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# Circulating Receptor Activator of Nuclear factor kB Ligand and triglycerides are associated with progression of lower limb arterial calcification in Type 2 Diabetes: a prospective, observational cohort study

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# Abstract

Background:

Lower limb arterial calcification is a frequent, underestimated but serious diabetic complication. The DIACART study is a prospective cohort study designed to evaluate the determinants of lower limb arterial calcification progression in 198 patients with Type 2 Diabetes.

Methods:

Lower limb arterial calcification score was determined by computed tomography at baseline and after a mean follow up of 31+/-4 months. Serum RANKL (Receptor Activator of Nuclear factor kB Ligand) and bone remodeling, inflammatory and glycation markers were measured at baseline. The predictive effect of these markers on calcification progression was analyzed by a multivariate linear regression model.

Results:

At baseline, mean+/-SD and median lower limb arterial calcification scores were, 2364+/- 5613 and 527 respectively and at the end of the study, 3739 +/- 6886 and 1355 respectively. In multivariate analysis, progression of lower limb arterial log calcification score was associated with (β coefficient [slope], 95%CI, p-value) baseline log(calcification score) (1.02, 1.00–1.04, $p < 0.001$), triglycerides (0.11, 0.03–0.2, $p = 0.007$), log(RANKL) (0.07, 0.02–0.11, $p = 0.016$), previous ischemic cardiomyopathy (0.36, 0.15–0.57, $p = 0.001$) and duration of follow up (0.04, 0.01–0.06, 0.004).

Conclusion:

In patients with Type 2 Diabetes, lower limb arterial calcification is a frequent and major pathological process which can progress rapidly. Circulating RANKL and triglycerides are independently associated with the progression of lower limb arterial calcification. These results open new therapeutic perspectives in peripheral diabetic calcifying arteriopathy.

Trial registration: NCT02431234


# Background

Arterial calcification affects a wide variety of patients, but is particularly prevalent with ageing and in patients with diabetes or kidney failure(*1*). Lower limb arterial calcification is a highly prevalent complication of Type 2 Diabetes (up to 50%) and is independently associated with cardiovascular morbidity, renal failure and mortality(*2, 3*). This increase morbi-mortality associated with peripheral arterial calcification is probably due to consequences of vascular calcified deposits which will increase arterial wall stiffness and will induce systolic hypertension and diastolic hypotension. This, in turn, will favor the onset of hypertension and left ventricular hypertrophy and congestive heart failure. In small vessels, this increased rigidity will impair distal flow and will promote organ failure (renal, hepatic,

cerebrovascular,..). One major complication associated to lower limb arterial calcification is the increase risk of lower limb amputation. Guzman et al have shown that tibial arterial calcification is an independent risk factor of amputation in patients with diabetes (*4*). This increase risk could be explain by the association between lower limb calcification and peripheral occlusive calcification disease (*4–6*) and because arterial calcification is a risk factor of arterial revascularization failure(*7, 8*). Understanding the pathophysiology of progression of peripheral arterial calcification is important to open therapeutic perspectives.

For a long time, the mechanism of arterial calcification has been considered to be a passive process resulting in the precipitation and deposition of hydroxyapatite crystals. But vascular calcification is now considered as a complex and actively controlled molecular process, involving the differentiation of vascular smooth muscle cells and other cells into osteoblast-like cells(*9*). Several pathophysiological pathways are thought to be involved in this phenomenon of “arterial ossification”, such as dysregulation of calcium/phosphate homeostasis and imbalance between inhibitors and inducers of osteochondrogenesis (*5, 9, 10*). Among them, there is growing evidence that the OPG(osteoprotegerin)/RANKL(Receptor Activator of Nuclear factor κB ligand) signaling axis is central to lower limb arterial calcification(*11*). Especially, Ndip et al have shown that RANKL expression is colocalized in areas displaying arterial calcification and that human vascular smooth cells cultured in serum of patients with high circulating RANKL concentration showed accelerated osteoblastic differentiation and mineralization(*12*). Other pathways are also suspected such as i) matrix modification by mechanical stress and hypertension, ii) induction of calcification by lipids, inflammation, glucose, advanced glycation end-products (AGEs) and their soluble receptor (sRAGE)(*9, 13–15*). Neuropathy has also been involved in vascular calcification(*5*).

The DIACART study is the first prospective study designed to quantify lower limb arterial calcification progression in Type 2 Diabetes and to look at potential determinants of its progression. The primary objective was to determine if RANKL pathway could be associated with the progression of lower limb calcification in Type 2 Diabetes. Secondary objectives were to assess association between others proteins of bone remodeling, markers of inflammation and glycation and the progression of lower limb arterial calcification.

