Cardiovascular Diabetology

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High inflammatory burden may link non-albuminuric diabetic kidney disease to carotid atherosclerosis

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Abstract

Background Individuals with diabetic kidney disease (DKD) exhibit markedly elevated cardiovascular (CV) risk, which may differ across DKD phenotypes. Non-albuminuric DKD (NA-DKD) has been proposed as a phenotype with high CV risk. Inflammation may contribute to the link between DKD and cardiovascular disease, but inflammatory patterns across distinct DKD phenotypes remains insufficiently characterized. This study evaluated an extensive panel of inflammatory markers in DKD and examined their relationship with the presence of carotid atherosclerotic plaque, particularly focusing on NA-DKD.

Methods A total of 180 adults with type 2 diabetes were stratified into patients with DKD (n = 132) and control group without DKD (n = 48) and subsequently in the different DKD phenotypes according to glomerular filtration rate (eGFR) and urinary to albumin creatinine ratio (UACR): albuminuric DKD (A-DKD, UACR ≥ 30 mg/g and eGFR ≥ 60 ml/min/1.73 m^2; n = 46), NA-DKD (UACR < 30 mg/g and eGFR < 60 ml/min/1.73 m^2; n = 44), albuminuric and low eGFR DKD (UACR ≥ 30 mg/g and eGFR < 60 ml/min/1.73 m^2, n = 42). Participants underwent carotid and kidney ultrasonography, arterial stiffness assessment, and measurement of 37 inflammatory biomarkers. Logistic regression models adjusted for major confounders were used to assess associations between inflammatory markers and carotid atherosclerosis.

Results Individuals with DKD showed a higher prevalence of carotid plaques in comparison to controls (61.8 vs 39.6%, $P = 0.008$), a trend toward higher pulse wave velocity (11.45 ± 4.02 vs 10.11 ± 3.69 m/s, $P = 0.082$), and higher renal resistive index (0.75 ± 0.09 vs 0.71 ± 0.08, $P = 0.016$). Multiple inflammatory biomarkers—including soluble tumor necrosis factor receptors (sTNF-Rs)—were higher in DKD than in controls. When considering DKD phenotypes, sTNF-R1 was higher in NA-DKD in comparison to both controls and individuals with A-DKD. In the overall population, several inflammatory biomarkers correlated with estimated eGFR but not with UACR. Among participants with DKD,

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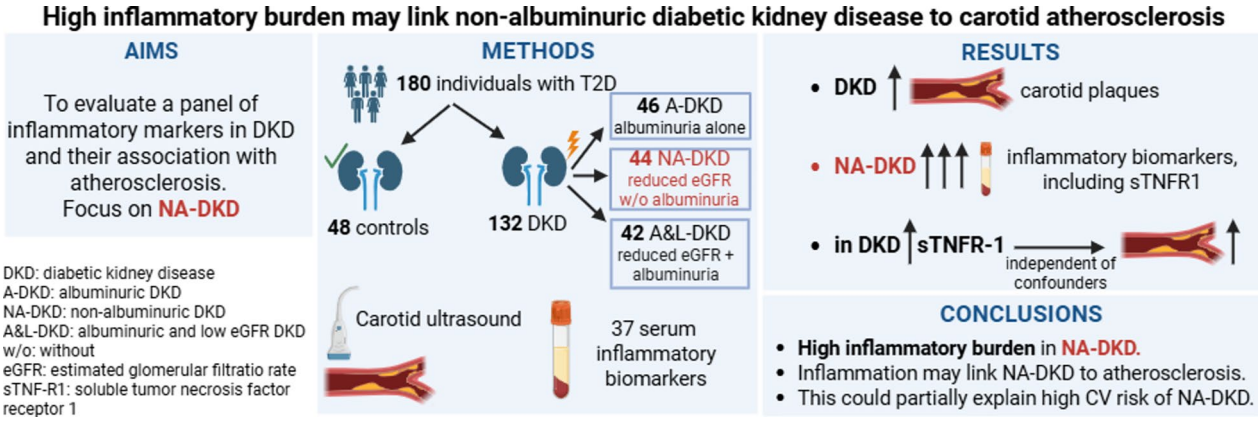
TNF-R1 levels in the top tertile group were independently associated with the presence of carotid plaques (OR 4.92, 95% CI 1.41–17.18, $P=0.010$, q value = 0.0494).

Conclusions This study showed a higher inflammatory burden in DKD and particularly in NA-DKD. sTNF-R1 was associated with the presence of carotid atherosclerotic plaque, and this could partially explain the elevated cardiovascular risk associated with this phenotype.

Keywords Type 2 diabetes, Diabetic kidney disease, Inflammation, Tumor necrosis factor receptors, Atherosclerosis, Cardiovascular risk

Graphical abstract



Research insights

What is currently known about this topic?

- Inflammation is a shared mechanism between DKD and atherosclerosis
- The differential inflammatory profile of distinct DKD phenotypes is insufficiently characterized
- Individuals with non-albuminuric diabetic kidney (NA-DKD) disease are at high cardiovascular risk

What is the key research question?

- Is there a relationship between inflammatory profile in NA-DKD and atherosclerosis?

What is new?

- In DKD, soluble tumor necrosis factor receptor 1 is associated with carotid atherosclerosis
- NA-DKD exhibits a worse inflammatory profile than albuminuric DKD.
- Several inflammatory biomarkers are independently associated with glomerular filtration rate (GFR)

How might this study influence clinical practice?

- Routinely GFR evaluation could help finding individuals at higher cardiovascular risk

Introduction

Diabetic kidney disease (DKD) represents a multifaceted complication of diabetes with high clinical impact, attributable not only to its potential for progression to end-stage renal disease, but, more critically, to the markedly increased risk of adverse cardiovascular outcomes [1, 2]. The complex nature of DKD is reflected in its heterogeneous clinical presentation, with the increasing prevalence of a phenotype characterized by reduced estimated glomerular filtration rate (eGFR) in the absence of albuminuria, known as non-albuminuric DKD (NA-DKD), whose cardiovascular risk in comparison to the classical albuminuric DKD (A-DKD) is still debated [3, 4].

