Original Article

ASSOCIATION OF FGF-2 CONCENTRATIONS WITH ATHEROMA PROGRESSION IN CHRONIC KIDNEY DISEASE PATIENTS

Running title: Subclinical Atheromatosis progression and FGF-2 in CKD

Category: Chronic Kidney Disease

Milica Bozic[1] PhD, Angels Betriu[1] MD, PhD, Marcelino Bermudez-Lopez[1] MD, PhD, Alberto Ortiz[2] MD, PhD, Elvira Fernandez[1] MD, PhD and Jose M Valdivielso[1] PhD on behalf of the NEFRONA investigators.

Address for correspondence:

José Manuel Valdivielso, PhD. E-mail: valdivielso@medicina.udl.es

Elvira Fernandez MD, PhD. E-mail: efernandez@irblleida.cat

[1]Vascular and Renal Translational Research Group, IRBLleida, Rovira Roure 80, Lleida, and [2]IIS Fundación Jiménez Díaz, Autonomous University of Madrid. REDinREN del ISCIII, Spain.

Word count: 3344

ABSTRACT

Background and objectives: Atherosclerosis is highly prevalent in chronic kidney disease (CKD). The rate of progression of atherosclerosis is associated with cardiovascular events. Fibroblast growth factor 2 (FGF-2) is a member of the FGF family with potentially both protective and deleterious effects in the development of atherosclerosis. The role of circulating FGF-2 levels in the progression of atherosclerosis in CKD is unknown.

Design, setting, participants, & measurements: Multicenter, prospective observational study in 481 CKD patients form the NEFRONA cohort. We determined the presence of atheroma plaque in 10 arterial territories by carotid and femoral ultrasounds. Progression of atheromatosis was defined as an increase in the number of territories with plaque after 24 months. Plasma levels of FGF-2 were measured by multiplex analysis. A multivariable logistic regression analysis was performed to determine whether plasma FGF-2 levels were associated with atheromatosis progression.

Results Average age of the population was 61 years. The percentage of patients in each CKD stage was 51% in stage 3, 41% in stage 4-5 and 8% in dialysis. 335 patients (70%) showed plaque at baseline. Atheromatosis progressed in 289 patients (67%). FGF-2 levels were similar between patients with or without plaque at baseline (79 vs 88 pg/ml), but lower in patients with atheromatosis progression after 2 years (78 vs 98 pg/ml. p<0.01). In adjusted analyses, higher plasma FGF-2 was associated with lower risk of atheromatosis progression (OR 0.86 (95% CI 0.76-0.96) per 50 pg/mL increment). Analysis of FGF-2 in tertiles showed that atheroma progression was observed for 102 participants in the lowest tertile of FGF-2 (reference group), 86 participants in the middle tertile of FGF-2 (adjusted OR 0.70, 95% CI 0.40-1.20) and 74 participants in the lowest tertile of FGF-2 (adjusted OR 0.48, 95% CI 0.28-0.82).

Conclusions: Low FGF-2 levels are independently associated with atheromatosis progression in CKD.

Comentado [CJASN1]: Please insert a description of the measurement method for FGF-2 after describing the study population and before describing the measurement of atheroma. Word count must stay below 300 – consider shortening the background to provide sufficient room

Comentado [CJASN2]: Please include the number and proportion of participants with atheroma progression

INTRODUCTION

Chronic kidney disease (CKD) is a growing public health problem worldwide and, over the past decade, it has developed into an area of intensive clinical and epidemiological research. Patients with CKD, even at early stages, demonstrate higher risk for the development of cardiovascular disease (CVD), [1, 2] enhancing morbidity and mortality in this population.[3] A variety of traditional cardiovascular risk factors such as hypertension, diabetes, dyslipidemia[2] and non-traditional variables such as endothelial cell dysfunction, [4] inflammation, [2] low vitamin D levels [5, 6] or hyperphosphatemia [7-9] begin to act very early in the course of CKD, accelerating the pathogenesis of cardiovascular disease. Taking into account that traditional and CKD-specific risk prediction equations explain only a portion of the cardiovascular disease associated with CKD, the search for new risk factors for cardiovascular disease in CKD patients continues.

Atherosclerotic cardiovascular disease is highly prevalent in CKD patients [7] and its progression is closely associated with the progression of CKD.[10] The role of atherosclerosis in the increased cardiovascular mortality in CKD patients seems clear, although its contribution in late stages is controversial. Indeed, there are differences in the type of cardiovascular events and the associated factors along the progression of CKD. Thus, in early stages, there is a high mortality rate due to ischemic events, related to atherosclerosis.[11] However, in dialysis patients most of the deaths seem to be caused by heart failure and sudden cardiac death, although subclinical atherosclerosis appears to be influencing the higher susceptibility of the myocardium of those patients to electrolyte

imbalances.[12] Indeed, atheroma burden also independently predicts cardiovascular events in dialysis, and the rate of atherosclerotic plaque formation predicts cardiovascular events in those patients.[13] Therefore, the determination of factors that affect atherosclerosis progression in CKD could lead to therapeutic targets specific for CKD patients.

Fibroblast growth factor 2 (FGF-2 or bFGF) is a member of a large family of heparin-binding proteins[14] that has an important role in the pathogenesis of atherosclerosis, but it also has cardioprotective actions. FGF-2 affects essential biological activities of vascular cells such as differentiation, proliferation and migration, showing a dual role in the cardiovascular system. The expression of FGF-2 and its receptors in a normal vessel wall is highly beneficial for the maintenance of the vascular homeostasis and protection of endothelial cells. [15] However, FGF-2 and its receptors play a role in the inflammatory process, intimal thickening and intra-plaque angiogenesis,[16] stimulating proliferation and migration of vascular smooth muscle cells (VSMC) [14, 17] and development of vasa vasorum in atherosclerotic lesions,[18] therefore accelerating atherosclerotic plaque growth.

There are scarce clinical data investigating the role of circulating levels of FGF-2 in atherosclerosis. Only few studies have demonstrated that levels of FGF-2 did not correlate with carotid obstruction grade[19] or coronary artery calcification in the general population.[4] However, to date, no investigation has been conducted analyzing the role of circulating FGF-2 levels in atheromatosis progression. In the present work, we analyzed the association between FGF-2 and progression of atheromatosis burden in a subpopulation of the the NEFRONA study.

METHODS

Design & study population

The protocol of the study was approved by the ethics committee of each hospital and all patients were included after signing informed consent. This research followed the principles of the Declaration of Helsinki. The design and objectives of the NEFRONA study have been already published in detail.[20, 21] Briefly, 2445 CKD patients (937 in CKD stage 3, 820 in stage 4-5 and 688 in dialysis) without a history of previous cardiovascular disease who were 18-75 years of age were enrolled from 81 Spanish hospitals between October 2009 and June 2011, with a scheduled follow-up visit after 24 months. Patients from the NEFRONA study who had hemodynamically significant stenotic carotid plaque, ankle-brachial index (ABI) <0.7 at baseline, a cardiovascular event or received a renal allograft along the 2 year's follow up, or died after the first ultrasound exploration were excluded from the follow-up. Thus, 1555 were followed for 24 months. Out of those, 481 had available plasma samples to measure FGF-2 levels. Samples from the other patients had already been spent to measure other biomarkers in sub-studies within the NEFRONA study.[22, 23] In any case, both populations did not differ in most of the parameters of the study, although there was a lower proportion of patients in dialysis than in the total sample. Consequently, high sensitivity C-reactive protein (hsPCR) levels were lower and 25(OH)D, total and LDL cholesterol levels were higher in the analyzed sample (Supplemental table S1).

