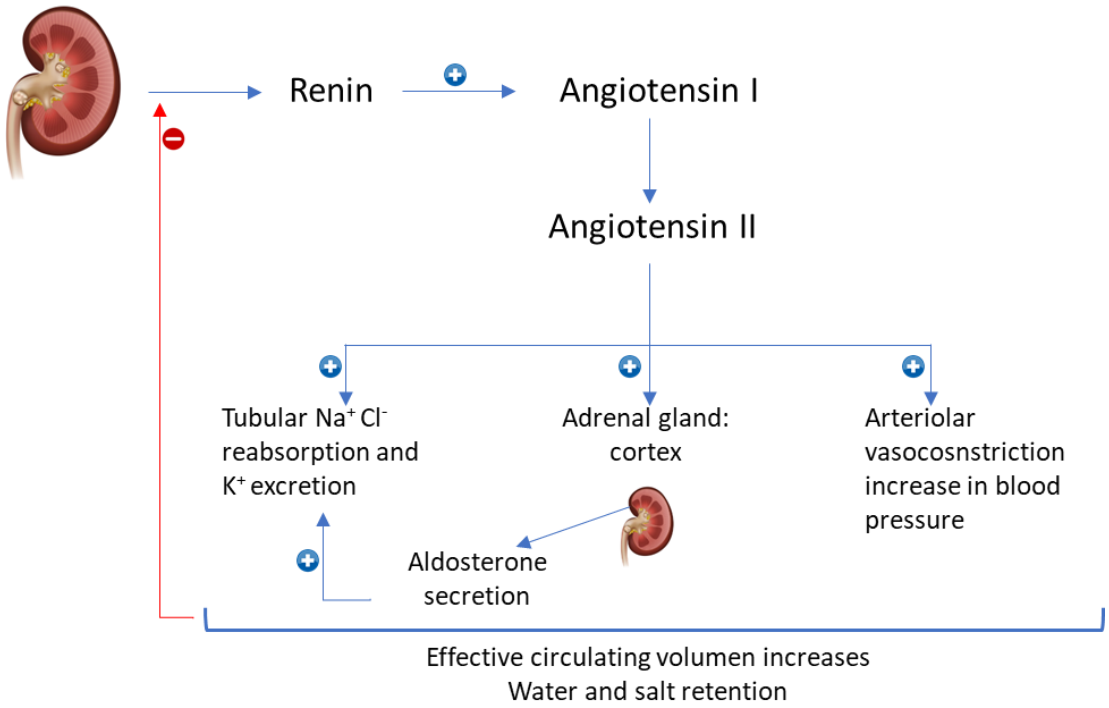

Figure 6. Renin-angiotensin-aldosterone system.

Renovascular hypertension is typically thought to be caused by angiotensin, at least in its early stages. Renin levels do, however, frequently fall low in people with African ancestry and in elderly hypertensive patients. Angiotensin II levels are frequently low in older individuals (Williams et al., 2014).

Chronic injury to renal structures activates an inflammatory response (Abumoawad et al., 2019). Inflammatory cells infiltrate the affected areas, releasing cytokines and growth factors promoting smooth muscle cell proliferation and production of extracellular matrix proteins. The mechanism is the development of arteriolosclerosis and the acceleration of the production of atherogenesis (Figure 7). Arteriolosclerosis is characterised by hypertrophy, hyperplasia and hyalinisation of the media and is most evident in small arterioles, predominantly in the eyes and kidneys (Kashgarian, 1985). In the kidneys, the changes result in arteriolar lumen constriction, with an increase in total peripheral vascular resistance, implying that hypertension leads to the development of more hypertension (Sadykhov et al., 2022). Likewise, once the diameter of the arteries begins to decrease. The additional shortening, however slight, reduces the lumen of the arteries to a greater extent than when acting on arteries of normal diameter.

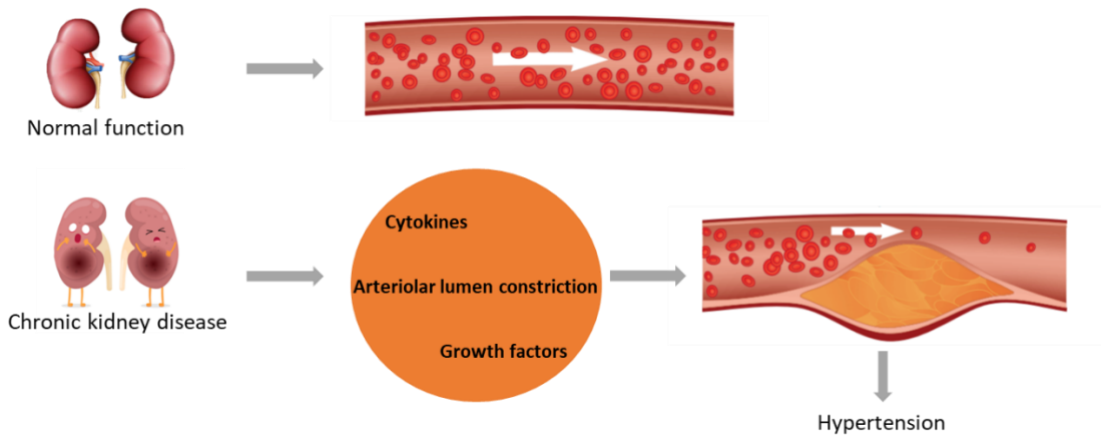

Figure 7. Atherosclerosis in CKD.

As a result, the buildup of scar tissue (fibrosis) within the kidney causes a progressive loss of functional nephrons and the replacement of healthy tissue with damaged tissue, which causes a progressive decline in kidney function (Simeoni et al., 2021). Albumin in particular can be filtered into the urine as a result of glomerular damage to the kidney and scarring. Mild

proteinuria is usually a symptom of nephrosclerosis, when overt proteinuria is present it is a sign of severe glomerular disease.

In addition, nephrosclerosis may be influenced by additional factors, such as diabetes and specific genetic predispositions. In type 2 diabetes, which is more common, the risk of hypertension is particularly high as insulin resistance can alter the function of blood vessels and increase blood pressure (Petrie et al., 2018).

The onset and development of nephrosclerosis may be sped up by the interaction of these factors with hypertension.

2.1.1.2. Clinical evolution of Nephrosclerosis

CKD known as nephrosclerosis has a clinical progression that is influenced by factors such as the severity of hypertension and comorbidities. It is important to point out how the progression of the disease can be different between patients, and how each individual experiences a different clinical profile. Some individuals may progress rapidly, while others may have a more indolent course with a slower deterioration of renal function. The presence of comorbidities, such as diabetes, hypertension or CV disease, may further complicate the clinical course and impact outcomes.

Disease progression can be classified into different stages, each stage being associated with different clinical manifestations depending on the level of tissue damage. The progression and stages are similar to the general CKD stages.

In the early stages of nephrosclerosis, patients may be asymptomatic and the disease may pass undetected. Routine medical check-ups or incidental findings during diagnostic tests for other conditions may reveal minor abnormalities in kidney function or the presence of proteinuria (excess protein in the urine). Blood pressure may be elevated, but controllable with lifestyle modifications and possibly a single antihypertensive drug.

As nephrosclerosis progresses, patients may begin to experience symptoms such as increased blood pressure, decreased urine output or changes in urinary characteristics such as blood in the urine (haematuria). Proteinuria may become more pronounced, indicating worsening kidney damage. Kidney function may gradually decline, and blood tests may show elevated levels of waste products such as creatinine and urea.

In advanced stages of nephrosclerosis, the clinical manifestations become more pronounced. Blood pressure may be difficult to control even with multiple antihypertensive medications. Renal function continues to decline and symptoms such as fatigue, fluid retention and difficulty concentrating may occur. Patients may require more intensive treatment, including dietary restrictions, fluid control and medication to treat complications such as anaemia and bone mineral disorders.

Finally, in ESKD, the kidneys have lost the majority of their function. Kidney function is severely impaired and patients require renal replacement therapies such as dialysis or kidney transplantation. ESKD is characterised by significant symptoms such as severe fatigue, fluid overload, electrolyte imbalances and complications related to the accumulation of waste products in the body.

2.1.1.3. Risk factors of Nephrosclerosis

Nephrosclerosis means that, in most cases, kidney damage develops more slowly than in other kinds of CKD. Kidney damage increases CV risk, which in turn accelerates the progression of end-stage renal disease (Robles et al., 2010, 2020). However, this vicious circle occurs in a significant proportion of cases due to the high prevalence of the disease and the significant inter-individual variability in its progression. Therefore, there is a need to identify other risk factors for nephrosclerosis to enable early detection, individualised treatment and the reduction of the enormous economic burden of renal replacement therapy and CKD.

An individual is more likely to develop a disease or experience a negative health event if certain traits or situations are present. It is important to remember that these risk factors simply increase the likelihood of developing nephrosclerosis, rather than causing it. Multiple risk factors can also increase the risk of chronic kidney disease. These risk factors act biologically to cause inflammation, endothelial dysfunction, lipid accumulation and atherosclerotic plaque development in the context of nephrosclerosis. Risk elements include the following items.

The main risk factor for nephrosclerosis is high blood pressure. The walls of the renal blood vessels undergo excessive stress as a result of hypertension. Endothelial dysfunction and inflammation result from the damage that this continuous stress causes to the cells lining the vessels. As a result, lipids and inflammatory cells enter the vessel walls in an influx which

leads to blockages and atherosclerotic plaques. This leads to oxygen and nutrient insufficiency and decreased renal blood flow (Zoccali, 2006).

Diabetes, particularly type 2 diabetes, is an important risk factor for nephrosclerosis (Cunillera-Puértolas et al., 2022). Diabetes is often a consequence of obesity, although it can also be found in lean people with hypertension, insulin resistance is usually associated with obesity. Endothelial dysfunction is a significant antecedent and modulator of atherosclerosis which has been demonstrated in both pre-diabetes and hypertension, as well as the pro-inflammatory and metabolic effects of obesity and insulin resistance (Petrie et al., 2018). Diabetes can also damage mesangial cells, which preserve the shape and function of renal capillaries. As a result, the vessels can become clogged and the kidneys gradually deteriorate.

Tobacco use is often a risk factor for many diseases, in particular for the one we are studying, several toxic chemicals in tobacco smoke damage endothelial cells and accelerate the development of atherosclerotic plaque. In addition, smoking increases free radical production and systemic inflammation (Popolo et al., 2013), which accelerates the progression of nephrosclerosis and vascular damage.

Increased concentrations of blood lipids, such as triglycerides and cholesterol, can accelerate the onset of nephrosclerosis (Park et al., 2000). The walls of the renal blood vessels become overloaded with lipids from the CV system, leading to an inflammatory reaction and the development of atherosclerotic plaques. The functionality of the kidneys is compromised because these plaques prevent the organs from receiving enough blood. Insulin resistance, ongoing inflammation and endothelial dysfunction are linked to obesity or overweight in relation to this whole mechanism. Each of these elements contributes to increased lipid accumulation in the walls of the renal blood vessels, which promotes the progression of kidney disease (Hao et al., 2007).

The risk of a person developing chronic kidney disease may also be higher if their family has a history of the condition. The likelihood of a person developing nephrosclerosis has been linked to a number of genetic factors (Li et al., 2020).

2.1.1.4. Cardiovascular risk

One of the most remarkable characteristics of nephrosclerosis is the chronic damage produced in the small blood vessels of the kidneys, it can lead to a number of CV events due to a strong relationship with hypertension and also because of the interaction between renal

and CV health. It is important to note that nephrosclerosis and its associated CV complications depend on several factors that have already been mentioned in previous sections, such as the severity of kidney damage, the degree of hypertension, the presence of other coexisting diseases and individual genetic factors. Moreover, even after starting renal replacement therapy mortality rates are higher in this group of patients.

Nephrosclerosis-caused chronic hypertension can result in hypertensive heart disease. This includes conditions like left ventricular hypertrophy (Said et al., 2014), where the left ventricle of the heart thickens and enlarges as a result of an increase in workload, and heart failure with preserved ejection fraction, where the heart's ability to pump is impaired. Nephrosclerosis patients have an increased risk of heart attacks (Said et al., 2014). The likelihood of arterial blockages that can result in decreased blood flow to the heart muscle is increased by the presence of hypertension, atherosclerosis, and impaired kidney function. The risk of stroke is increased in people with nephrosclerosis due to the increased prevalence of hypertension and atherosclerosis(Toyoda et al., 2005). This population is especially prone to ischemic strokes, which are brought on by blood clots or plaque buildup. Nephrosclerosis-related chronic kidney disease can upset the electrolyte-body balance, which can cause cardiac arrhythmias (irregular heartbeats). Complications like blood clots and strokes are made more likely by these arrhythmias.

Atherosclerosis, i.e., the development of fatty deposits (plaques) in arteries, and nephrosclerosis frequently coexist (Tobe et al., 2006). Coronary artery disease, peripheral artery disease, and carotid artery disease are just a few of the conditions that are classified as atherosclerotic CV disease and can result in decreased blood flow to different body parts. Heart failure, in which the heart's capacity to pump blood effectively is hampered, can result from the interaction of hypertension, atherosclerosis, and kidney dysfunction (Figure 8). Heart failure caused by nephrosclerosis can cause fluid retention, breathlessness, and a decline in quality of life (Said et al., 2014). Peripheral artery disease, which is characterized by reduced blood flow to the extremities, can result from vascular damage. This may lead to discomfort, restricted movement, and a higher risk of tissue damage. The risk of aortic aneurysms (weakening and ballooning of the aorta) and aortic dissections (tearing of the layers of the aortic wall) can increase due to structural changes in blood vessels brought on by nephrosclerosis. These factors collectively can raise the danger of sudden cardiac death (Shamseddin et al., 2011).

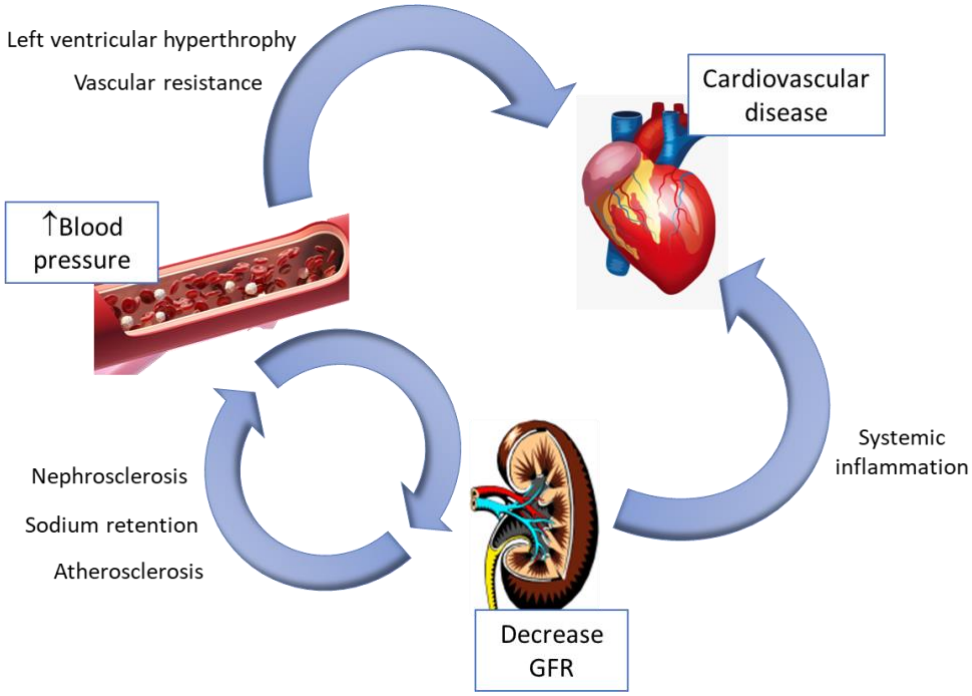

Figure 8. Cardiovascular risk in nephrosclerosis.

2.2. Biomarkers of Chronic Kidney Disease

Biomarkers play a crucial role in the diagnosis, prognosis and monitoring of disease progression, as well as being useful in assessing response to treatment. As a progressive disease, the use of biomarkers helps clinicians to understand and treat the disease better. Some important biomarkers commonly used in the assessment of chronic kidney disease are presented below.

2.2.1. Glomerular filtrate

The assessment of glomerular filtration rate (GFR) is key to the evaluation of renal function in current clinical practice. It corresponds to the volume of plasma from which a substance is eliminated renally in its totality per unit of time. Accurate measurement of GFR requires the use of exogenous substances (inulin, 57Cr-EDTA, 99mTc-DTPA, iothalamate, iohexol, etc.) and their determination both in blood and urine; it is a measurement that requires a complicated methodology usually not available in the laboratory. Therefore, its use is restricted to specific clinical situations such as dose adjustment of a highly toxic drug (García-

Maset et al., 2022; Holness et al., 2015; Soveri et al., 2014). It is for this reason that GFR (eGFR) is estimated from serum creatinine concentrations.

Several formulas for estimating GFR have been developed that are a combination of analytical, demographic and/or anthropometric variables. For example, MDRD (Modification of Diet in Renal Disease Equation) (Levey et al., 2003), the Cockcroft-Gault equation (Cockcroft et al., 1976) or CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration Equation) (Levey et al., 2009). This CKD-EPI equation is currently the most commonly used method to calculate eGFR. It has been found to be more accurate and precise in estimating kidney function, as well as being better at predicting mortality and CV risk. Because of its superior performance, it has been selected as the preferred equation in the KDIGO guidelines (García-Maset et al., 2022; Levey et al., 1999). The values used in this equation differ based on sex, serum creatinine level, and age of the patient; these variations are detailed in Table 2 (Levey et al., 2009).

Table 2. Variation of creatinine levels in adults by gender

Gender	Serum creatinine level	GFR equation
Woman	≤ 0,7 mg/dL	$144x(Crea/0,7)^{-0,329}x0,993^{age}$
	> 0,7 mg/dL	$144x(Crea/0,7)^{-1,209}$ $x0,993^{age}$
Man	≤ 0,9 mg/dL	$141x(Crea/0,9)^{-0,411}$ $x0,993^{age}$
	>0,9 mg/dL	$141x(Crea/0,9)^{-1,209}$ $x0,993^{age}$
Crea: Serum creatinine (mg/dL); Age (by years)		

Despite its limitations, such as being less accurate in extreme body mass index, advanced age, and different ethnicities, creatinine is commonly used to measure kidney function. To overcome these drawbacks, cystatin C has been introduced as a new marker. This protein is produced by nucleated cells and is not influenced by factors like age or gender that affect creatinine levels. However, it also has limitations related to thyroid function or inflammation (Levey et al., 1999). To enhance diagnostic accuracy and precision for estimating glomerular filtration rate (eGFR), a combined equation called CKD-EPIcreatinine+cystatin has been developed (see Table 3). This equation incorporates both serum creatinine and cystatin concentration values while considering all the factors present in the original formula based

solely on creatinine concentrations. In most cases in clinical practice, the preferred choice for eGFR calculation remains the CKD-EPI-creatinine equation. However, when dealing with borderline situations where there may be greater uncertainty regarding accuracy due to various factors mentioned before [extreme Body Mass Index (BMI), advanced age, etc.], using CKD-EPI-creatinine + cystatin becomes more appropriate according to recent studies (García-Maset et al., 2022).

Table 3. Estimation rate of GFR by CKD-EPcreatinina+cystatin in adults

Gender	Serum creatinine and cystatin concentrations	GFR equation
Woman	Crea ≤ 0,7 mg/dL Cys ≤ 0,8 mg/L	$130 \times (Crea/0,7)^{-0,248} \times (Cys/0,8)^{-0,375} \times 0,995^{age}$
	Crea ≤ 0,7 mg/dL Cys > 0,8 mg/L	$130 \times (Crea/0,7)^{-0,248} \times (Cys/0,8)^{-0,711} \times 0,995^{age}$
	Crea > 0,7 mg/dL Cys ≤ 0,8 mg/L	$130 \times (Crea/0,7)^{-0,601} \times (Cys/0,8)^{-0,375} \times 0,995^{age}$
	Crea > 0,7 mg/dL Cys > 0,8 mg/L	$130 \times (Crea/0,7)^{-0,601} \times (Cys/0,8)^{-0,711} \times 0,995^{age}$
Man	Crea ≤ 0,9 mg/dL Cys ≤ 0,8 mg/L	$135 \times (Crea/0,9)^{-0,207} \times (Cys/0,8)^{-0,375} \times 0,995^{age}$
	Crea ≤ 0,9 mg/dL Cys > 0,8 mg/L	$135 \times (Crea/0,9)^{-0,207} \times (Cys/0,8)^{-0,711} \times 0,995^{age}$
	Crea > 0,9 mg/dL Cys ≤ 0,8 mg/L	$135 \times (Crea/0,9)^{-0,601} \times (Cys/0,8)^{-0,375} \times 0,995^{age}$
	Crea > 0,9 mg/dL Cys > 0,8 mg/L	$135 \times (Crea/0,9)^{-0,207} \times (Cys/0,8)^{-0,711} \times 0,995^{age}$

Crea: Serum creatinine concentration (mg/dL); Cys: Cystatin C concentration (mg/L); age (by years)

2.2.2. Albuminuria/Proteinuria

Following the KDIGO guideline ('KDIGO Guideline', 2022), a high urine protein or albumin concentration, together with GFR, is the baseline for the diagnosis and classification

of kidney disease. Albuminuria is the accepted reference for screening, classification and prognosis of chronic kidney disease (Levey et al., 2020).

A healthy adult should eliminate less than 150 mg of protein in urine daily, of which less than 30 mg should be albumin. The presence of proteinuria is associated with a negative progression of CKD. The appearance of proteinuria may be a marker, mediator or cause of nephropathy progression. According to several studies, an increase in the severity of albuminuria affects comorbidity or increased CV risk. Furthermore, albuminuria has a renal toxic effect, contributes to the loss of nephron mass and may induce inflammation (Bover et al., 2008; Murton et al., 2021).

There are several ways to assess albuminuria, with the most common method being the albumin-to-creatinine ratio (ACR). Table 4 shows the classification of CKD according to albuminuria levels: A1 for normal to mild increase, A2 for moderate increase and A3 for significant increase. The ACR is determined by analysing a single urine sample, preferably collected from the first morning urine. It can be expressed as mg/mmol or mg/g. Another method to determine albuminuria is the albumin excretion index (AEI), which measures how much albumin is excreted in 24-hour urine collection. Although AEI offers greater sensitivity in predicting albuminuria, it is not commonly used due to practical limitations associated with accurately collecting all of the urine needed for measurement. In cases where directly measuring albuminuria is not possible, a reasonable approximation can be made using urine dipsticks. Nevertheless, this approach loses some accuracy ('KDIGO Guideline', 2022) and should only be used when more precise methods are unavailable or not feasible (Zhang et al., 2018).

Table 4. Categorization of Albuminuria Levels

Category	AEI (mg/24h)	ACR (mg/mmol)	ACR (mg/g)	Clinical relevance
A1	<30	<3	<30	Normal to mild increase
A2	30-300	3-30	30-300	Moderate increase
A3	>300	>30	>300	Major increase

2.2.3. New biomarkers of kidney disease

The development of new biomarkers, in urine, plasma or serum, should focus both on finding robust early markers and on markers of specific types of kidney injury. The study of specific biomarkers would allow the identification of kidney damage, and should reflect the underlying pathophysiological processes of kidney damage, namely changes in renal function, tubulointerstitial damage, endothelial dysfunction and inflammation, and/or CV risk (Lousa et al., 2021).

According to the review by Lousa et al (Lousa et al., 2021), several new biomarkers have been identified over the last 20 years as promising candidates for the treatment of CKD, namely Beta-trace protein (BTP) and Beta2 microglobulin, Klotho, neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule-1 (KIM-1), N-acetil-D-glucosaminidasa (NAG), liver-type fatty acid-binding protein (L-FABP), uromodulina (UMOD), asymmetric dimethylarginine (ADMA), fetuina-A, as well as markers of inflammation and metabolic profiles (Figure 9).

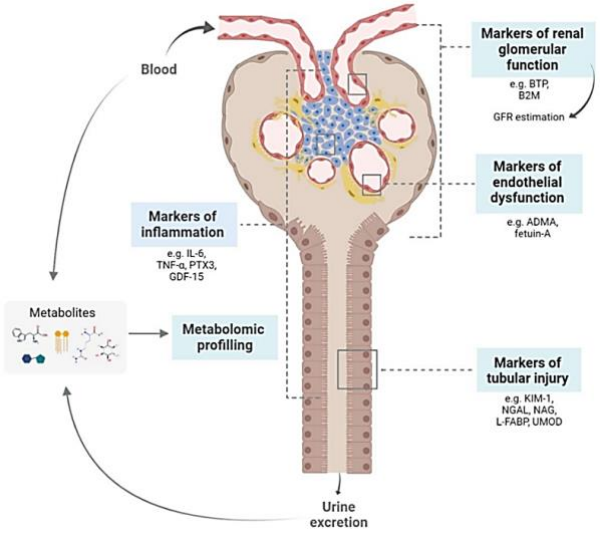

Figure 9. Biomarkers of chronic kidney disease according to anatomical location and/or site of production (Lousa et al., 2021).