Among the pathogenetic mechanisms involved in DKD, inflammation exerts a prominent role [5]. Thus, the interest for inflammatory biomarkers in DKD has been increasing alongside with accumulating evidence that novel nephroprotective drugs – namely sodium-glucose cotransporter 2 inhibitors (SGLT2i) and non-steroidal mineralocorticoid receptor antagonists – exert anti-inflammatory effects on the kidney, which may partially account for their beneficial effects [6–9]. However, since the distinct phenotypes of DKD exhibit several clinical and histopathological differences [4], their inflammatory profiles may also differ, although current data remain insufficient.

Intriguingly, low grade inflammation may represent a shared pathogenetic mechanisms with atherosclerosis, in which immune system plays a pivotal role [10, 11]. This

could therefore provide a mechanistic basis for explaining part of the increased cardiovascular risk associated with DKD.

The aim of this study was to assess a panel of inflammatory biomarkers in a cohort of individuals with DKD and to assess their association with atherosclerosis. We particularly focused our attention on NA-DKD, considering the increasing prevalence of this phenotype as the current trend of DKD epidemiology.

Material and methods

Study subjects

A total of 180 individuals with type 2 diabetes, who attended our Department of Internal Medicine for diabetes and cardiovascular risk evaluation at Garibaldi Nesima Hospital, Catania, Italy, were recruited for this study.

The inclusion criteria were established diagnosis of type 2 diabetes and age range 45–75 years, whereas exclusion criteria included: autoimmune diseases, malignancies, corticosteroids therapy, and history of alcohol and/or drug abuse.

All the participants underwent a complete physical examination, a review of their clinical history, smoking status, and medications.

Body mass index (BMI) was calculated as follows: weight (kg)/[height (m)]^2. Blood pressure was measured with a calibrated sphygmomanometer after 10 min of resting. Venous blood samples were drawn in the morning after overnight fasting. In addition, a sample of spot urine was also collected.

Biochemical analysis

Plasma glucose, serum total cholesterol, high-density lipoprotein cholesterol (HDL-c), and triglycerides (TG) were measured using available enzymatic methods. Glycated haemoglobin (HbA_{1c}) was assessed via high performance liquid chromatography using a National Glycohemoglobin Standardization Program and was standardized to the Diabetes Control and Complications Trial (DCCT) assay reference [12]. Chromatography was performed using a certified automated analyzer (HLC-723G7 hemoglobin HPLC analyzer; Tosoh Corp.) (normal range 4.25–5.9%).

Low density lipoprotein cholesterol (LDL-c) was estimated through the Friedwald formula: total cholesterol (mg/dl) – HDL-c (mg/dl) – TG (mg/dl)/5. eGFR was assessed with the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation [13] and albuminuria was determined as mean values of two measurements in a period of 3–6 months of UACR in spot urine samples as recommended in current guidelines [14]. Albuminuria was measured through immunoturbidimetric method (Alinity ci, Abbott Diagnostics, IL, USA).

Study groups

Participants, taking into account UACR and eGFR, were classified as controls (UACR < 30 mg/g and eGFR ≥ 60 ml/min/1.73 m^2) and DKD (UACR ≥ 30 mg/g and/or eGFR < 60 ml/min/1.73 m^2). Then, participants with DKD were split in the different phenotypes: albuminuric DKD (A-DKD; UACR ≥ 30 mg/g and eGFR ≥ 60 ml/min/1.73 m^2), NA-DKD (UACR < 30 mg/g and eGFR < 60 ml/min/1.73 m^2), albuminuric and low eGFR DKD (A&L-DKD; UACR ≥ 30 mg/g and eGFR < 60 ml/min/1.73 m^2).

Carotids ultrasound examinations

All ultrasound examinations were conducted by a single experienced physician, blinded to the patients' clinical and laboratory data, using a high-resolution B-mode ultrasound system. Longitudinal B-mode images (60 Hz, 128 radiofrequency lines) of the common carotid arteries, obtained 2 cm below the carotid bulb, were captured with a high-precision echo-tracking device (MyLab Alpha, Esaote, Maastricht, NL) coupled with a high-resolution linear array transducer (13 MHz). These images were used to assess the presence of atherosclerotic plaques – defined as the presence of a focal structure encroaching into the lumen having a thickness > 1.5 mm measured from the media–adventitia interface to the intima–lumen interface in accordance to current recommendation – and measure intima-media thickness (IMT) as previously described [15].

Pulse wave velocity

Pulse wave velocity (PWV) was measured using the SphygmoCor CvMS system (AtCor Medical, Sydney, Australia), which employs a tonometer to record pressure waves at two sites: the common carotid artery (proximal) and the femoral artery (distal). The onset of the pulse wave was determined from a simultaneously recorded electrocardiogram. PWV was calculated as the distance travelled by the pulse wave divided by the time delay between the two recording sites. To ensure reliability and account for a full respiratory cycle, the final PWV value was derived from the average of 10 consecutive waveforms as previously described [16].

Renal resistive index (RRI)

The Doppler spectrum of the intrarenal arteries was analysed to calculate the renal resistive index (RRI), defined as the difference between peak systolic (maximum) and end-diastolic (minimum) flow velocities, divided by the peak systolic velocity [17]. Measurements were obtained using a convex transducer connected to a high-precision echo-tracking device (MyLab Alpha, Esaote, Maastricht, NL). The mean of three separate measurements was used for analysis.

