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**ORIGINAL ARTICLE****Observational Study**

- 517** Risk factors and urinary biomarkers of non-albuminuric and albuminuric chronic kidney disease in patients with type 2 diabetes  
*Korbut AI, Klimontov VV, Vinogradov IV, Romanov VV*
- 534** Type 1 diabetes loci display a variety of native American and African ancestries in diseased individuals from Northwest Colombia  
*Gomez-Lopera N, Alfaro JM, Leal SM, Pineda-Trujillo N*

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## Observational Study

## Risk factors and urinary biomarkers of non-albuminuric and albuminuric chronic kidney disease in patients with type 2 diabetes

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## Abstract

## BACKGROUND

A number of recent studies indicate a transformation in the natural course of chronic kidney disease (CKD) in type 2 diabetes (T2D) patients: an increasing prevalence of declined renal function without proceeding to the accompanying elevation of albuminuria. It has been suggested that albuminuric and non-albuminuric CKD patterns could be different in their phenotypes and pathogenic mechanisms.

## AIM

To identify the risk factors and biomarkers of albuminuric and non-albuminuric patterns of CKD in patients with T2D.

## METHODS

Three hundred sixty patients with T2D duration  $\geq 10$  years were included in this observational cross-sectional study. The associations of a panel of demographic and clinical characteristics, complications, comorbidities, and metabolic and hematology parameters with albuminuric and non-albuminuric CKD patterns were analyzed. The urinary excretion of nephrin and podocin, two podocyte-specific markers, and WAP-four-disulfide core domain protein 2 (WFDC-2), a marker of tubulointerstitial fibrosis, was determined by ELISA in comparison with healthy controls.

## RESULTS

Non-albuminuric CKD was associated with age  $\geq 65$  years ( $P = 0.0001$ ), female

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sex ( $P = 0.04$ ), diabetes duration  $\geq 15$  years ( $P = 0.0009$ ), and the use of diuretics ( $P = 0.0005$ ). Male sex ( $P = 0.01$ ), smoking ( $P = 0.01$ ), waist-to-hip ratio  $>1.0$  ( $P = 0.01$ ) and hemoglobin A1c (HbA1c)  $> 8.0\%$  ( $P = 0.005$ ) were risk factors for elevated albuminuria not accompanied by a decrease in estimated glomerular filtration rate (eGFR). Duration of diabetes  $\geq 15$  years and the use of calcium channel blockers were risk factors for albuminuria with decreased eGFR (both  $P = 0.01$ ). In multivariate logistic regression analysis, age, HbA1c, female sex and diuretics were significant predictors for reduced eGFR, while waist-to-hip ratio, HbA1c and male sex were associated with elevated urinary albumin-to-creatinine ratio (UACR). Excretion of nephrin and podocin was increased in patients with albuminuria, regardless of decline in renal function ( $P < 0.001$ ), correlating positively with UACR. The urinary excretion of WFDC-2 was markedly higher in men than in women ( $P < 0.000001$ ). Men with T2D demonstrated increased WFDC-2 levels independently of the CKD pattern (all  $P < 0.05$ ). In T2D women, WFDC-2 excretion was increased in those with reduced renal function ( $P \leq 0.01$ ), correlating negatively with eGFR.

## CONCLUSION

The data provide further evidence that albuminuric and non-albuminuric CKD phenotypes correspond to different pathways of diabetic kidney disease progression.

**Key words:** Diabetes mellitus; Chronic kidney disease; Albuminuria; Glomerular filtration rate; Podocytes; Risk factors; Biomarkers

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**Core tip:** In this study, we demonstrate the differences in clinical and laboratory characteristics between albuminuric and non-albuminuric phenotypes of chronic kidney disease (CKD) in patients with long-term type 2 diabetes. Different risk factors are found for three different CKD phenotypes. We also show the diversity in the urinary excretion of nephrin and podocin, two slit diaphragm proteins, and of WAP-four-disulfide core domain protein 2, a tubulointerstitial fibrosis marker, between different CKD phenotypes. The results further support the notion that albuminuric and non-albuminuric CKD phenotypes are different in their pathophysiology and clinical characteristics.

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## INTRODUCTION

The increasing prevalence of diabetes around the world and changes in diabetes management have transformed the epidemiology of chronic kidney disease (CKD) in recent years. In many countries, including the United States, diabetes is responsible for over 40% of new cases of end-stage renal disease (ESRD), surpassing other causes to become the leading driver of the renal impairment<sup>[1]</sup>. Despite the fact that the prevalence of CKD among adult patients with diabetes remains stably high, a transformation in its natural course has been recorded. According to the classical paradigm, albuminuria is an early indicator of diabetic kidney disease. A number of recent studies have documented an increasing proportion of diabetic patients in whom a reduction in renal function develops without a preceding or concomitant increase in albuminuria<sup>[1-3]</sup>. This tendency is most evident in type 2 diabetes (T2D); the proportion of the non-albuminuric CKD (NA-CKD) pattern in this type of disease currently ranges from 40% to 70%<sup>[4]</sup>.

The causes for the shift in the natural course of diabetic kidney disease are not fully understood. Among others, the wide use of renin-angiotensin system blockers; advances in antihyperglycemic, antihypertensive, and hypolipidemic therapy; and

smoking cessation are discussed<sup>[2,5]</sup>. New classes of antihyperglycemic agents, including glucagon-like peptide-1 (GLP-1) analogs, dipeptidylpeptidase-4 (DPP-4) inhibitors, and sodium-glucose cotransporter-2 (SGLT-2) inhibitors, have demonstrated a distinct antialbuminuric effect in clinical trials<sup>[6-9]</sup>. Accordingly, the growing use of these agents in clinical practice may cause a reduction in the prevalence of albuminuria among diabetic patients.

Presently, little is known about the clinical phenotypes and pathophysiology of albuminuric and NA-CKD patterns in diabetes. A growing body of evidence indicates that these patterns demonstrate significant differences in natural course and outcomes. Even if a more favorable situation in terms of the risk of ESRD, NA-CKD is clearly associated with cardiovascular disease and its risk factors<sup>[4]</sup>. Accordingly, the non-albuminuric phenotype might be related to macroangiopathy instead of microangiopathy and/or be the consequence of repeated and/or unresolved episodes of acute kidney injury, even of a mild degree<sup>[10]</sup>. When comparing renal biopsy findings associated with normo-, micro-, or macroalbuminuria in T2D patients with glomerular filtration rate (GFR) less than 60 mL/min/1.73 m<sup>2</sup>, typical glomerular changes were revealed mostly in patients with elevated albuminuria. In those with NA-CKD, predominant interstitial and vascular changes were more frequent findings<sup>[11]</sup>. It was speculated that non-albuminuric renal impairment represents a different pathway to the loss of renal function compared to albuminuric one.

Podocyte injury has been identified as a pivotal event resulting in proteinuric kidney disease, glomerulosclerosis, and loss of renal function<sup>[12]</sup>. During filtration, plasma passes through a sieve consisting of a fenestrated endothelium and a broad basement membrane before it reaches the most unique part, the slit diaphragm, a specialized type of intercellular junction that connects neighboring podocyte foot processes. When podocytes become stressed, irrespective of the causative stimulus, they undergo foot process effacement and loss of slit diaphragms – two key steps leading to proteinuria<sup>[13]</sup>. It was demonstrated that not only proteinuria but also tubulointerstitial lesions should be assessed to predict rapid GFR decline in patients with T2D who have overt proteinuria<sup>[14]</sup>. Moreover, it was reported that interstitial fibrosis, tubular atrophy and interstitial inflammation, but not glomerular lesions, are significant predictors for renal prognosis in T2D patients with overt proteinuria<sup>[15]</sup>. In a recent study, interstitial fibrosis and tubular atrophy score, as well as glomerular basement membrane thickness, were independent predictors for renal replacement therapy initiation in T2D patients<sup>[16]</sup>. Taking into account the results of morphological investigations, the assessment of urinary markers of podocyte and interstitial involvement may provide further information on the development of different CKD phenotypes. This study aimed to identify the risk factors, as well as the markers of podocyte and interstitial involvement, in albuminuric and NA-CKD in patients with T2D.

## MATERIALS AND METHODS

### *Ethical issues*

The study protocol was approved by the Ethical Committee of the Research Institute of Clinical and Experimental Lymphology – branch of the Institute of Cytology and Genetics, Siberian Branch of Russian Academy of Sciences. All patients provided their written informed consent prior to the inclusion.

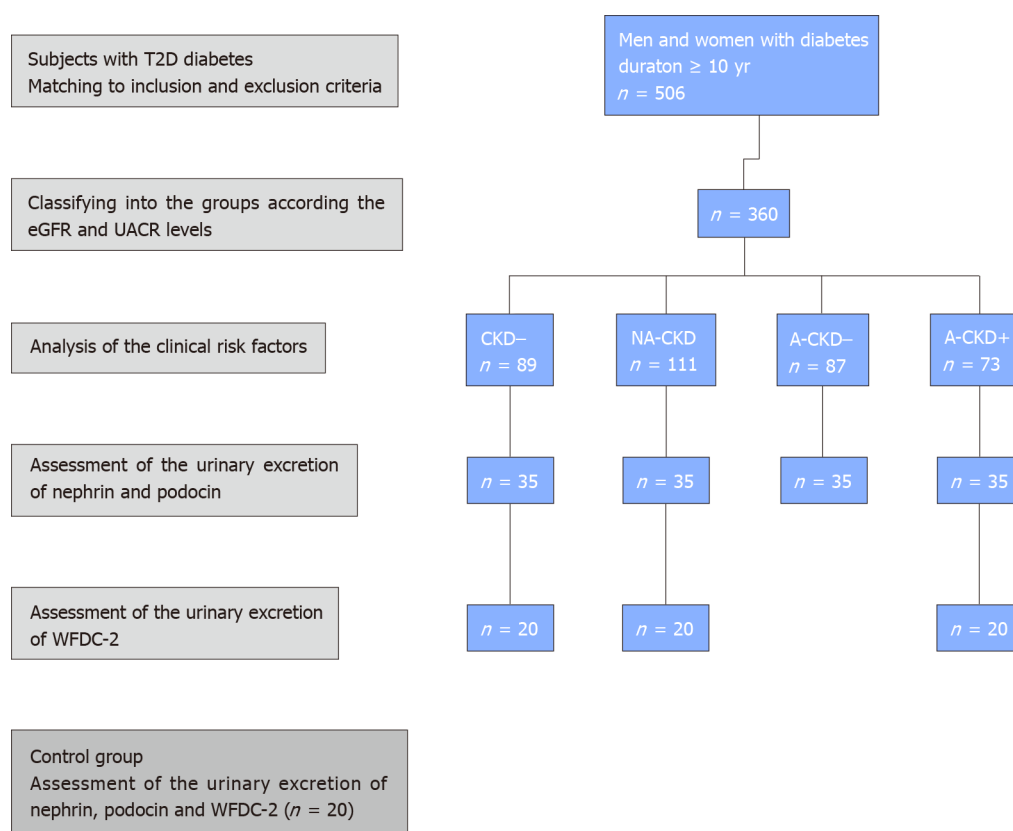
### *Design*

The design of this observational, single-center, cross-sectional study is presented in **Figure 1**. Adult men and women with T2D duration of at least 10 years from the date of diagnosis were included. Non-diabetic CKD, ESRD, urinary tract infection, ketoacidosis or hyperosmolar state at the time of the survey, treatment with DPP-4 inhibitors, GLP-1 receptor agonists and/or SGLT-2 inhibitors for three months prior to inclusion, malignant neoplasms, inflammatory or autoimmune diseases in the medical history, and a high-protein diet acted as exclusion criteria.

### *Subjects*

Five hundred six potentially eligible T2D patients who met the inclusion criteria were selected. After evaluation for exclusion criteria, 360 patients, 100 men and 260 women, from 43 to 88 years of age (median 66 years), were included in the analysis. Twenty individuals who had no history of diabetes, obesity or cardiovascular disease, including 13 women and 7 men, from 50 to 74 years of age (median 62.5 years), acted as controls in the study of urinary biomarkers.

### *Methods*



**Figure 1 The design of the study.** The study was designed as an observational, single-center, cross-sectional study. Adult men and women with type 2 diabetes (T2D) duration of at least 10 years from the date of diagnosis were included ( $n = 506$ ). After evaluation for exclusion criteria, 360 patients were included in the analysis. Patients were divided into four groups according to their estimated glomerular filtration rate (eGFR) and urinary albumin-to-creatinine ratio (UACR) levels. Individuals with  $\text{eGFR} \geq 60 \text{ mL/min} \times 1.73 \text{ m}^2$  and  $\text{UACR} < 3.0 \text{ mg/mmol}$  were recorded as patients without chronic kidney disease (CKD) signs (CKD- group). Those with  $\text{eGFR} < 60 \text{ mL/min} \times 1.73 \text{ m}^2$  and  $\text{UACR} < 3.0 \text{ mg/mmol}$  were assigned to the non-albuminuric chronic kidney disease group. Patients with  $\text{eGFR} \geq 60 \text{ mL/min} \times 1.73 \text{ m}^2$  and  $\text{UACR} \geq 3.0 \text{ mg/mmol}$  were defined as albuminuric with preserved renal function (A-CKD- group). Individuals with  $\text{eGFR} < 60 \text{ mL/min} \times 1.73 \text{ m}^2$  and  $\text{UACR} \geq 3.0 \text{ mg/mmol}$  comprised the albuminuric CKD group (A-CKD+). All patients underwent clinical examination, which included an evaluation of diabetes control and in-depth screening/monitoring of complications and comorbidities. The set of clinical risk factors was estimated for each CKD pattern. Urinary excretion of nephrin and podocin, two podocyte-specific markers, and WAP-four-disulfide core domain protein 2, a marker of tubulointerstitial fibrosis, was assessed in T2D patients and the control group (20 subjects without a history of diabetes, obesity or cardiovascular disease). CKD: Chronic kidney disease; eGFR: Estimated glomerular filtration rate; NA-CKD: Non-albuminuric chronic kidney disease; T2D: Type 2 diabetes; UACR: Urinary albumin-to-creatinine ratio; WFDC-2: WAP-four-disulfide core domain protein 2; CKD-: The group of individuals with estimated glomerular filtration rate  $\geq 60 \text{ mL/min} \times 1.73 \text{ m}^2$  and urinary albumin-to-creatinine ratio  $< 3.0 \text{ mg/mmol}$ ; NA-CKD: Non-albuminuric chronic kidney disease, the group of individuals with estimated glomerular filtration rate  $< 60 \text{ mL/min} \times 1.73 \text{ m}^2$  and urinary albumin-to-creatinine ratio  $< 3.0 \text{ mg/mmol}$ ; A-CKD-: Group of patients with estimated glomerular filtration rate  $\geq 60 \text{ mL/min} \times 1.73 \text{ m}^2$  and urinary albumin-to-creatinine ratio  $\geq 3.0 \text{ mg/mmol}$ ; A-CKD+: Group of individuals with estimated glomerular filtration rate  $< 60 \text{ mL/min} \times 1.73 \text{ m}^2$  and urinary albumin-to-creatinine ratio  $\geq 3.0 \text{ mg/mmol}$ .