In this thesis we have focused on inflammation, in particular on the role of prostaglandins in kidney damage. Several studies suggest that activation of inflammatory processes in the early stages of CKD leads to deterioration of renal function and propose that assessment of inflammatory markers could help in earlier diagnosis of CKD. In addition, inflammation is a risk factor for CKD-associated morbidity and may contribute to CV mortality in CKD patients.

2.3. Prostaglandin E2 metabolic pathway

Certain cell surface receptor molecules also have the ability to detect hydrophobic or lipophilic compounds that function as hormones. The main set of signalling molecules that fall into this category are known as prostaglandins.

Prostaglandins are derived from arachidonic acid, a 20-carbon fatty acid that is usually found covalently attached to the glycerol backbone of the phospholipids which make up the plasma membrane. Initially, arachidonic acid is released, by hydrolysis, through the action of the enzyme phospholipase A2 (PLA_2). Once released, oxygenation by cyclooxygenase (COX) begins, giving rise to prostaglandin G2 endoperoxide (PGG_2). Prostaglandin G/H synthase has the catalytic sites of cyclooxygenase which converts arachidonic acid to PGG_2, which is acted upon by the enzyme peroxidase and reduced to the unstable intermediate, PGH_2, responsible for the formation of different types of prostanoids such as prostaglandin E2 (PGE2), which, as we describe below, is the main prostaglandin involved in renal injury (Patrignani et al., 2015; Rimon et al., 2010).

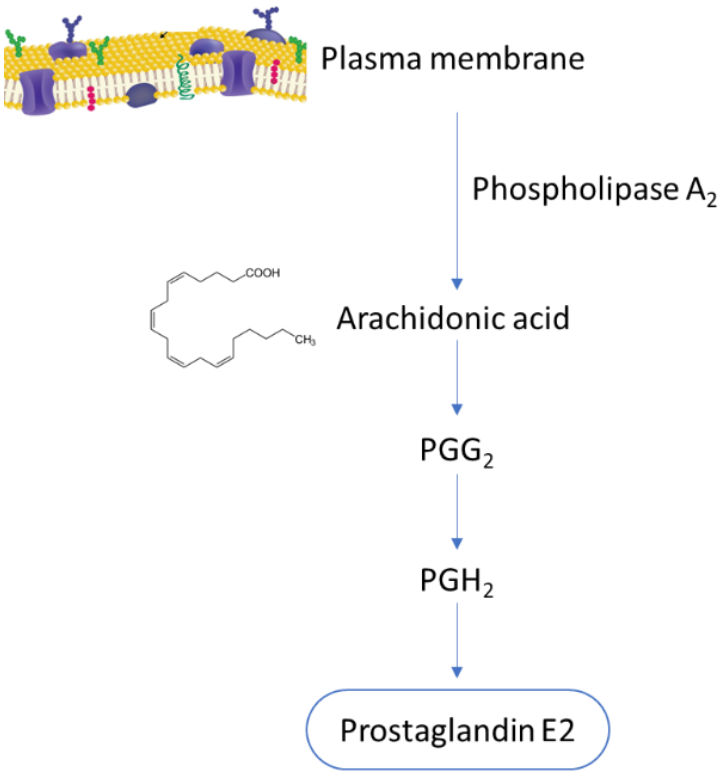

Figure 10. Prostaglandin E2 metabolic pathway.

2.3.1. Physiological functions of prostaglandin E2

Prostaglandin E2 (PGE2) serves as the primary arachidonic acid metabolite in the kidney (Wang et al., 2018), governing both renal homeostasis and pathological mechanisms such as inflammation, hyperfiltration, fibrosis, apoptosis, and the activation of the renin-angiotensin-aldosterone system (RAAS) (Francois et al., 2007; Nasrallah et al., 2014, 2016). The synthesis of PGE2 is directly regulated by cytosolic synthase (cPGES) and two microsomal synthases (mPGES1 and mPGES2), whose activities have been linked to renal dysfunction and elevated blood pressure (Gao et al., 2016; Regner, 2012).

The cyclooxygenase (COX), the principal enzymatic catalyst in prostanoid synthesis, is known to possess two main isoforms, COX-1 and COX-2 (Akyazi et al., 2013). COX-1 was initially referred to as physiological or constitutive due to its presence in the majority of tissues and its nearly constant expression (Kirkby et al., 2013; Kummer et al., 2002). Its primary function lies in maintaining a basal rate of prostanoid biosynthesis within the organism, facilitating swift and short-lived increases as required. In contrast, COX-2 is found in fewer tissues, yet its production surges approximately 20-fold in the presence of inflammatory stimuli, leading to its classification as a pathological or inducible enzyme (Barudzic et al., 2013; Kirkby et al., 2013; Kummer et al., 2002).

COX-1 is present within the glomeruli and is also localized in both the medullary and cortical regions of the collecting ducts. Furthermore, it is found in the afferent and efferent arterioles, contributing to the maintenance of the essential physiological functions of kidney, including the regulation of hemodynamic and GFR (DeMaria et al., 2003; Kummer et al., 2002; Moore et al., 2015; Nantel et al., 1999). This specific isoform is responsible for the generation of PGE2 and PGD2, which counteract the vasoconstrictive effects initiated by angiotensin II (Ang II) and inhibit the release of norepinephrine, respectively. Consequently, these prostanoids facilitate vasodilation, augmenting organ perfusion and causing a redistribution of blood flow from the renal cortex to the intramedullary nephrons. Additionally, PGE2, along with PGF2α, demonstrates diuretic and natriuretic effects, while PGE2, akin to PGI2, counterbalances the effects of vasopressin (Batlouni, 2010).

Despite the initial classification of COX-2 as pathological, it is also found to be constitutively present in the kidneys (Ahmetaj-Shala et al., 2015; DeMaria et al., 2003). Investigations involving COX-2 knockout rats have unveiled a significant impairment in renal development, indicating that this isoform plays a pivotal role in kidney maturation (Zhang et al., 1997). COX-2 is predominantly located in regions of utmost importance for renal function,

primarily within the medullary region and to a lesser extent in the cortical region (Ahmetaj-Shala et al., 2015). Specifically, it is expressed in the smooth muscle cells of afferent and efferent arterioles, the endothelium (including straight venules and vasa recta), the renal artery, interstitial fibroblasts, and podocytes in healthy kidneys (Khan et al., 1998). It is also detected in the thick ascending limb of the Henle loop and the macula dense, which mediates the interaction between glomerular filtration and proximal reabsorption. Moreover, it exerts control over the levels of sodium and potassium ions in the distal tubule lumen through the renin-angiotensin-aldosterone system, further underlining its significance in renal regulation (DuBois et al., 1998; S. Hao et al., 2014; Kaminska et al., 2014; Nantel et al., 1999; Nørregaard et al., 2015).

2.3.2. Role of prostaglandin E2 in kidney function

Prostaglandin E2 (PGE2) exerts a significant and multifaceted influence on kidney function. As a lipid mediator arising from arachidonic acid metabolism, it acts through specific EP receptors, yielding protective and regulatory functions within the kidneys that impact various facets of renal physiology.

PGE2 encourages vasodilation in renal blood vessels through EP receptor interaction, relaxing afferent arteriole smooth muscle cells and enhancing blood flow towards glomeruli. This vasodilation sustains a steady glomerular filtration rate by controlling blood flow into glomerular capillaries. Hypovolemia entails a reduction in blood volume, primarily stemming from excessive water loss within the kidney or haemorrhage, resulting in a decline in arterial pressure that may lead to hypovolemic shock and potentially fatal outcomes (Batlouni, 2010; Kummer et al., 2002). During hypovolemia, when sodium levels are depleted, COX-2 expression increases in the cortical region and diminishes in the medullary region, instigating the activation of the renin-angiotensin-aldosterone system. Juxtaglomerular cells secrete renin, which aids in Ang II formation. Ang II constricts the efferent arteriole along with norepinephrine, elevating intraglomerular hydrostatic pressure. Aldosterone secretion is also triggered, promoting sodium reabsorption and prompting the release of antidiuretic hormone (ADH), enhancing water and urea reabsorption. Simultaneously, angiotensin stimulates the synthesis of renal vasodilator prostaglandins (afferent arteriole), which restores the tubule glomerular feedback mechanism, thereby re-establishing glomerular filtration rate and safeguarding normal blood flow to prevent acute renal functional decline (Batlouni, 2010; Curiel et al., 2013; Kummer et al., 2002; Lipsky, 2000; Lomas et al., 2015).

Renal pathology is characterized by an inflammatory response and oxidative stress (Liu et al., 2015), with heightened COX-2 expression exacerbating inflammation. Utilizing a selective COX-2 inhibiting nonsteroidal anti-inflammatory drugs (NSAID), or Coxib, may prove beneficial for renal function by mitigating oxidative injury (Liu et al., 2015; Nørregaard et al., 2015; Wang et al., 2015). Specifically, parecoxib has demonstrated renal protection (Neto et al., 2015). Similarly, a study employing indomethacin, a non-selective NSAID, and rofecoxib showcased enhanced renal function and reduced proinflammatory cytokine levels (Feitoza et al., 2008). Finally, a COX-2 selective inhibitor drug, was also shown to exhibit preventive effects against renal damage (Suleyman et al., 2015).

In addition, in a hypertensive state, COX-2 expression also experiences an increase; however, unlike hypotension, this upregulation takes place in the medullary tissues, aiming to mitigate the vasoconstrictive and hypertension effects induced by Ang II. This mechanism seeks to restore sodium and water regulation while maintaining medullary blood flow (Gonzalez et al., 2014). In this context, the administration of any NSAIDs worsens hypertension and should be steered clear of. The inhibition of PGE2 by drugs leads to sodium retention, which further amplifies the adverse process (Harris, 2013). NSAIDs can inhibit PG synthesis, potentially resulting in a reduction of renin release and subsequent decrease in blood pressure for certain individuals. However, it should be noted that elderly individuals or those with already low levels of renin may not experience the compensatory decline in renin-mediated vasoconstriction along with the loss of PGI2- and PGE2-induced vasodilation leading us to suggest additional mechanisms could be activated by individual genetic components which may be influencing the increase in blood pressure (Nasjletti, 1998; Wilson et al., 2006).

Participating in immune response and inflammation modulation within kidneys, PGE2 exhibits context-dependent behavior, both promoting and suppressing inflammation (Song et al., 2015). In pathological contexts, this activation leads to the generation of proinflammatory cytokines (such as interleukin-1 and TNF-α) and reactive oxygen species (ROS), which hold a crucial role in renal physiopathology (Feng et al., 1995; Jia et al., 2015). Moreover, positive feedback mechanisms operating through signalling pathways contribute to the advancement and progression of the disease (Feng et al., 1995; Fujihara et al., 2003; Jia et al., 2015). Authors emphasize that inflammation serves as a causal factor that collaborates with renal lesions, particularly concerning PGE2. While PGE2 contributes to the maintenance of renal homeostasis in physiological conditions (Hao et al., 2007; Jia et al., 2015; Kinsey et al., 2013), it might be accountable for triggering a detrimental process in pathological situations (Jia et al., 2015).

The reason for the wide array of PGE2 functions in different clinical settings can be attributed to its interaction with four different receptors: EP1, EP2, EP3 and EP4 (Jia et al., 2015; Nasrallah et al., 2014). Thus, the regulation of natriuresis, diuresis and blood pressure are controlled by EP1. On the other hand, EP2 and EP4 receptors stimulate adenylate cyclase, leading to increased levels of cyclic adenosine monophosphate (cAMP) which promotes water reabsorption. The presence of EP4 has been linked to a protective function, as demonstrated by experiments with mice lacking EP4 that developed fibrosis and showed elevated levels of various inflammatory markers (Nakagawa et al., 2012). The interaction of PGE2 with EP4 is also responsible for maintaining podocyte integrity and reducing the occurrence of proteinuria in diabetes (Faour et al., 2010). However, other research suggests that activation of EP4 may have detrimental consequences such as glomerulosclerosis and tubulointerstitial fibrosis in diabetes (Mohamed et al., 2013). In contrast, EP3 has the opposite effect, as it mainly facilitates body water homeostasis, especially in situations of high salinity (Hao et al., 2016).

2.3.3. Genetics variants in prostaglandin E2 pathway

Genetic differences in the prostaglandin E2 (PGE2) pathway can influence how the body produces, metabolises and interacts with PGE2. This has potential effects on a number of processes including both normal physiological functions and abnormal pathological conditions. A significant number of these genetic variations occur in single nucleotide polymorphisms (SNPs), which involve changes in individual building blocks of DNA known as nucleotides. These alterations can influence the structure, function or expression levels of proteins. When specific genetic variants appear within the PGE2 pathway, they contribute to unique responses of individuals to PGE2 itself and thus affect several aspects of physiology and pathology. These genetic factors play a role in determining susceptibility not only to inflammatory disorders, but also influence pain perception along with CV risk, among other outcomes specifically affected by PGE (Nakagawa et al., 2012). Investigation of these different genetic variants helps to uncover the underlying mechanisms of disease and, at the same time, can inform personalised treatment approaches relevant to effective disease management by taking into account each individual sufferer's unique biological traits. In this section we will explore some specific examples of particular genetic variants associated with this essential pathway (Figure 11). The published human population genetic variants for each part of this pathway are summarised in tables 5, 6 and 7 of this section.

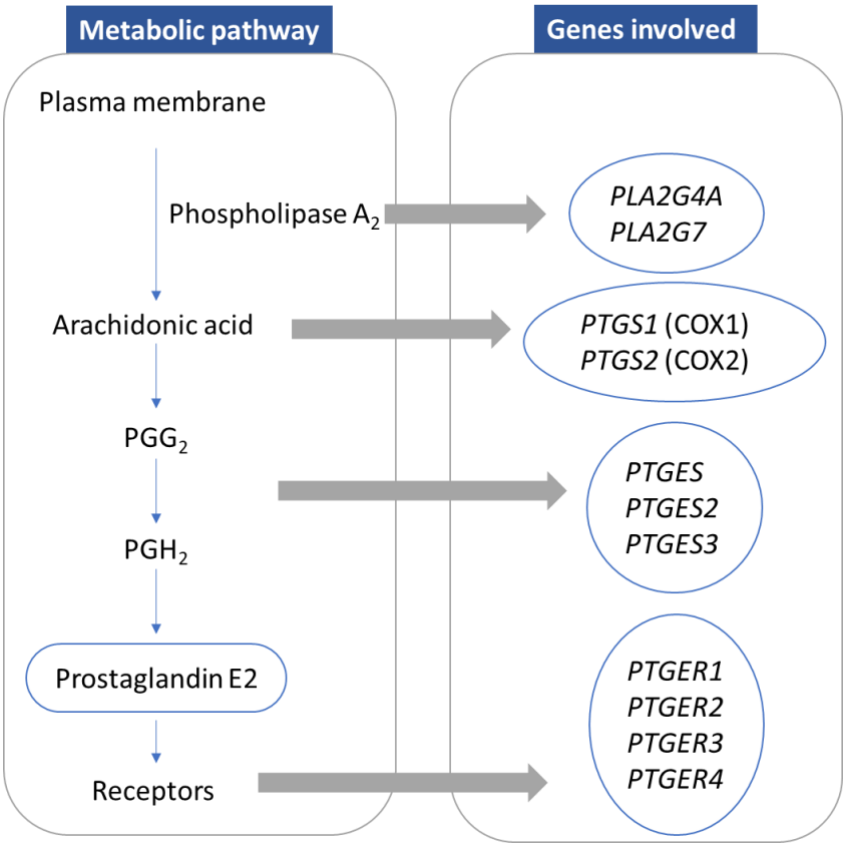

Figure 11. Main genes participating in the prostaglandin E2 pathway

The presence of certain genes in lipid metabolism, particularly those associated with fatty acid synthesis and metabolism, can indirectly impact the amount of arachidonic acid available for PGE2 production. Variations in these genes can affect levels of precursor fatty acids, thereby affecting the production of PGE2. Plasma lipoprotein-associated phospholipase A2 (Lp-PLA2) is an enzyme encoded by the *PLA2G7* gene. It has been found to convert low-density lipoproteins (LDL) into various substances that promote inflammation. Increased levels of Lp-PLA2 have been observed in conditions like vascular endothelial dysfunction, inflammation and necrotic core formation in atherosclerotic plaques. Because of this, it is being considered as a potential marker for predicting CV diseases related to atherosclerosis (Huang et al., 2020). Additionally, certain genetic variants within the *PLA2G7* gene have shown associations with both Lp-PLA2 activity and risk of developing atherosclerosis (Santoso et al., 2017; Suchindran et al., 2010). Another important gene called scavenger receptor class B1(*SCARB1*) (Manichaikul et al., 2018), which plays a role in cholesterol transport via high-density lipoproteins (HDL), also seems to be associated with variations in Lp-PLA2 activity and susceptibility to CV disease (Koenig et al., 2021; Ma et al., 2018; Manichaikul et al., 2018). There is another form of phospholipase A known as cytosolic

calcium-dependent phospholipase A alpha (cPLAα), which produced by the *PLA4GA* gene was found to be activated through increased cellular calcium concentration. It releases arachidonic acid lead into synthesis of proinflammatory compounds the overexpression of cPLAα (Bonetti et al., 2020; Clark et al., 1991; Elinder et al., 1997). In addition, certain SNPs present in the phospholipase A2 gene (*PLA24GA*), at an early stage of the initiation of the enzymatic cascade, are associated with an increased risk of infarction according studies (Hartiala et al., 2012).

Table 5. Summary of published human population genetic variants in phospholipase-related genes

Sample	Ethnicity	Outcome	Results	References
Meta-analysis	Meta-analysis	Risk of developing atherosclerosis	R92H SNP in *PLA2G7* gene might contribute to increased risk of clinical atherosclerosis	(Santoso et al., 2017)
6.668 subjects	Caucasian	Risk of developing atherosclerosis	rs1051931 in *PLA2G7* gene is linked with Lp-PLA2 activity and risk of developing atherosclerosis	(Suchindran et al., 2010)
2 brothers	Blacks	Risk of CV disease	Demonstrates the existence of loss-of-function *SCARB1* variants and their clinical relevance	(Koenig et al., 2021)
Meta-analysis	Meta-analysis	Risk of CV disease	Polymorphism rs5888 had a negative association with coronary heart disease	(Ma et al., 2018)
7.806 subjects	Multi-Ethnic	Risk of CV disease	*SCARB1* rs10846744 is significantly associated with Lp-PLA2 activity, atherosclerosis, and CVD events	(Manichaikul et al., 2018)

Genetic variability could influence the functionality of COX-1 and COX-2 genes, affecting prostaglandin levels. Variations in these genes can lead to changes in enzyme efficiency or increased expression of COX-2, leading to increased PGE2 synthesis during inflammatory responses. Several clinical studies have found a link between these genetic variations and CV risk. In particular, two specific polymorphisms (rs5277 and rs20417) within the COX-2 gene are associated with an increased likelihood of developing coronary heart disease as well as a likelihood of suffering major CV events (Liu et al., 2017; Ross et al., 2014). In addition, variants such as rs20417, rs689466 located in the promoter region of COX-2 along with another variant called rs3842787 found within COX-1 have been linked to cerebrovascular

incidents and development of vulnerable carotid plaque (Oliveira-Filho et al., 2015; Yi et al., 2016, 2017).

Table 6. Summary of published human genetic variants in genes leading to prostaglandin E2 (PGE2) synthesis

Sample	Ethnicity	Outcome	Results	References
1544 subjects	Various	Risk of CV disease	*COX2* rs5277 C allele increases the risk of left main coronary artery lesion	(Liu et al., 2017)
49.232 subjects	Various	Risk of CV disease	Polymorphim rs20417 within the *COX2* gene is associated with coronary heart disease	(Ross et al., 2014)
928 subjects	Caucasians and Hispanics	Cerebrovascular disease	Three SNPsx in *COX2* (rs19545927, rs20417, and rs201231411) were associated with brain cerebrovascular disease	(Oliveira-Filho et al., 2015)
850 subjects	Chinese	Acute ischemic stroke	The interactions of rs3842787 (*COX1*) and rs20417 (*COX2*) were associated with aspirin resistance	(Yi et al., 2016)
687 subjects	Chinese	Acute ischemic stroke	Polymorphim rs20417 in *COX2* is linked to cerebrovascular incidents and carotid plaque	(Yi et al., 2017)

PTGER1-4 genes are responsible for encoding receptors which mediate effects of PGE2. Variations in these *PTGER1-4* genes can affect the efficiency in which these receptors bind to PGE2 and transmit signals. These different variants have the potential to cause changes in cellular reactions to PGE2, affecting processes such as inflammation, vasodilation and PGE2-facilitated immune responses. The EP1 receptor gene has been found to be involved in hyperfiltration, albuminuria and kidney damage. Deletion or inactivation of this gene may be protective against these conditions (Makino et al., 2002; Suganami et al., 2003). The EP2 receptor is mainly located in the vascular and interstitial compartments of the kidney. Studies in mouse have shown deletion of *PTGER2* gene leads to salt-sensitive hypertension (Kennedy et al., 1999). Researchers such as Bigler et al. have discovered several SNPs in PGE2 receptors (EP1-4) as well as in PGE2 synthase, which may affect the function of the protein, but these authors have not yet thoroughly examined their association with specific

health problems (Bigler et al., 2007). In particular, a SNP known as rs17197 within the *PTGER2* gene has been linked to essential hypertension (Sato et al., 2007).

Table 7. Summary of published human genetic variants in genes coding for PGE2 Receptors

Sample	Ethnicity	Outcome	Results	References
600 subjects	Caucasian	Risk of atherothrombosis	rs708494 and rs708495 in *PTGER2* reduced risk of ischemic stroke	(Hegener et al., 2006)
3.971 subjects	Hispanic	Risk of CV disease	Association between rs12746200 with risk of CV disease	(Hartiala et al., 2012)

2.3.4. Prostaglandin E2 as a therapeutic target

Maintaining a delicate equilibrium in the effects of PGE2 plays a pivotal role in upholding regular kidney function and overall homeostasis. Disruption in the levels of PGE2 can play a contributory role in kidney disorders like renal inflammation, hypertension, and disturbances in sodium and water equilibrium. Interventions utilizing pharmacological agents that target the PGE2 pathway, like NSAIDs, can yield therapeutic benefits, while also bearing the potential for unintended consequences on kidney function. Consequently, comprehending the intricate functions of PGE2 within kidney physiology is imperative for formulating precise therapeutic approaches and effectively managing renal conditions.

NSAIDs are a class of pharmacological drugs used to treat inflammation. NSAIDs, once consumed over a prolonged period of time, can decrease prostaglandins in the kidney and cause hypertension (Romero & Reckelhoff, 1999). In addition, NSAID use has been associated with an increased risk of CV problems due to its impact on PGs, suggesting that PGs play a role in the development of heart disease (Fanelli et al., 2017). This information highlights how inflammation contributes to nephrosclerosis. Consequently, inflammatory changes occur at an early stage of CKD, underscoring the importance of controlling the inflammatory response as a means of slowing kidney damage (O'Sullivan et al., 2017; Rodrigues-Diez et al., 2021). The initial generation of NSAIDs inhibited PG synthesis through non-specific pathways involving both COX-1 and COX-2. However, prolonged administration of these drugs led to severe side effects such as renal and gastrointestinal toxicity (Chakraborti et al., 2010). Recently, novel NSAIDs have been developed that

selectively inhibit COX-2, such as Celecoxib or Rofecoxib (Gilroy & Colville-Nash, 2000). In addition, antagonists and agonists targeting several PG receptors have been formulated to regulate their signalling and actions, with satisfactory results in a variety of animal models, for example, the EP4 antagonist and their efficacy in mouse models of diseases such as autoimmune encephalitis or arthritis (Esaki et al., 2010).

Objectives

3. Objectives

The **main objective** of this thesis was to identify genetic biomarkers of cardiorenal function in patients with vascular nephropathy.

The **specific objectives** of this study are:

1. To determine the variability of 10 genes of the PGE2 pathway in patients diagnosed with nephrosclerosis and healthy controls using two complementary approaches:
    a. Genotyping of tag-SNPs, which define the variability of the pathway studied in Caucasian Europeans.
    b. Genotyping of variants in this pathway that have been previously associated with cardiorenal function.
2. To retrospectively determine the association of the identified genetic polymorphisms with clinical variables and relevant events extracted from the clinical histories of the patients:
    a. Renal function parameters (creatinine clearance, proteinuria, etc.)
    b. Blood pressure traits
    c. CV events in the four-year follow-up, which included: acute myocardial infarction, acute coronary syndrome, coronary catheterization requiring angioplasty, coronary bypass, typical angina with positive stress tests, sudden death, cerebrovascular accident, peripheral arterial disease, aortic aneurysm, and lower limb ischemia.
3. To develop models that, integrating clinical, demographic and genetic variables, help to predict the clinical evolution of the patient and the appearance of unfavourable events.

Results and Discussion

4. Results and Discussion

Three scientific papers have been included in the compilation of this thesis. These articles present a comprehensive set of results that have been thoroughly discussed in order to clarify the issues introduced at the beginning of each study. All the objectives outlined in the previous section were met.

The three studies were conducted with the same population. Given the extensive array of genes comprising the PGE2 metabolic pathway, a strategic choice was made to segment it into three distinct major sections. This approach aimed to enhance the precision of our evaluation and facilitate comprehensive discussions concerning findings obtained. The clinical and demographic variables of the study cohort are shown in table 8. Whole blood samples were drawn from the participants recruited and were genotyped by allelic discrimination using TaqMan® OpenArray Genotyping (Waltham, MA, USA).