Quantification of serum inflammatory biomarkers

Cytokine concentrations were quantified using the Bio-Plex® platform (Bio-Rad, Hercules, CA, USA). The Bio-Plex Pro™ Human Inflammation Panel 1, 37-Plex Assay was employed to measure the following analytes: A proliferation-inducing ligand/tumor necrosis factor ligand superfamily member 13 (APRIL/TNFSF13), B-cell activating factor/tumor necrosis factor ligand superfamily member 13B (BAFF/TNFSF13B), soluble cluster of differentiation (CD) 30/tumor necrosis factor receptor superfamily member 8 (sCD30/TNFRSF8), soluble CD163 (sCD163), chitinase-3-like 1 (CHI3L1), glycoprotein 130 / soluble Interleukin-6 receptor subunit β (gp130/sIL-6Rβ), interferon α2, interferon β (IFN-β), interferon γ, interleukin 2, soluble interleukin-6 receptor subunit α, Interleukin-8, Interleukin-10, Interleukin-11, Interleukin-12 subunit p40, Interleukin-12 heterodimer (p35 + p40), Interleukin-19, Interleukin-20, Interleukin-22, Interleukin-26, Interleukin-27 subunit p28, Interleukin-28A/Interferon λ-2, Interleukin-29/Interferon λ-1, Interleukin-32, Interleukin-34, Interleukin-35, Lymphotoxin-like, inducible expression, and competing with Herpes Simplex virus glycoprotein D for herpesvirus entry mediator, a receptor expressed on T lymphocytes / tumor necrosis factor ligand superfamily member, Matrix metalloproteinase-1 (MMP-1), Matrix metalloproteinase-2 (MMP-2), Matrix metalloproteinase-3 (MMP-3), osteocalcin, osteopontin, pentraxin-3, soluble Tumor Necrosis Factor Receptor 1 (sTNF-R1), soluble Tumor Necrosis Factor Receptor 2 (sTNF-R2), Thymic Stromal Lymphopoietin, and Tumor Necrosis Factor-like weak inducer of apoptosis/tumor necrosis factor ligand superfamily member 12 (TWEAK/TNFSF12), following the manufacturer's instructions. Briefly, serum samples were thawed on ice, centrifuged at 1,000 × g for 15 min and subsequently at 10,000 × g for 10 min, then diluted 1:4 in assay diluent. Each well of a 96-well plate was preloaded with 50 µL of fluorescently dyed magnetic microspheres covalently bound to specific antibodies. Standards, controls, or diluted samples (50 µL) were added and incubated for 1 h at room temperature (RT) with shaking (850 rpm), followed by three washes with wash buffer. Next, 25 µL of biotinylated detection antibody was added and plates were incubated for 30 min at RT under the same shaking conditions. After three additional washes, 50 µL of streptavidin–phycoerythrin conjugate was added and incubated for 10 min at RT with shaking, followed by a final wash step. Immunocomplexes were resuspended in 125 µL of assay buffer. Fluorescence was acquired on a Bio-Plex® 200 analyzer (Bio-Rad) and analyzed with BioPlex Manager™ Software v6.2 (Build 175; Bio-Rad). Median fluorescence intensity values were used for quantitative analysis.

All the cytokines with missing values due to concentration under detection limits of the assay were excluded from the analysis.

Statistical analysis

Statistical comparisons of clinical and biomedical parameters were performed using Stat View 6.0 for Windows. The data are presented as mean ± SD, median (1st–3rd quartile), or absolute number (percentage) as appropriate. The distributional characteristics of variables were assessed using the Kolmogorov–Smirnov test. Before inclusion in regression models, numerical variables that were not normally distributed were logarithmically transformed to reduce skewness. The comparison between study groups was performed with t-test for continuous, parametric variables, Mann–Whitney for continuous, non-parametric variables, and χ^2 test for categorical variables. Q-values for the comparison of inflammatory biomarkers were computed using the Benjamini–Hochberg method to control for false discovery rate (FDR). As sensitivity analyses age- and sex-standardized levels of inflammatory biomarkers were computed, employing marginal means from multivariate linear regression models adjusted for age and sex.

When the population was split according to different DKD phenotypes, the comparison between study groups was performed with ANOVA for continuous, parametric variables, Kruskal–Wallis for continuous, non-parametric variables and χ^2 test for categorical variables. The Bonferroni post hoc test for multiple comparisons was also performed. All tests were two-sided and a P value < 0.05 was considered significant.

For cytokines with higher expression in the DKD group, multiple regression models were fitted in the whole population to assess the association of cytokine levels (dependent variable) with clinical and laboratory data (age, sex, active smoking, known diabetes duration, BMI, systolic blood pressure, HbA1c, eGFR, UACR > 30 mg/g, and LDL-c).

Furthermore, to identify variables associated with the presence of carotid plaques, we fitted two Firth logistic regression models. Model 1 included age, sex, active smoking, known diabetes duration, BMI, systolic blood pressure, HbA1c, UACR > 30 mg/g, eGFR, and LDL-c as independent variables. Model 2 included all covariates from Model 1, and each inflammatory biomarker that showed higher concentrations in the DKD group was entered separately, one at a time, as a categorical variable defined by membership in the highest concentration tertile group. Q-values for the associations between carotid plaque and inflammatory biomarkers were computed using the Benjamini–Hochberg method to control for FDR.

As sensitivity analysis, we refitted model 2 with sTNF-R1 employing log-transformed sTNF-R1 instead of being in the higher tertile group of sTNF-R1 levels. Furthermore, we refitted the model:1) incorporating therapy with SGLT2i and therapy with renin–angiotensin–aldosterone system (RAAS) blockers as covariates; 2) incorporating history of cardiovascular disease (CVD) and therapy with statins.

The variance inflation factor (VIF) was used to check for multicollinearity among the predictor variables in multiple regression analysis. Any variable with a VIF that exceeded 4 was excluded from the model. No variable was detected with a VIF greater than 4.