All patients underwent clinical examination, which included an evaluation of diabetes control and in-depth screening/monitoring of complications. Routine laboratory measurements, including glycated hemoglobin A1c (HbA1c), total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglycerides and uric acid, were performed on AU480 Chemical Analyzer (Beckman Coulter, United States) with commercially available cartridges. The HbA1c levels were measured by turbidimetric immunoinhibition method. A kinetic enzymatic method was applied for determination the levels of lipids and uric acid. Three fasting and three 2-h postprandial blood glucose values were obtained daily from each patient in three-day series. The measurements were performed by One Touch Verio® (Johnson and Johnson/Lifescan, United States) glucose meter. A complete blood count was performed on a hematology analyzer (Analyticon Biotechnologies AG, Germany). Concentrations of fibrinogen, soluble fibrin monomer complex (SFMC) and D-dimer were evaluated on the hemostasis analyzer system (Instrumentation Laboratory, United States).

The levels of creatinine in serum and urine were determined on AU480 Chemical Analyzer (Beckman Coulter, United States) by modified kinetic Jaffe's method. The estimated GFR (eGFR) was calculated by the CKD-EPI formula (2009). Urinary albumin was determined in three morning urine samples by immunoturbidimetry on AU480 Chemical Analyzer (Beckman Coulter, United States) in accordance with the manufacturer's instructions. The mean albumin concentration (mg) was adjusted to

the excreted creatinine (mmol) and is expressed as the urinary albumin/creatinine ratio (UACR). Urinary excretion of total protein was assessed by colorimetric method with pyrogallol red-molybdate complex on AU480 Chemical Analyzer (Beckman Coulter, United States).

In this study, we assessed the urinary excretion of nephrin and podocin, two podocyte-specific markers, and of WAP-four-disulfide core domain protein 2 (WFDC-2), a marker of tubulointerstitial fibrosis. Both nephrin and podocin are expressed on the surface of podocytes, acting as components of the slit diaphragm complex; accordingly, these molecules are used as markers of dysfunction and injury of podocytes<sup>[17-19]</sup>. The increase in urinary excretion of nephrin and podocin in patients with diabetes was reported in a number of studies<sup>[17,20-22]</sup>. WFDC-2, also known as human epididymal protein-4 (HE-4), is expressed by myofibroblasts<sup>[23]</sup>. Focal and low expression of WFDC-2 is found in the distal convoluted tubule of the kidney<sup>[24]</sup>. It was shown that WFDC-2 suppressed the activity of multiple proteases, including serine proteases and matrix metalloproteinases, and specifically inhibited their capacity to degrade type I collagen<sup>[23]</sup>. Recently, serum WFDC-2 has been validated as a clinical marker of renal fibrosis<sup>[25,26]</sup>.

The morning urine samples for the biomarker assay were centrifuged, and the supernatants were separated and stored at -80 °C until analysis. Repeated freeze-thaw cycles were avoided. The concentrations of nephrin, podocin and WFDC-2 in the urine were assessed by ELISA using commercially available kits (Cloud-Clone Corp., United States, catalog/serial No. SEA937Hu/5A788AAD51, SEA938Hu/6E430916C8 and SEA241Hu/8D970EE435, respectively), in accordance with the manufacturer's instructions. The results were adjusted to urinary creatinine and compared to the control.

In-depth screening/monitoring of diabetic complications and associated conditions was performed in all patients. Diabetic retinopathy was diagnosed by ophthalmologist with a comprehensive dilated eye examination. Coronary artery disease was defined as myocardial infarction, unstable angina, coronary revascularization procedure, or transient myocardial ischemia in medical history, and/or abnormal result of exercise ECG testing or stress echocardiography. Chronic heart failure was assessed by New York Heart Association functional classification taking into account the limitation to physical activity, the results of physical examination and echocardiography. Carotid atherosclerosis and peripheral artery disease was verified by duplex ultrasound.

### Statistical analysis

The statistical software package Statistics 12.0 (Dell, United States) was used to analyze the results. Quantitative data are presented as medians (lower quartiles; upper quartiles). Frequencies are presented as percentages (%). The normal distribution was determined by the Kolmogorov-Smirnov test. Because most of the quantitative were not distributed normally, non-parametric multiple comparisons of mean ranks were used to assess the statistical significance of differences between groups by continuous characteristics. The statistical significance of differences in discrete parameters between groups was analyzed using the  $\chi^2$  test. A difference was defined as significant if the *P* value was less than 0.05. Spearman rank correlation analysis was applied to test the association between variables. To assess the contribution of the investigated parameters to declining eGFR and development of albuminuria, a multiple logistic regression was used. The contribution of the factor was defined as significant if the standard deviation of the coefficient  $\beta$  did not exceed the coefficient  $\beta$  and the *P* value was less than 0.05. To assess the significance of the studied factors, the odds ratio, 95% confidence interval (CI), and *P* value were calculated using MedCalc 18.11.6 (MedCalc Software, Belgium). The influence of the factor was determined to be significant when the boundaries of the 95%CI were located on the same side of 1.0 and the *P* value was less than 0.05.

## RESULTS

### Clinical and laboratory characteristics of T2D patients with different CKD patterns

Patients were divided into four groups according to their eGFR and UACR levels. The individuals with eGFR  $\geq 60$  mL/min  $\times 1.73$  m<sup>2</sup> and UACR  $< 3.0$  mg/mmol were recorded as patients without CKD signs (CKD- group). Those with eGFR  $< 60$  mL/min  $\times 1.73$  m<sup>2</sup> and UACR  $< 3.0$  mg/mmol were assigned to the NA-CKD group. Patients with eGFR  $\geq 60$  mL/min  $\times 1.73$  m<sup>2</sup> and UACR  $\geq 3.0$  mg/mmol were defined as albuminuric with preserved renal function (A-CKD- group). Finally, the individuals with eGFR  $< 60$  mL/min  $\times 1.73$  m<sup>2</sup> and UACR  $\geq 3.0$  mg/mmol comprised

the albuminuric CKD group (A-CKD+). The demographic and clinical characteristics of these groups are presented in [Table 1](#).

Women made up a high proportion of NA-CKD patients ( $P < 0.001$  compared with the CKD- group), while the proportion of men was highest in the A-CKD group ( $P = 0.012$  compared with the CKD- group). Patients from the NA-CKD and A-CKD+ groups were older than CKD- and A-CKD- patients (all  $P < 0.01$ ). Patients with NA-CKD demonstrated the lowest waist-to-hip ratio (WHR) among the examined T2D patients. In contrast, A-CKD- patients had the largest WHR values. There were no differences between the groups in body mass index (BMI). The percentage of current smokers was highest in the A-CKD- group.

Patients with reduced renal function (NA-CKD and A-CKD+ groups) had longer diabetes durations than those without. The prevalence of diabetic retinopathy tended to be higher in A-CKD- and A-CKD+ patients, though the differences between the groups were not statistically significant. Despite the fact that the prevalence of coronary artery disease did not differ between the groups, myocardial infarction occurred more frequently in individuals with albuminuria (A-CKD- and A-CKD+ groups). In contrast, carotid atherosclerosis and a history of cerebrovascular events (stroke or transient ischemic attack) were most prevalent in patients with decreased eGFR (NA-CKD and A-CKD+ groups). The prevalence of peripheral artery disease was highest in A-CKD+ patients.

Most patients in our cohort were insulin-treated ([Table 1](#)). The duration of insulin therapy was longest in NA-CKD patients. Interestingly, the daily insulin dose in this group was lowest. All patients received antihypertensive agents, mostly in combinations. The highest frequency of the use of diuretics was observed among NA-CKD patients, while the highest rate of treatment with dihydropyridine calcium channel blockers (nifedipine or amlodipine) was found in the A-CKD+ group. Statins and antiplatelet agents were more commonly prescribed to NA-CKD and A-CKD+ patients.

Laboratory parameters of T2D patients depending on their CKD status are summarized in [Table 2](#). The highest HbA1c and 2h-postprandial blood glucose levels were observed in the A-CKD- group. The NA-CKD group was characterized by the lowest HbA1c values. Serum uric acid was increased significantly in CKD patients compared to those without, but no dependence on the CKD pattern was observed. As expected, the red blood cell (RBC) count was decreased in patients with reduced renal function (NA-CKD and A-CKD+ groups) compared to those without. The lowest hemoglobin levels were found in the NA-CKD group. The erythrocyte sedimentation rate (ESR) was increased significantly in all CKD groups compared to the CKD- group. No differences in other hematological parameters were revealed. The A-CKD- patients demonstrated significantly increased levels of plasma SFMC compared to CKD- individuals. Fibrinogen and D-dimer levels did not differ between the groups.

### **Risk factors for CKD patterns**

The risk factors for different CKD patterns are presented in [Table 3](#). Age  $\geq 65$  years, duration of T2D  $\geq 15$  years, female sex, and the use of diuretics were significant risk factors for NA-CKD. On the other hand, male sex, smoking, WHR  $> 1.0$  and HbA1c  $> 8.0\%$  significantly increased the risk of A-CKD-. Diabetes duration  $\geq 15$  years and the use of dihydropyridine calcium channel blockers were associated with A-CKD+. In multiple logistic regression analysis, age, HbA1c, female sex and treatment with diuretics were significant predictors of decreased eGFR ([Table 4](#)). Meanwhile, WHR, HbA1c and male sex predicted elevated albuminuria ([Table 5](#)).

### **Urinary biomarkers in T2D patients with different patterns of CKD**

The excretion of nephrin and podocin was increased significantly in all diabetic groups compared to control (all  $P < 0.05$ , [Figure 2](#)). The CKD- and NA-CKD groups did not differ in the levels of urinary excretion of nephrin or podocin. Patients with elevated albuminuria (A-CKD- and A-CKD+ groups) demonstrated an increase in the excretion of both markers compared to the CKD- group (A-CKD-:  $P = 0.001$  and  $P = 0.006$ , respectively; A-CKD+:  $P = 0.04$  and  $P = 0.002$ , respectively) and the NA-CKD group (A-CKD-:  $P = 0.000003$  and  $P = 0.0003$ , respectively; A-CKD+:  $P = 0.04$  and  $P = 0.00007$ , respectively).

The urinary excretion of WFDC-2 in men was 9.2 times higher than in women ( $P < 0.000001$ ). Accordingly, sex differences in marker excretion were taken into account when evaluating the results ([Figure 3](#)). Men of the CKD-, NA-CKD and A-CKD+ groups demonstrated increased excretion of WFDC-2 compared to the nondiabetic control ( $P = 0.04$ ,  $P = 0.01$  and  $P = 0.009$ , respectively). However, there were no significant differences in this marker between diabetic groups. In women, WFDC-2 excretion was increased markedly in the NA-CKD and A-CKD+ groups compared to control ( $P = 0.01$  and  $P = 0.0007$ , respectively) and to patients without CKD ( $P = 0.01$