We performed a genetic analysis to identify variations in the genes responsible for prostaglandin PGE2 receptors (*PTGER1-4*). The objective was to establish connections between these genetic variants, along with other significant non-genetic risk factors, and the occurrence of nephrosclerosis. This research aimed at enhancing both diagnosis and treatment strategies for this complication.

Regarding the genetic analysis, 96 tag-SNPs representing the genetic variability of specific regions within the 4 genes coding for EP1-4 (6 SNPs in *PTGER1*, 8 SNPs in *PTGER2*, 73 SNPs in *PTGER3*, and 9 SNPs in *PTGER4*) were genotyped. Among these SNPs, one in *PTGER2* and six in *PTGER3* did not conform to Hardy-Weinberg equilibrium and were consequently, excluded from subsequent analyses.

After the Hardy-Weinberg analysis, associations between the remaining 89 SNPs and the risk of nephrosclerosis were examined in all inheritance models. Ten of these SNPs, all located

Table 8. Clinical and demographic characteristics of the population of this thesis

	Control (N=716)	CKD3 (N=307)	CKD4-5 (N=123)	CKD 5D (N=63)	Total (N=1209)	p-value Control vs. CKD	p-value between CKD groups
Age (yrs)	60 (51-68)	67 (61-71)	64 (57-71)	61 (50-68)	62 (54-69)	6.2E-17	1.05E-04
Males (%)	359 (50.2)	211 (68.7)	80 (65.0)	43 (68.3)	693 (57.4)	7.87E-10	0.758
Ethnicity							
Caucasian	709 (99.0)	304 (99.0)	120 (97.6)	58 (92.1)	1191 (98.5)	0.065	0.003
Others	7 (1.0)	3 (1.0)	3 (2.4)	5 (7.9)	18 (1.5)		
Weight (kg)	75.2 (66-85.8)	80.3 (72-88.6)	78.6 (67.5-87.2)	75 (64.8-86)	77.3 (67.6-86.7)	1.1E-05	0.030
BMI	30.4 (26.4-93)	30.3 (27.5-34.6)	28.8 (25.8-33.1)	27 (24.2-31.1)	30 (26.5-37.5)	1.6E-04	7.0E-06
Hypertension	405 (56.6)	293 (95.4)	121 (98.4)	60 (95.2)	879 (72.8)	1.95E-61	0.333
DM	55 (7.7)	74 (24.1)	32 (26.0)	8 (12.7)	169 (14.0)	4.23E-14	0.100
Hyperlipidemia	175 (35.9)	184 (67.2)	91 (74.0)	39 (61.9)	489 (51.6)	7.43E-24	0.203
Smoking							
Non-smoker	328 (45.8)	124 (40.4)	53 (43.1)	26 (41.3)	531 (43.9)	0.255	0.929
Current-smoker	125 (17.5)	53 (17.3)	22 (17.9)	13 (20.6)	213 (17.6)		
Former-smoker	263 (36.7)	130 (42.3)	48 (39.0)	24 (38.1)	465 (38.5)		
Systolic lood pressure (mmHg)	136 (124-149)	145 (132-160)	146 (133.5-161)	139 (130-155)	139 (127-154)	3.08E-14	0.162
Dyastolic blood pressure (mmHg)	81 (74-88)	81 (75-89)	79 (73-88)	80 (70-90)	81 (74-88.5)	0.838	0.410
Pulse pressure (mmHg)	54 (46-64.5)	64 (51-77)	64 (55-76.5)	60 (50-70)	57 (48-70)	5.6E-20	0.123
Total cholesterol (mg/dL)	200.7 (180-222.8)	185 (159.8-213.3)	175.5 (148.5-203)	160.5 (137.8-185.5)	192 (165-216)	1.9E-17	1.9E-05
CV events	9 (1.3)	25 (8.1)	10 (8.1)	6 (9.5)	50 (4.1)	1.33E-09	0.933
Creatinine (mg/dL)	0.8 (0.7-1)	1.5 (1.3-1.7)	2.9 (2.5-3.6)		1 (0.8-1.6)	1.06E-102	3.09E-40
Albumin/Creatinine (mg/g)	6.4 (4-41.2)	37.1 (7.5-195.1)	212.1 (46-601)		33.4 (5.1-182.4)	1.27E-11	3.6E-05
eGFR (ml/min/1.73 m²)	88.8 (78.7-100)	45 (37.4-50.8)	21 (16.2-25.6)		71.2 (40.2-90.1)	2.31E-143	4.39E-56

in the *PTGER3* gene, exhibited a significant association with the disease risk (Table 3 in Article 1). They were collectively included in a multiple regression model with backward elimination, controlling for other covariates. Four SNPs remained in the final model, of which two, rs11209730 and rs10399704, continued to exhibit a statistically significant association with the risk of nephrosclerosis [OR = 1.45 (1.07-1.95), p = 0.016, and OR = 0.71 (0.51-0.99), p = 0.041, respectively]. The genetic variability at the *PTGER3* gene locus has not been extensively studied, but it is established that the administration of EP3 antagonists in vivo leads to increased COX-2-mediated PGE2 expression in the kidney, while receptor activation decreases PGE2 levels (Hao et al., 2016). This is pertinent to the development of nephrosclerosis, as PGE2 plays a significant role in kidney disease by influencing albuminuria, growth/fibrosis, and the activation of the renin-angiotensin-aldosterone system (RAAS) (Nasrallah et al., 2016). Another hypothesis posits that CKD risk is modulated by the impact of genetic variability on the direct actions of EP3, such as vasoconstriction (Liu et al., 2020), water balance (Wang et al., 2008), regulation of renal blood flow (Audoly et al., 2001), or maintenance of renal cell integrity (Kvirkvelia et al., 2013).

Concerning the renal function of the 430 patients with nephrosclerosis, the genetic influence of the 89 SNPs was analysed with adjustments made for sex, age, ethnicity, hypertension, diabetes, and CKD stage. Notably, ten variants in *PTGER3*, predominantly situated in the proximal region of the gene, displayed a significant association with eGFR, exhibiting p-values ranging from 0.0003 to 0.038. Conversely, associations with the albumin/creatinine ratio were less pronounced and were also limited to *PTGER3*. The first two variants of the gene, namely rs61777096 and rs6656853, were the only ones that significantly impacted both eGFR and proteinuria. While the precise genetic consequences in this domain remain speculative, it is highly probable that the presence of functional polymorphisms linked to these tag-SNPs may exert a substantial influence on eGFR values, given that one of the key functions of EP3 is to mitigate hyperfiltration through afferent arteriole constriction (Sato et al., 2007; Tang et al., 2000).

As mentioned earlier, there is very limited information regarding the clinical impact of these SNPs in *PTGER3*. To our knowledge, there is only one report indicating an association of rs2268062 with hypertension in the general population, although this finding could not be replicated in a validation sample (Sõber et al., 2009). In our study sample, we did not find an association between this SNP and hypertension. It is important to note that the high prevalence of hypertension, affecting 95.7% of our nephrosclerosis patients, makes it more challenging to identify genetic markers for this trait in this particular cohort compared to a

general population setting. Except for *PTGER4* rs16870224, all variants affecting blood pressure traits were found to be located in *PTGER3*, primarily in a distal region of the gene, as shown in figure 2 of the first article. This observation aligns with prior in vitro data and animal studies. Rodent models have demonstrated that the administration of selective EP3 agonists induces an acute and significant increase in arterial pressure (Zhang et al., 2000), and the pressor action of PGE2 is mediated through EP3 (Swan et al., 2011). Moreover, our findings indicate that only variants in *PTGER3* are relevant for blood pressure regulation. Additionally, it has been reported that the expression levels of EP3 in renal resistance vessels and aorta are considerably higher compared to other EP receptors, and its vasopressor effects surpass the vasodilator effects of EP2 and EP4 (Zhang et al., 2000). This further underscores the significance of certain *PTGER3* SNPs identified in this study, along with the tagged regions. Notably, some of these variants showed significant associations with both nephrosclerosis risk and clinical variables in the renal patients. Specifically, among the 89 polymorphisms examined, rs11209708 was found to increase the risk of nephrosclerosis, and it also had an impact on eGFR and pulse pressure values. Nevertheless, it is important to acknowledge that the overall effects of PGE2 mediated by EP receptors are exceedingly intricate and contingent on numerous factors beyond genetics, including additional hormonal signalling (angiotensin II, endothelin, etc.) and individual clinical status (Nasrallah et al., 2014).

All participants in the study were monitored for a duration of four years, with a median follow-up period of approximately 47 months (range =7-54). Within this time frame, there were a total of 41 CV events reported among patients with nephrosclerosis, accounting for an incidence rate of around 8.3%. In contrast, only nine such events occurred among control subjects representing about1.3% [OR=7.13 (3.4-14.8), p-value = 1 .32e-09].

To streamline the number of SNPs for analysis in the survival study, we conducted a preliminary association analysis of the 89 variants with the risk of experiencing a CV event under the chosen genetic model. From this analysis, we identified five SNPs that displayed significant associations. Among these, the rs7533733 GG genotype exhibited the highest odds ratio (OR) and the lowest p-value [OR = 2.65 (1.28-5.46), p = 0.01]. Subsequently, Kaplan-Meier analysis of these five polymorphisms indicated that carriers of the *PTGER1* rs2241360 T variant allele had a better event-free CV survival compared to homozygous wild-type carriers (log-rank p = 0.014). Conversely, patients homozygous for the *PTGER3* rs7533733 G variant allele experienced a higher number of events than AA/AG carriers (log-rank p = 0.007). In subsequent Cox regression models, accounting for other confounding

variables, the differences between the genotypes of the two SNPs remained statistically significant (p = 0.029 and 0.011, respectively). There is substantial evidence supporting the hypothesis that alterations in these genes may lead to modified CV risk. For instance, the activation of these two receptors increases intracellular Ca^{2+} via phospholipase C, in contrast to EP2 or EP4, which do not influence $Ca2^{+}$ levels. Voltage-dependent Ca^{2+} channels are widely distributed in the body and play a pivotal role in maintaining vascular tone. Indeed, numerous studies have demonstrated an association between CV disease and intracellular calcium dysregulation (Anzawa et al., 2012; Chopra et al., 2009; Kumar et al., 2018). Furthermore, PGE2 is present in mouse atherosclerotic plaques, where it can enhance platelet aggregation, an effect mediated solely by EP3 (Fabre et al., 2001; Gross et al., 2007). In fact, mice lacking EP3 exhibit reduced thrombosis severity following arachidonic acid administration (Ma et al., 2001), and atherothrombosis mechanical plaque rupture is significantly decreased when platelets lack EP3 (Gross et al., 2007).

After that, we performed a genetic analysis to identify variants in the genes responsible for the first step of the PGE2 pathway. Eighty-six SNPs were analysed: 5 in *SCARB1*, 17 in *PLA2G7*, and 64 in *PLA2G4A*, representing the genetic variability of these 3 genes. These genes are involved in the synthesis of pro-inflammatory mediators which trigger the signalling cascade from the membrane. *PLA2G4A* gene encodes calcium-dependent cytosolic phospholipase, *PLA2G7* gene encodes plasma lipoprotein-associated phospholipase A2 which transforms low-density lipoprotein into a proinflammatory mediators and *SCARB1* gene encodes a primary receptor for selective transportation of cholesterol and also a typical locus for CV disease.

Six SNPs significantly differed ($p < 0.05$) from the Hardy-Weinberg equilibrium in the control population and were therefore ruled out from further analyses. Two out of the five SNPs analysed in the *SCARB1* gene and three additional variants in *PLA2G4A* demonstrated significant associations with nephrosclerosis risk after accounting for important variables such as age, sex, body mass index, ethnicity, diabetes status, and blood pressure. The two identified *SCARB1* variants (rs10846744 and rs838880) exhibited considerably lower p-values compared to the *PLA2G4A* SNPs. Their odds ratios (OR) were 0.66 (95% CI: 0.51-0.87; p = 0.003) and 1.48 (95% CI: 1.11-1.96; p=0.007), respectively. The encoded protein by *SCARB1* known as SR-BI receptor plays a crucial role in selective lipid transfer from HDL to cells while also facilitating internalization of oxidized phospholipids according to previous studies conducted by (Gillotte-Taylor et al., 2001). Native HDL does not possess similar effects on inflammatory cytokine production like oxidized HDL has been

linked to increased inflammation-causing properties observed in various renal cells including mesangial cells (Gao et al., 2014; Zhang et al., 2010). Additionally, expression pattern of SR-BI is comparatively higher in certain cell types, indicating a potential regulatory role in nephrosclerosis. With regard to the three *PLA2G4A* variants associated with nephrosclerosis risk, two were on the verge of statistical significance, whilst the third, rs78178583, displayed a lower p-value. This is an intronic A to C substitution found near the gene promoter (ENSR00000980) for ENSR00000934514. It's reasonable to speculate that this genetic variability may influence cPLA2 expression levels and subsequently impact CKD risk since cPLA2 expression has been associated with kidney damage in humans as well as animal models (Montford et al., 2016). One potential mechanism could be that this enzyme triggers AA release from the membrane, which then leads to synthesis of proinflammatory mediators like PGE2 a key mediator involved in causing kidney injury (Francois et al., 2007).

Nephrosclerosis and atherosclerosis share common risk factors and etiopathogenic mechanisms (Meyrier, 1996). In this study, we examined atherosclerosis parameters in association with genetic variability of genes included. The left and right common carotid arteries were explored anterolaterally, posterolaterally and mediolaterally. The analysis used the mean of the three measurements from the right and left to obtain a single common carotid intima-media thickness (ccIMT) variable. The ccIMT values were significantly higher in patients than in controls, as were the presence of atheromatous plaques (79.4% vs. 55.9%; p = 3.06E-14) and the total number of plaques identified, indicating a substantially higher incidence of atherosclerosis in CKD patients compared to control subjects. This could be attributed to the replacement of renal parenchyma by collagen and scar tissue in CKD patients, while control subjects show thickening and medial thickening of the arteries. Moreover, it has been proposed that increased atherosclerosis contributes to the glomerulosclerosis observed in CKD patients (Ticy et al., 1996).

Furthermore, we observed a slight but significant inverse correlation between ccIMT values and estimated glomerular filtration rate (eGFR) values in the entire study population (r = -0.181, p = 2.15E-07). In the nephrosclerosis cohort, the genetic association analysis revealed a core region of the *PLA2G4A* gene locus, tagged from rs10578509 to rs17526478 (positions 1:186873127-186882025), showing the most significant associations with glomerular filtration rate and proteinuria values. This finding is consistent with a previous Japanese study reporting an inverse correlation between ccIMT and eGFR values (Kawamoto et al., 2008), supporting the connection between kidney disease and CV issues. However, the observed correlation, while statistically significant, was weak and requires cautious interpretation.

Within the group of patients with nephrosclerosis, we observed associations between *PLA2G7* variants and the presence of atheroma plaques (7 tag-SNPs) and the number of plaques detected (6 tag-SNPs), with p-values ranging from 0.0004 to 0.047. The most notable associations with atherosclerosis measurements and *PLA2G4A* gene variability were observed with the variable "presence of atheromatous plaques", particularly for a distal region of the gene from rs72709847 onwards, which showed p-values between 0.002 and 0.040. In addition, six tag-SNPs (one in *PLA2G7* and five in *PLA2G4A*) had a significant impact on the risk of accelerated ccIMT progression (calculated by subtracting ccIMT at baseline from ccIMT at 24 months for each side) in patients with nephrosclerosis after adjusting for confounding variables. Notably, patients carrying the *PLA2G7* rs9472836-A gene showed the strongest association with a lower risk of progression [OR = 0.39 (0.19-0.80), p = 0.006].

Although information on the effect of *PLA2G4A* variants on atherosclerosis measurements is limited, there are previous reports linking *PLA2G7* SNPs to atherosclerosis. For instance, Wu et al. (Wu et al., 2020) reported that the A379V polymorphism was associated with carotid plaque formation, although not with plaque vulnerability, in patients with ischemic stroke. Similarly, Santoso et al. (Santoso et al., 2017) conducted a meta-analysis involving over 12,000 patients and identified two non-synonymous SNPs significantly associated with the risk of clinical atherosclerosis.

Our study followed 1,209 participants for a median of 47 months (range: 7-54). Among patients with nephrosclerosis, 8.3% (41 cases) experienced CV events, whereas only 1.3% (9 cases) were reported in control subjects [OR = 7.13 (95% CI: 3.4-14.8), p = 1.32e-09]. While *SCARB1* and *PLA2G7* did not harbour relevant SNPs, eight homozygous variant genotypes in the *PLA2G4A* gene significantly affected CV risk (p-values between 0.002 and 0.049). These SNPs were subjected to Kaplan-Meier analysis and then included in Cox regression models adjusting for classic CV risk factors. The results showed that the AA genotype of rs932476 significantly decreased CV event-free survival [estimated mean = 49.59 (95% CI: 47.97-51.21) vs. 51.81 (95% CI: 49.93-51.78) months for AG/GG carriers, p = 0.041,]. Additionally, carriers of the rs6683619 AA genotype also had inferior survival compared to subjects carrying the CC/CA genotypes [46.46 (95% CI: 41.00-51.92) vs. 51.17 (95% CI: 50.25-52.08) months, p = 0.022]. The main function of cPLA2, the enzyme encoded by *PLA2G4A*, is the release of arachidonic acid from the membrane, initiating a cascade of proinflammatory mediators, as well as mediating platelet aggregation (D'Emmanuele di Villa Bianca et al., 2013; González et al., 2021). Increasing evidence supports the involvement of

PLA2G4A in CV disease, as polymorphisms in this gene have been associated with myocardial infarction and coronary artery disease (Hartiala et al., 2011, 2012). Our findings are consistent with this background, and suggest that cPLA2 could be a useful therapeutic target. Indeed, cPLA2 inhibitors are currently being investigated in inflammatory disease (Nikolaou et al., 2019).

A receiver operating characteristic curve (ROC) analysis was performed to assess the predictive power of these eight variants for CV risk. Interestingly, the area under the ROC curve (AUC) values for the curves with classical and genetic-only risk parameters were quite similar ($p = 0.972$). However, the addition of both types of information resulted in a superior AUC of 79.1% (95% CI: 73.1-85.1) compared to the classical model ($p = 0.047$). Extending the analysis to both controls and patients, the combined model resulted in an elevated AUC of 84.4% (95% CI: 79.5-89.3), which was not significantly different from the model containing exclusively classical risk factors ($p = 0.135$). It is of note that the predicting improvement after adding genetic information was more substantial in patients with nephrosclerosis than in the overall study sample, as the incidence of classical risk factors is already high in the former. The association of *PLA2G7* with CVD remains controversial in the literature (Casas et al., 2010; Garg et al., 2018; Hou et al., 2009; Zheng et al., 2011). Some studies have failed to find loss-of-function alleles associated with disease risk (Gregson et al., 2017), and clinical trials with darapladib (Lp-PLA2 inhibitor) have not demonstrated consistent benefit in preventing major coronary events (White et al., 2014; O'Donoghue et al., 2014). This suggests that the genetic profile of CV risk in patients with nephrosclerosis may differ from that of the general population.

Finally, we performed a genetic analysis to identify clinically relevant variants in genes coding for cyclooxygenases COX1 (*PTGS1*), COX2 (*PTGS2*) and synthases in the cyclooxygenase pathway (*PTGES*, *PTGES2* and *PTGES3*). A total of 38 tag-SNPs (10 SNPs of *PTGS1*, 11 SNPs of *PTGS2*, 7 SNPs of *PTGES*, 2 SNPs of *PTGES2* and 8 SNPs of *PTGES3*) were screened for to determine gene variability. The *PTGES3* rs78343990 exhibited a significant deviation from the Hardy-Weinberg equilibrium ($p < 0.05$), and as a result, its findings were excluded from further consideration.

In the risk analysis, three SNPs in *PTGS2* [rs2066826, OR 0.72 (95% CI: 0.54-0.98), $p = 0.032$, rs4648268, OR 1.46 (95% CI: 1.03-2.07), $p = 0.032$, and rs20417, OR 0.70 (95% CI: 0.53-0.93), $p = 0.013$] showed significant associations with the risk of developing nephrosclerosis. These findings align with previous literature reports suggesting that elevated

COX2 levels in patients with chronic kidney disease (CKD) contribute to the disease process by mediating vascular smooth muscle cell calcification and other mechanisms (He et al., 2020). The rs20417 SNP is a G-765C base change in the promoter of gene region, which has been shown to enhance COX2 transcription (Szczeklik et al., 2004). This effect could lead to increased PGE2 synthesis and, consequently, higher inflammatory activity in the kidney, consistent with the observed elevated risk of CKD. Among the remaining significant SNPs, only the variant rs2066826 has reports on its potential clinical impact, as it has been associated with type 2 diabetes mellitus (Konheim et al., 2003), atherosclerosis (Rudock et al., 2009), and cancer (Wang et al., 2015).

Regarding the association of the 37 SNPs with blood pressure, two consecutive SNPs in the *PTGES* gene, rs11790782 and rs2241270, demonstrated a significant association with higher systolic blood pressure and diastolic blood pressure [OR=2.86 (95% CI: 0.36-5.37), p = 0.026] and [OR=2.77 (95% CI: 0.64-4.92), p = 0.011]. Dysfunction of the *PTGES* gene, which encodes microsomal prostaglandin E synthase-1, as observed in knockout mice, has been shown to promote PGI2 production, suggesting a redirection of the accumulated PGH2 substrate. This redirection prevents numerous detrimental PGE2-dependent mechanisms, such as vascular remodelling, stiffness, and endothelial dysfunction (Avendaño et al., 2018), which could explain the modulation of blood pressure traits shown by these variants.

The analysis of genetic associations with atherosclerosis measurements in the CKD group was performed using linear or binary regression analysis. Most notably, carriers of *PTGS1* rs10306194 exhibited higher values for three parameters: ccIMT progression [OR = 1.90 (95% CI: 1.07-3.36), p = 0.029], presence of carotid plaque [OR = 1.79 (95% CI: 1.06-3.01), p = 0.026], and atherosclerosis severity score (p = 0.041). This tag-SNP, marking the distal region of the gene coding for COX1, has not been extensively studied, although one report considered it clinically relevant, showing a strong association with acute urticaria/angioedema (Jurado-Escobar et al., 2021). This polymorphism induces an A to T change in the 3'-untranslated region (UTR) of *PTGS1*, the functional relevance of which remains to be elucidated. It is well-known that 3'-UTRs play crucial roles in mRNA stability, localization, and expression, as well as in protein-protein interactions. Therefore, alterations in this region may affect gene regulation and determine specific pathological conditions (Mayya & Duchaine, 2019).

In the context of survival analysis, we examined the impact of the 37 tag-SNPs on CV event-free survival in the patient cohort. Remarkably, three SNPs, namely rs4648268 (*PTGS2*),

rs2958155 (*PTGES3*), and rs11300958 (*PTGES3*), emerged as independent risk factors for CV events in this population. The Kaplan-Meier curves and Cox regression models revealed their significant association with CV events: rs4648268 [OR = 0.31 (0.09-0.99), p = 0.049], rs2958155 [OR = 4.41 (1.15-5.04), p = 0.020], and rs11300958 [OR = 2.20 (1.16-4.18), p = 0.016].

When we incorporated these variants into a model that already included classical risk factors (age, sex, hypertension, diabetes, ethnicity, and CK disease stage), the predictive power of the model significantly improved, resulting in an increased AUC from 84.7% to 87.3% (p = 0.031). As in the case of the phospholipases study, this improvement was more pronounced within the patient group than in the entire population of controls and patients. This discrepancy might be attributed to the relatively low incidence of CV disease cases in the control group (only nine cases) and the high prevalence of classical CV risk factors within the patient group, implying that genetic information can play a relevant role in predicting CV events in nephrosclerosis patients.

Finally, to explore potential interactions between tag-SNPs modifying CV risk, we assessed epistasis associations through pairwise statistical analysis. Three SNP-pair interactions were identified: two between variants in *PTGS2* (COX2) and *PTGS1* (COX1) rs4648268-rs1213265 ($p < 0.01$) and rs5275-rs1238420 ($p < 0.01$) and one between SNPs located in PGE2 synthases: *PTGES2* rs17445108-*PTGES3* rs884115 ($p < 0.01$). The effect of SNP rs4648268 in COX2 on atherosclerosis severity score (BSS) experienced by patients with nephrosclerosis was more pronounced when this variant was combined with another SNP in COX1. Such interactions between SNPs located in different genes of a relevant metabolic pathway may synergistically enhance their effects, as observed in aging pathways and other routes, significantly influencing CV risk more profoundly than individual SNPs (Li et al., 2020; Ukraintseva et al., 2021).

Conclusions

5. Conclusions

In this work, we have studied a large group of nephrosclerosis patients and controls and show how genetic variability in genes in the PGE2 pathway may be relevant not only for susceptibility to the disease, but also for several important phenotypic traits in these patients. The specific conclusions we have extracted from the articles presented in this volume, in accordance with the objectives we set out to achieve, are as follows:

I. Study on PGE2 receptor genes

1. Two *PTGER3* SNPs, rs11209730 and rs10399704, showed a significant association with the risk of nephrosclerosis.
2. A proximal region of *PTGER3* (from genomic position 1:70854192 to 1:70905737) was tagged as relevant for eGFR values in nephrosclerosis patients.
3. Carriers of the *PTGER1* rs2241360 T variant had better CV event-free survival than wild-type individuals, whilst *PTGER3* rs7533733 GG carriers had lower event-free survival than AA/AG patients.