Determination of FGF-2 in plasma samples

FGF-2 levels were measured in duplicate in frozen plasma samples after the end of the recruitment period. The detection was performed with multiplex kits (Milliplex MAP, Merck Millipore), an assay specially designed to identify 10 different biomarkers in a small sample by an Elisa-like method. The assay uses internally color-coded microspheres coated with specific primary antibodies. After a molecule from a test sample is captured by the bead, an additional biotinylated antibody, built to identify another epitope of the molecule, is introduced. Thus, the emission of the bead identifies the compound and the intensity if the second antibody quantifies the amount of the compound. The multiplex panel included FGF2, Eotaxin, GM-CSF, Fractalkine, IFN-gamma, MDC, IP-10, MCP-1, MIP-1Beta and VEGF. The purpose of the study was to identify whether those analytes, which have been previously associated to cardiovascular disease, were associated with atherosclerosis progression in CKD. Out of those, only FGF2 showed an association with the outcome, after Bonferroni´s correction for multiple testing.

In order to determine whether the method of recollection of the samples was interfering with the results, we measured in the multiplex kit 40 samples of in-house patients frozen immediately after extraction and compared the results against the same samples stored at 4ºC for 24 hours (in order to mimic the conditions of the samples during the shipment from the extraction place to the biobank in which they were stored). The Pearson's correlation coefficient was 0.99 ($p<0.001$) showing that the handling of the samples before storage did not affect the levels of FGF2. The inter-assay coefficient of variability of this analyte is 4.8%.

Comentado [CJASN3]: As requested by the reviewers and Associate Editor, more information on the assay is needed in the text, including the other analytes measured, reliability data (interassay CVs), storage conditions/freeze-thaw, duplicate measurements, etc.

Please create a separate sub-section within your methods section devoted specifically to measurement of FGF-2. This sub-section should be placed immediately after the first sub-section describing the study population.

Total word count must remain below 3000. Some areas to delete text are suggested to make room for important details on measurement of FGF-2

Comentado [U4R3]: Done

Clinical data and Laboratory examinations

Current health status, medical history, baseline cardiovascular risk factors and drug use information was obtained at baseline. A physical examination was performed, consisting of anthropometric measures, standard vital tests and ABI measurements as previously described.[24] A pathological ABI was described as ≤ 0.9 or ≥ 1.4. Biochemical data were obtained from a routine fasting blood test within three months from the vascular study. Previous and current smokers were classified as ever smokers. Diagnosis of dyslipidemia was obtained from the clinical history. Glomerular filtration rate (GFR) was estimated using the Modification of Diet in Renal Disease Study formula (MDRD-4).

Ultrasound Imaging

B-mode ultrasound of the carotid and femoral arteries was performed using the Vivid apparatus (General Electric) equipped with a 6-13 MHz broadband linear array probe as previously described. [25] Briefly, ultrasound imaging was performed with the subjects in a supine position and the head turned 45º contralateral to the side of the probe to evaluate carotid plaques. The presence of atheromatous plaques was defined as a IMT > 1.5 mm protruding into the lumen, according to the ASE Consensus Statement[26] and the Mannheim IMT Consensus.[27]

The presence of atheromatous plaques was explored in 10 territories (both internal, bulb and common carotids, and both common and superficial femoral arteries)

by a single reader in blinded mode, using semi-automatic software (EchoPAC Dimension, General Electric Healthcare). Therefore, a score from 0 to 10 was assigned to every patient both at basal and at the follow up exploration two years later; 0 meaning no plaques in any territorie and 10 the presence of atheroma plaque in all 10 territories explored. To assess intraobserver reliability, a sample of 20 individuals was measured 3–5 times on different days by a reader unaware of patients' clinical history. An intraclass correlation coefficient of 0.8 was obtained for plaque assessment, indicating very good intraobserver reliability. Intima-media thickness (IMT) was only measured in arterial regions without plaque and calculated as the average between left and right sides. When a territory presented with a plaque, IMT value was censored to be 1.5 mm in that territory. The average of the IMT value of the 10 territories was calculated and presented as the patient's IMT.

Evaluation of Progression

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 MESA study.[28] The analysis was performed in 10 arterial territories, yielding a score of 1 to 10 both, at the baseline (SB) and at the follow up (SFU) visit. The outcome variable (atheromatosis progression) was defined as an increase in the number of territories with plaque with respect to the basal visit. Thus, if SFU - SB > 0 the patient was assigned to the group of progressors. If SFU - SB = 0 the patient was assigned to the group of non-progressors. In no patient a negative value of (SFU - SB) was observed.

Statistical analysis

Univariable relationship between the levels of the ten compounds measured in the multiplex analysis with plaque presence and progression at 24 months was analyzed by Student's t test for normally distributed variables, and Mann-Whitney's test in non-parametric analysis. In order to account for the multiple testing problem, Bonferroni's correction was applied. Thus, only *p* values equal or lower than 0.005 were considered statistically significant in the univariate analysis. Significant variables in univariable analyses and potential confounders were used to develop appropriate multivariable logistic regression models. A forward step procedure was used to build the multivariable model, including the variable showing maximum contribution identifying those patients with 24-month atheromatosis progression, according to the likelihood ratio test (LRT). Those variables without a statistically significant contribution, but modifying in more than 10% the value of the coefficients of any of the significant variables when removed from the model, were considered confounders and included in the final model. Possible first degree interactions between FGF-2 and different parameters were tested. A statistical significance level of 0.05 was used. A sensitivity analysis using FGF-2 as a categorical variable was also built. All analyses were made using a standard statistical package (SPSS 24.0).

RESULTS

The characteristics of the population, grouped by tertiles of FGF-2, are shown in Table 1. The median age was 61 years, and the median (p25-p75) plasma FGF-2 levels were 85 (55-138)pg/mL. Table 2 shows the univariable analysis of factors associated with

atheromatosis progression. Of the 289 patients that progressed, 157 (54%) did so in one territory, 75 (26%) in two, 36 (13%) in three, 14 (5%) in four, 4 (1%) in five, 2 (0.7%) in six and 1 (0.3%) seven new territories were affected after 24 months. The univariable analysis (table 2) showed that patients in whom atherosclerosis progressed were significantly older, more frequently smokers and with a higher percentage of patients with diabetis and hypertension and had higher blood levels of triglycerides, glucose and hsCRP. Furthermore, the IMT values and the percentage of patients with plaque at baseline was also higher in progressors.