**Table 1 Clinical characteristics of type 2 diabetic individuals with different patterns of chronic kidney disease**

Parameter	CKD- (n = 89)	NA-CKD (n = 111)	A-CKD- (n = 87)	A-CKD+ (n = 73)
General clinical parameters				
Sex, M/F, n (%)	20/69	13/98	45/42	22/51
Age, yr	22.5/77.5	11.7/88.3 <sup>afh</sup>	51.9/48.1 <sup>ch</sup>	30.1/69.9 <sup>e</sup>
BMI, kg/m <sup>2</sup>	64 (58; 67)	68 (64; 73) <sup>bfi</sup>	63 (59; 68) <sup>i</sup>	67 (61; 77) <sup>ae</sup>
WHR	33.4 (28.7; 36.9)	32.6 (29.4; 37.2)	33.6 (30.1; 38.2)	33.4 (30.0; 36.8)
Smoking, n (%)	0.97 (0.94; 1.03)	0.94 (0.89; 0.99) <sup>fh</sup>	1.04 (0.97; 1.11) <sup>a</sup>	0.98 (0.95; 1.07)
Diabetes duration, yr	7 (7.9)	6 (5.4)	18 (20.9) <sup>a</sup>	3 (4.1)
Diabetic complications and comorbidities	15 (12; 19)	18 (15; 25) <sup>cd</sup>	15 (13; 20)	18 (14; 22) <sup>a</sup>
Diabetic retinopathy, n (%)				
Arterial hypertension, n (%)	62 (69.7)	74 (66.7)	65 (74.7)	57 (78.1)
Coronary artery disease, n (%)	85 (95.5)	111 (100)	87 (98.9)	73 (100)
Myocardial infarction in anamnesis, n (%)	41 (46.1)	58 (52.3)	46 (52.9)	41 (56.2)
Chronic heart failure (NYHA class III-IV), n (%)	7 (7.9)	19 (17.1)	20 (23.0) <sup>b</sup>	17 (23.3) <sup>b</sup>
Carotid atherosclerosis, n (%)	5 (5.6)	7 (6.3)	11 (12.6)	4 (5.5)
Cerebrovascular event in anamnesis, n (%)	15 (16.9)	51 (45.9) <sup>c</sup>	33 (37.9) <sup>a</sup>	40 (54.8) <sup>c</sup>
Peripheral artery disease, n (%)	6 (6.7)	13 (11.7) <sup>a</sup>	5 (5.8)	11 (15.1) <sup>a</sup>
Treatment	60 (67.4)	84 (75.7)	59 (67.8)	57 (78.1) <sup>a</sup>
Metformin, n (%)				
Sulfonylurea, n (%)	61 (68.5)	64 (57.7)	56 (64.4)	43 (58.9)
Insulin, n (%)	29 (32.6)	31 (27.9) <sup>g</sup>	21 (24.1)	10 (13.7) <sup>b</sup>
Duration of insulin therapy, yr	74 (83.1)	94 (84.7) <sup>g</sup>	76 (87.5)	70 (95.9) <sup>a</sup>
Daily insulin dose, IU	6 (4; 10)	10 (7; 13) <sup>cf</sup>	6 (3; 10)	8 (3; 11)
Daily insulin dose, IU/kg	52 (36; 72)	46 (34; 62) <sup>g</sup>	56 (40; 78)	60 (42; 74)
RAS blockers, n (%)	0.60 (0.40; 0.80)	0.55 (0.40; 0.70)	0.60 (0.40; 0.80)	0.63 (0.45; 0.90)
Diuretics, n (%)	67 (75.3)	93 (83.8)	69 (79.3)	61 (83.6)
Calcium channel blockers, n (%)	36 (40.4)	73 (65.8) <sup>adg</sup>	38 (43.7)	35 (47.9)
Antiplatelet agents, n (%)	27 (30.3)	38 (34.2) <sup>g</sup>	34 (39.1)	36 (49.3) <sup>a</sup>
Statins, n (%)	46 (51.7)	78 (70.3) <sup>bd</sup>	50 (57.5) <sup>g</sup>	57 (78.1) <sup>be</sup>
	28 (31.5)	59 (53.2) <sup>bd</sup>	31 (35.6) <sup>g</sup>	39 (53.4) <sup>be</sup>

<sup>a</sup>*P* < 0.05,<sup>b</sup>*P* < 0.01,<sup>c</sup>*P* < 0.001 *vs* CKD-,<sup>d</sup>*P* < 0.05,<sup>e</sup>*P* < 0.01,<sup>f</sup>*P* < 0.001 *vs* A-CKD-,<sup>g</sup>*P* < 0.05,<sup>h</sup>*P* < 0.01,

<sup>i</sup>*P* < 0.001 *vs* A-CKD+ ( $\chi^2$  test for discrete parameters and multiple comparisons of mean ranks for continuous parameters). BMI: Body mass index; WHR: Waist-to-hip ratio; CKD-: The group of individuals with estimated glomerular filtration rate  $\geq 60$  mL/min  $\times 1.73$  m<sup>2</sup> and urinary albumin-to-creatinine ratio < 3.0 mg/mmol; NA-CKD: Non-albuminuric chronic kidney disease, the group of individuals with estimated glomerular filtration rate < 60 mL/min  $\times 1.73$  m<sup>2</sup> and urinary albumin-to-creatinine ratio < 3.0 mg/mmol; A-CKD-: Group of patients with estimated glomerular filtration rate  $\geq 60$  mL/min  $\times 1.73$  m<sup>2</sup> and urinary albumin-to-creatinine ratio  $\geq 3.0$  mg/mmol; A-CKD+: Group of individuals with estimated glomerular filtration rate < 60 mL/min  $\times 1.73$  m<sup>2</sup> and urinary albumin-to-creatinine ratio  $\geq 3.0$  mg/mmol.

and *P* = 0.0007, respectively), while no difference between the CKD- group and the nondiabetic control was found.

In diabetic patients, urinary excretion of nephrin and podocin correlated positively with UACR (*r* = 0.47, *P* = 0.000001 and *r* = 0.43, *P* = 0.00001, respectively). Nephrin excretion demonstrated positive relationships with age (*r* = 0.27, *P* = 0.0007), diabetes duration (*r* = 0.31, *P* = 0.0002), and SFMC (*r* = 0.37, *P* = 0.001); at the same time, podocin excretion was related to age only (*r* = 0.27, *P* = 0.0004). No relationships between podocyte-specific markers and eGFR were found (*r* = 0.47, *P* = 0.000001 and *r* = 0.43, *P* = 0.00001, respectively). In women with diabetes, the excretion of WFDC-2 correlated with serum creatinine levels (*r* = 0.45, *P* = 0.001), eGFR (*r* = -0.50, *P* = 0.001) and UACR (*r* = 0.45, *P* = 0.001). In males, WFDC-2 correlated with BMI and WHR (*r* = 0.52, *P* = 0.01 and *r* = 0.53, *P* = 0.04, respectively), but not with UACR (*r* = 0.18, *P* > 0.05) or eGFR (*r* = -0.13, *P* > 0.05).

**Table 2** Laboratory parameters of type 2 diabetic individuals with different patterns of chronic kidney disease

Parameter	CKD- (n = 89)	NA-CKD (n = 111)	A-CKD- (n = 87)	A-CKD+ (n = 73)
Renal tests				
Serum creatinine, $\mu\text{mol/L}$	76 (67.3; 86.8)	111 (99.1; 124) <sup>cf</sup>	85.8 (76.1; 96.5)	116 (97.8; 144) <sup>cf</sup>
eGFR, $\text{mL}/\text{min} \times 1.73 \text{ m}^2$	77 (69; 87)	52 (46; 56) <sup>cf</sup>	72 (66; 84) <sup>i</sup>	51 (46; 55.8) <sup>c</sup>
UACR, $\text{mg}/\text{mmol}$	0.5 (0.3; 0.9)	0.7 (0.4; 1.0) <sup>fi</sup>	8.3 (4.8; 36.7) <sup>c</sup>	11.4 (5.6; 42.1) <sup>c</sup>
Urinary protein excretion, $\text{mg}/\text{day}$	65 (50; 100)	70 (50; 140)	170 (90; 530) <sup>ci</sup>	200 (130; 520) <sup>ci</sup>
Biochemistry				
HbA1c, %	8.4 (7.5; 10.1)	8.1 (7.2; 9.5) <sup>f</sup>	9.7 (8.5; 11.2) <sup>bg</sup>	8.6 (7.5; 9.8)
HbA1c, $\text{mmol}/\text{L}$	68 (58; 87)	65 (55; 80) <sup>f</sup>	83 (69; 99) <sup>bg</sup>	70 (58; 84)
Fasting blood glucose, $\text{mmol}/\text{L}$	8.9 (6.8; 10.2)	8.8 (6.5; 10.1)	9.5 (8.0; 12.8) <sup>a</sup>	9.6 (7.7; 12.0)
2h-postprandial blood glucose, $\text{mmol}/\text{L}$	10.7 (9.0; 13.7)	11.7 (8.9; 14.0)	13.1 (9.9; 15.0) <sup>a</sup>	11.3 (10.0; 14.0)
Total cholesterol, $\text{mmol}/\text{L}$	5.1 (4.5; 5.9)	5.1 (4.3; 6.0)	4.9 (4.1; 6.0)	5.3 (4.1; 6.4)
LDL-cholesterol, $\text{mmol}/\text{L}$	3.3 (2.7; 3.9)	3.2 (2.5; 4.0)	3.1 (2.5; 3.8)	3.2 (2.5; 4.1)
HDL-cholesterol, $\text{mmol}/\text{L}$	1.2 (1.0; 1.4)	1.3 (1.1; 1.5) <sup>e</sup>	1.2 (1.0; 1.3)	1.1 (1; 1.4)
Triglycerides, $\text{mmol}/\text{L}$	1.6 (1.3; 2.2)	1.6 (1.1; 2.4)	1.8 (1.2; 2.9)	1.8 (1.3; 2.8)
Uric acid, $\mu\text{mol}/\text{L}$	279 (218; 349)	327 (269; 381) <sup>a</sup>	324 (276; 376) <sup>a</sup>	349 (272; 390) <sup>a</sup>
Hematology				
Hemoglobin, $\text{g}/\text{L}$	137 (130; 144)	129 (123; 140) <sup>bd</sup>	138 (126; 147)	133 (123; 143)
RBC, $\times 10^{12}/\text{L}$	4.8 (4.5; 5.0)	4.5 (4.2; 4.8) <sup>ad</sup>	4.7 (4.5; 5.1) <sup>g</sup>	4.5 (4.1; 4.9) <sup>b</sup>
WBC, $\times 10^9/\text{L}$	6.5 (5.7; 8.0)	6.7 (5.7; 7.8)	6.6 (5.3; 7.9)	6.9 (5.7; 8.0)
Platelets, $\times 10^9/\text{L}$	238 (199; 270)	234 (195; 270)	233 (191; 281)	229 (189; 273)
ESR, $\text{mm}/\text{h}$	16.5 (10; 23)	22 (15; 31) <sup>b</sup>	22.5 (15.5; 29.5) <sup>b</sup>	23 (18; 33) <sup>c</sup>
Coagulation tests				
Fibrinogen, $\text{g}/\text{L}$	4.4 (3.9; 5.5)	4.4 (3.9; 5.1)	4.5 (3.8; 5.7)	4.1 (3.7; 5.1)
SFMCs, $\text{mg}/\text{dL}$	5.5 (3.5; 15)	12 (7; 16)	14 (8; 23) <sup>a</sup>	12.5 (7; 21)
D-dimer, $\text{ng}/\text{mL}$	263 (235; 303)	287 (239; 351)	271 (232; 304)	290 (254; 363)

<sup>a</sup> $P < 0.05$ ,<sup>b</sup> $P < 0.01$ ,<sup>c</sup> $P < 0.001$  vs CKD-,<sup>d</sup> $P < 0.05$ ,<sup>e</sup> $P < 0.01$ ,<sup>f</sup> $P < 0.001$  vs A-CKD-,<sup>g</sup> $P < 0.05$ ,

<sup>i</sup> $P < 0.001$  vs A-CKD+ ( $\chi^2$  test for discrete parameters and multiple comparisons of mean ranks for continuous parameters, estimated glomerular filtration rate,  $\text{mL}/\text{min} \times 1.73 \text{ m}^2$ ). LDL: Low-density lipoprotein; HDL: High-density lipoprotein; UACR: Urinary albumin-to-creatinine ratio; eGFR: Estimated glomerular filtration rate; HbA1c: Hemoglobin A1c; RBC: Red blood cell; WBC: White blood cell; SFMC: Soluble fibrin monomer complex; CKD-: The group of individuals with estimated glomerular filtration rate  $\geq 60 \text{ mL}/\text{min} \times 1.73 \text{ m}^2$  and urinary albumin-to-creatinine ratio  $< 3.0 \text{ mg}/\text{mmol}$ ; NA-CKD: Non-albuminuric chronic kidney disease, the group of individuals with estimated glomerular filtration rate  $< 60 \text{ mL}/\text{min} \times 1.73 \text{ m}^2$  and urinary albumin-to-creatinine ratio  $\geq 3.0 \text{ mg}/\text{mmol}$ ; A-CKD-: Group of patients with estimated glomerular filtration rate  $\geq 60 \text{ mL}/\text{min} \times 1.73 \text{ m}^2$  and urinary albumin-to-creatinine ratio  $\geq 3.0 \text{ mg}/\text{mmol}$ ; A-CKD+: Group of individuals with estimated glomerular filtration rate  $< 60 \text{ mL}/\text{min} \times 1.73 \text{ m}^2$  and urinary albumin-to-creatinine ratio  $\geq 3.0 \text{ mg}/\text{mmol}$ .

## DISCUSSION

### Key findings

The results of this study demonstrate the characteristics of different CKD course patterns in patients with long-term T2D. First, by matching a panel of clinical and laboratory parameters of T2D patients who had an increase in albuminuria or a decrease in eGFR, or both deviations, with those without, we identified the risk factors for these CKD patterns. Second, we showed some features in the urinary excretion of biomarkers, reflecting podocyte and interstitium involvement, in patients with T2D and different patterns of CKD. The data provide further evidence that albuminuric and NA-CKD phenotypes correspond to different pathways of diabetic kidney disease progression.