II. Study on phospholipase genes

1. *SCARB1* rs10846744 and rs838880 showed significant OR for nephrosclerosis risk.
2. *PLA2G4A* and *PLA2G7* harboured several variants associated with atherosclerosis measurements in the patients, namely ccIMT, presence of plaques, number of plaques detected and ccIMT progression
3. Eight SNPs in *PLA2G4A* were independent risk factors for CV events in nephrosclerosis patients and their addition to a predictive model for CV risk significantly improved its predicting ability compared to that of a classic risk model.
4. *PLA2G4A* rs932476AA and rs6683619AA genotypes were associated with lower CV event-free survival after controlling for confounding variables

III. Study on the cyclooxygenase system

1. Three SNPs in *PTGS2* (coding for COX2), namely rs2066826, rs4648268 and rs20417 were associated with nephrosclerosis risk
2. Two tag-SNPs (rs11790782 and rs2241270) in *PTGES* were linked to higher systolic and diastolic pressure
3. *PTGS1* (COX1) rs10306194 was associated with higher ccIMT progression, presence of carotid plaque and atherosclerosis severity.

4. The addition of three variants, namely *PTGS2* rs4648268, *PTGES3* rs2958155 and *PTGES3* rs11300958, to a predictive model for CV events containing classic risk factors in nephrosclerosis patients significantly enhanced its statistical power

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6. References

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Genetic Variants in PGE2 Receptors Modulate the Risk of Nephrosclerosis and Clinical Outcomes in These Patients

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Abstract: Prostaglandin E2 (PGE2) is a major actor mediating renal injury. We aimed to determine genetic variability in the genes coding for its receptors (*PTGER1-4*) and study associations with nephrosclerosis risk and clinical outcomes. We identified 96 tag-SNPs capturing global variability in *PTGER1-4* and screened 1209 nephrosclerosis patients and controls. The effect of these variants was evaluated by multivariate regression analyses. Two *PTGER3* SNPs, rs11209730 and rs10399704, remained significant in a backward elimination regression model with other non-genetic variables (OR = 1.45 (1.07–1.95), $p = 0.016$ and OR = 0.71 (0.51–0.99), $p = 0.041$, respectively). In the nephrosclerosis patients, a proximal region of *PTGER3* was tagged as relevant for eGFR (p values for identified SNPs ranged from 0.0003 to 0.038). Two consecutive *PTGER3* SNPs, rs2284362 and rs2284363, significantly decreased systolic ($p = 0.005$ and $p = 0.0005$), diastolic ($p = 0.039$ and $p = 0.005$), and pulse pressure values ($p = 0.038$ and 0.014). Patients were followed for a median of 47 months (7–54) to evaluate cardiovascular (CV) risk. Cox regression analysis showed that carriers of the *PTGER1*rs2241360 T variant had better CV event-free survival than wild-type individuals ($p = 0.029$). In addition, *PTGER3*rs7533733 GG carriers had lower event-free survival than AA/AG patients ($p = 0.011$). Our results indicate that genetic variability in PGE2 receptors, particularly EP3, may be clinically relevant for nephrosclerosis and its associated CV risk.

Keywords: nephrosclerosis; PGE2; EP receptors; cardiovascular risk

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1. Introduction

Chronic kidney disease (CKD), whose prevalence has increased by an alarming 30% in the last 30 years [1], is now present in approximately 10% of the population, making this disease a global healthcare issue that is predicted to be the fifth cause of death worldwide by 2040 [2]. Among the pathological processes involved, nephrosclerosis usually refers to chronic renal insufficiency in a hypertensive and/or aging patient in the absence of other renal pathologies [3]. Although progression to end-stage renal disease (ESRD) is uncommon, the impact of the disease on the global cardiovascular (CV) risk is a major concern [4].

Chronic reduction of prostaglandins in the kidney, such as that caused by non-steroidal anti-inflammatory drugs (NSAIDs), may result in hypertension [5]. In addition, NSAIDs have been shown to increase the incidence of CV problems, indicating the involvement of prostaglandins (PG) in the pathogenesis of CV diseases [6]. This background highlights the role of inflammation in nephrosclerosis. Thus, there are inflammatory changes in the initial stages of CKD and the control of inflammatory response is key to delaying kidney

damage [7,8]. One of the most important inflammatory pathways is the cycloxygenase (COX)-mediated synthesis of PG, of which PGE2 is the main renal metabolite and a major actor mediating renal injury [9,10]. PGE2 actions are conducted through the activation of four different G-protein-coupled receptors (EP1-4), which may cause vasodilation/constriction and influence renal blood flow and hemodynamics [11]. These receptors are also involved in a variety of damaging mechanisms, such as hyperfiltration, fibrosis, apoptosis, oxidative stress, or inflammation [12].

The susceptibility to CKD is significantly influenced by genetics [13,14]; therefore, a plausible hypothesis is that the presence of functional variants in the genes that code for PGE2 receptors (*PTGER1-4*), given the aforementioned role of this PG, may favor the onset of nephrosclerosis and/or affect clinical outcomes. Despite the existence of a few reports linking some of these single nucleotide polymorphisms (SNPs) to hypertension [15,16] and acute coronary syndrome [17], their effect on CKD patients remains untested. Our aim was to examine patients diagnosed with nephrosclerosis and control subjects to determine whether variability in the four genes coding for PGE2 receptors (defined by tag-SNPs) may be associated with the risk of this disorder and/or clinical outcomes in these patients.

2. Results

Clinical and demographic characteristics of the population are shown in Table 1. The study included 1209 subjects, 716 controls, and 493 patients with nephrosclerosis (stage 3 or higher). Statistical differences between control and CKD groups were observed for age, sex, weight, BMI, hypertension, diabetes, hyperlipidemia, blood pressure, cholesterol, occurrence of CV events, creatinine, albumin-to-creatinin ratio, albuminuria, and eGFR. In addition, differences were also observed among CKD groups regarding age, ethnicity, weight, BMI, cholesterol, serum creatinine, albumin-to-creatinin ratio, albuminuria, and estimated glomerular filtration rate (eGFR) (Table 1).

Table 1. Clinical and demographic characteristics of the population of study. Median (range) or count (percentages) are shown.

	Control (n = 716)	CKD 3 (n = 307)	CKD 4–5 (n = 123)	CKD 5D (n = 63)	Total (n = 1209)	p Value (Control vs. CKD)	p Value (between CKD Groups)
Age (yrs)	60 (51–68)	67 (61–71)	64 (57–71)	61 (50–68)	62 (54–69)	6.2×10^{-17}	1.05×10^{-4}
Males (%)	359 (50.2)	211 (68.7)	80 (65.0)	43 (68.3)	693 (57.4)	7.87×10^{-10}	0.758
Ethnicity							
Caucasian	709(99.0)	304 (99.0)	120 (97.6)	58 (92.1)	1191 (98.5)	0.065	0.003
Others	7 (1.0)	3 (1.0)	3 (2.4)	5 (7.9)	18 (1.5)		
Weight (kg)	75.2 (66–85.8)	80.3 (72–88.6)	78.6 (67.5–87.2)	75 (64.8–86)	77.3 (67.6–86.7)	1.1×10^{-5}	0.030
BMI	30.4 (26.4–93)	30.3 (27.5–34.6)	28.8 (25.8–33.1)	27 (24.2–31.1)	30 (26.5–37.5)	1.6×10^{-4}	7.0×10^{-6}
Hypertension	405 (56.6)	293 (95.4)	121 (98.4)	60 (95.2)	879 (72.8)	1.95×10^{-61}	0.333
DM	55 (7.7)	74 (24.1)	32 (26.0)	8 (12.7)	169 (14.0)	4.23×10^{-14}	0.100
Hyperlipidemia	175 (35.9)	184 (67.2)	91 (74.0)	39 (61.9)	489 (51.6)	7.43×10^{-24}	0.203
Smoking							
Non-smoker	328 (45.8)	124 (40.4)	53 (43.1)	26 (41.3)	531 (43.9)	0.255	0.929
Current-smoker	125 (17.5)	53 (17.3)	22 (17.9)	13 (20.6)	213 (17.6)		
Former-smoker	263 (36.7)	130 (42.3)	48 (39.0)	24 (38.1)	465 (38.5)		
Systolic blood pressure (mmHg)	136 (124–149)	145 (132–160)	146 (133.5–161)	139 (130–155)	139 (127–154)	3.08×10^{-14}	0.162
Diastolic blood pressure (mmHg)	81 (74–88)	81 (75–89)	79 (73–88)	80 (70–90)	81 (74–88.5)	0.838	0.410
Pulse pressure (mmHg)	54 (46–64.5)	64 (51–77)	64 (55–76.5)	60 (50–70)	57 (48–70)	5.6×10^{-20}	0.123
Total cholesterol (mg/dL)	200.7 (180–222.8)	185 (159.8–213.3)	175.5 (148.5–203)	160.5 (137.8–185.5)	192 (165–216)	1.9×10^{-17}	1.9×10^{-5}
Cardiovascular events	9 (1.3)	25 (8.1)	10 (8.1)	6 (9.5)	50 (4.1)	1.33×10^{-9}	0.933
Creatinine (mg/dL)	0.8 (0.7–1)	1.5 (1.3–1.7)	2.9 (2.5–3.6)		1 (0.8–1.6)	1.06×10^{-102}	3.09×10^{-40}
Albumin/creatinine (mg/g)	6.4 (4–41.2)	37.1 (7.5–195.1)	212.1 (46–601)		33.4 (5.1–182.4)	1.27×10^{-11}	3.6×10^{-5}
eGFR (ml/min/1.73 m^2)	88.8 (78.7–100)	45 (37.4–50.8)	21 (16.2–25.6)		71.2 (40.2–90.1)	2.31×10^{-143}	4.39×10^{-56}

BMI, body mass index; DM, diabetes mellitus; eGFR, estimated glomerular filtration rate.

2.1. Genetic Associations with the Risk of Nephrosclerosis

The overall call rate and reproducibility percentages of the genotyping analysis were 97.8 and 99.71, respectively. One SNP in *PTGER2* and six in *PTGER3* were not in Hardy–Weinberg equilibrium and hence they were ruled out from subsequent analyses.

The results of the univariate analyses testing associations between the remaining 89 SNPs and the risk of nephrosclerosis in all models of inheritance are shown in Supplementary Materials Table S1. After controlling for confounding variables, ten SNPs, all in the *PTGER3* gene, were significantly associated with the risk of the disease (Table 2).

Table 2. Association of *PTGER3* polymorphisms with the risk of nephrosclerosis. Odds ratios with 95% confidence intervals (OR) were adjusted by sex, age, ethnicity, diabetes and hypertension.

Polymorphism	Genotype	Control *n* (%)	CKD *n* (%)	OR	*p* Value
rs11209708	A/A A/G-G/G	558 (80.5) 135 (19.5)	369 (77.4) 108 (22.6)	1.41 (1.01–1.97)	0.043
rs6424411	A/A A/G-G/G	330 (48.0) 358 (52.0)	203 (42.7) 272 (57.3)	1.32 (1.01–1.74)	0.045
rs475468	G/G G/A-A/A	254 (37.0) 432 (63.0)	207 (43.1) 273 (56.9)	0.72 (0.54–0.95)	0.018
rs6679370	A/A A/G-G/G	201 (29.1) 490 (70.9)	166 (34.4) 316 (65.6)	0.71 (0.53–0.95)	0.022
rs11209730	A/A A/G-G/G	475 (66.6) 238 (33.4)	306 (62.7) 182 (37.3)	1.38 (1.04–1.83)	0.024
rs2250312	C/C C/T-T/T	311 (43.7) 401 (56.3)	191 (38.9) 300 (61.1)	1.41 (1.07–1.84)	0.013
rs2268062	T/T T/C-C/C	209 (29.3) 504 (70.7)	171 (34.9) 319 (65.1)	0.69 (0.51–0.92)	0.011
rs61778876	A/A A/G	646 (93.4) 46 (6.6)	460 (95.4) 22 (4.6)	0.54 (0.31–0.95)	0.03
rs2300175	C/C C/T-T/T	207 (29.0) 507 (71.0)	170 (34.7) 320 (65.3)	0.69 (0.52–0.92)	0.012
rs10399704	G/G G/A-A/A	163 (23.4) 534 (76.6)	139 (29.2) 337 (70.8)	0.72 (0.52–0.98)	0.035

The predictive power of the identified SNPs was evaluated with ROC curves in models including or not relevant non-genetic covariates. In spite of a slight increase in the AUC value (80.2% vs. 78.6%) in favor of the combined genetics/classic model, the difference was not statistically significant ($p = 0.379$, Supplementary Figure S1).

Next, we included these 10 SNPs together in an adjusted multiple regression model with backward elimination controlling for the aforementioned covariates. Four SNPs remained in the final model, two of which, rs11209730 and rs10399704, still showed a statistically significant association with the risk of nephrosclerosis (OR = 1.45 (1.07–1.95), $p = 0.016$ and OR = 0.71 (0.51–0.99), $p = 0.041$, respectively, Table 3).

Table 3. Multivariate logistic regression model for the risk of nephrosclerosis.

	B	SE	Wald	OR	CI	*p* Value
Sex	0.748	0.15	25.87	2.11	(1.58–2.82)	3.65×10^{-7}
Diabetes	1.119	0.21	27.39	3.06	(2.01–4.65)	1.66×10^{-7}
Hypertension	3.077	0.27	127.95	21.70	(12.73–36.98)	1.15×10^{-29}
rs11209708 A/G-G/G	0.348	0.18	3.75	1.42	(1.00–2.01)	0.053
rs6424411 A/G-G/G	0.251	0.15	2.95	1.29	(0.97–1.71)	0.086
rs11209730 A/G-G/G	0.37	0.15	5.83	1.45	(1.07–1.95)	0.016
rs10399704 G/A-A/A	−0.345	0.17	4.16	0.71	(0.51–0.99)	0.041
Constant	−3.573	0.34	111.52	0.03		4.54×10^{-26}

B, regression coefficient; SE, standard error; OR, odds ratio; CI, 95% confidence interval.

2.2. Impact of Polymorphisms on Renal Function and Blood Pressure Traits

The influence of the 89 SNPs on the renal function of the 430 nephrosclerosis patients who were not in dialysis was analyzed adjusting by sex, age, ethnicity, hypertension, diabetes, and CKD stage. Ten variants in *PTGER3*, mainly located in the proximal region of the gene, showed a significant association with eGFR, with p values ranging from 0.0003 to 0.038 (Figure 1). Associations with the albumin-to-creatinine ratio were far less noticeable and also only observed in *PTGER3* (Figure 1). The two first variants in the gene, rs61777096 and rs6656853, were the only ones to significantly affect both eGFR and proteinuria. Mean eGFR and albuminuria values displayed by the patients with significant differences between genotypes are shown in Supplementary Table S2.

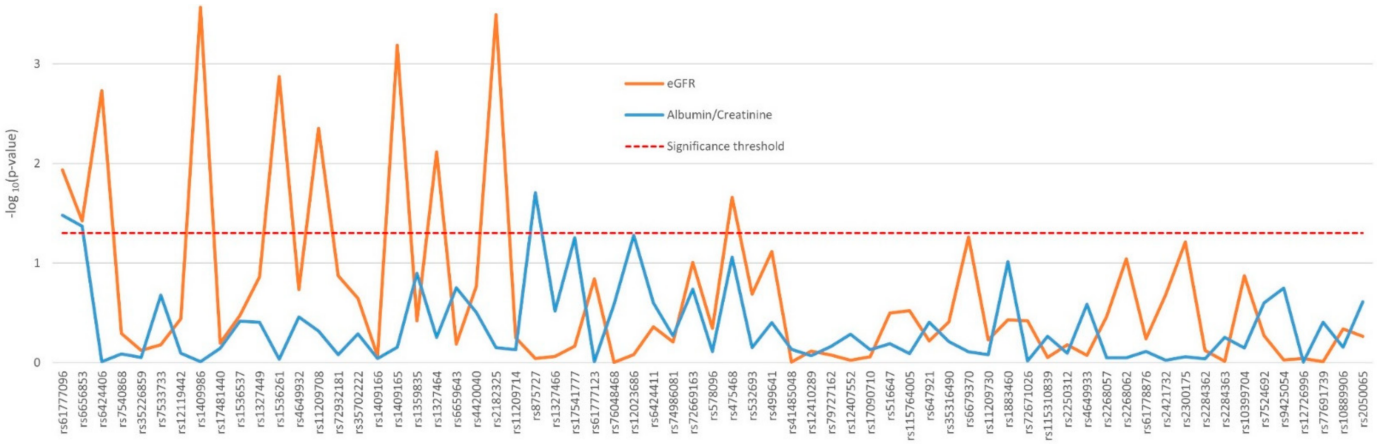

Figure 1. Effect of genetic variability in the *PTGER3* gene determined by tag-SNPs on the renal function of nephrosclerosis patients.

Except for *PTGER4* rs16870224, all the variants found to affect blood pressure traits—namely, systolic (SBP) and diastolic blood pressure (DBP) and pulse pressure—were located in *PTGER3*, mostly in a distal area of the gene (Figure 2). Supplementary Table S3 shows the differences across genotypes observed in the nephrosclerosis patients. Most notably, two consecutive tag-SNPs, rs2284362 and rs2284363 were found to significantly decrease SBP (p = 0.005 and p = 0.0005, respectively), DBP (p = 0.039 and p = 0.005), and pulse pressure values (p = 0.038 and 0.014, Figure 2).

Figure 2. Effect of genetic variability in the *PTGER3* gene determined by tag-SNPs on blood pressure traits of nephrosclerosis patients.

2.3. Association of PTGER Variants with the Incidence of Cardiovascular Events

Participants were followed for a median of 47 months (range 7–54), in which a total of 50 CV events were reported, nine in the control group (1.3%), and 41 (8.3%) in the nephrosclerosis patients (OR = 7.13 (3.4–14.8), $p = 1.32 \times 10^{-9}$). Among the CKD patients, those with CV events were significantly older and predominantly males. These and other characteristics of the patients experiencing or not CV events are summarized in Table 4.

Table 4. Parameters of interest in nephrosclerosis patients experiencing or not cardiovascular events. Median (range) or count (percentages) are shown.

Variable	No CVE	CVE	*p* Value
Age (years)	66 (59–71)	67 (64–72.5)	0.014
Sex (male)	300 (66.4)	34 (82.9)	0.019
Ethnicity			
Caucasian	441 (97.6)	41 (100)	0.381
Others	11 (2.4)	0	
Weight	79.8 (69.7–88)	81.5 (70.9–91.5)	0.568
BMI (kg/m^2)	29.8 (26.5–33.4)	29.7 (26.1–33)	0.923
Hypertension	435 (96.2)	39 (95.1)	0.480
DM	100 (22.1)	14 (34.1)	0.064
Hyperlipidemia	* 284 (67.8)	30 (73.2)	0.302
Smoking			
Non-smoker	190 (42.0)	13 (31.7)	
Current-smoker	77 (17.0)	11 (26.8)	0.224
Former-smoker	185 (49.9)	17 (41.5)	
Pulse pressure (mmHg)	62 (51–75.4)	67 (57.3–78.3)	0.096
Systolic lood pressure (mmHg)	144 (132–160)	145 (134–162)	0.642
Dyastolic blood pressure (mmHg)	81 (74–89)	77.5 (70.8–88.8)	0.245
Chronic kidney disease stage			
Stage 3	282 (62.4)	25 (61.0)	
Stage 4–5	113 (25.0)	10 (24.4)	0.933
Dialysis	57 12.6)	6 (14.6)	
Total cholesterol (mg/dL)	181 (154.7–209)	165 (139.5–212)	0.065
Creatinine (mg/dL)	1.7 (1.4–2.4)	1.7 (1.3–2.3)	0.689
Albumin/Cr (mg/g)	68.9 (9.9–294.3)	173.6 (25.5–470.6)	0.171
eGFR (mL/min/1.73 m^2)	38.2 (26.6–47.7)	37.4 (25–47)	0.705
Glucose (mg/dL)	100.5 (90–114)	102 (96.5–123.5)	0.134
Calcium (mg/dL)	9.4 (9.1–9.7)	9.5 (9.1–9.7)	0.947
Sodium (mEq/L)	141 (139–142)	141 (139–142)	0.951
Potassium (mEq/L)	4.7 (4.4–5.1)	4.8 (4.4–5)	0.946

* Dyslipemia data was missing in 33 individuals.

In order to narrow down the number of SNPs to be analyzed in the survival study, we carried out a previous analysis of the crude association of the 89 variants with the risk of experiencing a CV event under the selected genetic model. Five SNPs resulted in significant associations, with the rs7533733 GG genotype displaying the highest OR and the lowest *p* value (OR = 2.65 (1.28–5.46), $p = 0.01$, Table 5). Kaplan–Meier analysis of these five polymorphisms revealed that carriers of the *PTGER1* rs2241360 T variant allele had better CV event-free survival than homozygous wild-type carriers did (log-rank $p = 0.014$), whilst patients homozygous for the *PTGER3* rs7533733 G variant allele experienced more events than AA/AG carriers (log-rank $p = 0.007$, Figure 3). After adjusting these results in a Cox regression model accounting for other confounding variables (see Supplementary Table S4 for details), the differences between genotypes in both SNPs remained statistically significant ($p = 0.029$ and 0.011, respectively).

Table 5. Crude risk analysis for the occurrence of cardiovascular events in nephrosclerosis patients.

Gene	Polymorphism	Model	Genotype	No CVE	CVE	OR	*p* Value
PTGER1	rs2241360	Dominant	CC CT/TT	316 (70.9) 130 (29.1)	34 (85.0) 6 (15.0)	0.43 (0.18–1.05)	0.037
PTGER3	rs7533733	Recessive	AG/AA GG	390 (86.5) 61 (13.5)	29 (70.7) 12 (29.3)	2.65 (1.28–5.46)	0.010
PTGER3	rs12119442	Dominant	G/G G/A-A/A	304 (69.7) 132 (30.3)	21 (51.2) 20 (48.8)	2.19 (1.15–4.18)	0.014
PTGER3	rs74986081	Dominant	GG GA/AA	333 (75.7) 107 (24.3)	34 (89.5) 4 (10.5)	0.37 (0.13–1.05)	0.035
PTGER3	rs2268057	Overdominant	CC/TT CT	254 (56.3) 197 (43.7)	16 (39.0) 25 (61.0)	2.02 (1.05–3.88)	0.025

CVE, cardiovascular event; OR, odds ratio with 95% confidence interval.

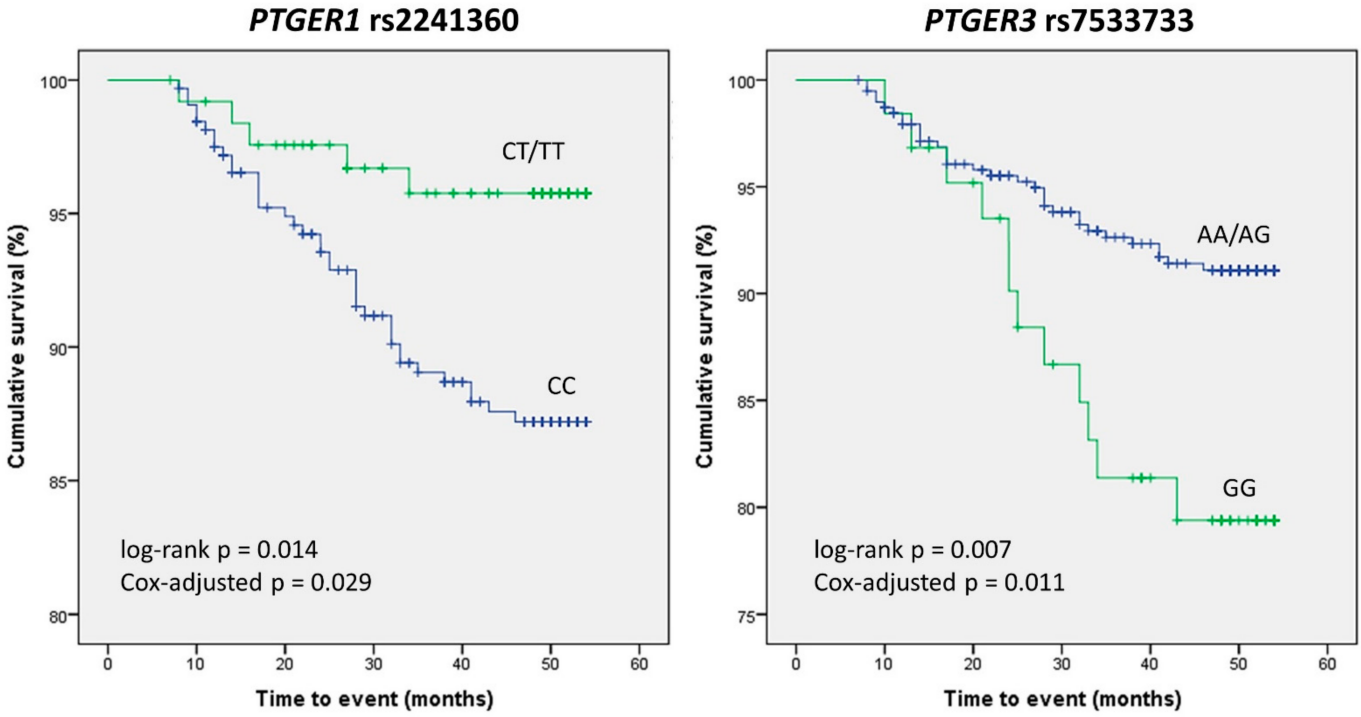

Figure 3. Kaplan–Meier curves depicting the occurrence of cardiovascular events in nephrosclerosis patients. Differences between genotypes of *PTGER1* rs2241360 and *PTGER3* rs7533733 are shown.