As seen in Figures 1A and B, FGF-2 levels did not differ among patients with or without plaque at baseline neither between patients with different atheromatosis burden. However, Figure 1C shows that FGF-2 levels were significantly lower in patients in which atherosclerosis progressed after 2 years of follow-up, compared with patients where the plaque burden remained stable. In addition, FGF-2 levels were significantly lower in patients with more advanced CKD stages (Figure 2).

The unadjusted analysis modeling atherosclerosis progression (Table 3, model 1) shows that higher levels of FGF-2, assessed as a continuous variable, are associated with lower progression of atherosclerosis. Adjustment for age and sex (model 2) and by several known risk factors for atheromatosis progression (model 3) show that the effect of FGF-2 remains unmodified. A sensitivity analysis using FGF-2 as a categorical variable (tertiles) yielded similar results. The possible interactions between FGF-2 levels and several parameters (including CKD stage) were tested and disregarded as nonsignificant. The

Comentado [CJASN5]: Spell out all abbreivations, including CKD and FGF, in figure legends

Comentado [U6R5]: Done

Comentado [CJASN7]: Use "higher" and "lower" instead of "decrease" and "increase" fo cross-sectional analyses. The latter imply change over time, which is not assessed here. Please review the entire manuscript thoroughly for consistency throughout

Comentado [U8R7]: Done

Comentado [CJASN9]: Consider deleting

Comentado [U10R9]: Sentences have been combined

Comentado [CJASN11]: Replace with non-causal language

Comentado [U12R11]: done

Hosmer and Lemeshow p values show an excellent goodness of fit of the logistic regression model, so the model is considered well calibrated.

DISCUSSION

The present work shows that FGF-2 levels are associated with atherosclerosis progression in CKD patients. This effect is independent of other parameters known to affect the progression of atherosclerosis in CKD like age, the degree of renal function, the levels of cholesterol or the smoking status. Therefore, FGF-2 could be considered as a marker and potential target in atherosclerosis progression in CKD patients.

The role of FGF-2 in atherosclerosis is unclear, and preclinical data show that beneficial and detrimental effects could coexist. On the one hand, FGF-2 may be deleterious due to its potential to increase plaque size. Thus, FGF-2 and FGF receptors are expressed in VSMC[29] and FGF-2 induces VSMC proliferation *in vitro*. [17] Furthermore, FGF-2 promotes VSMC migration by inducing matrix metalloproteases (MMP) 2 and 9 expression in baboon aortic explants[30] and by regulating TRAIL expression in mice undergoing the cuff injury model.[31] In addition, FGF-2 could regulate VSMC phenotypic transformation from the contractile to the synthetic phenotype, a necessary step in the early stages of atherosclerosis.[32] Thus FGF-2 has been shown to act synergistically with platelet-derived growth factor inducing the phenotypic change *in vitro*.[33] In any case, and although FGF-1 levels increase in atherosclerotic plaques versus normal artery tissue, FGF-2 levels have been reported to remain normal in human atherosclerotic artery samples.[29]

On the other hand, the effects of FGF-2 in endothelial cells point to a beneficial effect on atherosclerosis. Atherosclerotic disease is a progressive disorder that develops over a long period of time, triggered locally by endothelial dysfunction. FGF-2 increases proliferation and migration of endothelial cells[34] and, furthermore, maintains their integrity,[15, 35] antagonizing endothelial dysfunction. In addition, administration of FGF-2 improves endothelial function decreasing vascular endothelial adhesion molecule expression and macrophage infiltration early on in the course of experimental atherosclerosis.[36]

In vivo studies in experimental models of atherosclerosis have yielded controversial results. Thus, Che *et al*. showed that endothelial overexpression of FGF receptor 2 accelerates atherosclerosis in a transgenic mouse model.[37] Raj *et al*. also showed that pharmacological inhibition of FGF receptor signaling with a tyrosine kinase activity inhibitor attenuated atherosclerosis in apolipoprotein E null mice.[38] However, the effects observed in the former studies could be attributed to signaling of any of the 23 FGF species described so far. In experiments using specifically FGF-2, direct administration of the compound did not increase neointimal formation after balloon injury in pigs,[39] dogs,[40] rabbits[41] and it even decreased neointimal formation in a rat model of balloon injury [42] and in a rabbit model of poor distal runoff circulation. [43] In any case, the potential different effects of FGF-2 on experimental atherosclerosis could be explained by the different effect of the compound depending on the stage of evolution of plaque, from type I plaques with relatively normal histology and endothelial dysfunction, to complicated type VI plaques with thrombus. Thus, in early stages, increased FGF-2 levels

can prevent the formation of plaque by reducing endothelial dysfunction.[36] By contrast, in aadvanced lesions where endothelial dysfunction is no longer an issue, FGF-2 pro-mitogenic actions can induce plaque growth and even rupture by increasing the density of vasa-vasorum and MMP synthesis.[44, 45] In our study, lower FGF-2 levels are associated with the appearance of new plaques, but were not associated with the baseline presence of atheroma plaques, agreeing with a protective role of FGF-2 in the early stages of atheroma plaque formation. Indeed, clinical trials using FGF-2 as a treatment for revascularization have not shown any effect aggravating atherosclerotic disease.[46, 47]

Our results disclosing a higher odds ratio (OR) for atheromatosis progression in CKD stages 4-5 and dialysis than in stage 3 patients may appear discordant with those of Rigatto et al[48] observing lower progression of atheromatosis in later CKD stages. In our study, the OR for atheromatosis progression was higher in stages 4-5 and dialysis compared with stage 3 patients. However, the design of both studies differ, and those differences may account for the discrepancies. First, our cohort specifically excluded CKD patients with previous cardiovascular disease, while one third of patients in Rigatto's cohort, had a previous cardiovascular event. Furthermore, our study explored the femoral territories, which were not tested in Rigatto´s study. Finally, a possible interaction between FGF-2 levels and CKD stage could also explain the discrepancy, although that interaction was tested and disregarded as non-significant.

Another result from our study shows that FGF-2 levels are lower in more severe CKD stages, reaching very low levels in dialysis patients. There is little information about the role of FGF-2 in CKD. Experimental data show that FGF-2 could be a therapeutic tool to

reduce CKD. FGF-2 administration reduces functional and structural damage in experimental CKD.[49] Furthermore, FGF-2 mediates the improvement of renal function induced by the administration of mesenchymal stem cells in a rat model of CKD.[50] Thus, FGF-2 could have a potential role in the progression of CKD, although further research is warranted.

The main strength of this study is the relatively large number of patients with longitudinal observations, which allows us to make associations controlling for multiple confounders. A second strength is that the vascular exploration was performed by the same team and evaluated by a single reader. Nevertheless, our study also has some limitations. The main one is that, out of the total sample of the patients followed for atherosclerosis progression, only a portion had available plasma sample to measure FGF-2 levels. Therefore, the subpopulation might not be representative of the full cohort, as some parameters differed between patients with available sample and the rest. However, the number of patients is still high enough to adjust for many potential confounding factors. Furthermore, patients that died or suffered a cardiovascular event were also unavailable for the follow up visit, alongside patients that underwent a kidney transplant. Therefore, we can not exclude survival bias, since individuals with poor CV health were excluded. Another limitation of the study is the fact that the detection method is a multiplex assay, and results should be validated with a method specific for the FGF-2. Furthermore, the observational nature of the study and the unclear biologic function of the measured circulating FGF-2 are additional limitations.