Table 1 Clinical and metabolic characteristics of the study population

	Controls (n = 48)	DKD (n = 132)
Age, years	63.96 ± 7.30	67.48 ± 6.57
Sex, no. (%) of males	34 (70.8)	101 (77.1)
Active smokers, no. (%)	11 (22.9)	43 (32.6)
Known diabetes duration, years	12.49 ± 6.59	16.07 ± 9.91
History of CVD[§], no. (%)	10 (20.8)	40 (30.3)
BMI, kg/m^2	29.12 ± 5.18	28.39 ± 4.39
SBP, mmHg	131.98 ± 15.39	135.60 ± 13.99
DBP, mmHg	76.98 ± 10.35	79.06 ± 10.03
HbA_{1c}, %	7.16 ± 1.27	7.16 ± 1.28
Fasting glucose, mg/dl	134.12 ± 39.20	130.65 ± 40.41
eGFR, ml/min/1,73m^2	91.43 ± 13.26	63.44 ± 22.76
UACR, mg/g	10.00 [8.00 – 16.00]	47.50 [17.50 – 183.75]
Total cholesterol, mg/dl	155.40 ± 39.78	147.81 ± 33.42
HDL-c, mg/dl	46.50 ± 12.37	45.58 ± 11.54
LDL-c, mg/dl	84.84 ± 32.81	75.80 ± 31.56
Triglycerides, mg/dl	105.00 [79.00 – 152.75]	111.00 [82.00 – 151.00]
MEDICATIONS		
Metformin, no. (%)	47 (97.9)	94 (71.2)
Insulin, no. (%)	15 (31.2)	51 (38.6)
SGLT2 inhibitors, no. (%)	9 (18.8)	62 (47.0)
DPP4 inhibitors, no. (%)	6 (12.5)	11 (8.33)
GLP1-RAs, no. (%)	22 (45.8)	56 (42.4)
Sulphonylureas, no. (%)	2 (4.2)	2 (1.5)
Antithrombotics, no. (%)	31 (64.6)	71 (53.8)
Statins, no. (%)	34 (70.8)	105 (79.5)
ACE-i/ARBs, no. (%)	27 (56.2)	99 (75.0)
Other antihypertensive medications, no. (%)	21 (43.8)	100 (75.8)

Data are presented as mean ± SD, median (1st – 3rd quartile), or percentage and compared with t-test, Mann–Whitney, or χ^2 as appropriate. DKD: Diabetic kidney disease (UACR ≥ 30 mg/g and/or eGFR < 60 ml/min/1.63 m^2); CVD: cardiovascular disease; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; eGFR: estimated glomerular filtration rate; UACR: urinary albumin to creatine ratio; SGLT2: sodium-glucose transporter 2; DPP4: dipeptidyl-peptidase 4; GLP1-RAs: glucagon like peptide 1 – receptor agonists. ACE-i: angiotensin converting enzyme inhibitors; ARBs: Angiotensin receptor blockers. [§]history of CVD includes myocardial infarction, stroke, and peripheral artery disease

Results

General characteristics of the study population

According to eligibility criteria, 180 individuals with type 2 diabetes were included in this study. The study population was divided into two groups: 48 subjects without DKD (controls) and 132 subjects with DKD.

As shown in Table 1, participants with DKD were older and had a longer known diabetes duration in comparison to controls. Sex distribution, percentage of active smokers, BMI, and blood pressure were homogenous between study groups, as well as the glycaemic and lipids profile. As expected, individuals with DKD showed lower eGFR and higher UACR. As regards medications, people with DKD were more likely to receive SGLT2i and Angiotensin converting enzyme inhibitors (ACEi)/Angiotensin receptor blockers (ARBs) and less likely to receive metformin, in accordance with clinical guidelines. In the supplementary table S1 are shown general characteristics of the population stratified according to DKD phenotypes (A-DKD, n = 46; NA-DKD, n = 44; A&L-DKD, n = 42).

Inflammatory profile of the study population

As shown in Table 2, the DKD group, in comparison to controls, had higher serum concentrations of APRIL/TNFSF13, sCD30/TNFRSF8, MMP3, sTNF-R1, and sTNF-R2. After FDR correction, APRIL/TNFSF13 (q-value < 0.0001), sCD30/TNFRSF8 (q-value = 0.0049), MMP3 (q-value 0.0082), sTNF-R1 (q-value = 0004) remained significantly higher in the DKD group, but not sTNF-R2 (q-value = 0.085). Furthermore, there was a trend toward higher levels of osteopontin and gp130/sIL-6Rβ in the DKD group in comparison to controls. Adjusting cytokines levels for age and sex, the between-group differences remained consistent (Supplementary table S2).

When participants with DKD were split according to DKD different phenotypes (Fig. 1 and supplementary table S3), individuals with NA-DKD had higher levels of APRIL/TNFSF13 and sTNF-R1 in comparison to controls and those with A-DKD. Furthermore, NA-DKD showed higher levels of sCD30/TNFRSF8 and MMP3 than controls, whereas A-DKD showed levels of MMP3 and sCD30/TNFRSF8 comparable to controls.

Multiple regression models assessing the association between serum cytokines and clinical and laboratory characteristics in the whole study population

In multiple regression models, after adjusting for major confounders, APRIL/TNFSF13 (β = – 0.55, $P < 0.001$), MMP3 (β = -0.28, $P = 0.037$), sTNF-R1 (β = – 0.42, $P < 0.001$), and sTNF-R2 (β = – 0.26, $P = 0.013$) were all independently and inversely associated with eGFR, but not with UACR ≥ 30 mg/g. Conversely, sCD30/TNFRSF8 was associated with UACR ≥ 30 mg/g (β = 0.23, $P = 0.017$).

Table 2 Serum inflammatory biomarkers profile of the study population

	Controls (n = 48)	DKD (n = 132)
APRIL/TNFSF13, ng/ml	106.55 ± 36.54	143.81 ± 64.26
BAFF/TNFSF13B, ng/ml	4.31 [3.71–5.53]	4.31 [3.50–5.34]
sCD163, ng/ml	36.68 [30.57–48.08]	38.59 [29.30–48.18]
sCD30/TNFRSF8, pg/ml	300.82 [240.75–404.93]	414.07 [301.52–570.74]
CHI3L1, ng/ml	4.32 [3.18–5.38]	4.64 [3.46–5.69]
gp130/sIL-6Rβ, ng/ml	35.98 [32.46–40.19]	39.54 [34.04–44.63]
IFN-β, pg/ml	3.38 [2.70–4.64]	3.33 [2.34–4.30]
MMP1, ng/ml	0.79 [0.43–0.86]	0.76 [0.43–1.02]
MMP2, ng/ml	1.84 [1.37–3.94]	2.55 [1.462–3.87]
MMP3, ng/ml	3.02 [1.79–4.38]	4.09 [2.96–5.89]
Osteocalcin, ng/ml	2.05 [1.55–2.61]	2.21 [1.58–3.17]
Osteopontin, ng/ml	4.86 [3.54–6.51]	5.68 [4.02–8.49]
Pentraxin-3, ng/ml	3.78 [3.02–4.69]	3.84 [3.07–5.38]
sTNF-R1, pg/ml	806.25 [701.06–1033.94]	1184.21 [869.60–1527.64]
sTNF-R2, pg/ml	227.05 [205.01–243.92]	238.90 [210.55–266.22]
TWEAK/TNFSF12, pg/ml	134.62 [105.49–160.38]	124.75 [96.91–158.35]
CRP, mg/dl	0.31 [0.20–0.62]	0.28 [0.18–0.44]