3. Discussion

The evolution of kidney damage in nephrosclerosis is usually slower than in diabetic nephropathy. However, the high prevalence of the disease and the great interindividual variability in its progression result in a significant proportion of cases experiencing a vicious cycle: kidney damage worsens CV risk, which, in turn, increases disease progression to ESRD [18,19]. There is therefore a need for identifying additional risk factors for nephrosclerosis that may allow early detection, the individualization of treatments and the reduction of the enormous economic burden caused by CKD and renal replacement therapy.

Our findings showed that nine tag-SNPs, each representing a haplotype block in the *PTGER3* gene locus, and rs2268062, also in *PTGER3*, which was included in the study for being reportedly linked to hypertension [15], were significantly associated with the risk of nephrosclerosis. Variability in the *PTGER3* gene locus has not been extensively studied, and consequently there is no data regarding the functional impact of polymorphisms. We do know, however, that the administration of EP3 antagonists in vivo increases COX-2-mediated PGE2 expression in the kidney, whilst the receptor activation decreases PGE2 levels [20]. This is relevant to the development of nephrosclerosis, as PGE2 contributes significantly to kidney disease, as it is involved in albuminuria, growth/fibrosis, and

the activation of the renin–angiotensin–aldosterone system (RAAS) [21]. Therefore, it is tempting to speculate that the areas tagged by the identified variants might affect the function/expression of the receptor, which, in turn, would translate into altered levels of PGE2 in renal tissue, hence modifying the susceptibility to kidney damage. Another hypothesis is that the risk of CKD was modulated by the impact of genetic variability on the direct actions of EP3, e.g., vasoconstriction [22], water balance [23], regulation of renal blood flow [24], or maintenance of renal cell integrity [25]. Finally, changes in the susceptibility to nephrosclerosis could also be the result of changes in the susceptibility to the main risk factor, i.e., hypertension. As we mentioned above, the information about the clinical impact of these SNPs in *PTGER3* is very scarce. To our knowledge, there is only one report mentioning an association of rs2268062 with hypertension in the general population, although the observation could not be replicated in a validation sample [15]. In our sample, this SNP was not associated with hypertension, in fact, only rs2250312 in a recessive model appeared to be linked to this phenotype (data not shown). In any case, the fact that 95.7% of our nephrosclerosis patients had hypertension makes it much harder to identify genetic markers for this feature in this cohort than in a general population setting. On the other hand, the effect of the identified tag-SNPs was not profound enough to significantly improve a predictive model (calculated with ROC analysis) based on classic risk factors. The most probable reason is that the impact of nongenetic factors such as hypertension was as marked as to overshadow that of the genetic variants.

The study of the nephrosclerosis cohort identified several variants in the *PTGER3* gene associated with significant changes in renal parameters and blood pressure traits. In particular, the effect on eGFR was especially noticeable, with several SNPs tagging a proximal region of the gene (from genomic position 1:70854192 to 1:70905737) highly linked to altered filtration. Again, we can only speculate on the consequences of genetic variants in this area, but given that one of the main EP3 roles is to reduce hyperfiltration by constricting the afferent arteriole [11,26], it is more than likely that the presence of functional polymorphisms linked to these tag-SNPs may have a significant impact in eGFR values.

The study also tagged regions in *PTGER3* that were relevant for blood pressure. This is in line with previous in vitro data and animal studies. Thus, rodent models have shown that the administration of selective EP3 agonists results in an acute and significant rise in arterial pressure [27] and that the pressor actions of PGE2 are mediated by EP3 [28]. Furthermore, we found that only variants in *PTGER3* were relevant for blood pressure. Accordingly, it has been reported that the expression levels of EP3 in both renal resistance vessels and the aorta are much higher than those of other EP receptors, and that its vasopressor effects are far superior to EP2 and EP4 vasodilator properties [27]. Finally, another fact that highlights the relevance of some of the *PTGER3* SNPs identified in this study, and therefore that of the tagged regions, is that some of these variants were repeatedly pinpointed in both the risk analysis and the cohort study. Most notably, out of the 89 polymorphisms studied, rs11209708 was observed to increase the risk of nephrosclerosis as well as affecting eGFR and pulse pressure values. In any case, it should be mentioned that the overall effects of PGE2 that are mediated by EP receptors are particularly complex and depend on many factors aside from genetics—e.g., EP1–4 relative expression levels—additional hormonal signaling (angiotensin II, endothelin, etc.) or the individual clinical status [12].

CV mortality in nephrosclerosis is up to 20 times more frequent than in the general population [4], and even after renal replacement therapy, mortality rates are higher in patients with nephrosclerosis than in other CKD groups [18]. In this regard, two SNPs, located in the genes coding for EP1 and EP3, had a significant impact on the incidence of CV events in the nephrosclerosis cohort. There is ample evidence to support the hypothesis that changes in these genes can lead to an altered CV risk. For instance, the activation of these two receptors increases intracellular Ca^{2+} via phospholipase C, in opposition to EP2 or EP4, which have no effect on Ca^{2+}. Voltage-dependent Ca^{2+} channels are widely distributed throughout the body and play a critical role in the maintenance of vascular tone.

Indeed, substantial research has demonstrated the association between cardiovascular disease and the dysregulation of intracellular calcium [29–31]. Moreover, PGE2 is present in mouse atherosclerotic plaques, where it can potentiate platelet aggregation, an effect solely mediated by EP3 [32,33]. Indeed, mice lacking EP3 develop less severe thrombosis after administration of arachidonic acid [34] and atherothrombosis induced in vivo by mechanical rupture of the plaque is drastically decreased when platelets lack EP3 [32]. This background and the findings presented herein suggest that variability in the genes coding for EP3 and, to a lesser extent, EP1, may play a key role in the occurrence of CV events in nephrosclerosis patients.

This work has a number of limitations. First, a validation cohort was lacking; paradoxically, however, this was a consequence of one of the strengths of the study: the requirement of a specific nephrosclerosis diagnosis, which improved the homogeneity of the cohort by excluding patients with diabetic kidney disease. Another limitation is that the *PTGER3* gene was far more polymorphic than *PTGER1*, *2*, and *4* and therefore many more SNPs were needed to tag the entire gene locus. This could have resulted in more relevant results obtained. Finally, one limitation was inherent to the study design. We analyzed representative SNPs in a region of the genome with high linkage disequilibrium—i.e., their determination makes it possible to infer total genetic variability and identify phenotypic associations without genotyping the rest of SNPs in that area. However, there is a drawback, tag-SNPs are intronic variants and therefore we cannot speculate on their functional consequences as we could in the case, for instance, of a nonsynonymous polymorphism.

In a seminal review, Nasrallah et al. propose targeting PGE2 receptors as a potential new pharmacological mechanism to prevent kidney damage and dysfunction in CKD [12], although it is true that the actual benefits in vivo of this strategy are still a matter of debate [35]. To our knowledge, there are no previous reports on how the modulation of these PGE2 receptors could affect outcomes in renal patients. In this work, we have shown that genetic variability in the genes encoding for these receptors may indeed be relevant, not only for the susceptibility to nephrosclerosis, but also for several important phenotypic traits in these patients. Most importantly, given the high CV risk shown by individuals with the disease, we have identified two genetic variants in *PTGER1* and *PTGER3* that were solidly linked to the occurrence of CV events, including death. These findings strengthen the aforementioned hypothesis that PGE2 receptors may constitute valuable therapeutic targets in nephrosclerosis and point to certain areas in the genes loci that may be of interest in this regard. Notwithstanding, more data from independent cohorts, and especially in vitro studies that can characterize the functional consequences of SNPs in *PTGER*1-4, are warranted to confirm our findings.

4. Patients and Methods

4.1. Study Design

The study was designed as an observational, retrospective study on 493 patients diagnosed with nephrosclerosis and 716 controls. Patients' samples were obtained from two sources: (i) the NEFRONA repository, which archives biological samples that were collected in a former multicenter study of cardiovascular morbidity and mortality in Spanish subjects with CKD stage 3 or higher (see explanation below), including patients in dialysis [36]; and (ii) the Nephrology Service of the Badajoz University Hospital, where patients with the same characteristics were recruited over a four-year period.

KDIGO (Kidney Disease—Improving Global Outcomes, kdigo.org, accessed on 4 August 2021) is a global nonprofit organization developing and implementing evidence-based clinical practice guidelines in kidney disease. According to its guidelines, CKD is defined as abnormalities of kidney structure or function, present for more than three months, with implications for health. CKD is divided into five stages based on levels of kidney function assessed by the glomerular filtration rate, which was estimated using the Modification of Diet in Renal Disease (MDRD) equation. These stages are Stage 1: Mild kidney damage, eGFR 90 mL/min/1.73 m^2 or higher; Stage 2: Mild loss of kidney function, eGFR

60–89 mL/min/1.73 m^2; Stage 3a and 3b: Mild to severe loss of kidney function, eGFR 30–59 mL/min/1.73 m^2; Stage 4: Severe loss of kidney function, eG FR 15–29; and Stage 5: Kidney failure or close to failure, eGFR less than 15 mL/min/1.73 m^2. Therefore, our patients (stage 3 and higher) had all an eGFR lower than 60 mL/min/1.73 m^2.

Nephrosclerosis patients over 18 years of age were selected according to current diagnostic guidelines, i.e., those with biopsy alterations typical of vascular nephropathy or that met clinical criteria. These criteria were based on the absence of signs of other kidney diseases and the presence of data suggestive of the pathology (advanced age, long-standing hypertension, left ventricular hypertrophy, initially mild renal failure and proteinuria below 0.5–1 g/24 h). Patients with proteinuria higher than 1 g were biopsied to confirm the diagnosis. Proteinuria was defined as a value greater than 500 mg or albuminuria higher than 300 mg in 24 h urine. Diagnostic and prognostic stratification of patients were carried out using the KDIGO classification, the KDIGO table of risk of progression and the CONSORTIUM-CKD equation (Kidney Risk Failure; www.kidneyriskfailure.org, accessed on 4 August 2021). CV risk was defined as the likelihood of experiencing a fatal or non-fatal CV event in the four-year follow-up (54 months). CV events included acute myocardial infarction, acute coronary syndrome, coronary catheterization requiring angioplasty, coronary bypass, typical angina with positive stress tests, sudden death, cerebrovascular accident, peripheral arterial disease, aortic aneurysm, and lower limb ischemia.

Control subjects matched by sex and age with eGFR > 60 mL/min/1.73 m^2 were recruited from: (i) Badajoz University Hospital; (ii) Primary Care centers throughout the country in the case of samples from the NEFRONA repository; and (iii) the DNA repository of the Instituto de Salud Carlos III (www.bancoadn.org, accessed on 4 August 2021). Exclusion criteria included previous history of any CV event, transplantation of any organ, carotid artery surgery, active infection, pregnancy or life expectancy below one year. All subjects gave written consent for their participation in the study, which had been approved by the Ethics Committee of the participating institutions, and that was carried out in accordance with the Declaration of Helsinki and its subsequent revisions.

4.2. SNP Selection and Genotype Analysis

We retrieved the coding sequence and adjacent 3′ and 5′ UTR regions of the *PTGER1* (ENSG00000160951; HGNC:HGNC9593), *PTGER2* (ENSG00000125384, HGNC:HGNC:9594), *PTGER3* (ENSG00000050628; HGNC:HGNC9595), and *PTGER4* (ENSG00000171522; HGNC:HGNC9596) genes, coding for EP1–4 receptors, and identified tag-SNPs (polymorphisms that represent genetic variability in a certain area) with Haploview 4.2 (Cambridge, MA, USA). In order to capture common variations, we chose a pair-wise tagging with a minimum r^2 of 0.80 and a threshold for minor allele frequency of 10%. In addition to the tag-SNPs identified, we included two variants, rs17197 and rs2268062, with a reported impact on BP [15,16]. In this manner, 6 SNPs in *PTGER1*, 8 SNPs in *PTGER2*, 73 SNPs in *PTGER3*, and 9 SNPs in *PTGER4* were analyzed. The list of all evaluated SNPs is shown in Supplementary Table S1.

Whole blood samples (10 mL) were drawn from the participants recruited at the Badajoz University Hospital and stored at −80 °C until DNA purification, which was conducted with a standard phenol-chloroform extraction and ethanol precipitation. DNA samples were then stored at 4 °C in sterile plastic vials. In the case of participants recruited in the NEFRONA study, genetic material was obtained from biological samples stored at the REDinREN biobank [37] using QIAamp DNA Blood Kits (Hilden, Germany).

Genotyping was performed by allelic discrimination using TaqMan® OpenArray Genotyping (Waltham, MA, USA) with a customized panel on a QuantStudio™ 12K Flex Real-Time PCR System (Life Technologies, Carlsbad, CA, USA) in the Centro Nacional de Genotipado-Instituto de Salud Carlos III (CeGen-ISCIII; Madrid, Spain, www.cegen.org, accessed on 4 August 2021). A trio of samples from the Coriell Institute biorepository, with known genotypes, were included in each chip as quality control.

4.3. Statistical Analyses

Differences between quantitative variables were assessed by the Student's *t*/Mann–Whitney or ANOVA/Kruskal–Wallis tests, depending on the normality of the data and the number of groups compared. Categorical variables were compared with the Chi-square test. Logistic regression modeling was used to examine the influence of the SNPs on vascular CKD risk, adjusting for demographics and classic risk factors, namely age, sex, ethnicity, diabetes and hypertension, as formerly described [38]. After testing five inheritance models in the preliminary genetic analyses (codominant, dominant, recessive, overdominant, and log-additive), we decided to utilize the dominant model in the risk analysis, as we have done in former reports by our group [39,40], because the resulting genotype groups were the most balanced in terms of size and because it resulted in more significant associations. In order to examine the value of the studied SNPs in predicting susceptibility to CKD, receiving operating curves (ROC) were generated for models containing classic clinical and demographic risk factors adding or not the genetic information. The area under the curve (AUC) of these models were compared with the DeLong test. Genetic association analyses with clinical variables were performed with regression modelling adjusting by confounding variables.

The incidence of CV events and its association with the presence of SNPs was assessed by Kaplan–Meier curves, which were compared with the log-rank test. Cox regression modeling was carried out in order to evaluate the effect of additional covariates. Patients were followed up until the earliest of CV event, death, or end of study.

Statistical analyses were carried out with the *SNPassoc*, *pROC*, and *survival* packages in the R environment and the IBM SPSS statistical software (SPSS Inc., Chicago, IL, USA; version 22.0).

5. Conclusions

The determination of genetic variability of PGE2 receptors, and particularly that of EP3, may be useful to identify patients at risk of nephrosclerosis and/or the detection of patients with this disease that have a higher likelihood of experiencing CV events.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jpm11080772/s1, Figure S1. ROC curves analyzing the predictive power for a nephrosclerosis diagnosis of a combined model containing genetic and non-genetic variables (green curve) and a model including classic risk factors only (blue curve); Table S1. Univariate analysis of the association of *PTGER* polymorphisms with the risk of nephrosclerosis in each model of inheritance. Significant *p* values are in boldface type; Table S2. Differences in parameters of renal function shown by nephrosclerosis patients according to polymorphisms in the *PTGER3* gene; Table S3. Differences in blood pressure traits shown by nephrosclerosis patients according to polymorphisms in *PTGER* genes; Table S4. Cox regression analyses modelling the risk of cardiovascular events in nephrosclerosis patients according to relevant genotypes.

Author Contributions: L.M.G. recruited patients, carried out statistical analyses and drafted the manuscript; N.R.R. and J.M.V. helped with study design and the clinical evaluation of the patients; S.M.-Z. carried out genetic analyses; J.L.-G. participated in sample collection and clinical analyses; G.G. designed the study, searched for funding and wrote the final version of the paper. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Clinica Research Ethics Committee of Badajoz (no. 18002909, date of approval: 21 February 2018).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data underlying this article will be shared on reasonable request to the corresponding author.

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Tag-SNPs in Phospholipase-Related Genes Modify the Susceptibility to Nephrosclerosis and its Associated Cardiovascular Risk

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Nephrosclerosis patients have a high cardiovascular (CV) risk that is very often of more concern than the renal disease itself. We aimed to determine whether variants in phospholipase-related genes, associated with atherosclerosis and CV outcomes in the general population, could constitute biomarkers of nephrosclerosis and/or its associated CV risk. We screened 1,209 nephrosclerosis patients and controls for 86 tag-SNPs that were identified in the *SCARB1*, *PLA2G4A,* and *PLA2G7* gene loci. Regression models were utilized to evaluate their effect on several clinical parameters. Most notably, rs10846744 and rs838880 in *SCARB1* showed significant odds ratios (OR) of 0.66 (0.51–0.87), p = 0.003 and 1.48 (1.11–1.96), p = 0.007 for nephrosclerosis risk. *PLA2G4A* and *PLA2G7* harboured several SNPs associated with atherosclerosis measurements in the patients, namely common carotid intima media thickness (ccIMT), presence of plaques, number of plaques detected and 2-years ccIMT progression (significant p-values ranging from 0.0004 to 0.047). Eight SNPs in *PLA2G4A* were independent risk factors for CV events in nephrosclerosis patients. Their addition to a ROC model containing classic risk factors significantly improved its predictive power from AUC = 69.1% (61.4–76.9) to AUC = 79.1% (73.1–85.1%), p = 0.047. Finally, *PLA2G4A* rs932476AA and rs6683619AA genotypes were associated with lower CV event-free survival after controlling for confounding variables [49.59 (47.97–51.21) vs. 51.81 (49.93–51.78) months, p = 0.041 and 46.46 (41.00–51.92) vs. 51.17 (50.25–52.08) months, p = 0.022, respectively]. Variability in phospholipase-related genes play a relevant role in nephrosclerosis and associated atherosclerosis measurements and CV events.

Keywords: nephrosclerosis, atherosclerosis, cardiovascular risk, single nucleotide polymorphism, phospholipases

INTRODUCTION

Chronic kidney disease (CKD) is defined by abnormalities of the kidney structure and/or function for over 3 months with clinical consequences (Stevens and Levin, 2013). CKD increases the risk of all-cause and cardiovascular (CV) mortality (Ortiz et al., 2014) and has been predicted to be the fifth cause of death worldwide by 2040 (Foreman et al., 2018). Nephrosclerosis in particular, which usually refers to the presence of CKD in a hypertensive and aging patient in the absence of histological confirmation, has an enormous impact on the global CV risk (Robles et al., 2020), which often times is of more concern than the progression of the renal disease itself (Zoccali, 2006).

Plasma lipoprotein-associated phospholipase A2 (Lp-PLA2), encoded by the *PLA2G7* gene, transforms low-density lipoprotein (LDL) into a number of proinflammatory mediators. Increased Lp-PLA2 levels has been observed in vascular endothelial dysfunction, atherosclerotic plaque inflammation, and necrotic core formation in plaques, which has led to regard Lp-PLA2 as a predictive marker for atherosclerosis-related CV diseases (CVD) and a promising therapeutic target (Huang et al., 2020). A number of *PLA2G7* single nucleotide polymorphisms (SNPs) have been associated with Lp-PLA2 activity (Suchindran et al., 2010; Grallert et al., 2012) and atherosclerosis (Santoso et al., 2017). The activity of Lp-PLA2 has also been linked to the presence of genetic variants in the human scavenger receptor class B type (*SCARB1*) gene (Manichaikul et al., 2018), a primary receptor for selective transportation of cholesterol for high-density lipoprotein (HDL) and also a typical locus for CVD (Ma et al., 2018; Manichaikul et al., 2018; Koenig et al., 2021). In addition, calcium-dependent cytosolic phospholipase A2α (cPLA2α), a product of the *PLA2G4A* gene, is activated by calcium enabling arachidonic acid release thus leading to the synthesis of various proinflammatory factors (Clark et al., 1991). cPLA2α overexpression has been observed in atherosclerotic arterial wall, mainly in the intima in regions with an inflammatory infiltrate (Elinder et al., 1997) and has recently been revealed as a key factor for calcification in aortic valve interstitial cell cultures (Bonetti et al., 2020).

Our goal was to determine whether genetic variability in three candidate phospholipase-related genes, namely *PLA2G7*, *SCARB1* and *PLA2G4A*, was associated with atherosclerosis and the occurrence of CV events in a population of nephrosclerosis patients. Additionally, we screened a group of healthy individuals for the same SNPs to evaluate the putative role of these variants as risk factors for nephrosclerosis.

SUBJECTS AND METHODS

Study Subjects

The study comprised 1,209 participants, 493 patients diagnosed with nephrosclerosis and 716 controls, who were obtained from 1) the Nephrology Service at Badajoz University Hospital, 2) the NEFRONA repository, a collection of biological samples from Spanish renal patients (Arroyo et al., 2014) and 3) the Instituto de Salud Carlos III biobank (www.bancoadn.org). All patients had stage 3 or higher CKD, i.e., presented with an estimated glomerular filtration rate (eGFR) below 60 ml/min/1.73 m^2. These patients were all over 18 years of age and were selected among those with biopsy alterations typical of vascular nephropathy or that met clinical criteria, namely the presence of data indicative of the disease (advanced age, long-standing hypertension, left ventricular hypertrophy, initially mild renal failure and proteinuria below 0.5–1 g/24 h) and the lack of signs of other kidney pathologies. Control subjects had all eGFR >60 ml/min/1.73 m^2. Exclusion criteria included having experienced a CV event before the beginning of the study, organ transplantation, carotid artery surgery, pregnancy, active infection and life expectancy below 1 year.

All subjects gave written consent for their participation in the study, which had been approved by the Bioethics & Biosafety Committee of the University of Extremadura and the Clinical Research Ethics Committee of the Badajoz University Hospital, and that was carried out in accordance with the Declaration of Helsinki and its subsequent revisions.

Clinical Variables

An amount of more than 500 mg of protein (or more than 300 mg of albumin) in 24-h urine was defined as proteinuria (patients with proteinuria >1 g were biopsied for diagnosis confirmation). Renal function was estimated using the Modification of Diet in Renal Disease (MDRD) equation. Diagnostic and prognostic stratification of patients was carried out using the CONSORTIUM-CKD equation and the KDIGO classification and table of progression risk (www.kidneyriskfailure.org). CV risk was defined as the likelihood of experiencing a CV event (fatal or not) in a four-year follow-up. Patients were followed up until the earliest of CV event, death, or end of study. CV events included acute myocardial infarction, acute coronary syndrome, coronary catheterization requiring angioplasty, coronary bypass, typical angina with positive stress tests, sudden death, cerebrovascular accident, peripheral arterial disease, aortic aneurisma and lower limb ischemia.

Arterial Ultrasound

The presence of atheromatous plaques was assessed in 412 out of the 493 nephrosclerosis as described elsewhere (Abajo et al., 2017). In brief, explorations were carried out in ten different arterial territories with a high-resolution B-mode ultrasound (Vivid BT09, GE Healthcare, Waukesha, WI, United States) according to guidelines of the Mannheim IMT Consensus (Touboul et al., 2004) and the American Society of Echocardiography (Stein et al., 2008). In the case of intima media thickness (IMT), the left and right common carotid arteries (CCAs) were explored in the anterolateral, posterolateral, and mediolateral directions. IMT was defined as the distance between the leading edge of the lumen intima echo and the leading edge of the media-adventitia echo in the far wall. It was measured 1 cm from the bifurcation. Three longitudinal measurements of IMT were accomplished on the right and left CCAs. We used the mean of the three right and left measurements in the analysis. Atheromatous plaques were considered when IMT was larger than 1.5 mm protruding into

the lumen. Common carotid intima media thickness (ccIMT) progression was calculated for nephrosclerosis patients by subtracting ccIMT at baseline from ccIMT at 24 months for each side. Values were then averaged and expressed in mm changed per year. An atherosclerosis severity score ranging from 0 to 3 was created [0: Ankle Brachial Index (ABI) > 0.9 and ccIMT <90% reference interval; 1: ABI = 0.7–0.9 and/or ccIMT ≥90% reference interval; 2: presence of a carotid plaque with stenosis <125 cm/seg; 3: plaque with stenosis ≥125 cm/seg and/or ABI <0.7]. The ABI is a quick, noninvasive way to check for peripheral artery disease. The test compares the blood pressure measured at your ankle with the blood pressure measured at your arm. A low ankle-brachial index number can indicate narrowing or blockage of the arteries in your legs. A value of less than 0.9 is indicative of peripheral artery disease.

Genetic Analyses

We retrieved the coding sequence and adjacent 3′- and 5′-UTR regions of the *PLA2G7* (ENSG00000146070; HGNC:9040), *SCARB1* (ENSG00000073060; HGNC:1664) and *PLA2G4A* (ENSG00000116711; HGNC:9035) genes, and identified tag-SNPs (polymorphisms that represent genetic variability in a certain area of the gene locus) with Haploview 4.2. A pair-wise tagging with $r^2 \geq 0.80$ and a 5% threshold for minor allele frequencies (MAF) were established to capture common variants. Several of the tag-SNPs included had been previously related to CV outcomes, namely rs10846744, rs5888, and rs1051931. Overall, 86 SNPs were analyzed: 5 in *SCARB1*, 17 in *PLA2G7*, and 64 in *PLA2G4A*. **Supplementary Table S1** lists all the SNPs included in the present study.