In summary, lower FGF-2 levels are associated with atheromatosis progression in CKD patients. Further research is needed to determine whether FGF-2 could be a therapeutic target to reduce atheromatosis progression in renal patients.

Acknowledgments

This work was supported by the intramural program of the IRBLleida, the Instituto de Salud Carlos III (RETIC RD16/0009/0011, FIS PI16/01354) and FEDER funds. The authors would like to thank the NEFRONA team (Eva Castro, Virtudes María, Teresa Molí, Teresa Vidal, Meritxell Soria) and the Biobank of RedInRen for their invaluable support. The NEFRONA study investigator group is listed in the supplementary material.

REFERENCES

1. Coll, B, Betriu, A, Martinez-Alonso, M, Borras, M, Craver, L, Amoedo, ML, Marco, MP, Sarro, F, Junyent, M, Valdivielso, JM, Fernandez, E: Cardiovascular risk factors underestimate atherosclerotic burden in chronic kidney disease: usefulness of non-invasive tests in cardiovascular assessment. *NephrolDialTransplant,* 25**:** 3017-3025, 2010.
2. Balla, S, Nusair, MB, Alpert, MA: Risk factors for atherosclerosis in patients with chronic kidney disease: recognition and management. *Curr Opin Pharmacol,* 13**:** 192-199, 2013.
3. Olechnowicz-Tietz, S, Gluba, A, Paradowska, A, Banach, M, Rysz, J: The risk of atherosclerosis in patients with chronic kidney disease. *International Urology and Nephrology,* 45**:** 1605-1612, 2013.
4. Yeboah, J, Sane, DC, Crouse, JR, Herrington, DM, Bowden, DW: Low plasma levels of FGF-2 and PDGF-BB are associated with cardiovascular events in type II diabetes mellitus (diabetes heart study). *Dis Markers,* 23**:** 173-178, 2007.
5. Bozic, M, Alvarez, A, de Pablo, C, Sanchez-Nino, M-D, Ortiz, A, Dolcet, X, Encinas, M, Fernandez, E, Manuel Valdivielso, J: Impaired Vitamin D Signaling in Endothelial Cell Leads to an Enhanced Leukocyte-Endothelium Interplay: Implications for Atherosclerosis Development. *Plos One,* 10, 2015.
6. Valdivielso, JM, Coll, B, Fernandez, E: Vitamin D and the vasculature: can we teach an old drug new tricks? *Expert OpinTherTargets,* 13**:** 29-38, 2009.
7. Betriu, A, Martinez-Alonso, M, Vittoria Arcidiacono, M, Cannata-Andia, J, Pascual, J, Manuel Valdivielso, J, Fernandez, E, Study, N: Prevalence of subclinical atheromatosis and associated risk factors in chronic kidney disease: the NEFRONA study. *Nephrology Dialysis Transplantation,* 29**:** 1415-1422, 2014.
8. Bozic, M, Panizo, S, Sevilla, MA, Riera, M, Soler, MJ, Pascual, J, Lopez, I, Freixenet, M, Fernandez, E, Valdivielso, JM: High phosphate diet increases arterial blood pressure via a parathyroid hormone mediated increase of renin. *Journal of Hypertension,* 32**:** 1822-1832, 2014.
9. Martin, M, Valls, J, Betriu, A, Fernandez, E, Valdivielso, JM: Association of serum phosphorus with subclinical atherosclerosis in chronic kidney disease. Sex makes a difference. *Atherosclerosis,* 241**:** 264-270, 2015.
10. Gracia, M, Betriu, A, Martinez-Alonso, M, Arroyo, D, Abajo, M, Fernandez, E, Valdivielso, JM, Investigators, N: Predictors of Subclinical Atheromatosis Progression over 2 Years in Patients with Different Stages of CKD. *Clinical Journal of the American Society of Nephrology,* 11**:** 287-296, 2016.
11. Thompson, S, James, M, Wiebe, N, Hemmelgarn, B, Manns, B, Klarenbach, S, Tonelli, M, Network, AKD: Cause of Death in Patients with Reduced Kidney Function. *J Am Soc Nephrol,* 26**:** 2504-2511, 2015.
12. Shamseddin, MK, Parfrey, PS: Sudden cardiac death in chronic kidney disease: epidemiology and prevention. *Nature Reviews Nephrology,* 7**:** 145-154, 2011.
13. Benedetto, FA, Tripepi, G, Mallamaci, F, Zoccali, C: Rate of atherosclerotic plaque formation predicts cardiovascular events in ESRD. *Journal of the American Society of Nephrology,* 19**:** 757-763, 2008.
14. Nugent, MA, Iozzo, RV: Fibroblast growth factor-2. *Int J Biochem Cell Biol,* 32**:** 115-120, 2000.
15. Murakami, M, Nguyen, LT, Zhuang, ZW, Zhang, ZW, Moodie, KL, Carmeliet, P, Stan, RV, Simons, M: The FGF system has a key role in regulating vascular integrity. *J Clin Invest,* 118**:** 3355-3366, 2008.