### The risk factors for NA-CKD

The development of NA-CKD in patients with T2D was associated with older age ( $\geq 65$  years), female sex, longer diabetes duration ( $\geq 15$  years), and the use of diuretics. Age over 65 years was a risk factor for both albuminuric and NA-CKD patterns in our

**Table 3 Risk factors for different patterns of chronic kidney disease in patients with type 2 diabetes**

Risk factor	Pattern of CKD		
	NA-CKD (n = 111)	A-CKD- (n = 87)	A-CKD+ (n = 73)
Age ≥ 65 yr	3.16 (1.76-5.70) <i>P</i> = 0.0001	1.00 (0.55-1.80) <i>P</i> = 0.99	1.76 (0.94-3.28) <i>P</i> = 0.08
Duration of diabetes ≥ 15 yr	2.81 (1.53-5.17) <i>P</i> = 0.0009	1.63 (0.89-3.01) <i>P</i> = 0.12	2.32 (1.19-4.53) <i>P</i> = 0.01
Male sex	0.46 (0.21-0.98) <i>P</i> = 0.04	2.32 (1.20-2.48) <i>P</i> = 0.01	1.49 (0.74-3.01) <i>P</i> = 0.24
Female sex	2.19 (1.02-4.69) <i>P</i> = 0.04	0.43 (0.22-0.83) <i>P</i> = 0.01	0.67 (0.33-1.36) <i>P</i> = 0.24
Smoking	0.81 (0.25-2.60) <i>P</i> = 0.72	3.49 (1.31-9.28) <i>P</i> = 0.01	0.56 (0.13-2.34) <i>P</i> = 0.43
WHR >1.0	0.61 (0.22-1.65) <i>P</i> = 0.32	3.64 (1.32-9.99) <i>P</i> = 0.01	1.53 (0.57-4.10) <i>P</i> = 0.40
HbA1c > 8.0%	0.68 (0.38-1.20) <i>P</i> = 0.18	2.67 (1.35-5.27) <i>P</i> = 0.005	1.10 (0.58-2.09) <i>P</i> = 0.76
Treatment with diuretics	2.80 (1.56-5.00) <i>P</i> = 0.0005	1.10 (0.60-2.00) <i>P</i> = 0.76	1.30 (0.70-2.44) <i>P</i> = 0.41
Treatment with calcium channel blockers	1.20 (0.66-2.17) <i>P</i> = 0.56	1.47 (0.79-2.75) <i>P</i> = 0.22	2.23 (1.17-4.25) <i>P</i> = 0.01

The data are presented as odds ratio, 95% confidence interval and *P* value. CKD: Chronic kidney disease; CKD-: The group of individuals with estimated glomerular filtration rate  $\geq 60$  mL/min  $\times 1.73$  m<sup>2</sup> and urinary albumin-to-creatinine ratio  $< 3.0$  mg/mmol; NA-CKD: Non-albuminuric chronic kidney disease, the group of individuals with estimated glomerular filtration rate  $< 60$  mL/min  $\times 1.73$  m<sup>2</sup> and urinary albumin-to-creatinine ratio  $< 3.0$  mg/mmol; A-CKD-: Group of patients with estimated glomerular filtration rate  $\geq 60$  mL/min  $\times 1.73$  m<sup>2</sup> and urinary albumin-to-creatinine ratio  $\geq 3.0$  mg/mmol; A-CKD+: Group of individuals with estimated glomerular filtration rate  $< 60$  mL/min  $\times 1.73$  m<sup>2</sup> and urinary albumin-to-creatinine ratio  $\geq 3.0$  mg/mmol; HbA1c: Hemoglobin A1c; WHR: Waist-to-hip ratio.

cohort. This finding may be explained by the general tendency for GFR to decrease in elderly patients<sup>[27]</sup>, as well as the inverse dependence of eGFR on age when calculated using the CKD-EPI formula<sup>[28]</sup>.

In our patients, female sex was a risk factor for NA-CKD, which is consistent with previous studies<sup>[29,30]</sup>. It should also be taken into consideration that in women, who predominated in the NA-CKD group, the CKD-EPI formula gives lower eGFR values than in men when operating with equal creatinine levels. It was recently revealed that in healthy individuals, single-nephron GFR demonstrated no differences between men and women, but the total GFR values in women are typically lower than those in men due to fewer nephrons in the female kidney<sup>[31]</sup>. The duration of diabetes for 15 years or more increased the risk of NA-CKD and A-CKD+ phenotypes, affecting eGFR more than albuminuria.

The proportion of patients taking diuretics was highest in the NA-CKD group. The relationships between diuretics and CKD require cautious interpretation. On the one hand, diuretics, especially in high doses, can cause deteriorative effects on the renal tubulointerstitium by provoking metabolic acidosis<sup>[32]</sup>, activation of the renin-angiotensin system<sup>[33]</sup>, or hypokalemia<sup>[34]</sup>. Recent data indicate that the use of diuretics is associated with adverse renal outcomes, indicated by a decline in eGFR and an increasing risk of renal replacement therapy initiation in CKD patients. It was speculated that reduced GFR can be the result of episodes of lowering blood pressure, volume depletion and related acute renal injury induced by diuretics, especially when used in combination with other antihypertensive agents<sup>[35]</sup>. The worsening of renal function in elderly patients with chronic heart failure has been associated with high doses of loop diuretics<sup>[36]</sup>. The more frequent use of diuretics in patients with reduced renal function may be attributed to more prevalent and/or advanced arterial hypertension, heart failure or other fluid retention syndromes, which occur before the start of diuretic therapy. In our cohort, no differences in the prevalence of arterial hypertension or congestive heart failure were observed between the groups. The role of diuretics as factors modifying the CKD course requires further research.

**Table 4** Logistic regression model for estimated glomerular filtration rate < 60 mL/min × 1.73 m<sup>2</sup>,  $\text{logit}(P) = \ln[P/(1-P)]$ 

Parameter	Coefficient $\beta$	95%CI	P value
Constant	-3.5742	-6.1459, -1.0025	0.006
Age, years	+0.0751	0.0413, 0.1089	0.00001
HbA1c, %	-0.2277	-0.3645, -0.0908	0.001
Female sex (1 or 0)	+0.2277	0.0051, 0.5743	0.046
Use of diuretics (1 or 0)	-0.2521	-0.4895, -0.0143	0.04

Area under the receiver operating characteristic curve = 0.7441, P value for Kolmogorov-Smirnov statistics =  $2 \times 10^{-11}$ . CI: Confidence interval; HbA1c: Glycated hemoglobin; logit: Logit-function.

### **Risk factors for albuminuria not accompanied by eGFR reduction**

In our study, male sex, smoking, WHR > 1.0 and HbA1c > 8.0% were identified as risk factors for albuminuria not accompanied by a decrease in eGFR. This CKD pattern was more common in men. In multivariate logistic regression analysis, male sex was a significant risk factor for albuminuria. These data are in agreement with results of other research indicating an increased risk of albuminuria in men with T2D<sup>[37,38]</sup>.

The percentage of current smokers was highest in the A-CKD- group. The association between albuminuria and smoking has been reported previously<sup>[37,39,40]</sup>. It was demonstrated that smoking cessation contributes to the reduction of albuminuria in patients with newly diagnosed T2D<sup>[41]</sup>. The direct fibrogenic effect of tobacco smoke in the kidneys has been revealed in experimental CKD<sup>[42]</sup>. Other underlying mechanisms for the association between smoking and albuminuria have been proposed, including activation of the sympathetic and renin-angiotensin systems, an increase in blood pressure, changes in intraglomerular hemodynamics, progression of atherosclerotic changes, and activation of vascular-platelet interactions<sup>[43]</sup>.

As expected, poor glycemic control turned out to be a risk factor for UACR elevation. The albuminuric effect of hyperglycemia has been linked with the accumulation of advanced glycation end-products, which, through the activation of protein kinase C and nuclear factor- $\kappa$ B, enhance the synthesis of fibrogenic and proinflammatory factors in glomerular and tubular cells<sup>[44]</sup>. These changes lead to deterioration of the glomerular endothelium and podocytes<sup>[45]</sup> and impair albumin reabsorption in the proximal tubules<sup>[46]</sup>. Recent studies have indicated that suppression of autophagy under hyperglycemic conditions promotes podocytopathy and increases the permeability of the glomerular filter<sup>[47,48]</sup>.

The WHR was identified as another risk factor for albuminuria not accompanied by eGFR reduction. The relationship between abdominal obesity and albuminuria has been shown in a number of studies<sup>[37,49,50]</sup>. Hyperproduction of proinflammatory and fibrogenic cytokines, oxidative stress, and imbalances in adipokines are suggested as mechanisms of albuminuric effect in abdominal obesity<sup>[51]</sup>. The association between WHR and albuminuria could be mediated by insulin resistance. A study in *db/db* mice, a T2D model, showed that both albuminuria and glomerulosclerosis are related to insulin resistance<sup>[52]</sup>. A positive correlation has been found between homeostatic model assessment of insulin resistance index and the UACR values in patients with T2D<sup>[53]</sup>. Misregulation of epithelial proteins, such as nephrin and megalin, and activation of the mTOR/S6 kinase pathway seem to be involved in mediating the pathophysiology of insulin resistance, kidney hypertrophy, hyperfiltration and microalbuminuria<sup>[54]</sup>.

### **Risk factors for albuminuric CKD**

According to our results, diabetes duration and the use of calcium channel blockers were risk factors for albuminuric CKD. If the effect of the disease duration on the risk of CKD is quite natural, the relationships between dihydropyridines and CKD deserve discussion. On the one hand, initially more severe hypertension and the use of several antihypertensive drugs, there could be a combination of factors similar to the effects of diuretics, such as initially more severe arterial hypertension, and the use of several antihypertensive agents, which lead to a higher risk of arterial hypotension and prerenal acute kidney injury<sup>[35]</sup>. Another possible mechanism involves the dilatation of the *vas afference* and the intraglomerular hypertension that can be induced by nifedipine, or amlodipine, the most widely used L-type calcium channel blockers<sup>[55-57]</sup>. In our patient cohort, nifedipine and amlodipine were the only representatives of the class of calcium channel blockers. Meanwhile, observational studies have shown a reduction in albuminuria when patients were switched from L-

**Table 5** Logistic regression model for urinary albumin-to-creatinine ratio  $\geq 3.0$  mg/mmol,  $\text{logit}(P) = \ln[P/(1-P)]$ 

Parameter	Coefficient $\beta$	95%CI	P value
Constant	-8.1206	-13.1599, -3.0813	0.002
WHR	+5.1228	0.3920, 9.8535	0.03
HbA1c, %	+0.3570	0.1169, 0.5971	0.004
Male sex, (1 or 0)	+0.6725	0.1920, 1.1531	0.006

Area under the receiver operating characteristic curve = 0.7612, *P* value for Kolmagorov-Smirnov statistics = 0.00004. CI: Confidence interval; HbA1c: Glycated hemoglobin; logit: Logit-function; WHR: Waist-to-hip ratio.

type calcium channel blockers to T/L-type<sup>[58]</sup> or L/N-type ones<sup>[59]</sup>. According to meta-analyses, antialbuminuric activity has been shown for nondihydropyridine<sup>[60]</sup>, dihydropyridine L/N-, L/T-<sup>[61]</sup> and T-type<sup>[62]</sup> calcium channel blockers.

### Urinary excretion of nephrin and podocin

In this study, we found that urinary excretion of nephrin and podocin was increased significantly in individuals with long-term T2D and correlated positively with UACR. These data are in agreement with the notion that podocytopathy is a key factor leading to the development of proteinuria and glomerulosclerosis in diabetic kidney disease<sup>[12]</sup>. Elevated excretion of nephrin and podocin, which are expressed in podocytes exclusively, may reflect more severe podocyte injury in albuminuric patients. It was demonstrated that the loss of the podocytes correlates with the levels of proteinuria in diabetic nephropathy<sup>[63]</sup>. Thus, increased urinary excretion of nephrin and podocin can be a sign of podocytopathy, which is detected in diabetic kidney disease<sup>[64]</sup>. The correlation between these markers and the urinary concentrations of podocytes in diabetes has been shown previously<sup>[65]</sup>. In our study, the excretion of nephrin and podocin was increased dramatically in patients with elevated albuminuria, regardless of the concomitant decline in renal function, compared to patients without any signs of CKD or NA-CKD. This may indicate more advanced podocyte injury in T2D patients with albuminuric CKD.

### Urinary excretion of WFDC-2

The serum levels of WFDC-2 (HE-4) were validated previously as a marker of tubulointerstitial fibrosis<sup>[25,26]</sup>. In this study, we investigated for the first time the urinary excretion of WFDC-2 in individuals with T2D. First, we found that excretion of WFDC-2 in men was markedly higher (approximately 10 times) than in women. These findings could be explained by the sexual differences in the expression of this molecule. Besides the kidneys, in males WFDC-2 is expressed in the epithelial cells of the epididymal and seminal ducts and the glandular epithelium of the prostate, i.e., in the organs that are related to the urinary tract anatomically. In women, WFDC-2 expression is detected in the fallopian tubes, endometrium, and Bartholin's glands<sup>[24]</sup>, which do not contact directly with the urinary system.

