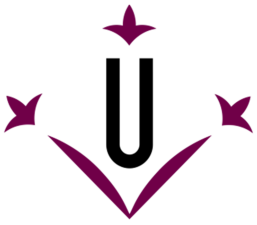

Soluble TWEAK as a predictor of atheromatosis progression in patients with chronic kidney disease

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Abstract

Soluble TNF-like weak inducer of apoptosis (sTWEAK) is a member of the TNF superfamily whose concentrations have been related with the presence of chronic kidney disease (CKD) and cardiovascular disease (CVD). We hypothesized that sTWEAK levels may relate to atherosclerotic burden (defined as the number of atherosclerotic plaques) and atheromatosis progression (defined as an increment in the number of atherosclerotic plaques). For that purpose, we have analyzed baseline sTWEAK serum concentrations in seven hundred CKD patients without any previous CV event from the NEFRONA Study, and their association with atherosclerotic burden and atheromatosis progression after 24 months of follow-up.

A continuous decrease in sTWEAK concentrations with an increase in the number of atherosclerotic plaques after 24 moths of follow-up was observed in the studied population. Multivariable linear regression analysis showed that age, blood pressure, HDL-c, and sTWEAK concentrations were independent predictors of atherosclerotic burden after 24 months of follow-up. In addition, sTWEAK concentrations were diminished in CKD patients in whom atheromatosis progression was observed. After adjustment for confounders, the odds ratio for atheromatosis progression in patients in the lowest versus the highest tertile of sTWEAK was 1.76 [95% confidence interval, 1.19-2.63; p=0.003].

In conclusion, lower sTWEAK concentrations at baseline are associated with atherosclerotic burden and atheromatosis progression in CKD patients free from clinical CVD. These data suggest that sTWEAK could serve as a biomarker to predict CV risk before clinical manifestations.

Cardiovascular disease (CVD) is the main cause of death in patients with chronic kidney disease (CKD), in which cardiovascular death is the main outcome [1]. Compared to the CVD mortality in the general population, the rate of deaths in CKD is 15 to ?? times higher than in non-CKD subjects [2]. The mechanisms for elevated CVD risk in CKD patients are very complex and may implicate changes in the vasculature. In this sense, it has been shown that increases in plaque burden have a strong predictive value assessing cardiovascular events in the general population [3], and the existence of accelerated atheromatosis in CKD population could play an important role in their higher cardiovascular mortality. However, little is known about the predictors of atheromatosis progression in patients with CKD.

Atherosclerosis is a multifactorial disease characterized by a chronic inflammatory response and excessive cell proliferation. Several cytokines contribute to atherosclerotic plaque development participating as inflammatory messengers [4]. One of these proinflammatory cytokines is tumor necrosis factor-like weak inducer of apoptosis (TWEAK). TWEAK, a member of the TNF superfamily, participates in several responses associated with atherosclerotic plaque development such as inflammation, proliferation, angiogenesis, matrix degeneration and thrombosis [5]. Different experimental studies have demonstrated that TWEAK, through is receptor Fn14, plays a key role in atherosclerotic plaque development, progression and subsequent rupture [6-8]. In addition, gain or loss-of function experiments have demonstrated that TWEAK participates in the development of experimental stroke, abdominal aortic aneurysm, heart failure and myocardial infarction [9-12].

TWEAK is expressed as a full-length, membrane-bound protein and then is proteolytically processed by furin, leading to the release of a 156-amino acid, 18 kDa soluble form (sTWEAK) [13]. sTWEAK was described as a potential biomarker being detected in lower amount in the secretome from carotid atherosclerotic plaques than in healthy walls [14]. Since then, the association of sTWEAK with CVD or CVD-related diseases has been extensively studied. sTWEAK concentrations are diminished in patients with coronary artery disease [15], type II diabetes [16], systolic heart failure [17], abdominal aortic aneurysm [18], and CKD [19]. However, the impact of circulating sTWEAK concentration on atheromatosis progression is unknown.

The National Observatory of Atherosclerosis in Nephrology (NEFRONA) Study was an observational multicenter prospective study designed to evaluate the prevalence and evolution of subclinical atheromatosis in CKD patients [20]. The NEFRONA Study has shown that patients in early CKD stages already have a higher prevalence of atherosclerotic plaques than those without CKD [21]. In addition, atheromatosis progression affects more than one half of the patients included in the NEFRONA study [22]. In this study, we analyzed the association between sTWEAK and atheromatosis progression in the NEFRONA population.

Results

Characteristics of the CKD population

Seven hundred CKD patients free from clinical CVD [age 62 (52-68) years; median (IQR); 61% men] were included in the study. Of patients, 68% have dyslipidemia, 93% have hypertension, 28% were diabetic, and 58% smokers. The demographic and clinical characteristics of the studied population according to CKD stages are summarized in Table 1. There were significant differences among the different CKD stages regarding age, gender, body mass index, presence of dyslipidemia, and sTWEAK concentrations.