Genetic material from controls and cases was purified from whole blood samples obtained from biobanks or directly from participants recruited at the Badajoz University Hospital. DNA purification was carried out by means of standard phenol-chloroform extraction followed by ethanol precipitation or by QIAamp DNA Blood Kits. DNA samples were then stored in plastic vials at 4°C until analysis.

Genotyping analyses were conducted with TaqMan® OpenArray using a customized panel on a QuantStudio™ 12K Flex Real-Time PCR System (Life Technologies, Carlsbad, CA, United States). Quality controls (sample trios from the Coriell Institute Biorepository) were included in all runs. Analyses were carried out at the Centro Nacional de Genotipado-Instituto de Salud Carlos III (CeGen-ISCIII; Madrid, Spain, www.cegen.org).

Statistical Analyses

Categorical variables were compared with the Chi-square test, whilst quantitative variables were compared by the Mann-Whitney or Kruskal-Wallis tests depending on the number of groups. The influence of the SNPs on the risk of nephrosclerosis and other clinical variables was assessed by regression modelling, adjusting for demographics and classic risk factors, namely age, sex, body mass index, ethnicity, diabetes, blood pressure and CKD stage, as formerly described (Valls et al., 2019; González et al., 2021). Kaplan-Meier curves for evaluating the influence of SNPs on CV events were compared with log-rank tests. Subsequently, a Cox regression procedure was carried out to include the effect of additional relevant covariates for those SNPs with log-rank p-values < 0.1. The predictive value of the SNPs regarding the risk of nephrosclerosis was evaluated with Receiving Operating Curves (ROC), which were generated for models with classic risk factors with or without genetic information. The DeLong test was used to detect differences between the area under the curve (AUC) of these models. The statistical power calculation was carried out with Quanto software v. 1.2.4 (USC, Los Angeles, CA, United States) by analyzing the frequency of carriers of allelic variants with an arbitrary effect size of 2.0 and a type-I error of 0.05. With the reported incidence of CKD and the available sample size available (n = 1209), the statistical power of the study to identify genetic associations ranged from 0.939 to 0.996 depending on the specific MAF. Statistical analyses were carried out with the IBM SPSS statistical software (SPSS Inc., Chicago, IL, version 22.0) and the *SNPassoc*, *pROC* and *survival* packages in the R environment.

RESULTS

Main demographic and clinical characteristics of the study population are listed in **Table 1**. 50.2% of controls and 67.7% of cases were males. Median (range) age values of controls and cases were, respectively, 60 (21–84) and 66 (24–89) years. As expected, nephrosclerosis patients showed a higher incidence of diabetes, hypertension and dyslipidemia ($p < 0.0001$ in all cases). Regarding biochemistry data, differences in glucose, potassium and cholesterol levels were also statistically significant between cases and controls ($p < 0.001$, $p < 0.0001$ and $p < 0.0001$, respectively).

Nephrosclerosis Risk Analysis

Genotyping was successful in 97.6% of the samples. Mean MAF in the whole study population was 22.6%. Six SNPs significantly differed ($p < 0.05$) from the Hardy-Weinberg equilibrium in the control population and were therefore ruled out from further analyses (**Supplementary Table S1**).

Under a dominant model (carriers vs. non-carriers), two of the five SNPs studied in the *SCARB1* gene and three more variants in *PLA2G4A* showed significant associations with the risk of nephrosclerosis after controlling for meaningful covariates, namely age, sex, body mass index, ethnicity, diabetes, and blood pressure (**Table 2**). The two identified *SCARB1* variants, rs10846744 and rs838880, showed p-values that were 10-fold lower than those of *PLA2G4A* SNPs, with odds ratios (OR) of 0.66 (0.51–0.87), $p = 0.003$ and OR = 1.48 (1.11–1.96), $p = 0.007$, respectively. None of the *PLA2G7* variants were associated with susceptibility to the disease.

Atherosclerosis Measurements

ccIMT median values were significantly higher in patients [0.77 (0.44–1.51) mm] than in controls [0.71 (0.4–1.28) mm; $p = 2.23E-07$], as it was the presence of atheromatous plaques (79.4% vs. 55.9%; $p = 3.06E-14$), the total number of plaques identified in ten different arterial territories [Median (range) = 3 (0–10) vs. 1

TABLE 1 | Demographic, biochemical, and clinical characteristics of the population of study. Median (range) or count (percentage) is shown.

		Controls		Nephrosclerosis		*p*-value
Sex	Males	359	50.2%	334	67.7%	<0.0001
	Females	356	49.8%	159	32.3%	
Age, years		60	(21–84)	66	(24–89)	<0.0001
Ethnicity	Caucasian	709	99.0%	482	97.8%	NS
	Other	7	1.0%	11	2.2%	
Weight, kg		75.15	(43.6–160)	79.95	(38–135)	<0.0001
Body mass index		28.25	(17.24–61.76)	29.34	(18.71–51.73)	<0.0001
Glucose (mg/dl)		97	(56–254)	101	(47–355)	<0.001
Calcium (mg/dl)		9.4	(5.6–10.6)	9.4	(6.2–10.7)	NS
Sodium (mEq/L)		141	(133–149)	141	(128–148)	NS
Potasium (mEq/L)		4.5	(3.5–5.9)	4.7	(3.2–7.5)	<0.0001
Cholesterol, mg/dl		201	(121–322)	180	(76–317)	<0.0001
HDL, mg/dl		52.0	(24.8–137)	47.0	(15.1–132)	<0.0001
LDL, mg/dl		125.0	(35–232.1)	103.0	(26–235)	<0.0001
Diabetes	No	660	92.3%	379	76.9%	<0.0001
	Yes	55	7.7%	114	23.1%	
Hypertension	No	310	43.4%	19	3.9%	<0.0001
	Yes	405	56.6%	474	96.1%	
Smoking	Non-smokers	327	45.7%	203	41.2%	NS
	Former smokers	263	36.8%	202	41.0%	
	Smokers	125	17.5%	88	17.8%	
Dyslipidemia	No	313	64.1%	146	31.7%	<0.0001
	Yes	175	35.9%	314	68.3%	
Creatinine, mg/dl		0.83	(0.50–1.28)	1.68	(0.32–8.17)	<0.0001
eGFR, ml/min		88.78	(12–149)	38.11	(5–60.74)	<0.0001
ACR, mg/g		6.40	(0.01–256)	72.62	(0.01–4,583.89)8	<0.0001

eGFR, estimated glomerular filtration rate; ACR, albumin-to-creatinine ratio; NS, non-significant.

TABLE 2 | Adjusted risk analysis for the risk of nephrosclerosis.

Gene	SNP	Genotype	Control, n (%)	CKD, n (%)	OR	*p*-value
SCARB1	rs838880	T/T	314 (45.5)	235 (49.5)	0.66 (0.51–0.87)	0.003
		T/C-C/C	376 (54.5)	240 (50.5)		
SCARB1	rs10846744	G/G	460 (66.7)	285 (60.3)	1.48 (1.11–1.96)	0.007
		G/C-C/C	230 (33.3)	188 (39.7)		
PLA2G4A	rs78178583	A/A	611 (85.7)	401 (82.2)	1.58 (1.09–2.29)	0.015
		A/C-C/C	102 (14.3)	87 (17.8)		
PLA2G4A	rs72709847	T/T	575 (80.8)	413 (84.8)	0.70 (0.49–0.99)	0.046
		T/A-A/A	137 (19.2)	74 (15.2)		
PLA2G4A	rs1569479	T/T	153 (22.1)	133 (28.1)	0.73 (0.53–1)	0.048
		T/G-G/G	538 (77.9)	340 (71.9)		

CKD, nephrosclerosis patients; OR, odds ratios with 95% confidence interval.

(0–9); $p = 1.48E\text{-}22$] and the severity score (mean = 1.77 ± 0.71 vs. 1.36 ± 0.82, $p = 4.83E\text{-}14$); overall indicating a far higher incidence of atherosclerosis in the CKD patients compared with control subjects.

The group of nephrosclerosis patients was analyzed to investigate associations with ccIMT, presence of plaques, total number of plaques and atherosclerosis score. *SCARB1* tag-SNPs did not show any relation to atherosclerosis in our study sample (data not shown) after adjusting for confounding variables (age, sex, body mass index, ethnicity, blood pressure, diabetes and CKD stage); however, *PLA2G7* variants were found to particularly affect the presence of atheromatous plaques (7 tag-SNPs) and the number of plaques detected (6 tag-SNPs), with *p*-values ranging from 0.0004 to 0.047 (**Figure 1**). Six of these variants, namely rs41273658, rs6899519, rs2216463, rs1421369, rs6915496, and rs9472836, were significantly associated with more than one of the four atherosclerosis measurements.

The impact of variability in *PLA2G4A* is depicted in **Figure 2**. In this case, observed associations were more noticeable with the variable "presence of atheromatous plaques" and particularly for a distal region of the gene starting at rs72709847, which showed *p*-values between 0.002 and 0.040 (**Figure 2**).

The median (range) ccIMT progression per year in the nephrosclerosis patients was 0.0125 (-0.22–0.45) mm and the threshold for accelerated progression (75th percentile) was 0.412 mm/year. Six tag-SNPs (one in *PLA2G7* and five in *PLA2G4A*) had a significant impact on the risk of accelerated

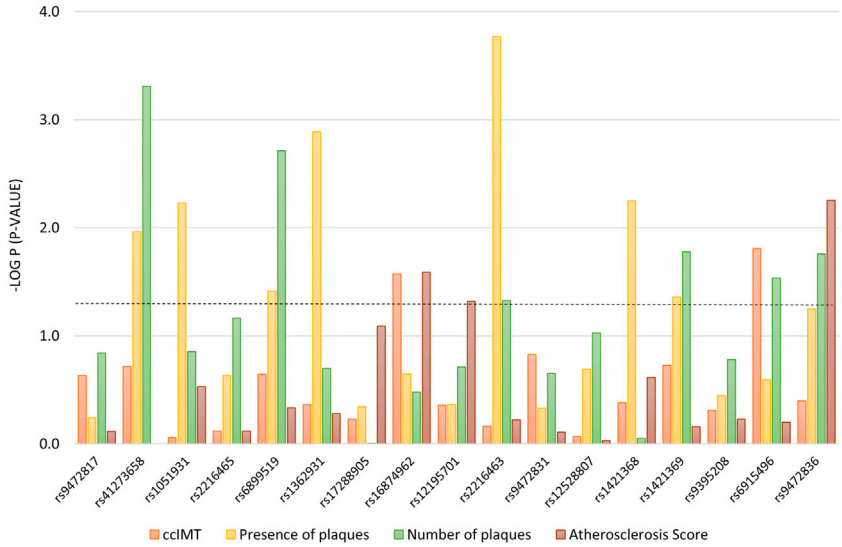

FIGURE 1 | Association between tag-SNPs in the *PLA2G7* gene with atherosclerosis-related measurements. The dotted line denotes the level of statistical significance. ccIMT, common carotid intima media thickness (mm).

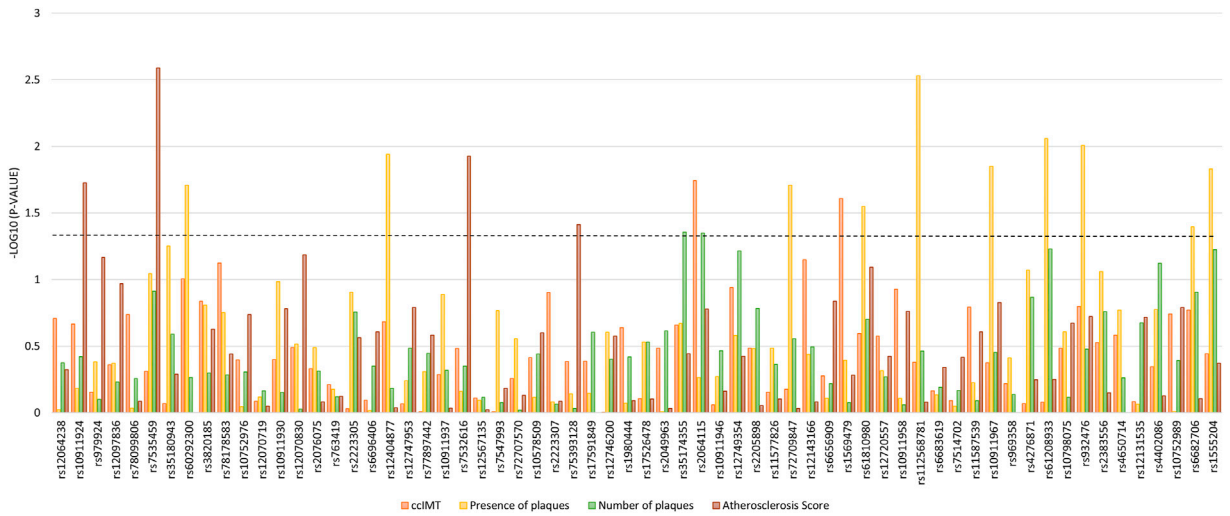

FIGURE 2 | Association between tag-SNPs in the *PLA2G4A* gene with atherosclerosis-related measurements. The dotted line denotes the level of statistical significance.

progression in nephrosclerosis patients after adjusting for confounding variables. Most notably, patients who carried the *PLA2G7* rs9472836-A variant allele showed the strongest association with lower risk of progression [OR = 0.39 (0.19–0.80), p = 0.006, **Table 3**].

Parameters of Renal Damage and Function

A mild but significant inverse correlation between ccIMT and eGFR values was observed in the whole population of study (r = -0.181, p = 2.15E-07) (**Supplementary Figure S1**). The genetic association analysis in the nephrosclerosis cohort revealed a central region of the *PLA2G4A* gene locus, tagged from rs10578509 to rs17526478 (positions 1:186873127-186882025) that displayed the most significant associations with glomerular filtration and proteinuria values (**Supplementary Figure S2**). Remarkably, rs2223307 and rs17591849 SNPs were linked to both parameters (p-values for associations with eGFR and albumin-to-creatinine ratios (ACR) were 0.033 and 0.042 for the rs2223307 and 0.036 and 0.016 for rs17591849). Of the remaining SNPs studied, only one variant in *SCARB1* and another in *PLA2G7* were associated with renal function (**Supplementary Table S2**).

TABLE 3 | Adjusted association analysis with the risk of accelerated ccIMT progression in nephrosclerosis patients.

Gene	SNP	Genotype	SP, n (%)	AP, n (%)	OR	p
PLA2G7	rs9472836	G/G	145 (68.4)	60 (84.5)	0.39 (0.19–0.80)	0.006
		G/A-A/A	67 (31.6)	11 (15.5)		
PLA2G4A	rs72707570	G/G	85 (41.3)	19 (27.1)	2.0 (1.08–3.69)	0.024
		G/C-C/C	121 (58.7)	51 (72.9)		
PLA2G4A	rs10578509	T/T	152 (76.4)	58 (86.6)	0.47 (0.21–1.03)	0.045
		T/del-del/del	47 (23.6)	9 (13.4)		
PLA2G4A	rs12746200	A/A	161 (80.1)	61 (89.7)	0.44 (0.19–1.05)	0.049
		A/G-G/G	40 (19.9)	7 (10.3)		
PLA2G4A	rs12143166	A/A	70 (34.8)	33 (49.3)	0.54 (0.31–0.-97)	0.039
		A/G-G/G	131 (65.2)	34 (50.7)		
PLA2G4A	rs112568781	A/A	152 (75.2)	43 (63.2)	1.84 (1.01–3.34)	0.048
		A/G-G/G	50 (24.8)	25 (36.8)		

SP, slow progression; AP, accelerated progression; OR, odds ratios with 95% confidence interval.

TABLE 4 | Demographic, biochemical and clinical characteristics of nephrosclerosis patients with or without cardiovascular events (CVE) recorded during the four-year follow-up.

	No CVE	*CVE*	*p*
Patients	452	41	
Men	300 (66.4%)	34 (82.9%)	0.02
Women	152 (33.-6%)	7 (17.1%)	
Age (years)	66 (24–89)	67 (53–78)	0.01
Weight (kg)	79.8 (38–135)	81.4 (52–125)	0.56
Body mass index	29.4 (18.71–51.73)	29.7 (20.31–48.83)	0.55
Waist circumference (cm)	100 (67–138)	104 (69–131)	0.36
Tobacco			
Nonsmokers	190 (42%)	13 (31.7%)	0.22
Former smokers	185 (40.9%)	17 (41.5%)	
Current smokers	77 (17%)	11 (26.8%)	
Hypertension	435 (96.2%)	39 (95.1%)	0.72
Diabetes Mellitus	100 (22.1%)	14 (34.1%)	0.08
Dyslipidemia	284 (67.8%)	30 (73.2%)	0.47
Chronic kidney disease			
Stage 5D	57 (12.6%)	6 (14.6%)	0.93
Stage 4–5	113 (25%)	10 (24.4%)	
Stage 3	282 (62.4%)	25 (60.9%)	
Biochemical findings			
Cholesterol (mg/dl)	181 (76–317)	165 (108–317)	0.06
Glucose (mg/dl)	100.5 (47–355)	102 (56–195)	0.13
eGFR (MDRD4)	38.2 (5–60.74)	37.4 (13–59.6)	0.7
Albumin/Cr (mg/g)	68.9 (0.01–4,391.65)	173.6 (0.21–4,583.89)	0.17

Cardiovascular events

The 1,209 participants in the study were subjected to follow-up for 4 years (median = 47 months, range = 7–54). Forty-one CV events (8.3%) were reported for nephrosclerosis patients vs. only nine (1.3%) for control subjects [OR = 7.13 (3.4–14.8), $p = 1.32$ e-09]. **Table 4** lists the main demographic and clinical characteristics of patients with or without CV events.

The results of genetic association analyses on the incidence of CV events in the nephrosclerosis patients under dominant and recessive inheritance models are shown in **Supplementary Table S3**. While *SCARB1* and *PLA2G7* did not harbor any relevant SNPs, eight homozygous variant genotypes in the *PLA2G4A* gene were found to significantly affect CV risk (p-values ranging from 0.002 to 0.049). We then carried out ROC analysis to establish the predictive power of these eight variants for CV risk. **Figure 3A** depicts the curves corresponding to predictive models including classic risk factors only, genetics only and both combined in the patients group. Interestingly, AUC values for the classic and genetics only curves were quite similar ($p = 0.972$), whilst the addition of both types of information resulted in a higher AUC of 79.1% (73.1–85.1) that was significantly improved compared to the classic model ($p = 0.047$). When we extended the analysis to both controls and patients (**Figure 3B**), the classic risk model gained more predictive power [AUC = 78.3% (71.9–84.6)]. This resulted in a high AUC of 84.4% (79.5–89.3) for the combined model but that was not significantly different from the one containing exclusively classic risk factors ($p = 0.135$).

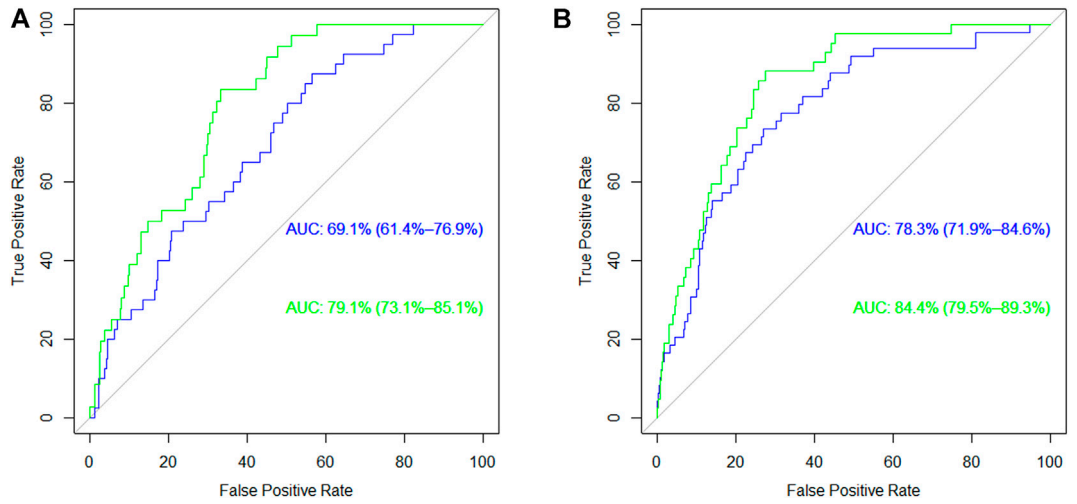

FIGURE 3 | Receiving Operating Curves for the risk of cardiovascular events in **(A)** nephrosclerosis patients and **(B)** the whole population of patients and controls. The blue line corresponds to the model with classic risk factors and the green line corresponds to the same model when genetic information is added. AUC, area under the curve.

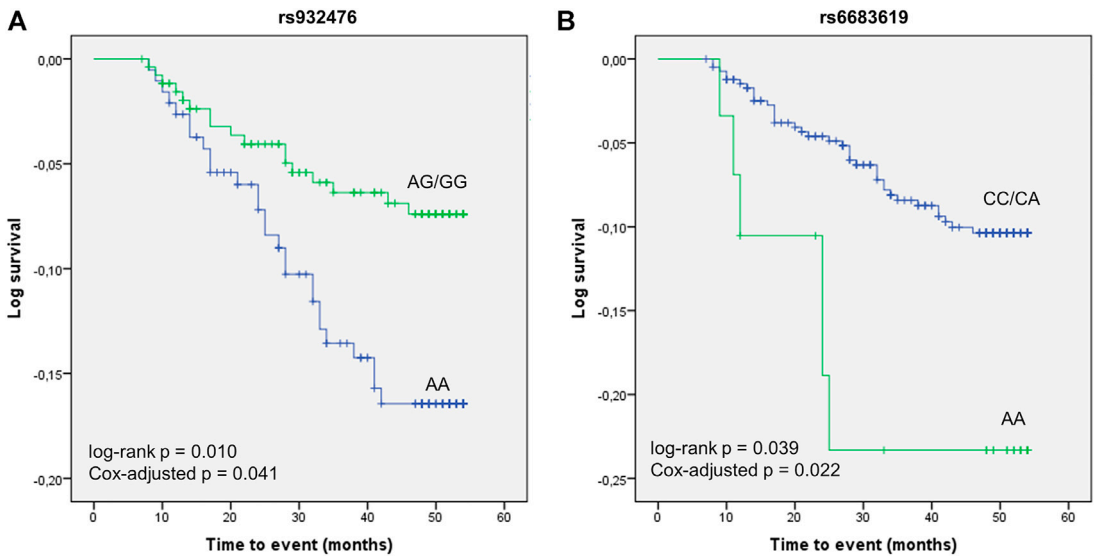

FIGURE 4 | Kaplan-Meier curves of the association of two *PLA2G4A* polymorphisms, rs932476 **(A)** and rs6683619 **(B)**, with CV event-free survival.

We next examined associations with CV event-free survival in the cohort of nephrosclerosis patients. Eight SNPs, all in the *PLA2G4A* gene, displayed log-rank *p*-values <0.1 and were subsequently subjected to Cox regression analysis. After adjusting for other CV risk factors, the AA genotype of rs932476 significantly decreased CV event-free survival [estimated mean = 49.59 (47.97–51.21) vs. 51.81 (49.93–51.78) months for AG/GG carriers, $p = 0.041$, **Figure 4A**]. In addition, carriers of the rs6683619 AA genotype also had lower survival than subjects harbouring the CC/CA genotypes [46.46 (41.00–51.92) vs. 51.17 (50.25–52.08) months, $p = 0.022$, **Figure 4B**].

DISCUSSION

There is a need to find new biomarkers in the field of CKD that can help identify patients at risk and that are able to predict how fast the disease is going to progress to ESRD. This need is pressing in nephrosclerosis, as these patients often find themselves within a vicious circle, where kidney deterioration worsens CV risk whilst, in turn, these CV implications accelerate disease progression. To our knowledge, this is the first time that the clinical impact of phospholipase-related SNPs has been evaluated in the nephrosclerosis setting, which is ideal for investigating

atherosclerosis measurements and CV outcomes. Indeed, nephrosclerosis and atherosclerosis go frequently hand in hand (Kawamoto et al., 2008; Erten et al., 2011). Furthermore, nephrosclerosis patients suffer from a CV mortality which is up to 20 times more frequent than in the general population (Foley et al., 1998) and have mortality rates higher than other CKD groups after renal replacement therapy (4).