Data are presented as mean ± SD or median (1st–3rd quartile) and are compared with t-test or Mann–Whitney as appropriate. DKD: Diabetic kidney disease (UACR ≥ 30 mg/g and/or eGFR < 60 ml/min/1.63 m^2); UACR: urinary albumin to creatine ratio; eGFR: estimated glomerular filtration rate; APRIL/TNFSF13: A proliferation-inducing ligand/tumor necrosis factor ligand superfamily member 13; BAFF/TNFSF13B: B-cell activating factor/tumor necrosis factor ligand superfamily member 13B; sCD30/TNFRSF8: soluble cluster of differentiation (CD) 30/tumor necrosis factor receptor superfamily member 8; sCD163: soluble CD163; CHI3L1: Chitinase-3-like 1; gp130/sIL-6Rβ: gp130 glycoprotein 130 / soluble Interleukin-6 receptor subunit β; IFN- β interferon β; MMP-1: Matrix metalloproteinase-1; MMP-2: Matrix metalloproteinase-2; MMP-3: Matrix metalloproteinase-3, sTNF-R1: soluble tumor necrosis factor receptor 1; sTNF-R2: soluble tumor necrosis factor receptor 2; TWEAK/TNFSF12: tumor necrosis factor-like weak inducer of apoptosis/tumor necrosis factor soluble factor 12

Cardiovascular risk profile and RRI of the study population

As shown in Table 3, participants in the DKD group showed higher prevalence of carotid atherosclerotic plaques in comparison to controls. Furthermore, they showed a trend toward higher PWV.

As concern RRI, individuals with DKD showed higher values than controls.

In the supplementary Table S4, cardiovascular risk profile parameters and RRI are shown according to DKD phenotypes. As concerns carotid plaque, there was a trend toward higher prevalence of plaque in A-DKD and NA-DKD, whereas people with A&L-DKD had significantly higher prevalence of carotid plaque than controls. RRI was higher in NA-DKD and A&L-DKD in comparison to both controls and A-DKD group.

Firth logistic regression models assessing the association between the presence of carotid atherosclerotic plaques and levels of serum cytokines in participants with DKD

Considering the subset of participants with DKD, in the first Firth logistic regression model (Supplementary Table S5), the presence of carotid atherosclerotic plaques was associated with HbA_{1c} (odds ratio [OR] 1.64 95% confidence interval [CI] 1.01 – 2.66, $P = 0.035$). In the second regression model including sTNF-R1 (Fig. 2) the presence of carotid atherosclerotic plaques was associated only with being in the top tertile group of sTNF-R1 levels (sTNF-R1 > 1412.95 pg/ml, OR 4.92 95%CI 1.41 – 17.18, $P = 0.010$). When included in model 2, none of APRIL/TNFSF13 (APRIL/TNFSF13 > 154.24 ng/ml, Supplementary table S6) and sCD30/TNFRSF8 (sCD30/TNFRSF8 > 506.17 pg/ml, Supplementary table S7), sTNF-R2 (sTNF-R2 > 255.28 pg/ml, Supplementary table S8), and MMP3 (MMP3 > 5.08 ng/ml, Supplementary table S9) were associated with carotid atherosclerotic plaque. After FDR correction, the association between sTNF-R1 and carotid plaque was still significant (q value = 0.0494).

Sensitivity analysis to confirm the association between carotid atherosclerotic plaques and sTNF-R1

When Firth logistic regression Model 2 was refitted using log-transformed sTNF-R1 concentrations (Supplementary Table S10), the association between sTNF-R1 and carotid plaque remained significant (OR 3.15, 95% CI 1.02– 11.87; P = 0.045).

When therapy with SGLT2 inhibitors and RAAS blockers was included in Model 2 with sTNF-R1 as a covariate (Supplementary Table S11), the association between carotid atherosclerotic plaque and sTNF-R1 was confirmed (OR 4.93, 95% CI 1.43 – 17.03; $P = 0.009$). This association was also confirmed (OR 7.19, 95% CI 1.78 – 28.99, $P = 0.004$), when history of CVD and therapy with statins were considered as covariates in model Model 2 (Supplementary Table S12).

Discussion

In this study, individuals living with type 2 diabetes underwent a comprehensive evaluation of inflammatory biomarkers alongside with cardiovascular risk assessment. Participants with DKD exhibited higher circulating levels of inflammatory mediators, including metalloproteinases, soluble TNF receptors, and cytokines from the TNF family. Specifically, APRIL/TNFSF13, MMP3, sTNF-R1, and sTNF-R2 were all associated with reduced eGFR but not with albuminuria. Furthermore, when considering DKD phenotypes, NA-DKD showed a worse inflammatory profile than A-DKD. Additionally, among the subgroup of participants with DKD, higher sTNF-R1