Baseline circulating levels of sTWEAK in the overall population was 409 (271-580) pg/mL. No differences in sTWEAK concentrations were observed in CKD patients with diabetes, hypertension or dyslipidemia compared with those without these cardiovascular risk factors. Current smokers (N=410) presented lower sTWEAK concentrations compared with non-smokers (N=292) [390 (262-562) *vs* 442 (288-599) pg/mL; median (IQR); p=0.019]. No differences were found according to the prescription of anti-hypertensive drugs, statins or anti-diabetic treatments.

Atheromatous plaque prevalence at baseline was 67.5% without significant differences between CKD stages. Patients with atherosclerotic plaques showed lower sTWEAK concentrations compared with those without plaques [375 (262-537) vs 505 (319-652) pg/mL; median (IQR); p<0.001].

sTWEAK and atheromatosis progression

We found a weak but significant correlation between baseline sTWEAK levels and c-IMT and ABI at 24 months (r=-0.102, p=0.008; and r=0.096, p=0.011; respectively). Furthermore, patients with severe atherosclerosis at 24 months showed reduced baseline sTWEAK levels as compared with the incipient atherosclerosis group [373 (262-537) vs 503 (340-646) pg/mL; median (IQR); p<0.001; Fig. 1A]. To clarify the link between sTWEAK and severity of atherosclerosis, we have analyzed baseline sTWEAK concentrations according with the number of territories with atherosclerotic plaques (10 territories studied). Baseline sTWEAK levels were reduced with increasing number of atherosclerotic plaques. Thus, subjects with 1-4 or ≥5 territories with plaques showed lower baseline sTWEAK concentrations compared with those without

plaques at 24 months (Fig. 1B). In addition, CKD patients with multiple territories affected (≥5) also presented lower sTWEAK levels than CKD patients with 1-4 territories with atherosclerotic plaques (Fig. 1B). To confirm that baseline sTWEAK could be a biomarker of atherosclerotic burden at 24 months of follow-up, a multivariable linear regression analysis was performed with the number of atherosclerotic plaques at 24 months as a dependent variable (Table 2). Age, SBP, DBP, HDL-c and sTWEAK concentrations were independent predictors of atherosclerotic burden at 24 months of follow-up (r=0.389).

In order to analyze the potential association between baseline sTWEAK concentrations and atheromatosis progression, CKD patients were divided in two groups: no atheromatosis progression (patients without increased number of atherosclerotic plaques at 24 months); and atheromatosis progression (appearance of new plaque/s at 24 months). The percentage of patients with atherosclerotic plaque/s increased from 67.5% to 80.5% at 24 months. Atheromatosis progression occurred in 58.4% of patients. Baseline potential factors predicting atheromatosis progression are summarized in Table 3. There were significant differences among patients with progression and no progression regarding age, gender, glucose concentrations, and the presence of hypertension, diabetes and/or dyslipidemia.

Table 4 shows univariate regression logistic analysis to assess predictors of atheromatosis progression in CKD patients. Low sTWEAK concentrations were defined as sTWEAK levels below the 33rd percentile (≤ 309 pg/mL) and high sTWEAK as its concentrations above 66th percentile (≥ 517 pg/mL). In univariate analysis, older age, gender, current smokers, presence of hypertension, diabetes and/or dyslipidemia, use of anti-diabetic drugs, and lowest sTWEAK tertile were predictors of atheromatosis progression. After that, multivariable logistic regression analysis including only variables that were statistically significant in the univariate analysis was performed to assess predictors of atheromatosis progression. Older age, current smoker, and lowest sTWEAK tertile were independent predictors of atheromatosis progression.

Discussion

In this work, we investigated sTWEAK serum levels as predictors of atheromatosis progression after 24 months of follow-up in a CKD population with or without cardiovascular risk factors but free from previous clinical CVD. We have observed that patients with severe atherosclerosis at 24 months showed lower sTWEAK concentrations compared with those with incipient atherosclerosis. In addition, we have observed that sTWEAK levels were associated with atherosclerotic burden after 24 months of follow-up. Finally, we observed for the first time an independent and significant association between low sTWEAK concentration and atheromatosis progression in CKD patients.