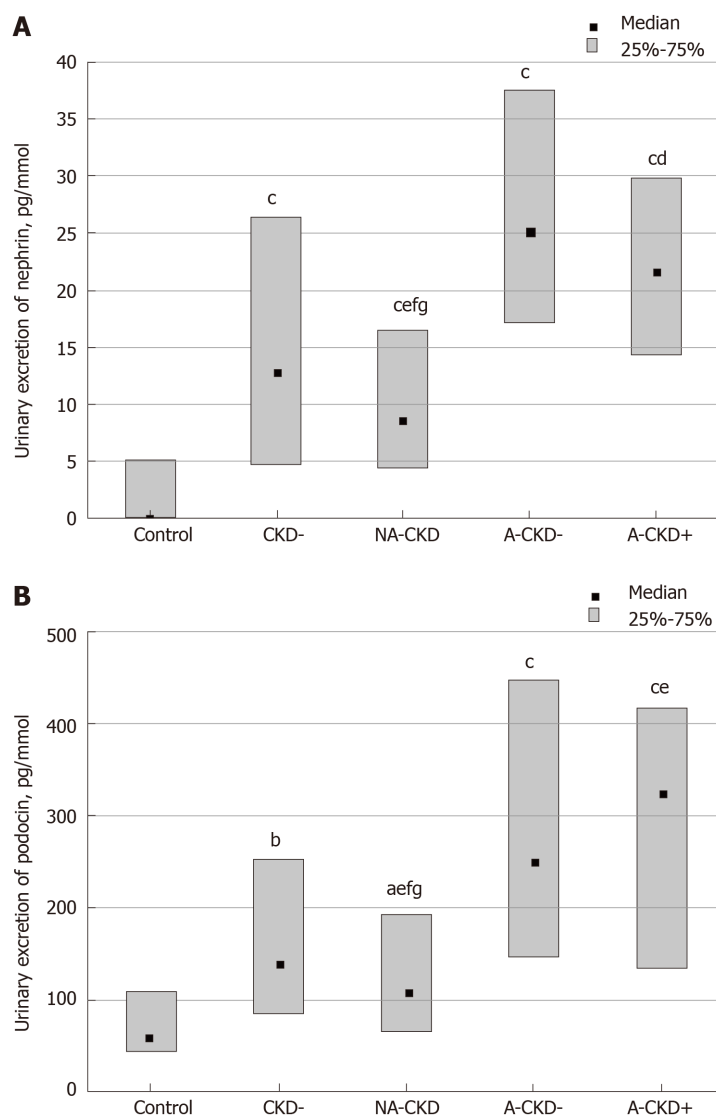
Excretion of WFDC-2 showed different relationships with CKD between men and women. In men with diabetes, excretion of WFDC-2 was increased in all groups, regardless of the presence or pattern of CKD. In women, WFDC-2 excretion was increased in the groups with declined eGFR only. The women had an inverse correlation between WFDC-2 and eGFR values and a direct correlation between WFDC-2 and UACR. At the same time, urinary excretion of WFDC-2 was not associated with excretion of nephrin or podocin. These data are in agreement with previous morphological studies indicating a close relationship between GFR and tubulointerstitial involvement rather than glomerulopathy<sup>[15,66]</sup>.

### Limitations

Our study is not without limitations. First, it is a cross-sectional study that does not prove causality. The natural intraindividual variability in eGFR and UACR values could be a source of some errors in classifying patients into groups. The recruitment of patients at one clinical center and the relatively small sample size could have led to a shift in the results of biomarker assessment with respect to the general diabetic population.

### The remarks for clinical practice and future research

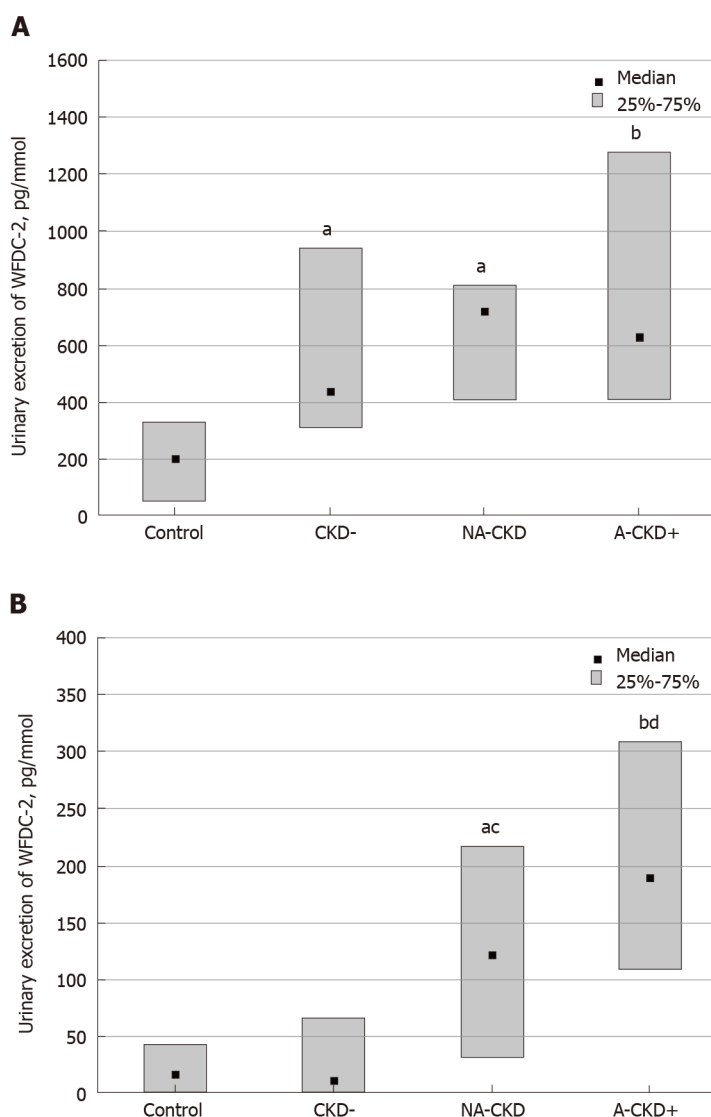
In this study, we demonstrate the differences in the clinical and laboratory



**Figure 2 Urinary excretion of podocyte-specific markers in patients with type 2 diabetes and different patterns of chronic kidney disease.** A: Nephrin; B: Podocin. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , <sup>c</sup> $P < 0.001$  vs non-diabetic control; <sup>d</sup> $P < 0.05$ , <sup>e</sup> $P < 0.01$  vs chronic kidney disease-group; <sup>f</sup> $P < 0.001$  vs albuminuric chronic kidney disease-group, <sup>g</sup> $P < 0.001$  vs albuminuric chronic kidney disease+ group (the test of multiple comparisons of mean ranks). CKD-: The group of individuals with estimated glomerular filtration rate  $\geq 60$  mL/min  $\times$  1.73 m<sup>2</sup> and urinary albumin-to-creatinine ratio  $< 3.0$  mg/mmol; NA-CKD: Non-albuminuric chronic kidney disease, the group of individuals with estimated glomerular filtration rate  $< 60$  mL/min  $\times$  1.73 m<sup>2</sup> and urinary albumin-to-creatinine ratio  $< 3.0$  mg/mmol; A-CKD-: Group of patients with estimated glomerular filtration rate  $\geq 60$  mL/min  $\times$  1.73 m<sup>2</sup> and urinary albumin-to-creatinine ratio  $\geq 3.0$  mg/mmol; A-CKD+: Group of individuals with estimated glomerular filtration rate  $< 60$  mL/min  $\times$  1.73 m<sup>2</sup> and urinary albumin-to-creatinine ratio  $\geq 3.0$  mg/mmol.

characteristics of albuminuric and NA-CKD in patients with long-term T2D. We found that female sex, older age, longer diabetes duration and diuretic use were associated with the NA-CKD phenotype. Meanwhile, male sex, smoking, abdominal obesity, and poor glycemic control were risk factors for albuminuria elevation not accompanied by a reduction in eGFR. It should be noted that some of the abovementioned risk factors are modifiable, especially those associated with albuminuria, which is important from clinical point of view. The predominant effect of antihyperglycemic drugs on albuminuria or GFR should be considered when choosing treatment for T2D patients with different CKD phenotypes.

To our knowledge, this is the first study addressing the diversity in urinary biomarkers in T2D patients with different CKD phenotypes. The different patterns of the shifts in the urinary excretion of the biomarkers of podocyte and tubulointerstitial involvement in albuminuric and NA-CKD give further support the notion that these phenotypes differ in their pathophysiology. A significantly more demonstrative increase in the excretion of nephrin and podocin in patients with elevated UACR compared to those without could suggest that albuminuric CKD is



**Figure 3 Urinary excretion of WAP-four-disulfide core domain protein 2 in individuals with type 2 diabetes and different patterns of chronic kidney disease.** A: Males; B: Females. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs non-diabetic control, <sup>c</sup> $P < 0.05$ , <sup>d</sup> $P < 0.01$  vs CKD- group (the test of multiple comparisons of mean ranks). CKD-: The group of individuals with estimated glomerular filtration rate  $\geq 60$  mL/min  $\times$  1.73 m<sup>2</sup> and urinary albumin-to-creatinine ratio  $< 3.0$  mg/mmol; NA-CKD: Non-albuminuric chronic kidney disease, the group of individuals with estimated glomerular filtration rate  $< 60$  mL/min  $\times$  1.73 m<sup>2</sup> and urinary albumin-to-creatinine ratio  $< 3.0$  mg/mmol; A-CKD-: Group of patients with estimated glomerular filtration rate  $\geq 60$  mL/min  $\times$  1.73 m<sup>2</sup> and urinary albumin-to-creatinine ratio  $\geq 3.0$  mg/mmol; A-CKD+: Group of individuals with estimated glomerular filtration rate  $< 60$  mL/min  $\times$  1.73 m<sup>2</sup> and urinary albumin-to-creatinine ratio  $\geq 3.0$  mg/mmol.

associated with more severe podocyte involvement. The increase in urinary excretion of WFDC-2 in women with T2D and decreased eGFR apparently indicates more advanced tubulointerstitial lesion. The assessment of the predictive value of the studied biomarkers in different phenotypes of CKD is a challenge for future research. Studies of the renoprotective potential of antihyperglycemic, antihypertensive and other therapeutic agents in different CKD phenotypes are urgently needed. In conclusion, the data provide further evidence that albuminuric and NA-CKD phenotypes correspond to different pathways of diabetic kidney disease progression.

## ARTICLE HIGHLIGHTS

### Research background

A number of researches show the heterogeneity of the natural course of chronic kidney disease (CKD) in patients with diabetes. Moreover, it has been shown that natural course of diabetic kidney disease is being transformed with increasing prevalence of declined renal function not accompanied by elevation of albuminuria. The trend is more evident in patients with type 2

diabetes (T2D). Currently, little is known about the mechanisms that determine the development of the albuminuric or nonalbuminuric phenotype of CKD. It was suggested that an increase in albuminuria may be a consequence of podocytopathy, while a decrease in renal function is associated with the involvement of tubulointerstitium.

### Research motivation

The main topic of this study is in-depth clinical characteristics and identification of the risk factors and biomarkers of albuminuric and non-albuminuric CKD phenotypes in patients with T2D. The results may provide further progress in understanding of individual differences in the natural course of diabetic kidney disease and generation differentiated approaches to prevention and treatment of this complication.

### Research objectives

The study aimed to identify the risk factors and urinary biomarkers of albuminuric and non-albuminuric CKD in patients with long-term T2D. Wherein, we tested the hypothesis that albuminuric and non-albuminuric CKD phenotypes correspond to different pathways of diabetic kidney disease progression.

### Research methods

Three hundred and sixty patients with T2D duration of at least 10 years from the date of diagnosis were included in this observational cross-sectional study. The associations of a panel of demographic and clinical characteristics, complications, comorbidities, and metabolic parameters with albuminuric and non-albuminuric CKD were analyzed. The urinary excretion of nephrin and podocin, two podocyte-specific markers, and WAP-four-disulfide core domain protein 2 (WFDC-2), a marker of tubulointerstitial fibrosis, was determined by ELISA in defined CKD phenotypes.

### Research results

In this study we identified the risk factors of three CKD phenotypes in T2D patients. According to our data, non-albuminuric CKD is associated with age  $\geq 65$  years, female sex, diabetes duration  $\geq 15$  years, and the use of diuretics. Male sex, smoking, waist-to-hip ratio  $> 1.0$  and HbA1c  $> 8.0\%$  are risk factors for elevated albuminuria not accompanied by a decrease in estimated glomerular filtration rate (eGFR). Duration of diabetes  $\geq 15$  years and the use of calcium channel blockers seem to be risk factors for albuminuria with decreased eGFR. We also found some differences in predictors of decreased eGFR and increased albuminuria. In multivariate logistic regression analysis, age, HbA1c, female sex and diuretics were significant predictors for reduced eGFR, while waist-to-hip ratio, HbA1c and male sex were associated with elevated urinary albumin-to-creatinine ratio (UACR). In accordance with the tested hypothesis, we found the differences in urinary biomarkers of podocyte and tubulointerstitium involvement in patients with different CKD phenotypes. Excretion of nephrin and podocin was increased in patients with albuminuria, regardless of decline in renal function, correlating positively with UACR. At the same time, in women, WFDC-2 excretion was increased in those with reduced renal function, correlating negatively with eGFR.

### Research conclusions

To our knowledge, this is the first study addressing the diversity in clinical characteristics and urinary biomarkers in T2D subjects with different CKD phenotypes. The results of this study provide new data on the risk factors and mechanisms of different variants of CKD in patients with long-term T2D. Firstly, by matching a panel of clinical and laboratory parameters of T2D patients who had an increase in albuminuria, a decrease in eGFR, or both deviations, with parameters in T2D patients with normoalbuminuria and preserved renal function, we showed the differences in profiles of the risk factors for CKD phenotypes. According to our data, non-albuminuric CKD phenotype is associated with age, female sex, diabetes duration, and the use of diuretics, whereas male sex, smoking, abdominal obesity and poor glycemic control are risk factors for elevated albuminuria. Secondly, we demonstrated some features in the urinary excretion of biomarkers, reflecting the podocyte and interstitial involvement, in patients with different CKD phenotypes. A significantly more demonstrative increase in the excretion of nephrin and podocin in patients with elevated UACR compared to those without could suggest that albuminuric CKD is associated with more severe podocyte involvement. The increase in urinary excretion of WFDC-2 in women with T2D and decreased eGFR apparently indicates more advanced tubulointerstitial fibrosis. The data provide further evidence that albuminuric and non-albuminuric CKD phenotypes correspond to different pathways of diabetic kidney disease progression. The diversity in the profiles of risk factors should be taken into account by clinicians in the management of diabetes.