## Methods

# Study design:

DIACART (for “DIAbète et Calcification ARTerielle”) study is a prospective monocentric cohort study(*5*). The recruitment period extended from February to October 2014. Inclusion criteria were: Type 2 Diabetes with at least history of coronary artery disease and/or peripheral arterial occlusive disease and/or age > 50 years for men and > 60 years for women. Exclusion criteria were: (1) an estimated glomerular filtration rate (GFR) by the modification of diet in renal disease equation < 30 ml/min, (2) a history of lower limbs angioplasty and/or bypass, (3) Type 1 Diabetes. The study was approved by the local ethics committee

and registered in ClinicalTrials.gov (Identifier: NCT02431234). All patients were informed about the study objectives and procedure. Participants gave their written informed consent to participation prior to inclusion.

# Study follow-up

At the inclusion visit and after a mean follow up of 31+/-4 months, all patients had a clinical evaluation, laboratory blood tests, and helicoidal CT (computerized tomography) scan. Patient interviews focused on comorbidities and personal disease history. Patients' medical records were reviewed to check clinical information and to record concomitant treatments. Ischemic cardiomyopathy was defined as a history of myocardial infarction, acute coronary syndrome or any surgical procedures undergone for coronary artery disease.

# Assessment of peripheral neuropathy

All clinical tests were performed by a physician not informed of calcification score and laboratory results. Peripheral neuropathy was assessed by the Neuropathy Disability Score (NDS)(*16*). NDS assesses vibration sensory on the great toe using 128-Hz tuning fork, temperature sensory on dorsum of the foot using tuning fork with beaker of ice or warm water, pinprick sensory applying pin near to big toe nail and Achilles reflex. Each sensory test scores 0 for normal and 1 for abnormal sensation, for each foot. Achilles reflex score 0 if they are present, 1 if they are present with reinforcement and 2 if they are absent, for each foot. NDS $\geq$ 6 allows the diagnosis of diabetic peripheral neuropathy.

# Laboratory evaluations

Blood and urine samples were collected after an overnight fast for the measurement of routine biochemistry tests, glucose, glycated hemoglobin (HbA1c), cholesterol (total and HDL), triglycerides, high-sensitivity C-reactive protein (hsCRP), calcium, phosphate, albuminuria and creatininuria.

Total OPG was measured by ELISA (ELISA MicroVue; Quidel Corporation), and soluble RANKL (sRANKL) concentration was evaluated using the human RANKL Single Plex kit from Millipore (Ref: HBN51K1RANKL; eBioscienceo). The minimum detectable concentration of sRANKL is 4.8 pg/mL and inter- and intra-assay precision is below 6% coefficient of variation with this kit. The OPG to sRANKL concentration ratio was calculated for each individual patient without any postanalytical modifications.

Circulating total adiponectin concentrations were measured in serum using an enzyme-linked immunosorbent assay kit (ALPCO, Eurobio, Paris, France) as recommended by the manufacturer. The lower detection limit was 0.4 µg/mL for total adiponectin. Inter-assay coefficients of variation of low and high human pool controls were 7.93% and 8.46% for total adiponectin, respectively.

Selected assays (including desphospho–uncarboxylated MGP (dp-ucMGP) and total-uncarboxylated MGP (t-ucMGP) assays) were performed after the samples had been frozen, stored at -80 °C and thawed. A dual-antibody ELISA was used to measure dp-ucMGP levels; the capture antibody was directed against the non-phosphorylated MGP sequence 3–15 (mAb-dpMGP; VitaK BV, Maastricht, The Netherlands) and

the detecting antibody was directed against the uncarboxylated MGP sequence 35–49 (mAb-ucMGP; VitaK BV). The same antibodies have already been used for immunohistochemical staining(*17, 18*). Intra-assay variability was 5,6% for dp-ucMGP and 8,9% for t-ucMGP, when inter-assay variability was 9,9% for dp-ucMGP and 11,4% for t-ucMGP. A competitive (single-antibody) ELISA was used to measure t-ucMGP levels, as described previously(*19, 20*).

Soluble RAGE (sRAGE) was measured on plasma samples with a commercially available ELISA kit (Quantikine Human RAGE Immunoassay, R&D Systems, Minneapolis, USA; see http://www.rndsystems.com/Products/DRG00 for detailed description of the measurement method).

Advanced Glycation End products (Carboxymethyllysine (CML), Methylglyoxal-derived hydroimidazolone1 (MG-H1) and pentosidine) were determined on serum samples by liquid chromatography coupled with tandem mass spectrometry (API4000 system ABSciex, Les Ulis, France)(*21*). AGEs concentrations were expressed as a ratio to total protein concentrations.