According to previous data in which low sTWEAK levels have been observed in patients with carotid atherosclerosis and CAD, we also showed that sTWEAK concentrations are reduced in CKD patients with atherosclerosis. Moreover, sTWEAK levels were even more reduced in CKD patients with severe atherosclerosis compared with those with incipient atherosclerosis. In this sense, sTWEAK concentrations have been related to different surrogate markers of atherosclerosis namely carotid intima/media thickness and endothelial dysfunction [14, 19, 23-26]. Thus, sTWEAK levels have been negatively associated with c-IMT in CKD or asymptomatic patients [14, 23-26]. However, this association was not confirmed in other cohorts and, indeed, positively association has been shown in transplanted CKD patients [27], which could be due to the presence of special therapy regimens used before and after renal transplantation. In addition, sTWEAK concentrations are strongly and independently correlated with flow-mediated dilation, suggesting a link between endothelial dysfunction and sTWEAK in CKD patients [19]. We have also observed a continuous decrease in sTWEAK concentrations with an increase in the number of atherosclerotic plaques after 24 moths of follow-up in the studied population. This is in agreement with previous data obtained from asymptomatic patients in which sTWEAK levels were related with the amount of atherosclerotic plaques [26]. Overall, these data indicate that sTWEAK could be a biomarker of atherosclerotic burden and severity in CKD patients.

Traditional cardiovascular risk factors are associated with the presence of atherosclerotic plaques and burden. However, few studies have analyzed the predictors of atheromatosis progression over time [28-29]. Atheromatosis progression has been recently analyzed in the NEFRONA Study after 24

months of follow-up, observing a high prevalence of atheromatosis progression in CKD patients [30]. In our sub-population, older age and current smoker were predictors of atheromatosis progression. This is according with data obtained from the MESA Study in which current smokers was also a strong predictor of new plaque formation [31]. More interestingly, our study also indicates that baseline sTWEAK concentrations may predict atheromatosis progression measured by increased number of territories with plaques in CKD patients.

The reasons by which the progression of atherosclerosis is associated with sTWEAK levels are unknown, but experimental evidence suggests a causal role. TWEAK is expressed in both the normal and pathological arterial wall, but Fn14 is almost absent in healthy arteries and its expression is highly upregulated in the carotid and femoral atherosclerotic plaques [33-34]. Binding of TWEAK to its receptor induces several responses in vascular and inflammatory cells. Thus, TWEAK increases adhesion molecules and proinflammatory cytokines expression in vascular cells and infiltrating macrophages, upregulates metaloproteinases activity and participates in prothrombotic responses and vascular calcification [5]. Experiments from animal models have demonstrated that TWEAK participates in development, progression and rupture of atherosclerotic plaques. Systemic injection of TWEAK increased atherosclerotic plaque size and inflammatory response in the aortic root of ApoE deficient mice [8]. In addition, genetic deletion of TWEAK reduced plaque progression and increased plaque stability in ApoE deficient mice [7]. Data from experimental models support that TWEAK and its functional receptor Fn14 are a promising target for the treatment of patients with different CVD. Treatment with the TWEAK neutralizing antibody or Fn14-Fc decoy protein has demonstrated a beneficial effect on the development and progression of atherosclerotic plaques in mice [6-7].

The mechanism(s) by which sTWEAK is reduced in CKD patients with atherosclerosis are unknown. The increment in Fn14 expression observed in pathological arterial wall could favor sTWEAK binding and retention in tissue. In addition, the presence of CD163, a scavenger receptor of TWEAK, in pathological tissues could facilitate TWEAK degradation by inflammatory macrophages, leading to low TWEAK levels [34]. In this sense, it has been reported that both sTWEAK and CD163 are expressed in an opposite trend in

human carotid atherosclerotic plaques [35]. However, this is a hypothesis that needs to be tested in further studies.

Strengths of this study are the relatively large sample size of CKD patients with longitudinal observations included in our study. In addition, this population included CKD patients from daily clinical practice with or without concomitant medication with the aim to have a representative population. Moreover, vascular exploration was done by the same team and evaluated by a single reader. However, some limitations of our study should be highlighted for a correct interpretation of the implications of our findings. There is an intentional bias, because only patients with no history of cardiovascular events were included, as the study was aimed to primary prevention of cardiovascular events. In addition, plaque volume and density was not measured.

In conclusion, our study shows that baseline sTWEAK concentrations could be an independent predictor for the development of atherosclerotic plaques in CKD patients.

Methods

Patients

Studied population included 700 patients (CKD 3-5 and dialysis) from the observational and multicenter NEFRONA study, recruited from October 2009 to June 2011 [20-21] and with 24 moths of follow-up. The NEFRONA study included male and female without history of CVD (angina pectoris, acute myocardial infarction, ischemic stroke, hemorrhagic stroke, abdominal aortic aneurysm and atherosclerosis), and ages ranged between 18 and 74 years old if they had CKD stage 3 or higher as defined by current guidelines (eGFR lower than 60 mL/min/1.73 m^2 estimated using the 4-variable Modification of Diet in Renal Disease (MDRD) equation]. Exclusion criteria were pregnancy, VIH infection, any type of organ transplantation, previous history of carotid artery disease, active infections, any hospitalization in the last month, and intercurrent illness that presumes absence of follow-up or survival expectation less than 1 year. The exclusion criteria were previous CV events, active infections (HIV, tuberculosis), pregnancy, having received any organ transplantation, and having a life expectancy of <1 year. For the prospective study at 24 months, renal parameters serum creatinine and eGFR), carotid/femoral echography, atheromatous disease (AD), ankle-brachial index (ABI), intima-media thickness (IMT), CV events and mortality were assessed.