Our results showed that two SNPs in the *SCARB1* gene, rs10846744, and rs838880, were significantly linked to the susceptibility to nephrosclerosis. The SR-BI receptor, encoded by *SCARB1*, is the primary mediator of the selective transfer of lipids from HDL into cells, and has also been shown to mediate the internalization of oxidized phospholipids (Gillotte-Taylor et al., 2001). In contrast to native HDL, oxidized HDL may increase the production of inflammatory cytokines. In this regard, oxidized HDL has been reported to enhance proinflammatory properties in mesangial and other renal cells, which has been pointed out as an important mechanism of CKD (Zhang et al., 2010; Gao et al., 2014). Moreover, SR-BI expression has been shown to be increased in animal models of chronic renal failure (Cho et al., 2010). It is therefore tempting to speculate that genetic variants affecting SR-B1 activity/expression could increase the transport of oxidized lipids into renal cells and hence constitute an explanation for the observed association with the increased nephrosclerosis risk. Other mechanisms are also possible. For instance, rs10846744 is a widely studied variant located in intron 1 of the *SCARB1* gene containing DNase I hypersensitivity clusters and enhancer-promoter histone markers. This SNP has not shown correlation with SR-BI expression (Manichaikul et al., 2012), rather, it has been proposed to mediate the enhanced expression of distant genes affecting apoptosis (Li et al., 2005) and endothelial (Mineo and Shaul, 2007) or inflammatory pathways (Suchindran et al., 2010), which could also contribute to the disease. Three variants in *PLA2G4A* also showed associations with the risk of nephrosclerosis, although rs72709847 and rs1569479 were at the border of statistical significance. The remaining tag-SNP, rs78178583, is an intronic A-to-C substitution located within the promoter flanking region of the gene (ENSR00000934514), and therefore it is plausible that it could be tagging genetic variability modulating cPLA2 expression, which could in turn affect the risk of CKD, since cPLA2 expression has been related to renal damage both in humans and animal models (Montford et al., 2016). A suggested mechanism would be that this enzyme releases AA from the membrane leading to the synthesis of proinflammatory mediators such as PGE2, a major actor mediating renal injury (Francois et al., 2007).

Nephrosclerosis and atherosclerosis share risk factors and similar etiopathogenic mechanisms (Meyrier, 1996). Renal parenchyma is replaced by collagen and scar tissue in the former, whilst the latter presents with eccentric and medial thickening of arteries. Indeed, it has been proposed that increased atherosclerosis contribute to the rate of glomerulosclerosis observed in these CKD patients (Tracy et al., 1996). In this regard, a Japanese study had reported an inverse correlation between ccIMT and eGFR values (Kawamoto et al., 2008), which we could also observe in our population and that it might support the connection between renal and CV disease. However, the observed correlation, although significant, was very weak and should be interpreted with caution. Our findings also show that variability in the *PLA2G4A* and *PLA2G7* gene loci was associated with several atherosclerotic features in nephrosclerosis patients, including the risk of an accelerated progression. Whilst there is no available information on the effect of *PLA2G4A* variants on atherosclerosis measurements, there are some previous reports linking *PLA2G7* SNPs to this pathology. Thus, (Wu et al., 2020) reported that the A379V polymorphism was associated with carotid plaque formation, but not plaque vulnerability, in ischemic stroke patients . In the same line, (Santoso et al., 2017) performed a meta-analysis including over 12,000 patients and identified two nonsynonymous SNPs significantly associated with the risk of clinical atherosclerosis . The results presented herein confirm the important role of phospholipases in atherosclerosis, highlight *PLA2G4A* as a genetic locus to be considered, and overall point at nephrosclerosis as an atherosclerosis-related pathology where the screening of phospholipase genes could be useful to identify patients more prone to a worse clinical course.

A remarkable finding of this study was that several tag-SNPs in *PLA2G4A* were independent CV risk factors in the nephrosclerosis patients. Moreover, they significantly enhanced the predictive power of a model containing standard, non-genetic risk factors. It should be noticed how the improvement after adding the genetic information was greater in the nephrosclerosis patients than in the whole study sample, as the incidence of classic risk factors in the former is already high. It is somewhat surprising that it was the *PLA2G4A* gene, and not *PLA2G7*, coding for the traditionally CVD-associated Lp-PLA2, that exerted the most noticeable influence on the incidence of CV events. However, it should be noted that the association of *PLA2G7* with CVD is still controversial (Hou et al., 2009; Casas et al., 2010; Zheng et al., 2011; Garg et al., 2018). For instance, a large study with over 45,000 coronary heart disease patients and 88,000 controls could not find any loss-of-function allele related to disease risk (Gregson et al., 2017). Likewise, clinical trials with darapladib (Lp-PLA2 inhibitor) have failed to show benefit on the prevention of major coronary events (White et al., 2014; O'Donoghue et al., 2014). On the other hand, it could also be that the CV risk genetic profile in nephrosclerosis patients was different from that occurring in the general population. Unfortunately, only eight events were registered in our control subjects and hence no formal analysis could be carried out to address this hypothesis.

The results from the survival study also pointed to *PLA2G4A* as a key genetic locus regarding CV risk in nephrosclerosis, with two tag-SNPs (rs932476 and rs6683619) significantly affecting event-free survival. In any case, it should be mentioned that the association with rs6683619 was only observed for a recessive model and hence it was based on a low number of events. There is a growing body of evidence supporting the involvement of *PLA2G4A* in CVD. The main function of cPLA2, the enzyme encoded by *PLA2G4A*, is the release of arachidonic acid from the membrane to initiate a cascade of pro-inflammatory mediators. In this regard, our group has previously reported

an association between SNPs in PGE2 receptors, at the end of this cascade, with the occurrence of CV events in CKD patients (González et al., 2021). In addition, cPLA2 have been shown to mediate platelet aggregation induced by homocysteinemia (d'Emmanuele di Villa Bianca et al., 2013) and by high doses of digoxin in atrial fibrillation that can be behind CV mortality in these patients (Pastori et al., 2018). Moreover, polymorphisms in *PLA2G4A* have also been related to myocardial infarction (Hartiala et al., 2012) and coronary artery disease (Hartiala et al., 2011). Overall, our results are in line with this background and indicate that the areas of *PLA2G4A* tagged by the SNPs presented herein hold the potential to be useful biomarkers for CV risk in nephrosclerosis patients. Furthermore, these findings suggest that cPLA2 may be an interesting therapeutic target and that novel cPLA2 inhibitors that are being currently developed for a variety of inflammatory diseases (Nikolaou et al., 2019) should also be evaluated for CVD.

In our cohort, significant sex-related differences were observed regarding the incidence of nephrosclerosis and the occurrence of CV events, with males showing higher risk for both outcomes. To establish whether the studied SNPs could, at least in part, be behind these differences, we analyzed their distribution both in CKD patients and in the whole population (data not shown). None of the SNPs with a relevant role in the risk of nephrosclerosis or in CV outcomes were differently distributed between men and women. Therefore, we found no evidence supporting a role of the studied variants in the observed sex-related differences in nephrosclerosis.

Our study has several limitations. First, we did not measure Lp-PLA2 activity, which has been related to CV outcomes (Huang et al., 2020) and could have helped identify putative mechanisms for the genotype-phenotype associations reported. Second, the *PLA2G4A* gene was far more polymorphic than *PLA2G7 and SCARB1* and consequently far more SNPs were necessary to tag the whole locus, which could have led to the obtention of more significant results. Third, our study design implies that the reported genotype-phenotype associations cannot, in general, be linked to a specific biochemical consequence of the SNP, as these were tag-SNPs, i.e., intronic variants that represent variability in a certain haplotype block. In contrast, one of the strengths of the study is precisely that by revealing clinically relevant tag-SNPs, we made it possible to infer total genetic variability in the tagged region of the gene locus and identify phenotypic associations without having to determine the rest of SNPs in that area. Another asset of the study was the uncommon homogeneity of the patient cohort, as CKD diagnosis was limited to nephrosclerosis, thus excluding patients with diabetic nephropathy, who are usually included together with nephrosclerosis patients in a single entity (Smith et al., 2020).

In summary, our results taken together indicate that phospholipase-related genes play a relevant role in nephrosclerosis and associated CV outcomes. We showed that *SCARB1* for the risk of nephrosclerosis, *PLA2G7* and, especially, *PLA2G4A* for the CV risk in these patients, are loci that harbor genetic variants whose identification could be of utility in the management of this CKD.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://figshare.com/, https://figshare.com/articles/dataset/Phospholipases_genes_dataset/17032403.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Bioethics and Biosafety Committee of the University of Extremadura Clinical Research Ethics Committee of the Badajoz University Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LG recruited patients, carried out statistical analyses and drafted the manuscript; NR, JA-L and JV helped with study design and the clinical evaluation of the patients; SM-Z carried out genetic analyses; JL-G participated in sample collection and clinical analyses; GG designed the study, searched for funding and wrote the final version of the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphar.2022.817020/full#supplementary-material

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Influence of variability in the cyclooxygenase pathway on cardiovascular outcomes of nephrosclerosis patients

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Nephrosclerosis patients are at an exceptionally high cardiovascular (CV) risk. We aimed to determine whether genetic variability represented by 38 tag-SNPs in genes of the cyclooxygenase pathway (*PTGS1*, *PTGS2*, *PTGES*, *PTGES2* and *PTGES3*) leading to prostaglandin E2 (PGE2) synthesis, modified CV traits and events in 493 nephrosclerosis patients. Additionally, we genotyped 716 controls to identify nephrosclerosis risk associations. The addition of three variants, namely *PTGS2* rs4648268, *PTGES3* rs2958155 and *PTGES3* rs11300958, to a predictive model for CV events containing classic risk factors in nephrosclerosis patients, significantly enhanced its statistical power (AUC value increased from 78.6 to 87.4%, p = 0.0003). Such increase remained significant after correcting for multiple testing. In addition, two tag-SNPs (rs11790782 and rs2241270) in *PTGES* were linked to higher systolic and diastolic pressure [carriers vs. non-carriers = 5.23 (1.87–9.93), p = 0.03 and 5.9 (1.87–9.93), p = 0.004]. *PTGS1*(COX1) rs10306194 was associated with higher common carotid intima media thickness (ccIMT) progression [OR 1.90 (1.07–3.36), p = 0.029], presence of carotid plaque [OR 1.79 (1.06–3.01), p = 0.026] and atherosclerosis severity (p = 0.041). These associations, however, did not survive Bonferroni correction of the data. Our findings highlight the importance of the route leading to PGE2 synthesis in the CV risk experienced by nephrosclerosis patients and add to the growing body of evidence pointing out the PGE2 synthesis/activity axis as a promising therapeutic target in this field.

Nephrosclerosis is an umbrella term that usually denotes the presence of renal impairment in an aging patient with hypertension, frequently with no histological confirmation[1]. This is a chronic kidney disease (CKD) that not only contributes greatly to progression to end-stage kidney disease (ESKD), but that also have an immense impact on global cardiovascular (CV) risk[2]. Classic CKD biomarkers only stand out when the disease is well under way and there is therefore a need for novel markers that can help in the early identification of patients at risk for adverse outcomes.

Arachidonic acid is metabolized by cyclooxygenases COX1 and COX2 to a variety of inflammatory mediators. Of these, PGE2 is the major arachidonic metabolite in the kidney[3] and is responsible for both renal homeostasis and pathological mechanisms such as inflammation, hyperfiltration, fibrosis, apoptosis or renin-angiotensin aldosterone system (RAAS) activation[4–6]. PGE2 production is directly governed by one cytosolic (cPGES) and two microsomal synthases (mPGES1 and mPGES2), whose activity has been related to renal function impairment and blood pressure elevation[7,8].

Genetics are known to play a role in CKD onset and development[9,10], accordingly, we hypothesize that the pathway leading to PGE2 synthesis and its actions may be a suitable candidate for identifying genetic variants relevant for CKD, particularly for its CV-associated impact. Indeed, we have recently shown how single nucleotide polymorphisms (SNPs) in the genes coding for PGE2 receptors[11] and phospholipase-related genes[12] are associated with CV outcomes in these patients. Our aim was therefore to identify tag-SNPs (variants that represent variability in a certain region of the gene locus) in five candidate genes of the cyclooxygenase pathway (Fig. 1),

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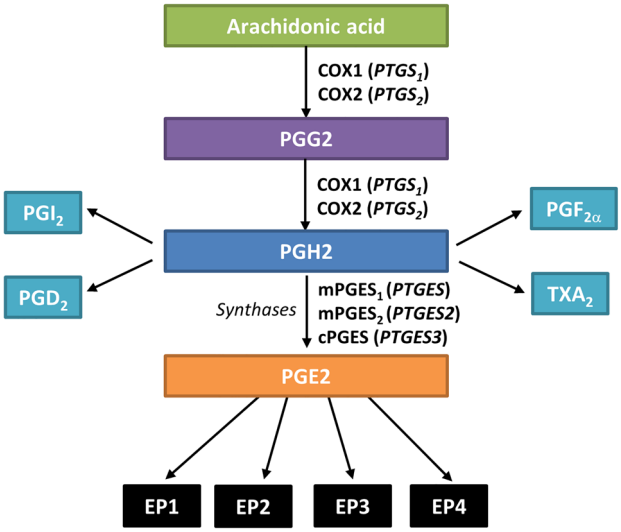

Figure 1. Overview of the cyclooxygenase-PGE2 pathway. Gene names are shown in parenthesis.

namely *PTGS1* (COX1), *PTGS2* (COX2), *PTGES* (mPGES1), *PTGES2* (mPGES2) and *PTGES3* (cPGES), and investigate putative associations with CV traits and events (CVE) in these patients. Additionally, we screened a group of individuals with normal renal function to identify associations with the risk of nephrosclerosis.

Results

Median age and interquartile range (IQR) of patients and controls were, respectively, 66 (12) and 60 (17) years, whilst the percentage of males in the same groups were 50.2% and 67.7%. The percentage of classic CV risk factors such as diabetes, hypertension, or dyslipidemia, was higher in the CKD patients than in the control group ($p < 0.0001$). These variables were therefore included, amongst others, in the regression models that were later applied. In contrast, the frequency of smokers was similar in both study groups. As expected, biochemistry data were also significantly different between patients and controls. Table 1 shows these and other demographic and clinical characteristics of the study groups stratifying patients by CKD stage.

PTGES3 rs78343990 showed a significant deviation from the Hardy–Weinberg equilibrium ($p < 0.05$) and its results were therefore disregarded. Minor allele frequencies (MAF) and successful genotyping rates for the analyzed loci ranged from 2.8 to 38.8% and from 95.2 to 99.8%, respectively.

Genetic associations with cardiovascular events. A four-year follow-up [median (IQR) = 47 (6) months] was carried out in the population of study that registered 41 and 9 CVE in the CKD patients (8.3%) and control group (1.3%), respectively. As expected, nephrosclerosis had a deep impact on CV risk [OR 7.13 (3.4–14.8), $p < 0.0001$]. Main features of individuals with and without CVE are listed in Table 2.

We analyzed the effect of the 38 tag-SNPs studied on CV event-free survival in the patients' cohort. Kaplan–Meier curves compared with the log-rank test showed that three SNPs, namely *PTGS2* rs4648268, *PTGES3* rs2958155 and *PTGES3* rs11300958, displayed suggestive associations (Fig. 2). Median survival for carriers vs. non-carriers of the three variants were, respectively, 52.79 (51.42–54.16) vs. 50.56 (49.47–51.64) months, $p = 0.030$; 49.97 (48.65–51.28) vs. 52.59 (51.15–53.42) months, $p = 0.013$; and 49.58 (48.00–51.17) vs. 51.86 (50.78–52.94) months, $p = 0.021$. The direction of the associations was maintained when analyses were adjusted by traditional CV risk factors (age, sex, BMI, diabetes, hypertension, and CKD stage) in Cox regression models, although corrected p-values were higher than the Bonferroni threshold. Hazard ratios for the variant genotypes were 0.31 (0.09–0.99), $p = 0.049$; 2.41 (1.15–5.04), $p = 0.02$ and 2.20 (1.16–4.18), $p = 0.016$ for rs4648268, rs2958155 and rs11300958, respectively. Supplementary Tables S1–S3 show the resulting Cox models for each SNP. We also performed a sub-analysis to re-assess these associations when only coronary events were considered. Survival analysis show that only PTGS2 rs4648268 remained linked to this subgroup of events ($p = 0.020$), as none of the patients carrying the T-variant allele experienced an event (Supplementary Fig. S1).

Next, we developed CV risk prediction models using Receiving Operating Characteristic curves (ROC) analysis, with the state variable being the occurrence of a CV event during follow-up. Figure 3A shows that, in the whole population of study, the addition of the aforementioned three SNPs to a model containing traditional CV risk factors (age, sex, hypertension, diabetes, ethnicity and CKD stage), slightly improved its predictive power from an AUC of 84.7% to 87.3% ($p = 0.031$). Interestingly, when the analysis was restricted to the patients' group, this improvement was far larger, as the addition of genetic information made the AUC increase from 78.6 to 87.4% ($p = 0.0003$, Fig. 3B). Such increase was still significant after Bonferroni correction for multiple testing.

In order to investigate whether any of the 38 tag-SNPs could interact with each other to modify CV risk, we also estimated associations of pairwise statistical epistasis (Fig. 4). Three SNP-pair interactions were identified, two between variants in *PTGS2* (COX2) and *PTGS1* (COX1), namely rs4648268-rs1213265 ($p < 0.01$) and

	Control (N = 716)	CDK3 (N = 307)	CDK4-5 (N = 123)	CDK 5D (N = 63)	p-value (control vs. CKD)	p-value (CKD groups)
Males (%)	359 (50.2)	211 (68.7)	80 (65)	43 (68.3)	< 0.0001	NS
Age (years)	60 (17)	67 (10)	64 (14)	61 (18)	< 0.0001	< 0.001
Ethnicity						
Caucasian	709 (99)	304 (99)	120 (97.6)	58 (92.1)	< 0.01	< 0.01
Other	7 (1)	3 (1)	3 (2.4)	5 (7.9)		
Weight (kg)	75.15 (19.83)	80.25 (16.55)	78.60 (19.70)	75 (21.2)	< 0.0001	< 0.05
BMI	28.25 (5.13)	29.97 (5.77)	28.75 (7.32)	26.96 (6.83)	< 0.0001	< 0.01
Glucose (mg/dL)	97 (20)	102.5 (25)	101.00 (21)	89 (21)	< 0.001	< 0.0001
Total cholesterol (mg/dL)	200.7 (43)	185 (54)	175.50 (55)	160.5 (48)	< 0.0001	< 0.0001
Cholesterol HDL (mg/dL)	52 (19.2)	47 (17)	46.9 (18.2)	45 (17)	< 0.0001	NS
Cholesterol LDL (mg/dL)	126.4 ± 32.24	110.81 ± 34.47	100.79 ± 34.73	89.55 ± 34.67	NS	NS
Calcium (mg/dL)	9.4 (0.5)	9.50 (0.6)	9.35 (0.8)	9.15 (0.8)	NS	< 0.0001
Potassium (mEq/L)	4.5 (0.5)	4.65 (0.6)	4.8 (0.8)	4.86 (0.6)	< 0.0001	< 0.05
Sodium (mEq/L)	141 (3)	141 (3)	141 (4)	138.5 (4)	NS	< 0.0001
ACR (mg/g)	6.4 (37.2)	37.11 (187.62)	212.11 (554.99)	–	< 0.0001	< 0.0001
eGFR (ml/min/1.73 m^2)	88.82 (21.34)	44 (13.74)	20.67 (9)	–	< 0.0001	< 0.0001
Cardiovascular events						
Yes	9 (1.3)	25 (8.1)	10 (8.1)	6 (9.5)	< 0.0001	NS
No	707 (98.7)	282 (91.9)	113 (91.9)	57 (90.5)		
Hypertension						
Yes	405 (56.6)	293 (95.4)	121 (98.4)	60 (95.2)	< 0.0001	NS
No	310 (43.4)	14 (4.6)	2 (1.6)	3 (4.8)		
DM						
Yes	55 (7.7)	74 (42.1)	32 (26)	8 (12.7)	< 0.0001	NS
No	660 (92.3)	233 (75.9)	91 (74)	55 (87.3)		
Smoking						
Non-smoker	327 (45.7)	124 (40.4)	53 (43.1)	26 (41)	NS	NS
Former-smoker	263 (36.8)	130 (42.3)	48 (39)	24 (38.1)		
Current-smoker	125 (17.5)	53 (17.3)	22 (17.9)	13 (20.6)		
Systolic pressure (mmHg)	133 (24)	145 (28)	146 (28)	139 (25)	< 0.0001	NS
Diastolic pressure (mmHg)	80 (14)	81 (14)	79 (15)	80 (20)	NS	NS
Pulse pressure (mmHg)	52 (16)	64 (26)	64 (22)	60 (20)	< 0.0001	NS

Table 1. Demographic and clinical parameters of the study population. This same population has also been analyzed in previous studies by our group[11,12]. *BMI* body mass index, *eGFR* estimated glomerular filtration rate, *DM* diabetes mellitus, *ACR* albumin-to-creatinine ratio, *NS* not significant.

rs5275-rs1238420 ($p < 0.01$), and one between SNPs located in PGE2 synthases: *PTGES2* rs17445108-*PTGES3* rs884115 ($p < 0.01$).

Genetic associations with cardiovascular-related variables of nephrosclerosis patients. The results of the association analyses between the 38 studied SNPs and blood pressure (BP) revealed that, most notably, two consecutive SNPs in the *PTGES* gene, which codes for a microsomal PGE2 synthase, rs11790782 and rs2241270, were associated with higher systolic BP (SBP), as revealed by linear regression analyses also considering age, sex, BMI, diabetes, ethnicity, and CKD stage. Mean difference values with 95% confidence interval for carriers vs. non-carriers of the two SNPs were 5.23 (1.87–9.93) mmHg, $p = 0.03$ and 5.9 (1.87–9.93) mmHg, $p = 0.004$ (Table 3). Interestingly, these same variant genotypes also increased diastolic BP (DBP) figures [2.86 (0.36–5.37), $p = 0.026$ and 2.77 (0.64–4.92), $p = 0.011$; Table 3). Two more SNPs, rs20417 and rs2302821 were also related to SBP and DBP, respectively (Table 3). No associations were observed regarding pulse pressure (data not shown).

Overall, atherosclerosis was far more prevalent in the nephrosclerosis patients than in control subjects. Indeed, patients had significantly higher values of: (i) mean atherosclerosis severity score (1.77 ± 0.71 vs. 1.36 ± 0.82, $p < 0.0001$); (ii) median (IQR) common carotid intima media thickness (ccIMT) [0.77 (0.22) vs. 0.71 (0.20) mm,

	No CVE	CVE	p-value
Males (%)	300 (66.4)	34 (82.9)	< 0.05
Age (years)	66 (12)	67 (9)	< 0.05
Ethnicity			
Caucasian	441 (97.6)	41 (100)	NS
Other	11 (2.4)	–	
Weight (kg)	79.8 (18.40)	81.5 (20.6)	NS
BMI	29.39 (6.07)	29.74 (6.99)	NS
Glucose (mg/dL)	100.5 (24)	102 (27)	NS
Total cholesterol (mg/dL)	181 (54)	165.5 (73)	NS
Cholesterol HDL (mg/dL)	47 (17)	41.55 (21.3)	< 0.01
Cholesterol LDL (mg/dL)	103 (45)	96.5 (56.5)	NS
Calcium (mg/dL)	9.4 (0.6)	9.5 (0.6)	NS
Potassium (mEq/L)	4.7 (0.7)	4.8 (0.6)	NS
Sodium (mEq/L)	141 (3)	141 (3)	NS
Albumin/creatinine (mg/g)	68.87 (284.37)	173.62 (445.07)	NS
eGFR (ml/min/1.73 m^2)	38.21 (21.02)	37.43 (22)	NS
Hypertension			
Yes	435 (96.2)	39 (95.1)	NS
No	17 (3.8)	2 (4.9)	
DM			
Yes	100 (22.1)	14 (34.1)	NS
No	352 (77.9)	27 (65.9)	
Smoking			
Non-smoker	190 (42)	13 (31.7)	NS
Former-smoker	185 (40.9)	17 (41.5)	
Current-smoker	77 (17)	11 (26.8)	
Systolic pressure (mmHg)	144 (28)	145 (28)	NS
Diastolic pressure (mmHg)	81 (15)	77.50 (18)	NS
Pulse pressure (mmHg)	62 (24)	67 (21)	NS

Table 2. Characteristics of individuals with and without cardiovascular events registered during the follow-up in the whole population of study. *BMI* body mass index, *eGFR* estimated glomerular filtration rate, *DM* diabetes mellitus, *NS* not significant.

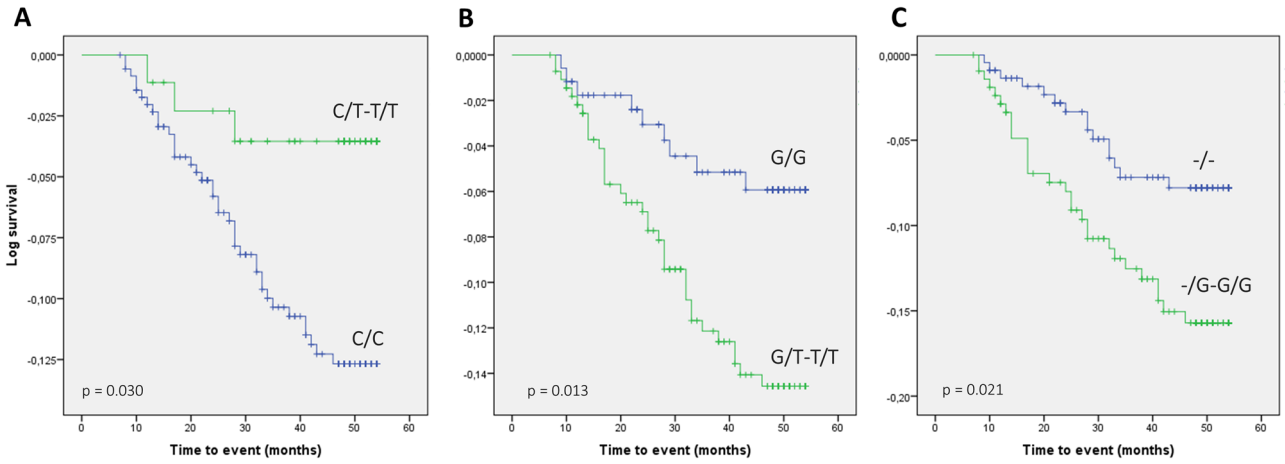

Figure 2. Kaplan–Meier curves for the association of *PTGS2* rs4648268 (**A**), *PTGES3* rs2958155 (**B**) and *PTGES3* rs11300958 (**C**) with cardiovascular event-free survival. Wild type homozygous vs. variant genotypes are depicted. P-values for the long-rank tests carried out for comparison of the different genotypes are shown. The rs11300958 polymorphism produces an insertion (G-allele).