### Research perspectives

Since our study has a cross-sectional design, it does not prove causality. Accordingly, significance of some identified risk factors needs further confirmation. In particular, the role of abdominal obesity, insulin resistance, diuretics and calcium channel blockers needs to be verified in prospective studies. The assessment of the predictive value of the studied biomarkers in albuminuric and non-albuminuric CKD phenotypes is a challenge for future research. The studies of the renoprotective potential of antihyperglycemic, antihypertensive and other therapeutic agents in different CKD phenotypes are urgently needed.

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## Observational Study

## Type 1 diabetes loci display a variety of native American and African ancestries in diseased individuals from Northwest Colombia

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## Abstract

## BACKGROUND

Type 1 diabetes (T1D) is a complex disease with a higher incidence in Europeans than other populations. The Colombians Living in Medellín (CLM) is admixed with ancestry contributions from Europeans, Native Americans (NAT) and Africans (AFR).

## AIM

Our aim was to analyze the genetic admixture component at candidate T1D loci in Colombian individuals with the disease.

## METHODS

Seventy-four ancestry informative markers (AIMs), which tagged 41 T1D candidate loci/genes, were tested by studying a cohort of 200 Northwest Colombia diseased individuals. T1D status was classified by testing for glutamic acid decarboxylase (GAD-65 kDa) and protein tyrosine-like antigen-2 auto-antibodies in serum samples. Candidate loci/genes included *HLA*, *INS*, *PTPN22*, *CTLA4*, *IL2RA*, *SUMO4*, *CLEC16A*, *IFIH1*, *EFR3B*, *IL7R*, *NRP1* and *RNASEH1*, amongst others. The 1,000 genome database was used to analyze data from 94 individuals corresponding to the reference CLM. As the data did not comply with a normal distribution, medians were compared between groups using the Mann-Whitney *U*-test.

## RESULTS

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Both T1D patients and individuals from CLM displayed mainly European ancestry (61.58 *vs* 62.06) followed by Native American (27.34 *vs* 27.46) and to a lesser extent the AFR ancestry (10.28 *vs* 10.65) components. However, compared to CLM, ancestry of T1D patients displayed a decrease of NAT ancestry at gene *EFR3B* (24.30 *vs* 37.10) and an increase at genes *IFIH1* (32.07 *vs* 14.99) and *IL7R* (52.18 *vs* 39.18). Also, for gene *NRP1* (36.67 *vs* 0.003), we observed a non-AFR contribution (attributed to NAT). Autoimmune patients (positive for any of two auto-antibodies) displayed lower NAT ancestry than idiopathic patients at the *MHC* region (20.36 *vs* 31.88). Also, late onset patients presented with greater AFR ancestry than early onset patients at gene *IL7R* (19.96 *vs* 6.17). An association analysis showed that, even after adjusting for admixture, an association exists for at least seven such AIMs, with the strongest findings on chromosomes 5 and 10 (gene *IL7R*,  $P = 5.56 \times 10^{-6}$  and gene *NRP1*,  $P = 8.70 \times 10^{-19}$ , respectively).

## CONCLUSION

Although Colombian T1D patients have globally presented with higher European admixture, specific T1D loci have displayed varying levels of Native American and AFR ancestries in diseased individuals.

**Key words:** Type 1 diabetes; Genetic admixture; Native American; Idiopathic; Colombia

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**Core tip:** We have tested the effect of genetic admixture in a set of Colombian patients with Type 1 Diabetes (T1D). We show that, although no differences between T1Ds and Colombians living in Medellín arose globally, there appear to be ancestry differences when looking at specific T1D loci/genes (*e.g.*, genes *EFR3B*, *IFIH1*, *IL7R* and *NRP1*). Also, when comparing patient ancestry according to the presence/absence of T1D-related auto-antibodies or age at onset of the disease, differences were also observed. The most striking differences in ancestry occurred outside the HLA region, which is considered the master risk locus in T1D and for autoimmune diseases overall. This in itself is a striking observation.

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## INTRODUCTION

Type 1 diabetes (T1D) is a heterogeneous disease with pathogenic processes and phenotypic characteristics that show marked variation. It is accepted that genetic effects are an important factor for this heterogeneity. *HLA* confers the major genetic susceptibility to T1D, contributing up to 50%; it is located on chromosome 6p21<sup>[1]</sup>. In addition, over 50 non-*HLA* genes (so far) increase susceptibility to T1D<sup>[2,3]</sup>. Recently, we have identified that *RNASEH1* gene variants associate with T1D in Northwest Colombia<sup>[4]</sup>. This gene, which is located on chromosomal region 2p25, has not thus far been associated elsewhere with the disease. A wide geographical variation in the incidence of T1D both among and within countries has been reported<sup>[5]</sup>. Incidence of T1D is higher in Europeans<sup>[6-8]</sup> than in Latin American countries<sup>[7,8]</sup>. Genetic admixture is a factor that influences allelic frequencies in a population; this, in part, may contribute to explaining the differences observed in T1D epidemiology.

Three studies in Latin America have tested the admixture effect on T1D. Two of these were carried out in Brazil<sup>[9,10]</sup> and the third in Cuba<sup>[11]</sup>. These three studies found that T1D patients are mostly of European descendant and not necessarily different than controls. Thus, one Brazilian study and the one from Cuba reported that patients carried a greater European component than their controls; this observation was established as a risk factor<sup>[9,11]</sup>.

In Colombia, the admixture process was produced differently in each region of the country. Populations in southern Colombia show higher values of Native American

ancestry (NAT, average 60%), whilst African (AFR) ancestry is more observed in the region of Chocó (average 68%) and the Caribbean coast (average 30%)<sup>[12-14]</sup>. On the other hand, northwest Colombia, inhabited by the “paisa” population, exhibits the highest percentage of European ancestry, which ranges in studies from 47-79%<sup>[15-19]</sup>. In Colombia, the admixture effect has been examined for some complex diseases such as type 2 diabetes<sup>[20]</sup>, asthma<sup>[21]</sup>, cancer<sup>[22,23]</sup>, dengue patients<sup>[24]</sup>, Alzheimer’s disease<sup>[17]</sup>, as well as for cardio-metabolic parameters<sup>[25]</sup>.

Although much of the work on the admixture effect on several phenotypes has been done in Latin America and Colombia, none has tested this effect on T1D in Colombian patients. Our purpose was to analyze the genetic admixture composition of a set of Colombian T1D patients, by testing previously reported admixture informative markers (AIMs) in the vicinity of previously reported T1D candidate genes/loci. Besides, two chromosomal regions of high relevance to T1D in our population were tested more thoroughly. These loci were *6p21* (*HLA*), which is globally accepted as the T1D master risk locus, and *2p25* (*RNASEH1*), which has been reported solely in Colombia, so far. We inferred individual patient proportions of European, AFR and NAT ancestry components. Although the European component was higher than the two other parental contributions in a global analysis, some loci are clearly non-Europeans in cases *vs* the reference population, or between T1D categories. This study shed light on the genetics of T1D in a Colombian population, and reinforces the importance of including different approaches when looking for T1D genetic architecture. This is suggested by finding no admixture differences in strongly associated T1D loci, such as *HLA* (*IDDM1*) or *IDDM2*. In contrast, a strong genetic admixture effect was observed for other loci not described as high determinants for developing T1D. For instance, this was the case for chromosomal regions *5p13.2* and *10p11.22*.

## MATERIALS AND METHODS

### Study population

The study group consisted of 200 Colombian individuals with T1D. Their age at onset was < 15 years. Diagnostic criteria were according to the American Diabetes Association<sup>[26]</sup>. Patients were considered as “Paisas” according to a self-reported questionnaire asking for their geographical origin back until their great-grandparents. Other questions included gender, age at onset, and other family members with autoimmune diseases.

Patients were identified in the main pediatric endocrinology institutes from Antioquia: Program of Pediatric Endocrinology (Universidad de Antioquia and Hospital San Vicente Fundación), IPS Universitaria, Universidad Pontificia Bolivariana, Instituto Antioqueño de Diabetes and Clínica Integral de Diabetes. This study was approved by the ethics committee of the Faculty of Medicine at Universidad de Antioquia. Informed consent was obtained from patients and their parents before drawing blood samples.

### Auto-antibodies testing

Two diabetes-related autoantibodies (AABs) were tested in sera samples from the 200 patients. These AABs were glutamic acid decarboxylase (GAD-65 kDa) and protein tyrosine-like antigen-2 (IA-2), as reported previously<sup>[4]</sup>. They were measured using a commercial ELISA-based kit (AESKULISA and LifeSpan BioSciences, Inc) according to the manufacturer's instructions. If a patient presented with at least one of these AABs, he/she was classified as autoimmune (T1AD), or was otherwise classified as idiopathic (T1BD).

### Genotyping and admixture estimation

Genomic DNA was isolated from peripheral blood samples using either the phenol-chloroform or salting out protocols. A set of 75 AIMs was tested in 200 T1D patient samples using the Competitive genotyping Allele-Specific PCR technology (KASP™), which was undertaken by the Company LGC Genomics Ltd. Details of this method can be obtained from <https://www.lgcgroup.com/genotyping/>.

The AIMs used have a high discriminatory power ( $\delta > 45\%$ ) among ancestral populations (Supplementary Table S1), which increases the statistical power for estimating individual ancestry. We selected these markers from Latino populations panels reported by Mao *et al*<sup>[27]</sup>, Galanter *et al*<sup>[28]</sup> and Ruiz-Linares *et al*<sup>[29]</sup>. The AIMs were distributed throughout the genome, tagging previously reported T1D candidate loci. However, we chose a higher density of markers for chromosome 2 (23 AIMs) where the *RNASEH1* gene is; and for chromosome 6 (18 AIMs) where the *HLA* region

is.

The 1,000 genome database was used to extract genetic information from 94 Colombians living in Medellin (CLM) for the 74 AIMs successfully typed (<ftp://ftp.1000genomes.ebi.ac.uk/vol1/ftp/release/20130502/>). These population individuals are from the same geographical region as the patients. We calculated allele and genotypic frequencies, and Hardy-Weinberg equilibrium (HWE) using PLINK v. 1.07<sup>[30]</sup>. In addition, we considered markers that were not in linkage disequilibrium with each other. We used markers with a genotyping rate higher than 95%, without significant deviations from HWE after a Bonferroni correction.

We estimated individual European, NAT and AFR ancestry proportions using ADMIXTURE software<sup>[31]</sup>. The proportions of each component were estimated using a supervised-learning strategy, providing the genotypes of 74 AIMs from reference populations AFR, European and NAT ( $k = 3$ ). We used 74/75 AIMs since one failed the PCR optimization.

To find the parental population allele frequencies, genotypes from 165 Europeans (Utah residents with ancestry from northern Europe and the West, named as CEU), and 165 AFR (Yoruba people in Ibadan, Nigeria, named as YRI) genotyped in the HapMap project were selected, which are deposited in the 1,000 genome database. Since we did not have access to NAT DNA samples or publicly available NAT genotype data on all 74 AIMs, we generated the genotypes of the 74 AIMs for 150 simulated individuals, according to the allele frequencies of NAT previously reported in the panels.

### Statistical analysis

Comparison between groups for continuous variables that did not comply with normal distribution was performed using the Mann-Whitney *U*-test. Thus, comparisons of ancestry medians between T1D subtypes (T1AD and T1BD) according to AABs, and individuals with early/late age at onset (*i.e.*  $\leq 5$  years or  $> 5$  years, respectively) were performed. In addition, these comparisons were also done to the CLM population. We performed these analyses for AIMs distributed across the set of candidate loci and independently for loci at different chromosomes. We ran all statistical analyses and graphs in the R package V3.3.3<sup>[32]</sup>. We also tested allelic association of these AIMs between T1D and CLM using PLINK 1.07<sup>[30]</sup>.

## RESULTS

### T1D versus CLM, our reference population

One out of 75 AIMs did fail the PCR optimization. Therefore, we tested a total of 74 AIMs in 200 T1D patients from Antioquia, Colombia. AIMs characteristics are shown in [Supplementary Table S1](#). Overall, the rate of genotyping was  $> 96\%$  for every AIM, and there was no deviation from the HWE, after Bonferroni correction for multiple testing ( $P = 6.75 \times 10^{-4}$ ). Also, as expected, none of the AIMs was in linkage disequilibrium with each other (data not shown).

The overall ancestral genetic makeup of the 200 T1D children showed a predominant proportion of European ancestry (EUR, Median = 61.58) followed by NAT ancestry (Median = 27.34), and AFR ancestry was found at a lower proportion (Median = 10.28, [Table 1](#) and [Figure 1](#)). [Figure 1](#) presents the ancestry distribution for the 200 T1D children studied here. It can be noticed that the European component is the prominent one. European ancestry ranged from 22% to 93%; the NAT ancestry ranged from 0 to 65%, and the AFR ancestry ranged from 0 to 40%.

Looking at the overall set of AIMs, and also at their distribution in specific loci, it was observed that diseased individuals of EUR ancestry had a median from 61.58-11.56. The lowest value was found for chromosome 5 AIMs ([Table 1](#)). NAT ancestry ranged from 52.18-24.30 in the diseased subjects. The highest value was found for gene *IL7R* AIMs (chromosome 5), and the lowest value was found for gene *EFR3B* AIMs (chromosome 2). The AFR component (AFR) ranged from 20.58 to 0.01. the lowest AFR ancestry was found for gene *IFIH1* AIMs (chromosome 2, [Table 1](#)). The wide ancestry variation across chromosomal regions is noticeable.