16. Liu, MH, Tang, ZH, Li, GH, Qu, SL, Zhang, Y, Ren, Z, Liu, LS, Jiang, ZS: Janus-like role of fibroblast growth factor 2 in arteriosclerotic coronary artery disease: atherogenesis and angiogenesis. *Atherosclerosis,* 229**:** 10-17, 2013.
17. Barillari, G, Iovane, A, Bonuglia, M, Albonici, L, Garofano, P, Di Campli, E, Falchi, M, Condò, I, Manzari, V, Ensoli, B: Fibroblast growth factor-2 transiently activates the p53 oncosuppressor protein in human primary vascular smooth muscle cells: implications for atherogenesis. *Atherosclerosis,* 210**:** 400-406, 2010.
18. Tanaka, K, Nagata, D, Hirata, Y, Tabata, Y, Nagai, R, Sata, M: Augmented angiogenesis in adventitia promotes growth of atherosclerotic plaque in apolipoprotein E-deficient mice. *Atherosclerosis,* 215**:** 366-373, 2011.
19. Porcu, P, Emanueli, C, Desortes, E, Marongiu, GM, Piredda, F, Chao, L, Chao, J, Madeddu, P: Circulating tissue kallikrein levels correlate with severity of carotid atherosclerosis. *Arterioscler Thromb Vasc Biol,* 24**:** 1104-1110, 2004.
20. Junyent, M, Martinez, M, Borras, M, Coll, B, Valdivielso, J, Vidal, T, Sarro, F, Roig, J, Craver, L, Fernandez, E: Predicting cardiovascular disease morbidity and mortality in chronic kidney disease in Spain. The rationale and design of NEFRONA: a prospective, multicenter, observational cohort study. *Bmc Nephrology,* 11, 2010.
21. Junyent, M, Martinez, M, Borras, M, Betriu, A, Coll, B, Craver, L, Marco, MP, Sarro, F, Valdivielso, JM, Fernandez, E: Usefulness of imaging techniques and novel biomarkers in the prediction of cardiovascular risk in patients with chronic kidney disease in Spain: The NEFRONA project. *Nefrologia,* 30**:** 119-126, 2010.
22. Fernandez-Laso, V, Mendez-Barbero, N, Valdivielso, JM, Betriu, A, Fernandez, E, Egido, J, Martin-Ventura, JL, Blanco-Colio, LM: Soluble TWEAK and atheromatosis progression in patients with chronic kidney disease. *Atherosclerosis,* 260**:** 130-137, 2017.
23. Anguiano, L, Riera, M, Pascual, J, Valdivielso, JM, Barrios, C, Betriu, A, Clotet, S, Mojal, S, Fernández, E, Soler, MJ, study, iftN: Circulating angiotensin converting enzyme 2 activity as a biomarker of silent atherosclerosis in patients with chronic kidney disease. *Atherosclerosis,* 253**:** 135-143, 2016.
24. Arroyo, D, Betriu, A, Valls, J, Gorriz, JL, Pallares, V, Abajo, M, Gracia, M, Manuel Valdivielso, J, Fernandez, E, Study, IN: Factors influencing pathological ankle-brachial index values along the chronic kidney disease spectrum: the NEFRONA study. *Nephrology Dialysis Transplantation,* 32**:** 513-520, 2017.
25. Coll, B, Betriu, A, Martinez-Alonso, M, Amoedo, ML, Arcidiacono, MV, Borras, M, Valdivielso, JM, Fernandez, E: Large artery calcification on dialysis patients is located in the intima and related to atherosclerosis. *ClinJ Am SocNephrol,* 6**:** 303-310, 2011.
26. Stein, JH, Korcarz, CE, Hurst, RT: Use of carotid ultrasound to identify subclinical vascular disease and evaluate cardiovascular disease risk: A consensus statement from the American society of Echocardiography carotid intima-media thickness task force (vol 21,pg 93, 2008). *Journal of the American Society of Echocardiography,* 21**:** 376-376, 2008.
27. Touboul, PJ, Hennerici, G, Meairs, S, Adams, H, Amarenco, P, Desvarieux, M, Ebrahim, S, Fatar, M, Hernandez, RH, Kownator, S, Prati, P, Rundek, T, Taylor, A, Bornstein, N, Csiba, L, Vicaut, E, Woo, KS, Zannad, F: Mannheim intima-media thickness consensus. *Cerebrovascular Diseases,* 18**:** 346-349, 2004.
28. Tattersall, MC, Gassett, A, Korcarz, CE, Gepner, AD, Kaufman, JD, Liu, KJ, Astor, BC, Sheppard, L, Kronmal, RA, Stein, JH: Predictors of Carotid Thickness and Plaque Progression During a Decade The Multi-Ethnic Study of Atherosclerosis. *Stroke,* 45**:** 3257-3262, 2014.
29. Brogi, E, Winkles, JA, Underwood, R, Clinton, SK, Alberts, GF, Libby, P: Distinct patterns of expression of fibroblast growth factors and their receptors in human atheroma and

nonatherosclerotic arteries. Association of acidic FGF with plaque microvessels and macrophages. *J Clin Invest,* 92**:** 2408-2418, 1993.
30. Kenagy, RD, Hart, CE, Stetler-Stevenson, WG, Clowes, AW: Primate smooth muscle cell migration from aortic explants is mediated by endogenous platelet-derived growth factor and basic fibroblast growth factor acting through matrix metalloproteinases 2 and 9. *Circulation,* 96**:** 3555-3560, 1997.
31. Chan, J, Prado-Lourenco, L, Khachigian, LM, Bennett, MR, Di Bartolo, BA, Kavurma, MM: TRAIL promotes VSMC proliferation and neointima formation in a FGF-2-, Sp1 phosphorylation-, and NFkappaB-dependent manner. *Circ Res,* 106**:** 1061-1071, 2010.
32. Gomez, D, Owens, GK: Smooth muscle cell phenotypic switching in atherosclerosis. *Cardiovasc Res,* 95**:** 156-164, 2012.
33. Chen, PY, Simons, M, Friesel, R: FRS2 via fibroblast growth factor receptor 1 is required for platelet-derived growth factor receptor beta-mediated regulation of vascular smooth muscle marker gene expression. *J Biol Chem,* 284**:** 15980-15992, 2009.
34. Tsuboi, R, Sato, Y, Rifkin, DB: Correlation of cell migration, cell invasion, receptor number, proteinase production, and basic fibroblast growth factor levels in endothelial cells. *J Cell Biol,* 110**:** 511-517, 1990.
35. Matsunaga, S, Okigaki, M, Takeda, M, Matsui, A, Honsho, S, Katsume, A, Kishita, E, Che, J, Jishan, C, Kurihara, T, Adachi, Y, Mansukhani, A, Kobara, M, Matoba, S, Matoba, Y, Tatsumi, T, Matsubara, H: Endothelium-targeted overexpression of constitutively active FGF receptor induces cardioprotection in mice myocardial infarction. *J Mol Cell Cardiol,* 46**:** 663-673, 2009.
36. Six, I, Mouquet, F, Corseaux, D, Bordet, R, Letourneau, T, Vallet, B, Dosquet, CC, Dupuis, B, Jude, B, Bertrand, ME, Bauters, C, Van Belle, E: Protective effects of basic fibroblast growth factor in early atherosclerosis. *Growth Factors,* 22**:** 157-167, 2004.
37. Che, J, Okigaki, M, Takahashi, T, Katsume, A, Adachi, Y, Yamaguchi, S, Matsunaga, S, Takeda, M, Matsui, A, Kishita, E, Ikeda, K, Yamada, H, Matsubara, H: Endothelial FGF receptor signaling accelerates atherosclerosis. *Am J Physiol Heart Circ Physiol,* 300**:** H154-161, 2011.
38. Raj, T, Kanellakis, P, Pomilio, G, Jennings, G, Bobik, A, Agrotis, A: Inhibition of fibroblast growth factor receptor signaling attenuates atherosclerosis in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol,* 26**:** 1845-1851, 2006.
39. Post, MJ, Laham, RJ, Kuntz, RE, Novicki, D, Simons, M: The effect of intracoronary fibroblast growth factor-2 on restenosis after primary angioplasty or stent placement in a pig model of atherosclerosis. *Clin Cardiol,* 25**:** 271-278, 2002.
40. Lazarous, DF, Shou, M, Scheinowitz, M, Hodge, E, Thirumurti, V, Kitsiou, AN, Stiber, JA, Lobo, AD, Hunsberger, S, Guetta, E, Epstein, SE, Unger, EF: Comparative effects of basic fibroblast growth factor and vascular endothelial growth factor on coronary collateral development and the arterial response to injury. *Circulation,* 94**:** 1074-1082, 1996.
41. Zhang, C, Yang, J, Feng, J, Jennings, LK: Short-term administration of basic fibroblast growth factor enhances coronary collateral development without exacerbating atherosclerosis and balloon injury-induced vasoproliferation in atherosclerotic rabbits with acute myocardial infarction. *J Lab Clin Med,* 140**:** 119-125, 2002.
42. Rutherford, C, Martin, W, Salame, M, Carrier, M, Anggård, E, Ferns, G: Substantial inhibition of neo-intimal response to balloon injury in the rat carotid artery using a combination of antibodies to platelet-derived growth factor-BB and basic fibroblast growth factor. *Atherosclerosis,* 130**:** 45-51, 1997.
43. Shoji, T, Yonemitsu, Y, Komori, K, Tanii, M, Itoh, H, Sata, S, Shimokawa, H, Hasegawa, M, Sueishi, K, Maehara, Y: Intramuscular gene transfer of FGF-2 attenuates endothelial