Each local ethics committee approved the study. The authors adhered to the declaration of Helsinki and patients were included after providing informed consent.

Clinical and biochemical data

At recruitment, the patients were asked to complete a questionnaire including clinical history of diabetes, hypertension and dyslipidemia; CV risk factors and medication use.

The itinerant teams collected the anthropometric parameters and blood samples. Blood samples were processed immediately after extraction and storage at -20º C. After that, samples were sent and stored at -80º C within 24h at the centralized biobank of the Spanish Network for Nephrological Research. Biochemical parameters were obtained from a routine fasting blood test. Serum sTWEAK concentrations were determined in duplicate with commercially

available ELISA kits (Bender MedSystems, Vienna, Austria). The same investigator measured all samples in a blinded manner (IIS-Fundación Jiménez Díaz). The minimum detectable level of sTWEAK was 10 pg/ml. Intra- and interassay coefficients of variation were 7.3 and 8.5%, respectively.

Atherosclerosis diagnosis

Subclinical atherosclerosis was evaluated as described previously [20]. Participants underwent a carotid and femoral ultra- sound to measure IMT, using the Vivid BT09 apparatus (General Electric instrument) equipped with a 6e13 MHz broadband linear array probe. The analysis of the presence of atheromatous plaques was performed by a unique reader in a blinded fashion, using the semi-automatic software EchoPAC Dimension (General Electric Healthcare). To assess the quality of the reading and the intra-observer reliability, a sample of 20 individuals was measured 3-5 times on different days. A kappa coefficient of 1 was obtained, indicating excellent intraobserver reliability.

Plaque presence was evaluated in a total of 10 territories: right common carotid arteries, right carotid bulb, right internal carotid arteries, left common carotid arteries, left carotid bulb, left internal carotid arteries, right common femoral arteries, right superficial femoral arteries, left common femoral arteries, and left superficial femoral arteries. Atheromatosis progression was defined as an increase in the number of territories showing a plaque with respect to the baseline visit as previously used in the Multi-Ethnic Study of Atherosclerosis (MESA) Study [31]. Patients were also classified in three groups according to the number of territories with plaques at 24 months: 0; 1 to 4; and ≥5.

Atherosclerotic disease (AD) was initially classified in four groups: AD 0: ABI >0.9 and IMT <80% reference interval; AD 1: ABI 0.7-0.9 and/or IMT ≥80% reference interval; AD 2: carotid plaque with stenosis <125 cm/s; and AD3: ABI <0.7 and/or carotid plaque with stenosis ≥125 cm/s [20-22]. For this study, AD was classified in two groups, incipient AD (combining AD 0 and 1) and severe AD (combining AD 2 and 3), as previously reported [36].

Statistical Analyses

All of the statistical analyses were performed using the SPSS 11.0 (SPSS, Chicago, IL) statistical package. Non-normally distributed variables were

expressed as median (IQR, expressed as the 25^{th} and 75^{th}), and normally distributed variables were expressed as mean±SD. Between-group comparisons were assessed for nominal variables with the X^2 test and by Mann-Whitney U or Kruskal-Wallis test. Multiple linear regression analyses using the number of territories with atherosclerotic plaques at 24 months as dependent variable was performed to identify independent predictor of atherosclerotic burden. Ninety-five percent confidence intervals (CI) were calculated for each comparison. p-value <0.05 was considered statistically significant.

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Author Contributions

F-L. V conducted the experiments. M-V. JL, and E. J, supervised and participated in the design of the study. V. JM, F. E, and B-C. LM coordinated the work, participated in the design of the study, wrote the manuscript and obtained funding for this work. All authors reviewed the manuscript.

None of the other authors have conflict of interest.

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Figure Legends

Figure 1: sTWEAK concentrations, atherosclerotic burden and atheromatosis progression in CKD patients.

A) Box plots showing the difference in baseline sTWEAK levels [median (IQR)] according to the stage of AD at 24 months of follow-up. Incipient AD (AD 0-1), N=218; Severe AD (AD 2-3), N=484.

B) Box plots showing the difference in baseline sTWEAK levels [median (IQR)] according to the number of atherosclerotic plaques at 24 months of follow-up. No plaques, N=137; 1-4 plaques, N=341; >5 plaques; N=224.

C) Box plots showing the difference in baseline sTWEAK levels [median (IQR)] according to atheromatosis progression at 24 months of follow-up. No progression, N=291; Progression, N=411.

Whiskers represent 10^{th} and 90^{th} percentiles.