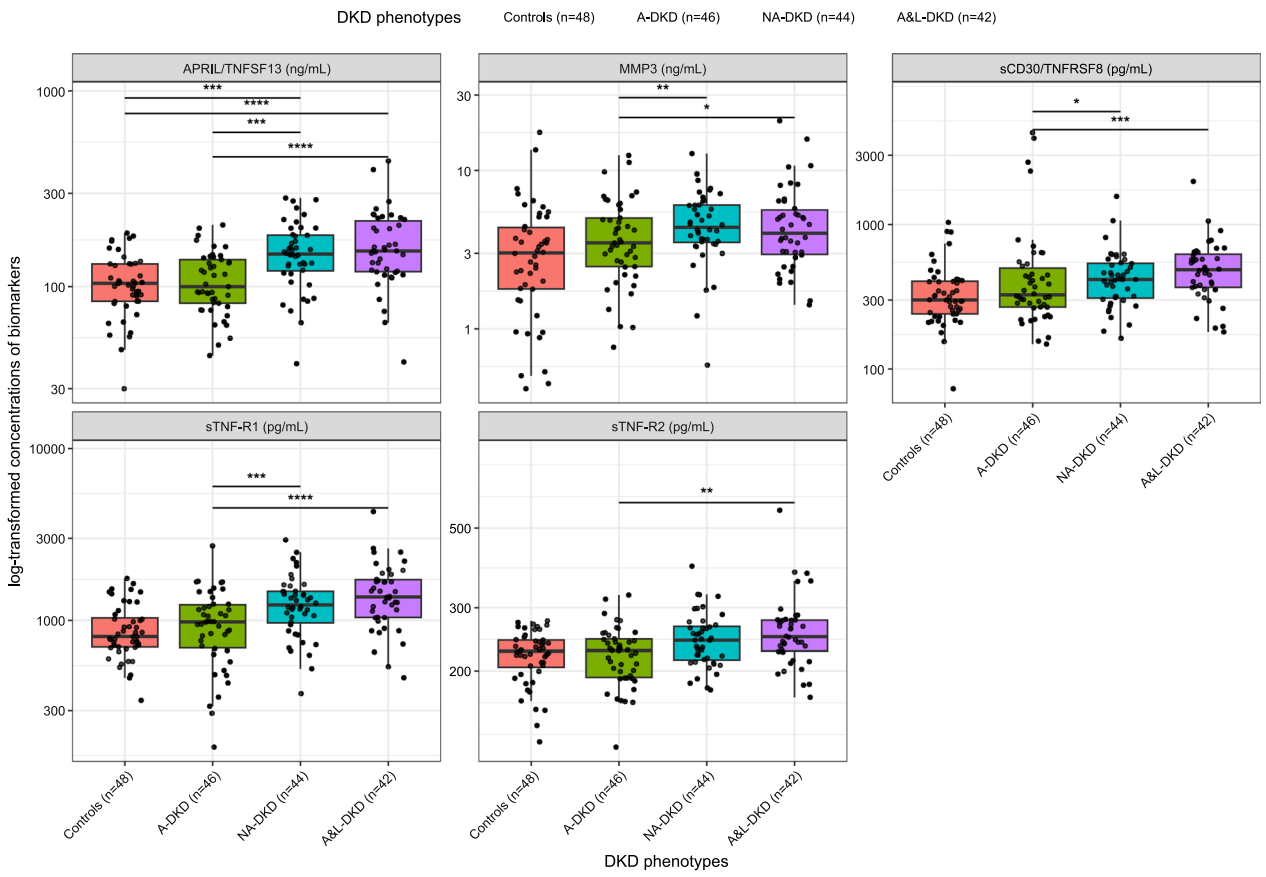


Fig. 1 Box plots of the serum concentrations of cytokines according to DKD phenotypes. X-axis shows diabetic kidney disease (DKD) phenotypes: controls (UACR < 30 mg/g and eGFR ≥ 60 ml/min/1.63 m^2); A-DKD: albuminuric diabetic kidney disease (UACR ≥ 30 mg/g and eGFR ≥ 60 ml/min/1.63 m^2); NA-DKD: non-albuminuric diabetic kidney disease (UACR < 30 mg/g and eGFR < 60 ml/min/1.63 m^2); A&L-DKD: albuminuric and low estimated glomerular filtration rate diabetic kidney disease (UACR ≥ 30 mg/g and eGFR < 60 ml/min/1.63 m^2). Y-axis shows the log-transformed serum levels of the following cytokines (pg/ml): APRIL/TNFSF13: A proliferation-inducing ligand/tumor necrosis factor ligand superfamily member 13; sCD30/TNFRSF8: soluble cluster of differentiation (CD) 30/tumor necrosis factor receptor superfamily member 8; MMP-2: Matrix metalloproteinase-2; MMP-3: Matrix metalloproteinase-3, sTNF-R1: soluble tumor necrosis factor receptor 1; sTNF-R2: soluble tumor necrosis factor receptor 2. The Bonferroni correction for multiple comparisons was applied. *$P < 0.05$; * $P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$

Table 3 Cardiovascular and renal profile of the study population

	Controls (n = 48)	DKD (n = 132)
PWV, m/s	10.11 ± 3.69	11.43 ± 4.02
Carotid plaques, no. (%)	19 (39.6)	82 (62.1)
IMT, mm	0.82 ± 0.14	0.86 ± 0.17
RRI	0.71 ± 0.08	0.75 ± 0.09

Data are presented as mean ± SD or percentage and compared with *t*-test or χ^2 as appropriate. *DKD* Diabetic kidney disease (UACR ≥ 30 mg/g and/or eGFR < 60 ml/min/1.63 m^2); *UACR* Urinary albumin to creatine ratio; eGFR: estimated glomerular filtration rate; *PWV* Pulse wave velocity; *IMT* Intima-media thickness; *RRI* Renal resistivity index

levels were associated with increased odds of carotid atherosclerosis.

The observation of higher levels of sTNF-R1 and sTNF-R2 in individuals with DKD aligns with previous evidence linking these biomarkers to kidney impairment. Carlsson et al. [18] reported higher odds of prevalent kidney disease among participants with higher circulating TNF receptors levels; other studies have confirmed TNF-R1 as a surrogate of renal structural and functional impairment in patients with type 2 diabetes with HbA1c > 6.5% and in Hispanic patients [19–21]. Furthermore, Gohda et al. [22] found, in a cohort of 314 individuals with type 2 diabetes without albuminuria, that both sTNF-R1 and sTNF-R2, but not TNF-α, were independently associated with NA-DKD. A meta-analysis including more than 11,000 individuals [23] further strengthened this evidence by identifying TNF receptors levels as predictors of DKD progression.