dysfunction and inhibits intimal hyperplasia of vein grafts in poor-runoff limbs of rabbit. *Am J Physiol Heart Circ Physiol,* 285**:** H173-182, 2003.
44. Sigala, F, Savvari, P, Liontos, M, Sigalas, P, Pateras, IS, Papalampros, A, Basdra, EK, Kolettas, E, Papavassiliou, AG, Gorgoulis, VG: Increased expression of bFGF is associated with carotid atherosclerotic plaques instability engaging the NF-κB pathway. *J Cell Mol Med,* 14**:** 2273-2280, 2010.
45. Mollmark, JI, Park, AJ, Kim, J, Wang, TZ, Katzenell, S, Shipman, SL, Zagorchev, LG, Simons, M, Mulligan-Kehoe, MJ: Fibroblast growth factor-2 is required for vasa vasorum plexus stability in hypercholesterolemic mice. *Arterioscler Thromb Vasc Biol,* 32**:** 2644-2651, 2012.
46. Kumagai, M, Marui, A, Tabata, Y, Takeda, T, Yamamoto, M, Yonezawa, A, Tanaka, S, Yanagi, S, Ito-Ihara, T, Ikeda, T, Murayama, T, Teramukai, S, Katsura, T, Matsubara, K, Kawakami, K, Yokode, M, Shimizu, A, Sakata, R: Safety and efficacy of sustained release of basic fibroblast growth factor using gelatin hydrogel in patients with critical limb ischemia. *Heart Vessels,* 31**:** 713-721, 2016.
47. Lederman, RJ, Mendelsohn, FO, Anderson, RD, Saucedo, JF, Tenaglia, AN, Hermiller, JB, Hillegass, WB, Rocha-Singh, K, Moon, TE, Whitehouse, MJ, Annex, BH, Investigators, T: Therapeutic angiogenesis with recombinant fibroblast growth factor-2 for intermittent claudication (the TRAFFIC study): a randomised trial. *Lancet,* 359**:** 2053-2058, 2002.
48. Rigatto, C, Levin, A, House, AA, Barrett, B, Carlisle, E, Fine, A: Atheroma Progression in Chronic Kidney Disease. *Clinical Journal of the American Society of Nephrology,* 4**:** 291-298, 2009.
49. Villanueva, S, Contreras, F, Tapia, A, Carreño, JE, Vergara, C, Ewertz, E, Cespedes, C, Irarrazabal, C, Sandoval, M, Velarde, V, Vio, CP: Basic fibroblast growth factor reduces functional and structural damage in chronic kidney disease. *Am J Physiol Renal Physiol,* 306**:** F430-441, 2014.
50. Villanueva, S, Ewertz, E, Carrión, F, Tapia, A, Vergara, C, Céspedes, C, Sáez, PJ, Luz, P, Irarrázabal, C, Carreño, JE, Figueroa, F, Vio, CP: Mesenchymal stem cell injection ameliorates chronic renal failure in a rat model. *Clin Sci (Lond),* 121**:** 489-499, 2011.

Table 1. Baseline characteristics of 481 participants in the NEFRONA cohort study according to plasma fibroblast growth factor-2 concentration .

	All (n=481)	Lower tertile (3-64 pg/mL) (n=160)	Middle tertile (65-119 pg/mL) (n=160)	Higher tertile (120-865 pg/mL) (n= 161)
Age, years	61 (52-68)	61 (53-68)	61 (50-68)	61 (50-68)
Male, N (%)	295 (61)	107 (67)	94 (59)	94 (58)
History of Smoking, N (%)	283 (59)	91 (61)	88 (55)	98 (61)
Dyslipidemia, N (%)	336 (70)	107 (67)	114 (71)	115 (71)
Diabetes Mellitus, N (%)	137 (29)	43 (27)	51 (32)	43 (27)
Hypertension, N (%)	442 (92)	150 (94)	147 (92)	145 (90)
SBP, mmHg	142 (20)	142 (20)	142 (20)	141 (20)
DBP, mmHg	81 (10)	81 (11)	81 (10)	81 (11)
Total Cholesterol, mg/dL	180 (156-207)	179 (148-206)	184 (165-209)	180 (157-206)
LDL-cholesterol, mg/dL	104 (85-126)	102 (75-124)	106 (93-128)	104 (87-128)
HDL-cholesterol, mg/dL	47 (38-59)	47 (39-58)	47 (37-61)	47 (39-61)
Triglycerides, mg/dL	127 (90-171)	133 (89-171)	133 (91-180)	117 (88-164)
Glucose, mg/dL	98 (88-114)	99 (87-111)	99 (90-129)	95 (89-112)
25-hydroxyvitamin D, ng/mL	16.7 (11.9-21.4)	16.1 (12.0-21.0)	17.5 (12.2-21.9)	15.7 (11.1-21.2)
IMT, mm	0.86 (0.67-1.1)	0.86 (0.71-1.14)	0.86 (0.65-1.1)	0.84 (0.64-1.1)
Hs-CRP, mg/L	2.12 (1.13-4.52)	2.12 (1.04-4.52)	1.97 (1.15-4.36)	2.16 (1.21-4.73)
CKD stage, N(%)				
CKD3	243 (51)	58 (36)	89 (56)	96 (60)
CKD4-5	199 (41)	71 (44)	67 (42)	61 (38)
Dialysis	39 (8)	31 (20)	4 (2)	4 (2)
Plaque present at baseline, N (%)	335 (70)	115 (72)	106 (66)	114 (71)
FGF-2 (pg/mL)	85 (55-138)	43 (26-55)	85 (73-100)	164 (138-204)

Dyslipidemia: Diagnosis of dyslipidemia in the clinical history. SBP: systolic blood pressure; DBP: diastolic blood pressure; LDL: low-density lipoprotein; HDL: high-density lipoprotein; Hs-CRP: high-sensitivity C-reactive protein; IMT: average intima/media thickness. Data are median (p25-p75) for quantitative variables non-normally distributed, mean (standard deviation) for normal variables and number (percentage) for categorical variables.

Comentado [CJASN13]: Retitle to more clearly describe the study population, e.g. "Baseline characteristics of 481 participants in the NEFRONA cohort study according to plasma fibroblast growth factor-2 concentration"

Comentado [U14R13]: Done

Comentado [U16R15]: Done

Comentado [CJASN15]: Please include the range of concentration of FGF-2 for each column here in the header

Comentado [CJASN17]: Define this covariate in methods and in the table footnote, or delete it if not needed

Comentado [U18R17]: Done

Comentado [CJASN19]: Please add rows for blood pressures to the table as requested by the Associate Editor

Comentado [U20R19]: Done

Table 2. Baseline characteristics of 481 participants in the NEFRONA cohort study according to atherosclerosis progression over the subsequent 2 years .