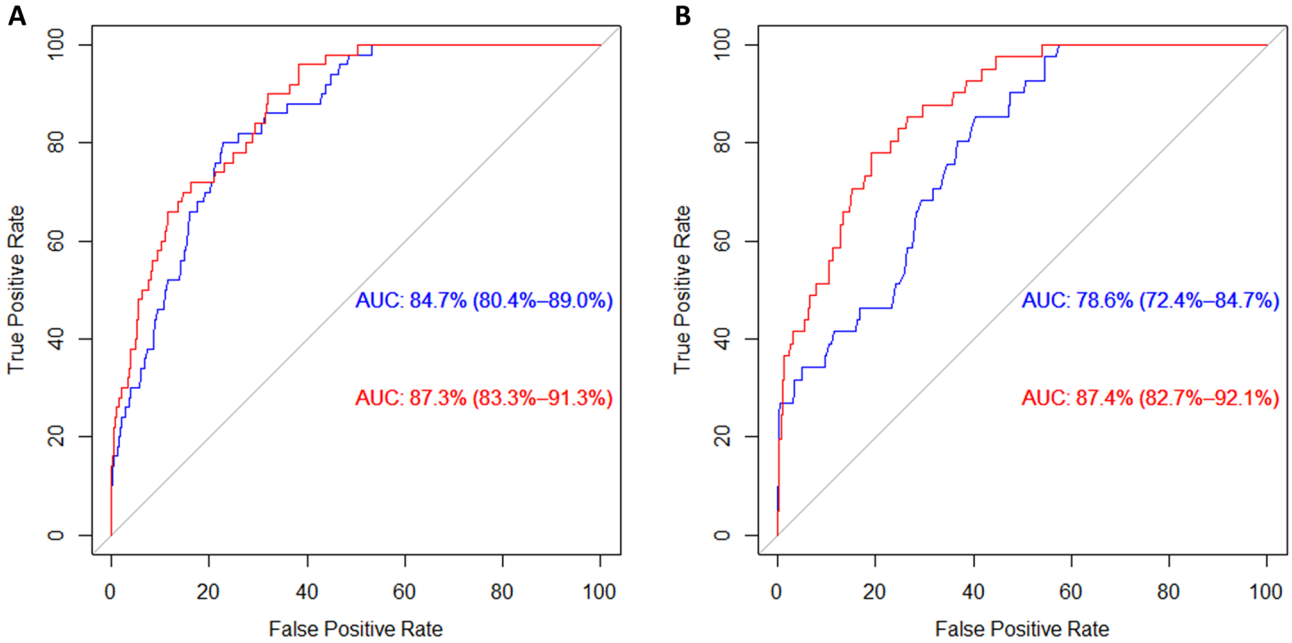

Figure 3. Receiving operating curves for the risk of cardiovascular events in (**A**) the whole population of patients and controls and (**B**) nephrosclerosis patients. The blue line corresponds to the model with classic risk factors and the red line corresponds to the same model when genetic information is added. *AUC* area under the curve.

$p < 0.0001$]; (iii) frequency of atheromatous plaques (79.4% vs. 55.9%; $p < 0.0001$); and (iv) median number of plaques detected [3 (4) vs. 1 (3); $p < 0.0001$]. The threshold for accelerated ccIMT progression in the nephrosclerosis patients was 0.412 mm/year.

The analysis of genetic associations with atherosclerosis measurements in the CKD group was carried out by linear or binary regression analyses (depending on the measured trait) controlling for age, sex, BMI, hypertension, diabetes, ethnicity, and CKD stage. Most notably, *PTGS1* rs10306194 carriers displayed higher values of three parameters, namely ccIMT progression [OR for accelerated progression = 1.90 (1.07–3.36), $p = 0.029$], presence of carotid plaque [OR = 1.79 (1.06–3.01), $p = 0.026$] and atherosclerosis severity score ($p = 0.041$; Table 4). Four more variants were found to be related to atherosclerosis (Table 4), albeit none of them affected more than one measurement. It should be noted, however, that none of the associations with BP traits or atherosclerosis remained significant after correction for the 37 SNPs analyzed.

Genetic associations with susceptibility to nephrosclerosis. Finally, we genotyped a control group to perform risk analyses adjusted by significant covariates (see "Methods") under a dominant model of inheritance, i.e., carriers vs. non-carriers. Three SNPs in *PTGS2* (coding for COX2) were associated with nephrosclerosis. These were rs2066826 [OR 0.72 (0.54–0.98), $p = 0.032$], rs4648268 [OR 1.46 (1.03–2.07), $p = 0.032$] and rs20417 [OR 0.70 (0.53–0.93), $p = 0.013$]. However, the addition of these SNPs to a ROC model containing other CKD risk factors did not improve the AUC of the curve significantly [84.6% (82.4–86.7) vs. 84.9% (82.8–87.0) for the combined standard-genetics model, $p = 0.156$; Supplementary Fig. S2].

Discussion

Patients with nephrosclerosis face renal impairment that worsens CV risk, while this increased risk speeds up disease progression. This vicious circle results in these patients not only having far higher CV mortality rates than the general population[13], but also higher rates than those shown by other CKD groups after dialysis or transplant[14]. This situation makes necessary the identification of new biomarkers that can help with the early identification of index patients.

One of the most interesting findings of the study is that three SNPs, one in COX2 and two in a PGE2 synthase, were independent CVE risk factors in the population of nephrosclerosis patients, as shown by Kaplan–Meier curves and Cox regression models. Furthermore, the addition of these variants to a model containing classic risk factors, significantly improved its predictive power. It should be noted that this improvement was greater in the patient group than when the whole population of controls and patients were considered. Two reasons may explain this. First, the incidence of CVE in controls was very low (only nine cases) and, second, standard CV risk factors are already highly prevalent in all nephrosclerosis patients and, therefore, genetic information can really make a difference in terms of the predictive ability of the model. Indeed, the AUC of the combined standard-genetic model was almost nine points higher. In this regard, we have recently reported similar findings for variants in phospholipase genes[12], located upstream in the same metabolic pathway leading to PGE2, as well as for SNPs present in PGE2 receptors[11]. When interpreting these findings, it should be taken into consideration

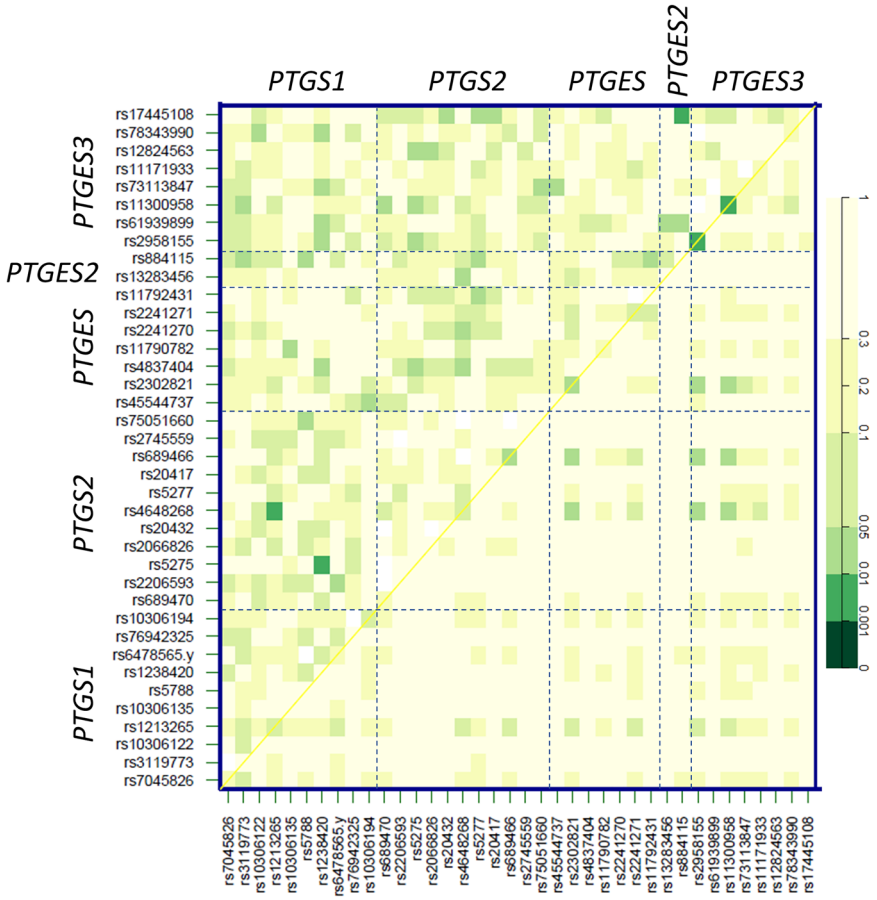

Figure 4. Interactions between genetic variants in the cyclooxygenase-PGE2 pathway and their association with cardiovascular events in nephrosclerosis patients. The upper triangle in the matrix contains p-values for the interaction (epistasis) log-likelihood ratio test. The lower triangle contains p-values from likelihood ratio test comparing the two-SNP additive likelihood to the best of the single-SNP models.

	Polymorphism		n (mean, mmHg)	Mean difference	p-value
SBP					
PTGS2	rs20417	C/C	332 (145.0)	4.28 (0.36 to 8.18)	0.032
		C/G-G/G	150 (148.8)		
PTGES	rs11790782	G/G	379 (145.4)	5.23 (0.51 to 9.94)	0.030
		G/A-A/A	90 (150.4)		
PTGES	rs2241270	C/C	344 (144.8)	5.9 (1.87 to 9.93)	0.004
		C/T-T/T	139 (149.8)		
DBP					
PTGES	rs2302821	A/A	392 (81.89)	− 2.89 (− 5.41 to − 0.38)	0.024
		A/C–C/C	88 (79.01)		
PTGES	rs11790782	G/G	379 (80.95)	2.86 (0.36 to 5.37)	0.026
		G/A-A/A	90 (82.99)		
PTGES	rs2241270	C/C	344 (80.77)	2.77 (0.64 to 4.92)	0.011
		C/T-T/T	139 (82.60)		

Table 3. Adjusted genetic association analysis with blood pressure in patients with nephrosclerosis. *SBP* systolic blood pressure, *DBP* diastolic blood pressure.

Progression of ccIMT			SP, n (%)	AP, n (%)	OR	p
PTGS1	rs10306194	C/C	155 (73.5)	41 (58.6)	1.90 (1.07–3.36)	0.029
		C/A-A/A	56 (26.5)	29 (41.4)		
PTGS2	rs2745559	C/C	133 (63)	54 (77.1)	0.46 (0.24–0.86)	0.012
		C/A-A/A	78 (37)	16 (22.9)		
Carotid plaque			**No, n (%)**	**Yes, n (%)**	**OR**	**p**
PTGS1	rs10306194	C/C	101 (76.5)	182 (64.3)	1.79 (1.06–3.01)	0.026
		C/A-A/A	31 (23.5)	101 (35.7)		
PTGES3	rs11300958	–/–	76 (59.8)	129 (47.1)	1.86 (1.15–3.01)	0.009
		–/G-G/G	51 (40.2)	145 (52.9)		
Severity score			**N, mean**	**Mean difference**	**p**	
PTGS1	rs10306194	C/C	236 (1.70)	0.15 (0.006–0.29)	0.041	
		C/A-A/A	115 (1.91)			
PTGES	rs45544737	G/G	318 (1.75)	0.24 (0.01–0.47)	0.041	
		G/A-A/A	34 (1.97)			
PTGES3	rs61939899	T/T	291 (1.80)	− 0.23 (− 0.42 to − 0.038)	0.019	

Table 4. Adjusted genetic association analysis with atherosclerosis measurements in patients with nephrosclerosis. *SP* slow progression, *AP* accelerated progression. These parameters have previously been analyzed in the same patients regarding their association with other gene sets in previous studies by our group[11,12].

that even though rs4648268 in PTGS2, and rs2958155 and rs11300958 in PTGES3 significantly correlated with CV outcomes, causality is still to be confirmed until an explanatory mechanism has been identified. Furthermore, different mechanisms could be involved for each SNP, as it seems to be inferred by the fact that rs4648268 correlated with coronary outcomes, whilst the other two variants did not.

We also performed epistasis analyses that revealed several SNP-pair interactions associated with CV risk in the nephrosclerosis patients. Interestingly, the aforementioned effect of rs4648268 in COX2 on CVE experienced by nephrosclerosis patients was enhanced when the variant occurred in combination with another SNP in COX1. It is somewhat logical that variants affecting genes participating in a certain pathway relevant for a given outcome may interact to enhance the effect produced by a single SNP. Indeed, it has repeatedly been shown how interactions between SNPs in aging pathways and other routes may influence CV risk more profoundly than individual SNPs in those same genes do[15–17].

With regard to CV traits, two consecutive SNPs in *PTGES*, rs11790782 and rs2241270, were associated with higher SBP and DBP values. These SNPs represent the variability occurring in a central area of the gene locus spanning 8.2 kb. *PTGES* codes for the microsomal prostaglandin E synthase-1, the downstream enzyme responsible for PGE2 synthesis. In this respect, it has been shown that a dysfunction of this gene, as seen in knockout mice, favors the production of PGI2 suggesting rediversion of the accumulated PGH2 substrate (see Fig. 1). In turn, this prevents numerous PGE2-dependent damaging mechanisms, such as vascular remodeling, stiffness, and endothelial dysfunction[18]. Therefore, it is tempting to speculate that variability in the area of *PTGES* tagged by these two variants might result in increased activity/expression of the encoded synthase, thus elevating PGE2 levels and, consequently, leading to higher BP values.

The analysis of associations with atherosclerosis measurements revealed that *PTGS1* rs10306194, tagging the distal region of the gene coding for COX1, was the most relevant SNP, as the variant genotypes were linked to increased presence of plaque in the carotid, higher atherosclerosis severity score and accelerated ccIMT progression. This tag-SNP has not been widely studied, but one report has regarded it as clinically relevant, after being solidly associated with acute urticaria/angioedema[19]. This polymorphism produces an A-to-T change in the 3′-untranslated (UTR) region of *PTGS1* whose functional relevance, however, is yet to be elucidated. In any case, 3′-UTRs sequences are known to be crucial for stability, localization and expression of mRNA, as well as for protein–protein interactions. Therefore, alterations in this region, such as that described herein, hold the potential to affect gene regulation and determine specific pathological conditions[20]. Indeed, several COX1 inhibitors (mimicking the deleterious effect of functional polymorphisms) are anticipated to be potential therapeutic agents for atherosclerosis[21,22]. In any case, the conclusions drawn from this particular set of results are less solid than those regarding the occurrence of CV events, as the reported associations lost statistical significance after Bonferroini correction of the results.

Finally, we also genotyped a group of individuals with normal renal function. Our findings showed that three SNPs in the gene coding for COX2 were associated with a modified risk of nephrosclerosis. In this regard, COX2 levels have been found to be elevated in patients with CKD and have been proposed to contribute to the disease process by mediating vascular calcification in vascular smooth muscle cells, among other mechanisms[23]. One of these three SNPs is rs20417, a G-765C base change in the gene promoter region that has been shown to potentiate COX2 transcription[24]. This effect would translate into a greater synthesis of PGE2 and hence increased inflammatory activity in the kidney compatible with the observed elevated CKD risk. This variant has been widely studied, but, in the renal setting, only one study has investigated its connection with CKD (diabetic nephropathy), albeit

with negative results[25]. Of the other two variants, only rs2066826 has reports on its putative clinical impact, having been associated with type 2 diabetes mellitus[26], atherosclerosis[27] and cancer[28]. This SNP was also studied in relation to allograft survival in kidney transplant, but no associations were observed[29]. In any case, it should be noted that the improvement caused by these SNPs when added to a ROC model containing standard CKD risk factors was negligible, and hence caution should be exerted when extrapolating these particular results.

Some limitations of the study were that the associations with blood pressure traits or atherosclerosis measurements did not survive Bonferroni correction for multiple testing, or that the definition of CVE was heterogeneous, including coronary heart disease manifestations along with other outcomes. In addition, the low incidence of CVE in the control group prevented genetic associations to be analyzed in these individuals. This could also be behind the small, though significant, improvement of predictive power shown by the combined clinical-genetic model in the whole population of study. Finally, it was difficult in general to link the reported genotype–phenotype associations to a particular consequence of a certain SNP, e.g. altered genetic expression or enzymatic activity. The reason is that we used tag-SNPs in the present study, i.e., intronic variants that account for the genetic variability in an area of the gene locus but that do not normally have a known functional impact. On the other hand, this design allows to point out certain regions of a gene whose variability is key for an outcome without having to screen for all the remaining variants. An additional strength of the study is that the patient cohort was uncommonly homogenous, as nephrosclerosis patients are usually combined with subjects with diabetic nephropathy and other conditions in more heterogeneous CKD groups[30].

This is the first time to our knowledge that the CV impact of index genetic variants in the cyclooxygenase route is evaluated in nephrosclerosis patients, a condition we think constitutes an ideal setting to study genetic associations with CV traits and events. Overall, our findings highlight the importance of this route in the extremely high CV risk experienced by nephrosclerosis patients and add to the growing body of evidence pointing out the PGE2 synthesis/activity axis as a promising therapeutic target in this field.

Subjects and methods

Study subjects. Controls (n = 716) and patients (n = 493) were recruited from three different sources, namely the NEFRONA repository, which is a collection of biological samples available for researchers that was created in a previous project on CV features of Spanish individuals[31]; the Nephrology Service of the Badajoz University Hospital (Badajoz, Spain); and the biobank at the Instituto de Salud Carlos III (ISCIII), which stores DNA samples from Spanish healthy subjects.

Control subjects had to present an eGFR over 60 ml/min/1.73 m^2 to be enrolled. Inclusion criteria for CKD patients were to be over 18 years of age, to present a stage 3 or higher renal impairment (eGFR < 60 ml/min/1.73 m^2) and to have histological findings compatible with vascular nephropathy or to meet clinical criteria (advanced age, long-term hypertension, left ventricular hypertrophy, initial mild renal failure and proteinuria < 0.5–1 g/24 h in the absence of other renal disease. On the other hand, the occurrence of a CV event (see definition below) before the start of the study, carotid artery surgery, transplantation, pregnancy, active infection and a life expectancy of less than 12 months were all considered exclusion criteria.

All participants provided written consent for their inclusion. The study was approved by the Ethics Committees of Badajoz University Hospital and the University of Extremadura and was carried out in accordance with the Declaration of Helsinki and its subsequent revisions.

Clinical variables. Diagnostic and prognostic stratification of patients was conducted with the KDIGO classification and table of progression risk and the CONSORTIUM-CKD equation. Kidney function was assessed by the Modification of Diet in Renal Disease (MDRD) equation. Proteinuria was considered when more than 500 mg protein (or 300 mg albumin) were found in 24-h urine samples. A biopsy was conducted to confirm diagnosis when proteinuria was over 1 g.

A total of 412 out of the 493 patients with nephrosclerosis were examined to identify signs of clinical or subclinical atherosclerosis as previously described[32]. Briefly, explorations were conducted in accordance to the American Society of Echocardiography[33] and the Mannheim IMT Consensus[34] with a B-mode ultrasound (Vivid BT09, GE Healthcare, Waukesha, WI, USA). IMT, a marker of subclinical atherosclerosis, was measured in the right and left common carotid arteries and defined as the distance between the leading edge of the lumen intima echo and the leading edge of the media-adventitia echo in the far wall. IMT > 1.5 mm protruding into the lumen was considered atheromatous plaque. ccIMT 24-month progression was also calculated in the nephrosclerosis patients and expressed as mm changed/year. The threshold for accelerated progression was set at the 75th percentile value. Finally, a severity score for atherosclerosis was established based on ccIMT measurements and ankle-brachial index (ABI). A score of 0 was assigned if ccIMT < 90% reference interval and ABI > 0.9; 1 was assigned when ccIMT ≥ 90% reference interval and/or ABI = 0.7–0.9; 2 when there was a carotid plaque with stenosis < 125 cm/seg; and 3 if the stenosis was ≥ 125 cm/seg and/or ABI < 0.7.

Follow-up was set at four years and patients were followed until the earliest of CV event, death, or end of study. CV risk was defined as the likelihood of experiencing a CV event, which included acute myocardial infarction, acute coronary syndrome, coronary catheterization requiring angioplasty, coronary bypass, typical angina with positive stress tests, sudden death, cerebrovascular accident, peripheral arterial disease, aortic aneurysm and lower limb ischemia. CV events were diagnosed by the responsible clinicians at the collaborating hospitals during follow-up; these data were included in the patients' electronic health records, from where we retrieved them to carry out the present study.

Genetic analyses. DNA was purified from 10-ml whole blood samples in the case of patients recruited at Badajoz University Hospital, following a standard procedure of phenol–chloroform extraction and ethanol

precipitation. Genetic material from biological samples stored at the NEFRONA repository and at the ISCIII biobank was extracted by QIAamp DNA Blood Kits and DNA was stored at 4° until analyzed. Genotyping was carried out at Centro Nacional de Genotipado (CeGen), Madrid, Spain, using a OpenArray customized panel on a QuantStudio™ 12 K Flex Real-Time PCR System (Life Technologies, Carlsbad, California, USA). Quality control was conducted by including sample trios with known genotypes from the Coriell Institute in all the analyses.

The study design called for the identification of tag-SNPs in the five genes of interest, namely *PTGS1* (Accession No. ENSG00000095303), *PTGS2* (ENSG00000073756), *PTGES* (ENSG00000148344), *PTGES2* (ENSG00000148334) and *PTGES3* (ENSG00000110958). For this, we retrieved genetic variability data for European populations from the 1000 genomes project (http://www.internationalgenome.org/) in *vcf* format and created *ped* files with the *vcf to ped converter* tool of Ensembl (https://www.ensembl.org/Homo_sapiens/Tools/VcftoPed). These *.ped* files were then analyzed with Haploview 4.2 software to assign tag-SNPs, considering a pair-wise tagging with $r^2 > 0.8$ and a MAF > 0.05. The complete list of 38 SNPs studied, with their corresponding alleles, MAF, and p-values for Hardy–Weinberg equilibrium test are shown in Supplementary Table S4.

Statistical analyses. Mean and standard deviation (SD) was used to describe parametric variables, whilst median and IQR in parenthesis was used for data not normally distributed. Chi-square tests were utilized to compare categorical variables. Quantitative variables were compared either by t-test or Mann–Whitney (2 groups) or by ANOVA or Kruskal–Wallis (> 2 groups) depending on the data distribution. Genetic associations with clinical variables were assessed by logistic regression adjusting for relevant covariates, namely sex, age, body mass index, ethnicity, diabetes, hypertension or CKD stage[11,35], which were chosen based on univariate analyses and/or clinical criteria (variables that had previously been associated with CKD). Genetic analyses were carried out under a dominant model of inheritance, i.e., carriers vs. non-carriers, because the resulting genotype groups were the most balanced in terms of size, as we have described in previous CKD studies[11,12].

CV event-free survival was calculated by Kaplan–Meier curves and the effect of the different genotypes was compared with the log-rank test. Additional Cox regression procedures were carried out to control for classic CV risk factors. The predictive value of the SNPs regarding the risk of nephrosclerosis and CVE was evaluated with ROC curves, which were generated for models with classic risk factors with or without genetic information. The DeLong test was used to detect differences between the area under the curve (AUC) of these models.

Statistical power calculations were conducted considering an arbitrary effect size of 2.0 and a type-1 error of 0.05. With the available sample size, the power to detect genetic associations with the disease ranged from 0.872 to 0.996 for the lowest and highest MAF values, respectively (Quanto software v. 1.2.4, USC, Los Angeles, USA). The threshold for statistically significant associations was set at $p < 0.05$. Bonferroni correction for the 37 SNPs assayed (one of them was not in Hardy–Weinberg equilibrium) lowered the significance threshold to 0.0013.

The *SNPassoc*, *pROC* and *survival* packages (R software) and IBM SPSS v.22.0 (SPSS Inc., Chicago, IL, v.22.0) were utilized for the statistical analyses.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Author contributions

L.M.G. recruited patients and carried out statistical analyses; N.R.R. and J.M.V. helped with study design and the clinical evaluation of the patients; S.M.-Z. and L.G.-R. participated in genetic analyses; J.L.-G. collaborated in sample collection and clinical analyses; G.G. designed the study, searched for funding and wrote the final version of the paper.

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Competing interests

The authors declare no competing interests.

Additional information

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