Consistent with these findings, metalloproteinases such as MMP3 have also been implicated in renal function decline. In a study including individuals with coronary artery disease, higher serum levels of MMP3 were associated with increased risk of a ≥ 25% eGFR reduction over a median follow-up of 8.5 years [24]. Similarly, Fu et al. reported that MMP3 levels increased progressively across chronic kidney disease stages, and that, in

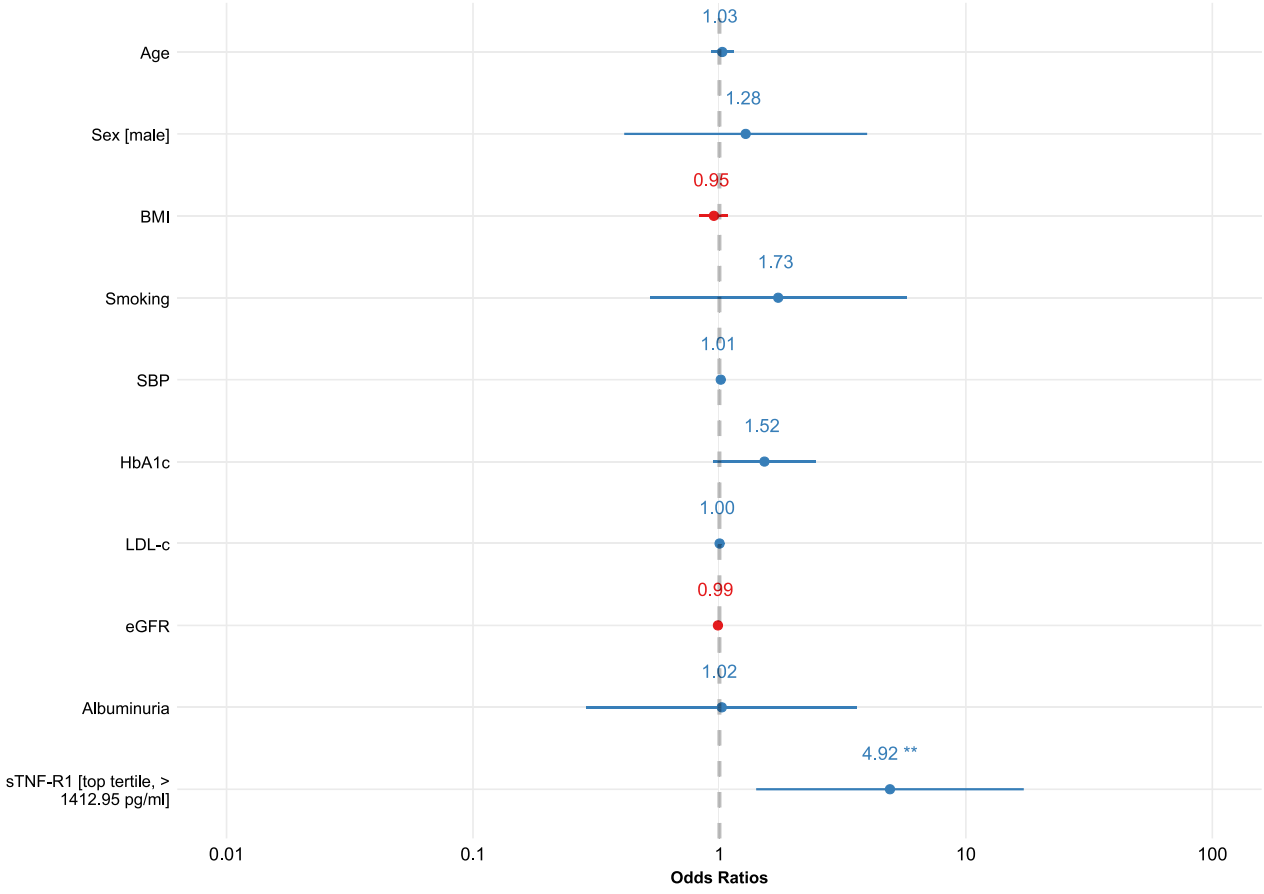


Fig. 2 Forest plots of Firth logistic regression analysis to evaluate association between presence of carotid plaques and levels of sTNF-R1 in participants with DKD. Forest plot showing Odds ratio and 95% Confidence interval of Firth logistic regression analysis with presence of carotid plaque as dependent variable (n. of plaque events: 82). The model is applied at the subgroup of participants with diabetic kidney disease (DKD; n = 132). Covariates: age, sex, body mass index (BMI), smoking status, systolic blood pressure (SBP), glycated haemoglobin (HbA1c), low density lipoprotein cholesterol (LDL-c), estimated glomerular filtration rate (eGFR), and presence of albuminuria. being in the top tertile group of soluble tumor necrosis factor receptor 1 (sTNF-R1 > 1412.95 pg/ml) for the DKD group. *$P < 0.05$; **$P < 0.01$. Red: odds ratio < 1.00; blue: odds ratio > 1.00

the early stages, individuals with higher MMP3 concentrations exhibited more severe renal impairment and inflammation [25].

The link between serum levels of metalloproteinases and TNF receptors with NA-DKD aligns with evidence that TNF-R1 and TNF-R2 are associated with interstitial fibrosis in other kidney diseases [26]. Moreover, Arthanarisami et al. [27], in preclinical models, showed an association of TNF-R1 and TNF-R2 levels with acute kidney injury predicting the transition to chronic kidney disease. Notably, both interstitial fibrosis and acute kidney injury to chronic kidney disease transition are features that have been associated with the pathogenesis of NA-DKD [28, 29]. Similarly, MMP3 has been associated with epithelial to mesenchymal transition and kidney fibrosis [30]. These observations related to serum biomarkers are also coherent with the ultrasound detection of higher RRI in people with DKD, a finding mainly driven by NA-DKD and A&L-DKD groups. Notably, RRI has been associated with histological kidney alterations, including interstitial fibrosis [31] and renal arteriosclerosis [32].