	No (N=192)	Yes (N=289)	p (no vs. yes)
Age, years	56 (42-65)	63 (56-69)	<0.001
Male, N (%)	109 (57)	186 (64)	0.10
Ever smoker, N (%)	100 (52)	183 (63)	0.01
Dyslipidemia, N (%)	126 (66)	210 (73)	0.11
Diabetes Mellitus, N (%)	40 (21)	97 (34)	0.002
Hypertension, N (%)	171 (89)	271 (94)	0.04
SBP, mmHg	137 (18)	145 (21)	<0.001
DBP, mmHg	80 (10)	81 (11)	0.29
Total Cholesterol, mg/dL	179 (156-202)	183 (157-213)	0.31
LDL-cholesterol, mg/dL	102 (85-123)	105 (86-129)	0.39
HDL-cholesterol, mg/dL	48 (40-61)	47 (37-58)	0.15
Triglycerides, mg/dL	109 (82-163)	137 (103-176)	0.001
Glucose, mg/dL	95 (86-105)	102 (91-123)	<0.001
25-hydroxyvitamin D, ng/mL	17.2 (11.8-22.3)	16.2 (11.9-20.7)	0.54
IMT, mm	0.72 (0.59-1.01)	0.92 (0.75-1.14)	<0.001
Hs-CRP, mg/L	1.87 (1.02-3.77)	2.49 (1.23-5.17)	0.01
CKD stage, N(%)			0.36
CKD3	104 (54)	139 (48)	
CKD4-5	72 (38)	127(44)	
Dialysis	16 (8)	23 (8)	
Plaque presence at baseline, N (%)	109 (57)	226 (78)	<0.001

Dyslipidemia: Diagnosis of dyslipidemia in the clinical history. SBP: systolic blood pressure; DBP: diastolic blood pressure; LDL: low-density lipoprotein; HDL: high-density lipoprotein; hs-CRP: high-sensitivity C-reactive protein; IMT: average intima/media thickness.

Data are median (p25-p75) for quantitative variables non-normally distributed, mean (standard deviation) for normal variables and number (percentage) for categorical variables.

Comentado [CJASN21]: Retitle to more clearly describe the study population, e.g. "Baseline characteristics of 481 participants in the NEFRONA cohort study according to atherosclerosis progression over the subsequent 2 years"

See also comments from Table 1 that apply here

Comentado [U22R21]: Done

Comentado [CJASN23]: Here and below p-value reporting is not consistent with CJASN requirements ("<0.01")

Comentado [U24R23]: Done

Table 3: Associations of plasma fibroblast growth factor-2 concentration with atherosclerosis progression in the NEFRONA cohort study .Upper row: Model using levels of FGF-2 as a continuous variable. Lower rows: Model using levels of FGF-2 as tertiles

	Model 1		Model 2		Model 3	
	OR	p	OR	p	OR	p
FGF-2 (per 50 pg/ml)	0.86 (0.76-0.96)	0.008	0.85 (0.75-0.95)	0.005	0.86 (0.76-0.96)	0.02
Hosmer-Lemeshow	0.935		0.759		0.218	
FGF-2 lowest tertile	Ref.		Ref.		Ref.	
FGF-2 middle tertile	0.61 (0.37-0.99)	0.05	0.65 (0.39-1.01)	0.11	0.70 (0.40-1.20)	0.20
FGF-2 highest tertile	0.42 (0.26-0.68)	<0.001	0.43 (0.26-0.72)	0.001	0.48 (0.28-0.82)	0.008
Hosmer-Lemeshow	1.00		0.189		0.691	

Model 1: Unadjusted analysis; Model 2: Adjusted by age and sex; Model 3: model 2 plus smoking status, diabetes, dyslipidemia, plaque presence at baseline, stage of chronic kidney disease, intima-media thickness, body mass index, serum levels of glucose, total cholesterol, high sensitive C-reactive protein, 25(OH) vitamin D and phosphate. All models included 431 patients (262 progressors and 169 non-progressors). Lowest tertile of FGF-2 included 143 patients (102 progressors and 41 non-progressors). Middle tertile of FGF-2 included 143 patients (86 progressors and 47 non-progressors) and highest tertile of FGF-2 included 145 patients (74 progressors and 71 non-progressors). Ref: Reference tertile.

Comentado [CJASN25]: Please revise title to clearly include exposure, outcome, and study population, e.g. "Associations of plasma fibroblast growth factor-2 concentration with atherosclerosis progression in the NEFRONA cohort study"

Please combine tables 3.1 and 3.2 into a single table, including rows for each FGF-2 tertile and a row (derived from parallel models as already done) for FGF-2 as a continuous variable. Delete all rows for covariates, continuing to list all of the covariates included in the footnote but not showing the OR for these covariates (which is not the focus of this paper). Call out any particularly important covariate associations with atheroma progression (e.g. CKD stage) in the text.

Please add a column for number of participants with progression (169 when evaluating FGF-2 as a continuous variable, but not stated yet for each FGF-2 tertile), as requested by the Associate Editor

Comentado [U26R25]: Done. Data for number of participants has been included in the footnote.

Comentado [CJASN27]: Consider reporting for a larger increment in FGF-2, e.g. per 50 ng/mL, for the reasons you describe in your response letter

Comentado [U28R27]: Done

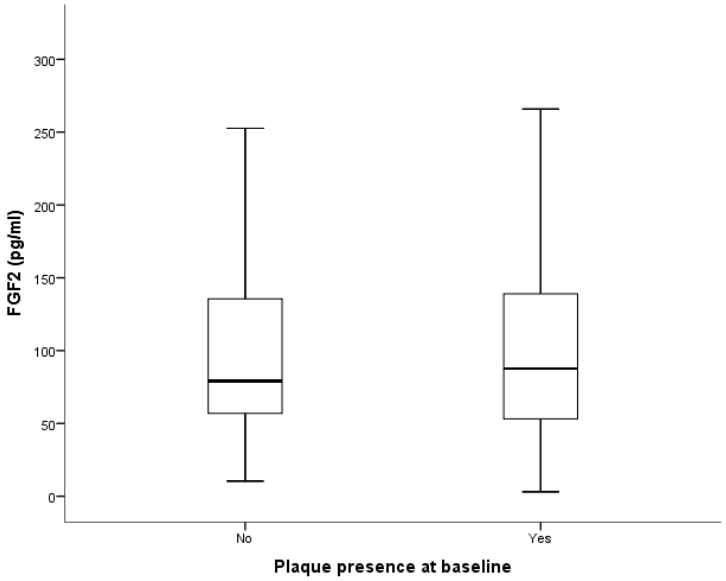
Figure 1: Plasma levels of fibroblast growth factor 2 (FGF-2) in the NEFRONA patients. **A)** Plasma levels in patients with atheromatosis vs. no atheromatosis at baseline. **B)** Plasma levels in patients with no atheromatosis, mild atheromatosis (1 to 3 territories with plaque) and severe atheromatosis (more than 3 territories with plaque. **C)** Plasma levels in patients in which atheromatosis progressed after two years vs. those in which remained stable.