Overall, the CLM reference population displayed a very similar ancestry distribution compared to T1D cases. Nonetheless, specific T1D loci presented marked differences between the two groups; one such difference was observed for the gene *EFR3B*, which presented with higher NAT in the CLM population ( $P = 0.02$ ), suggesting a protective role for developing T1D ([Table 1](#)). Also, at gene *IFIH1*, T1D patients presented with lower European ancestry ( $P = 0.05$ ), at the expense of a higher NAT component than in CLM ([Table 1](#)). Other differences between T1D and CLM were observed for the *IL7R* and *NRP1* genes (Chromosomes 5 and 10, respectively) as

**Table 1 Genetic ancestry of type 1 diabetes patients compared to Colombians living in Medellin control population**

Chromosomal region	Ancestry	T1D, median (IQR)	CLM, median (IQR)	P value <sup>1</sup>
Overall AIMs	EUR	61.58 (52.84-69.85)	62.06 (49.67-73.74)	0.675
	NAT	27.34 (21.36-34.05)	25.46 (16.12- 32.90)	0.106
	AFR	10.28 (4.0-16.83)	10.65 (6.05-16.75)	0.575
Chr2_EFR3B	EUR	60.27 (34.05-80.79)	47.88 (34.04-76.43)	0.189
	NAT	24.30 (0.01-51.24)	37.10 (1.61-62.49)	0.02
	AFR	7.17 (0.01-25.05)	0.01 (0.01-15.67)	0.06
Chr2_CTLA4	EUR	46.24 (16.82-69.52)	58.26 (30.82-82.79)	0.167
	NAT	27.96 (0.04-41.31)	25.01 (0.01-45.16)	0.829
	AFR	20.54 (0.01-41.72)	11.78 (0.01-36.59)	0.183
Chr2_RNASEH1	EUR	56.91 (31.33-73.91)	58.80 (32.75-78.43)	0.482
	NAT	27.41 (11.69-46.36)	24.81 (0.01-48.84)	0.241
	AFR	8.37 (0.01-28.24)	13.04 (0.01-23.87)	0.430
Chr2_IFIH1	EUR	42.42 (0.01-77.63)	52.01 (16.33-82.16)	0.05
	NAT	32.07 (0.01-56.74)	14.99 (0.01-41.35)	0.246
	AFR	14.99 (0.01-32.31)	17.83 (0.01-42.31)	0.181
Chr5_IL7R	EUR	11.56 (1.04-41.14)	24.75 (7.20-46.98)	$7.0 \times 10^{-3}$
	NAT	52.18 (3.74-98.06)	39.18 (0.04-52.80)	$1.0 \times 10^{-4}$
	AFR	15.21 (3.0-35.75)	33.89 (11.23-59.94)	$1.56 \times 10^{-5}$
Chr6_MHC	EUR	51.35 (32.92-70.32)	55.86 (32.61-71.21)	0.76
	NAT	23.28 (8.92-40.0)	21.61 (5.83-43.13)	0.835
	AFR	18.87 (1.63-36.08)	19.53 (0.3-34.11)	0.660
Chr10_NRP1	EUR	63.32 (23.70-63.32)	0.03 (0.001-20.34)	$2.2 \times 10^{-16}$
	NAT	36.67 (7.93-36.67)	0.003 (0.001-7.93)	$2.2 \times 10^{-16}$
	AFR	0.03 (0.001-30.91)	94.23 (53.94-99.99)	$2.2 \times 10^{-16}$

<sup>1</sup>Data from Mann-Whitney *U* test. AIMs: Ancestry informative markers; IQR: Interquartile range; CLM: Colombians living in Medellin from 1,000 genomes database; Chr: Chromosome; EUR: European; NAT: Native American; AFR: African; T1D: Type 1 diabetes.

follows.

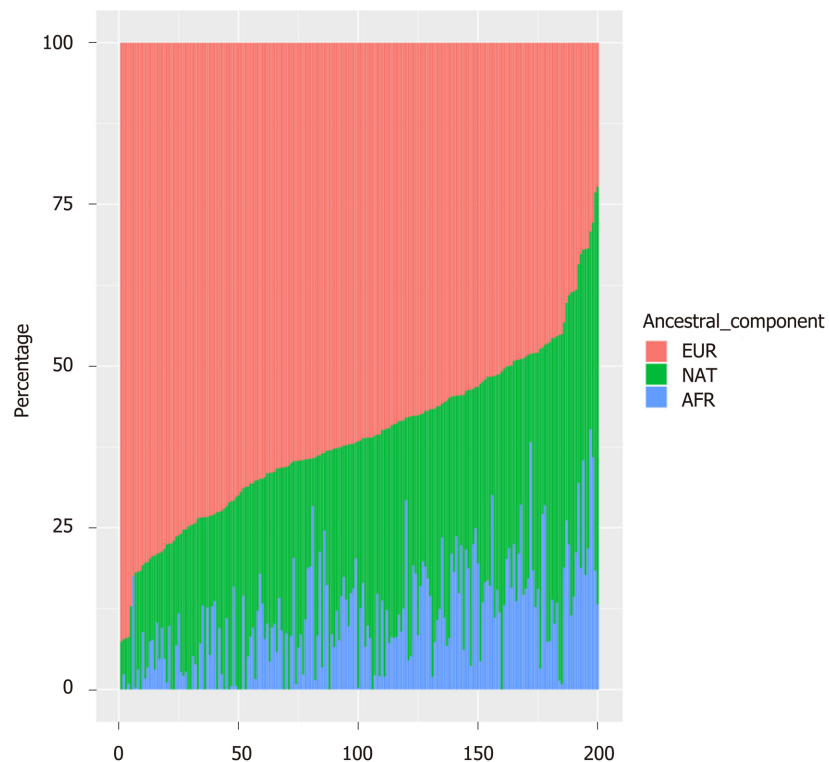
Chromosome 5 AIMs (gene *IL7R*) showed less European ( $P = 7.0 \times 10^{-3}$ ) and less AFR ancestries ( $1.56 \times 10^{-5}$ ) in diseased subjects than the CLM population; consequently, T1D patients had more NAT ancestry than CLM subjects at this chromosomal region ( $P = 1.0 \times 10^{-4}$ , Table 1). Regarding chromosome 10 AIMs (gene *NRP1*), it was observed that T1D patients presented a high European component compared to CLM (63.32 *vs* 0.03, Table 1). Conversely, patients presented an almost zero AFR component for this chromosomal region compared to CLM (0.03 *vs* 94.23, Table 1). Consequently, T1D patients displayed a predominance of NAT ancestry at this locus compared to CLM (36.67 *vs* 0.003, Table 1).

An exploratory association analysis showed that, after adjusting for admixture, seven markers were associated with T1D (Supplementary Table S2 and Table 2). The most significant findings were located on chromosomes 5 and 10 ( $P = 5.56 \times 10^{-6}$  and  $8.70 \times 10^{-19}$ , respectively). It is interesting that only one MHC marker (*rs2395656*) presented an association with the disease, and this happened with less strength in its association ( $P = 0.04$ ) than markers at chromosomes 5 and 10 (Table 2).

### **Ancestral components considering T1D subtypes (according to autoimmunity and age at onset)**

We stratified the T1D sample according to the presence (T1AD, autoimmune) or absence (T1BD, idiopathic) of diabetes-related AABs; we also stratified the patient group according to their age at onset, *e.g.*, early ( $\leq 5$  years) or late ( $> 5$  years). We found that 78% ( $n = 156$ ) of the patients had at least one T1D specific autoantibody (GAD-65 and IA-2), while the 22% remaining ( $n = 44$ ) were negative for these two antibodies. T1AD average age at onset was 8.25 years, whilst for T1BD it was 7.22. We did not find significant differences between men and women within these two groups (data not shown).

Over thirty percent ( $n = 61$ , 30.5%) of T1D individuals developed the disease before



**Figure 1** Ancestry proportions of 200 type 1 diabetes patients from Colombia. EUR: European; NAT: Native American; AFR: African.

the age of 5 years, with an average age at onset of 2.66 years. The remaining sample (69.5%,  $n = 139$ ) presented with an age at onset after 5 years, with a mean for this category of 10.28 years. As in the stratification by AABs, we did not find significant differences between men and women within the age at onset categories (data not shown). Regarding the autoimmune category, comparisons among ancestral genetic composition led to the identification of no differences for the 74 AIMs taken together (Table 3). However, looking at individual loci, it was observed that *MHC* AIMs present with lower NAT ancestry in the autoimmune subgroup ( $P = 0.019$ , Table 3).

In addition, when comparing diseased individuals in the autoimmune categories to CLM population, it was observed that gene *EF3B* AIMs present differences in their ancestral components (Supplementary Table S3). Thus, autoimmune patients presented with less NAT ancestry ( $P = 0.032$ ), whilst T1D idiopathic category presented with higher AFR ancestry ( $P = 0.016$ ). Regarding the age at onset categories, it was observed that the AFR ancestry is significantly higher in the late onset subgroup at gene *IL7R* AIMs ( $P = 0.023$ , Table 4). Comparing these two categories to the CLM population showed no significant differences for either the overall set of AIMs nor specific loci (Supplementary Table S3).

## DISCUSSION

T1D incidence differences among countries, mainly related to European *versus* non-Europeans, led us to assess whether our T1D patients had a predominantly European ancestral component or other. Our analyses were based on 74 AIMs located on previously reported T1D loci/genes. AIM deltas ( $\delta$ s) between the NAT, European and AFR populations indicated that they were appropriate discriminators. We found that T1D patients from northwest Colombia are predominantly of European ancestry, followed by NAT and AFR components. Proportion estimates of the three parental populations for this sample were consistent with those reported in previous studies for Colombians, but using different sets of markers<sup>[13,16,19,20,29]</sup>.

We also compared T1D children to CLM. Analyzing the overall set of AIMs found no statistically significant differences in the ancestral genetic component between the two groups. Comparable results were obtained by Gomes *et al*<sup>[10]</sup> in Sao Paulo-Brazil; they noted that the European component predominated in both T1D patients and controls, followed by AFR and NAT ancestry; however, no significant differences

**Table 2 Significant findings in an exploratory association analysis**

CHR	SNP	A1	MAF		OR <sup>a</sup>	95%CI	P value <sup>1</sup>	EMP1
			T1D	CLM				
2	rs798364	A	0.18	0.27	0.62	0.40-0.96	0.034	0.035
2	rs1606237	T	0.33	0.26	1.55	1.04-2.30	0.031	0.031
5	rs700164	T	0.56	0.35	2.38	1.62-3.48	5.56 × 10 <sup>-6</sup>	3.0 × 10 <sup>-6</sup>
6	rs9378428	C	0.38	0.48	0.61	0.42-0.89	0.010	0.018
6	rs2523747	G	0.42	0.30	1.68	1.13-2.51	0.010	0.012
6	rs2395656	G	0.23	0.28	0.63	0.41-0.98	0.040	0.041
10	rs3123687	G	0.15	0.86	0.04	0.02-0.08	8.70 × 10 <sup>-19</sup>	1.0 × 10 <sup>-6</sup>

<sup>1</sup>Odds ratio and *P* value adjusted for genetic admixture. This table extracts the significant findings shown in [Supplementary Table S2](#). CHR: Chromosome; A1: Minor allele; MAF: Minor allele frequency; CI: Confidence interval; EMP1: Empirical *P* value obtained by permutation tests; OR: Odds ratio; SNP: Single nucleotide polymorphism; T1D: Type 1 diabetes; CLM: Colombians living in Medellin.

between cases and controls were observed. For the contrary, a study conducted in ten Brazilian cities showed that T1D patients presented a higher percentage of European component than the healthy population<sup>[9]</sup>. Similarly, a study by Diaz-Horta *et al*<sup>[11]</sup> found a higher proportion of European component in cases than in controls. Even more, they found a risk association with the European ancestry.

Further analysis disaggregating the candidate loci tested led us to find a different ancestry composition for *MHC* AIMS. Lower NAT ancestry was observed in T1AD compared to T1BD patients (Table 3). Ancestry variation at the HLA region has been reported for Latin American populations. However, such variation has shown an excess of the AFR component in these populations, including CLM<sup>[16,33,34]</sup>. It has been suggested that the excess of the AFR component in the HLA region in Latin America is due to a positive selection orchestrated by the presence of infectious agents during the process of the conquest. The European conquerors brought to America, African and European diseases such as smallpox, measles, and influenza, which caused massive epidemics and were responsible for the extinction of many native populations<sup>[34]</sup>. Given this historical background, these AFR fragments could obtain a selective advantage, since the AFR populations have the most diverse repertoire in HLA<sup>[35,36]</sup>. However, the ancestry variation observed here shows that the European component is higher in autoimmune (T1AD) than T1BD, in combination with lower NAT in T1AD than T1BD (Table 3).

Another gene with remarkable findings is *IFIH1*. This observation is of particular interest to our population, since we had found in the past that SNP *rs10930046*, which is located at *IFIH1*, associates with T1D in our population<sup>[37]</sup>. This SNP has been reported as a rare variant in European populations (MAF = 0.02) related to Psoriasis<sup>[38]</sup>. Interestingly, we found in our previous study that this variant MAF = 0.3<sup>[37]</sup>. Therefore, such an allele frequency difference could have been speculatively explained by random genetic drift, involving over-representation of European chromosomes with such variants at the time of conquering Colombia. However, in the present study, evidence suggests that this allele frequency difference between populations might be a NAT contribution.

It is worth mentioning that *IFIH1* AIMS presented wide values for the AFR component comparing autoimmune to idiopathic patients (14.99 *vs* 0.01, Table 3), without reaching statistical significance. This was the case since the interquartile range overlapped between these two autoimmune categories. Neither gene *CTLA4* nor *RNASEH1* AIMS revealed significant contributions to T1D, either looking to the overall set of AIMS or in any of the loci/genes analyzed. Regarding *CTLA4*, this observation makes sense when related to our previous finding of no association of this gene variant with T1D<sup>[37]</sup>. However, a different situation holds for the *RNASEH1* gene.