Notably, in the present study higher sTNF-R1 levels were associated with increased odds of carotid atherosclerosis among individuals with DKD. Hints of a potential role for TNF-R1 in the development of atherosclerosis emerge from both preclinical and clinical research. In an elegant mouse grafting model, Zhang et al. compared carotid arteries from wild-type and *Tnfr1*$^{-/-}$ mice of varying ages. They observed that aged *Tnfr1*$^{-/-}$ mice exhibited a carotid atherosclerostic burden comparable to that of young animals and markedly lower than that of aged wild-type mice, supporting the role of TNF-R1 in age-related atherosclerosis development [33]. In the same study, the authors also identified single nucleotide polymorphisms of TNF-R1 gene that are associated with increased risk of atherosclerosis among individuals > 55 years from a cohort of subjects undergoing coronary angiography [33]. Clinical evidence further strengthens the link between TNF-R1 and cardiovascular

disease. In a Swedish cohort of individuals with type 2 diabetes, Carlsson et al. reported that TNF-R1 predicted incident cardiovascular events [18]. This association was also highlighted in a population of patients with stable coronary heart disease followed for 10 years [34]. Furthermore, insights from the CARLA study (Germany) suggests that increase in sTNF-R1 are associated with increased mortality risk, largely driven by cardiovascular mortality [35]. Although several biomarkers including sTNF-R1 have renal clearance, our finding of an association between sTNF-R1 and carotid plaque was independent of eGFR. Notably, although higher levels of specific biomarkers were observed in the DKD group, hs-CRP levels were homogeneous across study groups. One possible explanation is that biomarkers such as sTNF-R1 and MMP3 may reflect more specific pathways related to chronic low-grade inflammation, which have been suggested to be involved in kidney and vascular injury, whereas hs-CRP primarily reflects systemic acute-phase inflammatory responses. In this context, previous studies have reported that specific inflammatory cytokines, but not hs-CRP, were associated with or predictive of renal function decline [36–38].

This body of evidence about the role of inflammation in DKD and in the development of atherosclerosis, together with our observation of a worse inflammatory profile in the NA-DKD, may partially explain the high cardiovascular risk associated with this phenotype. Indeed, in a previous study we found that people with NA-DKD exhibit a worse vascular damage profile than those with albuminuria alone [39]. This finding has been reinforced by a longitudinal study of 2,306 individuals with type 2 diabetes, which showed that participants with NA-DKD had a significantly higher risk of cardiovascular events over a median follow-up of 2.8 years compared with controls, whereas those with albuminuria alone did not [40].

In this study, we also highlighted a trend toward higher PWV in the DKD group, indicating greater arterial stiffness. This finding aligns with previous evidence demonstrating both cross-sectional and longitudinal associations between arterial stiffness and DKD [41, 42]. Interestingly, the increase in arterial stiffness is evident across the continuum of glucose tolerance and in other metabolic diseases [43, 44]. Furthermore, anti-diabetic drugs may exert cardiovascular protective effect also by a positive role on decreasing arterial stiffness [45]. However, in our population the use of SLGT2i and GLP1-RA was limited, due to prescription issue within Italian public health system at the time of recruiting.

This study has several strengths and limitations. Among the strengths, we assessed the cardiovascular risk profile using well-validated measurements, and we measured a comprehensive panel of serum inflammatory biomarkers. Moreover, we applied sensitivity analyses to confirm the association we found between sTNF-R1 and carotid plaque and considered eGFR among covariates. However, the cross-sectional design prevents us from establishing longitudinal or causal relationships. In addition, renal biopsies samples were not available, but it would be interesting to consider histological changes to further characterize the changes due to the different phenotypes of DKD and involved inflammatory patterns for future investigations. Furthermore, although the association between carotid plaque and sTNF-R1 is significant, a certain variability is suggested by the 95%CI. Thus, this is an exploratory study and future prospective studies with a large sample size and hard clinical end points are needed to clarify the mediating role of inflammation in the association between different DKD phenotypes and cardiovascular disease.

Conclusions

In conclusion, our study highlighted a worse inflammatory profile in individuals with type 2 diabetes and DKD and particularly in NA-DKD. sTNF-R1 was associated with carotid atherosclerotic plaque, and this could partially explain the elevated cardiovascular risk associated with this specific DKD phenotype, but further longitudinal studies are needed to fully explore this field.

Abbreviations

A&L-DKD	Albuminuric and low estimated glomerular filtration rate diabetic kidney disease
ACE-i	Angiotensin converting enzyme inhibitors
A-DKD	Albuminuric diabetic kidney disease
APRIL/TNFSF13	A proliferation-inducing ligand/tumor necrosis factor ligand superfamily member 13
ARBs	Angiotensin receptor blockers
BMI	Body mass index
CD	Cluster of differentiation
CHI3L1	Chitinase-3-like 1
CI	Confidence interval
CVD	Cardiovascular disease
DKD	Diabetic kidney disease
eGFR	Estimated glomerular filtration rate
FDR	False discovery rate
gp130/sIL-6Rβ	Glycoprotein 130 / soluble Interleukin-6 receptor subunit β
HbA1c	Glycated haemoglobin
HDL-c	High-density lipoprotein cholesterol
IFN-β	Interferon β
IMT	Intima-media thickness
LDL-c	Low-density lipoprotein cholesterol
MMP1	Matrix metalloproteinase-1
MMP2	Matrix metalloproteinase-2
MMP3	Matrix metalloproteinase-3
NA-DKD	Non-albuminuric diabetic kidney disease
OR	Odds ratio
PWV	Pulse wave velocity
RAAS	Renin–angiotensin–aldosterone system
RRI	Renal resistive index
RT	Room temperature
sCD163	Soluble cluster of differentiation 163
sCD30/TNFRSF8	Soluble cluster of differentiation 30/tumor necrosis factor receptor superfamily member 8
SGLT2i	Sodium-glucose cotransporter 2 inhibitors
sTNF-R	Tumor necrosis factor receptor

TG	Triglycerides
TWEAK/TNFSF12	Thymic Stromal Lymphopoietin, and Tumor Necrosis Factor-like weak inducer of apoptosis/tumor necrosis factor ligand superfamily member 12
UACR	Urinary albumin to creatinine ratio
VIF	Variance inflation factor

Supplementary Information

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Supplementary material.

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Data availability
The dataset used and/or analysed during the current study is available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The study was in accordance with the Declaration of Helsinki and was approved by the local ethics committee (Comitato Etico Catania 2, protocol n. 270/C.E. 26 April 2022). Informed consent was obtained from each participant.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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