Figure 2: Plasma levels of fibroblast growth factor 2 (FGF-2) in patients with different stages of chronic kidney disease (CKD).

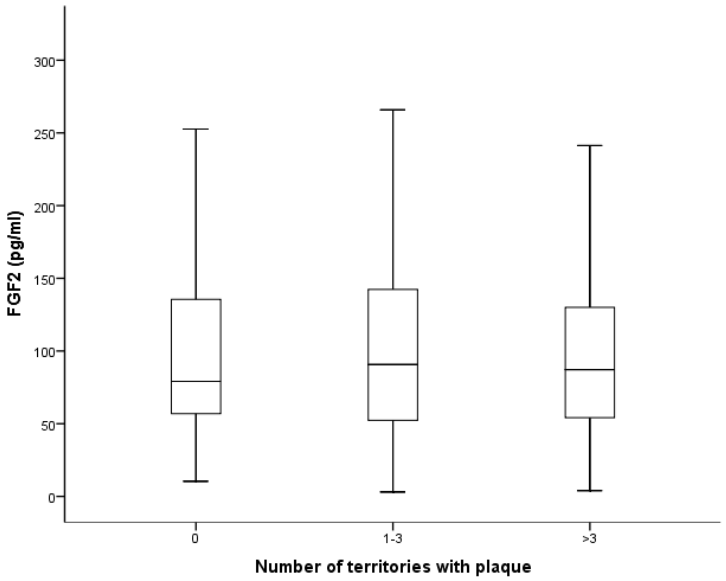
Comentado [CJASN29]: CJASN allows p-values up through 0.001. Presenting p-values as < 0.01 is not acceptable, as per CJASN guidelines. Please update the p-values in the figure appropriately

Comentado [U30R29]: Done

A


FGF2 (pg/ml)
300
250
200
150
100
50
0
No
Yes
Plaque presence at baseline

B


FGF2 (pg/ml)
300
250
200
150
100
50
0
0
1-3
>3
Number of territories with plaque

C

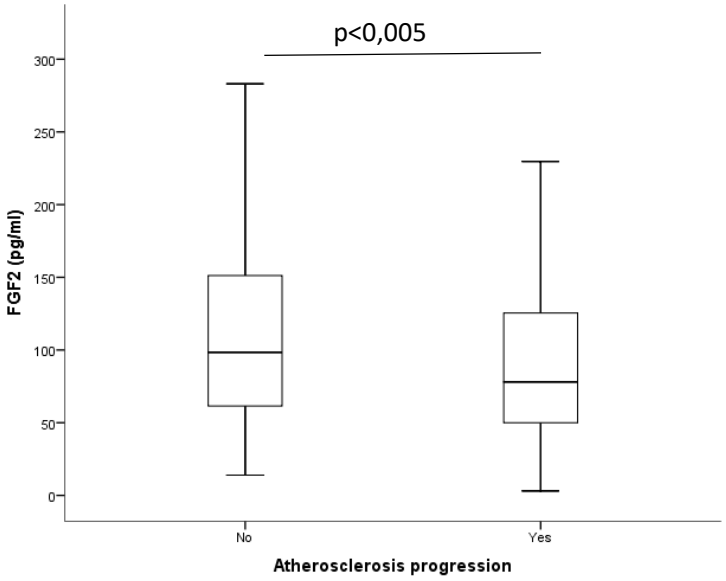

p<0,005
FGF2 (pg/ml)
300
250
200
150
100
50
0
No
Yes
Atherosclerosis progression

Figure 1

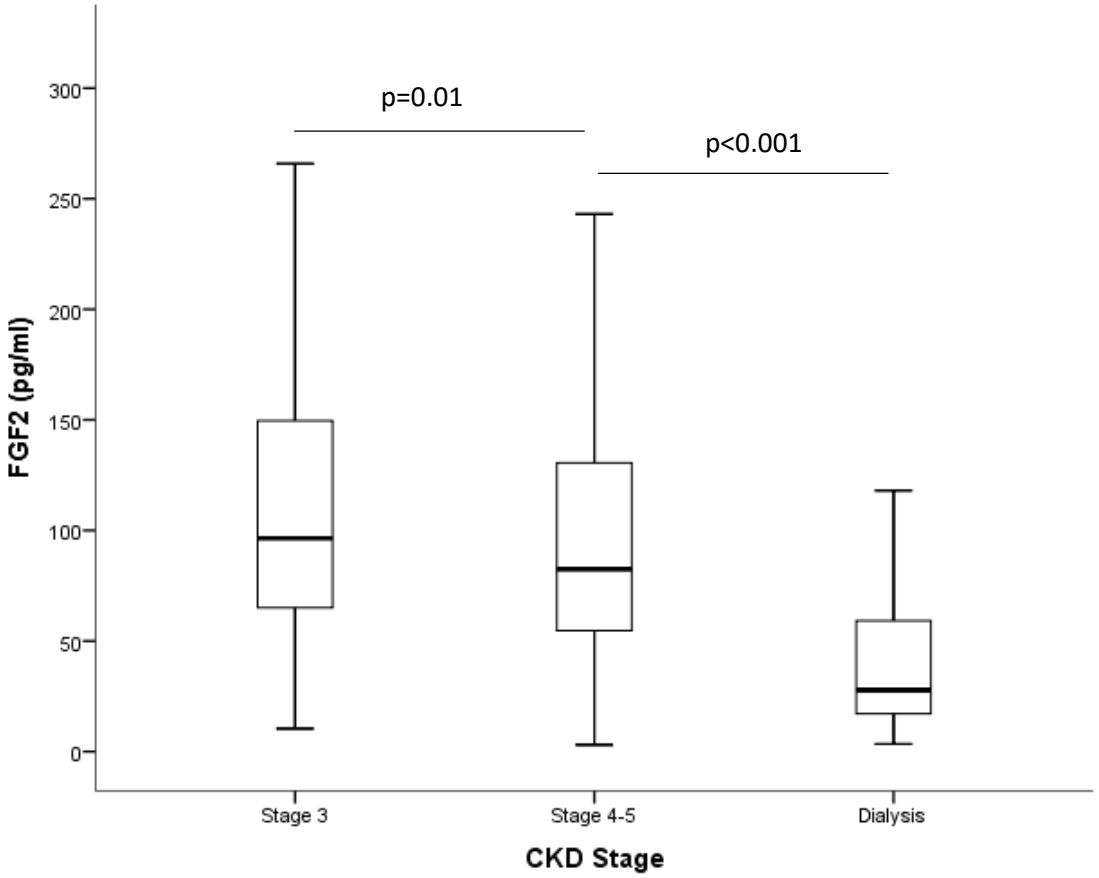

p=0.01
p<0.001
300
250
200
150
100
50
0
FGF2 (pg/ml)
Stage 3
Stage 4-5
Dialysis
CKD Stage

Figure 2