*RNASEH1* gene variants have thus far been associated with T1D only in the northwest Colombia population and not elsewhere in the world<sup>[4]</sup>. It has not even been reported in GWA studies using large sample sizes, albeit mostly of European origin<sup>[3]</sup>. Analyzing a larger sample size of T1D patients from this region in Colombia will allow us to conclude whether there really is an ancestry effect related to *RNASEH1* gene variants in T1D.

Unexpectedly, we found that ancestry for chromosomes 5 and 10 were sharply different between T1D patients and the CLM population (Tables 1 and 3). The former

**Table 3 Genetic ancestry for type 1 diabetes patients stratified according to autoimmunity**

Chromosomal region	Ancestry	T1AD, median (IQR)	T1BD, median (IQR)	P value <sup>1</sup>
Overall AIMs	EUR	61.92 (53.57-70.89)	59.79 (49.70-68.03)	0.333
	NAT	27.07 (20.82-33.48)	29.16 (21.57-37.06)	0.312
	AFR	10.19 (2.88-16.01)	11.54 (5.55-17.80)	0.344
Chr2_EFR3B	EUR	60.28 (36.24-83.66)	59.79 (32.34-80.01)	0.551
	NAT	25.16 (0.01-51.24)	21.92 (0.01-45.95)	0.979
	AFR	4.53 (0.01-23.32)	14.41 (0.01-27.36)	0.119
Chr2_CTLA4	EUR	53.34 (16.82-70.53)	41.16 (16.62-60.86)	0.231
	NAT	26.28 (4.55-41.31)	32.69 (9.35-50.25)	0.343
	AFR	20.54 (0.01-41.73)	19.47 (0.01-42.52)	0.764
Chr2_RNASEH1	EUR	58.96 (37.72-73.87)	47.50 (26.38-78.10)	0.506
	NAT	26.92 (14.19-45.66)	31.59 (0.01-51.98)	0.885
	AFR	6.85 (0.01-28.24)	11.71 (0.01-27.86)	0.657
Chr2_IFIH1	EUR	41.90 (0.01-76.72)	42.42 (0.01-67.93)	0.708
	NAT	32.56 (0.01-55.56)	27.39 (2.01-56.89)	0.985
	AFR	14.99 (0.01-42.31)	0.01 (0.01-36.48)	0.654
Chr5_IL7R	EUR	13.27 (1.02-43.73)	9.19 (1.77-33.67)	0.555
	NAT	50.94 (38.08-98.60)	53.12 (31.82-95.13)	0.984
	AFR	14.68 (3.17-33.94)	17.98 (2.75-41.01)	0.634
Chr6_MHC	EUR	52.31 (37.26-72.43)	45.73 (19.25-67.03)	0.087
	NAT	20.32 (7.12-37.06)	31.88 (17.65-44.62)	0.019
	AFR	18.50 (3.21-36.04)	21.13 (0.53-36.56)	0.905
Chr10_NRP1	EUR	63.32 (28.61-69.09)	63.31 (6.89-63.32)	0.092
	NAT	36.67 (7.93-36.67)	36.67 (7.93-59.79)	0.282
	AFR	0.25 (1e-05-26.0)	0.52 (1e-05-37.80)	0.848

<sup>1</sup>Mann-Whitney *U* test. AIMs: Ancestry informative markers; IQR: Interquartile range; Chr: Chromosome; EUR: European; NAT: Native American; AFR: African; T1AD: Autoimmune type 1 diabetes; T1BD: Idiopathic type 1 diabetes.

involves chromosomal region 5p13.2 (*IL7R*)<sup>[39]</sup>. This region was assessed with only one AIM, which clearly discriminates between NAT and non-NAT (Supplementary Table S1). As shown in Table 1, the T1D ancestry observed for this locus is confidently greater for NAT, at the expense of the two other ancestries. It is also apparent that AFR ancestry at this locus contributes to late onset of the disease (Table 4). Such results, in turn, should be taken with caution since this AIM does not clearly discriminate between EUR and AFR (Supplementary Table S1). Therefore, we cannot rule out the possibility that this effect is of European origin.

The second striking finding involves chromosomal region 10p11.22 (gene *NRP1*)<sup>[40]</sup>. Although the opposite ancestry contributions between T1D and CLM are evident (Table 1), it is worth keeping in mind that the only AIM (*rs3123687*) used for this locus is highly informative for AFR and non-AFR ancestries (*i.e.*, either EUR or NAT). Given this information, we are aware that the conclusion regarding greater NAT contribution in our study could eventually go towards greater EUR ancestry. Therefore we can only tell that the difference observed is non-AFR, but are not able to define whether it is European or NAT.

The actual SNPs reported as associated with disease in these two genes (*IL7R* and *NRP1*) have not yet been tested in the sample presented here. However, a test of association using the AIMs analyzed here, after adjusting for the admixture effect, revealed that AIM *rs700164* associates with affected status ( $5.56 \times 10^{-6}$ , Supplementary Table S2 and Table 2) and that similarly *rs3123687* strongly associates with the disease ( $P = 8.07 \times 10^{-19}$ , Supplementary Table S2 and Table 2) for *IL7R* and *NRP1* genes, respectively. A verification of this finding should be performed using the transmission disequilibrium test (TDT). The TDT is not susceptible to population structure issues, such as admixture. This analysis is to be done for the actual SNPs, as the parents for the patients presented here are available. Such association analyses should include choosing gene variants from the genetic variability in this set of patients, and should also consider the LD blocks observed in this population.

No ancestry differences were found overall when comparing T1AD to idiopathic (T1BD) (Table 3). T1AD, whose etiology and pathology are better characterized, has a

**Table 4 Genetic ancestry for type 1 diabetes patients stratified according to age at onset**

Chromosomal region	Ancestry	Early age at onset, ≤ 5 yr	Late age at onset, > 5 yr	P value <sup>1</sup>
Overall AIMs	EUR	62.06 (54.50-71.38)	61.12 (51.66-68.68)	0.420
	NAT	25.40 (18.14-33.55)	27.75 (21.86-34.35)	0.345
	AFR	11.09 (3.90-17.25)	10.19 (3.88-16.91)	0.927
Chr2_EFR3B	EUR	57.65 (34.05-82.17)	62.45 (42.04-80.67)	0.419
	NAT	25.16 (1.27-52.11)	24.30 (0.01-51.73)	0.607
	AFR	5.02 (0.01-27.29)	7.37 (0.01-23.32)	0.941
Chr2_CTLA4	EUR	58.49 (24.63-78.80)	46.09 (12.90-66.30)	0.180
	NAT	26.28 (3.98-39.82)	30.19 (4.55-42.10)	0.362
	AFR	18.05 (0.01-37.80)	20.68 (0.01-41.72)	0.701
Chr2_RNASEH1	EUR	56.84 (44.50-72.18)	56.91 (28.02-74.15)	0.672
	NAT	27.22 (0.01-31.22)	28.03 (8.15-48.58)	0.472
	AFR	3.38 (0.01-31.22)	8.62 (0.01-27.20)	0.917
Chr2_IFIH1	EUR	41.35 (0.01-66.94)	42.42 (4.44-76.73)	0.126
	NAT	33.05 (0.67-57.49)	29.05 (0.01-53.09)	0.339
	AFR	14.57 (0.01-42.31)	1.01 (0.01-42.31)	0.796
Chr5_IL7R	EUR	9.38 (0.33-41.70)	12.86 (1.73-43.11)	0.338
	NAT	53.83 (39.76-99.60)	50.94 (30.21-88.75)	0.197
	AFR	6.17 (1.01-29.03)	19.96 (9.6-37.36)	0.023
Chr6_MHC	EUR	51.67 (32.23-63.29)	52.07 (34.26-71.84)	0.596
	NAT	30.57 (13.47-43.21)	26.71 (7.69-37.39)	0.480
	AFR	15.03 (4.46-36.05)	20.52 (1.08-36.73)	0.118
Chr10_NRP1	EUR	63.32 (23.70-63)	63.32(43.07-69.08)	0.357
	NAT	36.68 (7.93-36.67)	36.67 (7.93-36.68)	0.797
	AFR	0.16 (0.001-39.1)	0.38 (0.001-63.3)	0.498

<sup>1</sup>Mann-Whitney *U* test. AIMs: Ancestry informative markers; IQR: Interquartile Range; Chr2: Chromosome 2; Chr6: Chromosome 6; EUR: European; NAT: Native American; AFR: African.

higher incidence in Europe<sup>[6]</sup>; on the contrary, T1BD is reported mainly in AFR and Asian countries<sup>[26]</sup>. Our results are different from those by Piñero-Piloña *et al*<sup>[41]</sup>, who reported a high incidence of T1BD in Mexican patients, whose predominant ancestral component was NAT. Our cohort presents a majority of autoimmune cases (78%) and, as described here, their predominant ancestry is of European contribution.

However, looking at chromosomal regions along the analysis stratified by age at onset of T1D, we found that patients with a late onset of the disease have a greater AFR component, which was more marked on chromosome 5 (Table 4). This suggests that AFR ancestry could be a risk factor for developing the disease at a late age in our population (over 2/3 of the sample had age at onset > 5 years), which can modify the metabolic phenotype of patients, and influence the risk of late complications of diabetes<sup>[42]</sup>.

Our study has an important limitation regarding the number and location of the AIMs. Thus, chromosomes 5 and 10 were tested with just a few such markers. It will be worth testing more AIMs nearby these two loci to further examine the differences revealed. Also, the reference population we used (CLM from the 1,000 genome database), although supposedly unaffected and older than our patients, were typed by a different method from the one we used to type our T1D cases. Nonetheless, both groups share comparable genetic ancestries.

Our study's strength is its population choice. As described, the northwest Colombia population is the one with a greater European component in the country<sup>[15-19]</sup>. Thus, our results make much more sense regarding the overall European contribution, together with the apparent unexplored NAT input to T1D, in addition to certain contributions of the AFR ancestry for late age at onset.

In conclusion, this study describes the ancestral genetic composition of 200 T1D patients from an admixed population from northwest Colombia. Consistently, we found a predominant proportion of European followed by NAT ancestry. No statistical difference was observed in the distribution of the proportions of ancestral genetic components between T1D patients and the CLM reference population. A variation in chromosomal segments derived from the parental populations was

observed when comparing individuals with T1AD *versus* T1BD, and those who had an early ( $\leq 5$  years) or late ( $> 5$  years) age at onset of the disease. These results demonstrate that the study of the genetic admixture provides new perspectives in the delineation of the genetic architecture underlying autoimmune diseases. Finally, performing a novel study in this sample, including unbiased distribution of AIMs through the whole genome, could help find undetected loci in previous studies, which would contribute to complete the T1D genetic architecture for our population. This will also contribute to making approaches, such as the polygenic risk score, become more accurate for these types of populations.

## ARTICLE HIGHLIGHTS

### Research background

Type 1 diabetes (T1D) is described as a disease predominantly in white populations. Subtypes of the disease are also more frequent in different ethnicities. Thus, the autoimmune form of the disease is observed more frequently in Caucasian countries, whilst the idiopathic form is more frequently observed in African and Asian countries. The patients included in this study are from Northwest Colombia. This is an admixed population originated by a three ethnic contribution. This population has been described as the most European in the country, followed by the Native American ancestry, and with its least significant component being African contribution.

### Research motivation

In this study, we looked at the genetic ancestry of a set of 200 diseased subjects from Northwest Colombia. We were interested in describing whether their global ancestry, as well as some specific genomic regions, were of which particular ancestry. Only a few of these types of studies have been reported in Latin American populations, and none have occurred in Colombia.

### Research objectives

We aimed at describing the ancestry composition of a cohort of Colombian patients with T1D. This description included both global analysis as well as specific tests on loci/genes previously related to the disease.

### Research methods

We studied 200 diseased subjects from Northwest Colombia. We tested 75 admixture informative markers (AIMs) distributed through a set of previously reported genes (or chromosomal regions) associated with T1D. The disease was classified as either autoimmune or idiopathic in the study subjects. This was done by testing two disease-related auto-antibodies (AABs). If at least one such AAB was present, then the disease was classified as autoimmune. We also classified the age at onset of the disease as early ( $\leq 5$  years) or late ( $> 5$  years). The reference population of Colombians living in Medellin (CLM) was compared to the set of patients presented here. We applied appropriate statistical tests given the non-normality of the data obtained.

### Research results

Seventy eight percent of the patients presented at least one AAB. Over two thirds (69.5%) of the subjects developed the disease after 5-years-old. There were no significant differences between genders among the affected individuals. Seventy four AIMs were successfully tested (one failed the PCR optimization). It was observed that both the diseased and CLM groups were predominantly of European ancestry (61.58 *vs* 62.06), followed by Native American (24.30 *vs* 37.10) and African ancestries (10.28 *vs* 10.65). In addition, specific genes such as *EFR3B*, *IFIH1*, *IL7R* and *NRP1* displayed differential Native American or African rather than European contributions. In addition, we found that autoimmune patients displayed lower Native American ancestry than idiopathic cases.

### Research conclusions

Our study shows that diseased individuals from Northwest Colombia are predominantly of European ancestry, followed by native American and African ancestries. Also, other European contributions were found for specific genes in our study.

### Research perspectives

MHC is expected to play the strongest role in T1D susceptibility. However, this was not the observation in our study. Our results suggest that different loci effect sizes might be at play in our admix population. This is inferred from the observation of the significance strength observed for MHC ancestry compared to other loci. Therefore, it would be worth testing AIMs in this sample (expanded with extra individuals from the same region in Colombia) throughout the whole genome. This way, it would be feasible to reveal differences in local ancestry either for known or unknown loci associated with T1D in our population. This would help complete the genetic architecture of the disease, particularly for our population. In turn, this would contribute to the knowledge of the disease biology, and would also make this sample population appropriate for applying approaches such as the polygenic risk score.

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