Total cholesterol and triglyceride concentrations were determined by an automated enzymatic method (Konelab, Thermoclinical Labsystems, Cergy Pontoise, France and Biomerieux, Marcy L'Etoile, France, respectively). HDL-cholesterol was determined by a direct method (Konelab). LDL-cholesterol was calculated using Friedewald's equation at triglycerides $\leq$ 3.9 mmol/L or directly measured when triglycerides > 3.9 mmol/L using a Konelab kit. Inter- and intra-assay coefficients of variation were 2.2% and 0.9%, 1.3% and 0.9%, 3.5% and 0.97%, 1.3% and 0.9%, respectively for total cholesterol, HDL-cholesterol, triglycerides and LDL-cholesterol, respectively.

Serum human Fetuin A was evaluated using ELISA kit (TECOmedical Group, France). Measurements of serum Fetuin A was performed according to instructions provided by Epitope Diagnostics (intra-assay coefficient of variation: <5.5%, inter-assay coefficient of variation: <6.8%; detection limit of the assay: 5.0 ng/mL).

Serum human C-terminal FGF23 (Fibroblast Growth Factor 23) was evaluated using ELISA kit (TECOmedical Group, France). Measurements of serum C-terminal human FGF23 was performed according to instructions provided by Immutopics (intra-assay coefficient of variation: <2.4%, inter-assay coefficient of variation: <4.7%; detection limit of the assay: 1.5 RU/mL).

IL-6 (Interleukine-6) was quantified using automated assays with Access 2 (Beckman Coulter Inc, Villepinte, France) according to the manufacturer's instructions automated. IGF-1 (Insulin like Growth Factor 1), 25-hydroxyvitamin D, intact parathyroid hormone (iPTH) were measured by a single step chemiluminescence sandwich method on the Liaison XL (DiaSorin) analyzer. Because IGF-1 decreases with age, a standardized IGF-1 score was calculated as previously described [IGF-1 score = (log [IGF-1 (micrograms per liter)] + 0.00625 × age − 2.555)/0.104](*22*).

# Assessment of insulin resistance

Insulin resistance was evaluated by the TyG index (triglycerides glucose index), which was calculated as ln(fasting triglycerides [mg/dL]*fasting glucose [mg/dL]/2)(*23*).

# Imaging for below-knee arterial calcification score.

Below-knee artery calcification score was obtained after scanning with a 128-slice multidetector dual source CT-scanner (Somatom Definition Flash, Siemens Healthcare, Erlangen, Germany) without contrast, from the base of the patella down to the ankle. Three mm cross-sectional slices were analyzed. Analysis was performed by radiologists who were blinded to the results of color duplex ultrasonography, laboratory tests or clinical examination, using a commercially available software package (Heartbeat CaScore, Philips Healthcare, Eindhoven, Netherlands). On cross-sectional images, areas of calcification along below-knee arteries with a density ≥ 130 Hounsfield units attenuation and a surface > 1mm2 were identified semi automatically. Calcification score, determined according to the method described by Agatston et al., was obtained separately for each of the main below-knee arteries (distal popliteal, anterior tibial, posterior tibial and peroneal arteries) and summed to obtain the total calcification score(*24*). Below-knee artery calcification score was performed at the inclusion visit and at the end of the study(*5*).

# Statistical analysis

Quantitative variables were described by their mean, standard deviation, median and interquartile range. Qualitative variables were described by their frequency and percentage. The effect of baseline serum RANKL on the progression of calcification of leg arteries at the end of the follow-up was analyzed using univariable and multivariable linear regression model with arterial calcification score at the end of study as the response variable. Distributions of biological parameters were checked graphically and those with a log-normal distribution were subsequently transformed before any analysis to improve normality. This model was adjusted on baseline cardiovascular risk factors and other factors known to be associated with vascular calcification (age, gender, tobacco use, hypertension, waist circumference, BMI, triglycerides, total cholesterol, HDL-cholesterol, LDL-cholesterol, ApoA1, urinary albumin-creatinine ratio, HbA1c, lower limb log calcification score at baseline, hsCRP, parathormone, glomerular filtration rate (MDRD), and duration of follow up). Cook distance was used for the detection of highly influential observations on the coefficient estimates. A backward stepwise variable selection procedure based on the Akaike Information Criterion was used to select the final multivariate model. Coefficient estimates were provided with their corresponding 95% confidence intervals.

The effects of other bone remodeling factors, markers of inflammation and glycation (Calcium, iPTH, OPG, MGP, IGF-1, Fetuin A, FGF-23, hsCRP, IL-6, carboxyméthyllysine, MG-H1, pentosidine, RAGE) were evaluated with multivariate linear regression model using the same procedure.

Significance was defined as p-values less than 0.05. Statistical analyses were performed using R 3.5.1 (http://www.R-project.org).

# Sample size

It was assumed that the correlation between the logarithm of the serum RANKL level and the logarithm of the artery calcification score at 2 years, adjusted on the covariates, is about 0.20 (or an $R^2$ of 0.04). In addition, it was assumed that the multivariate linear model (based on RANKL and associated risk factors) will explain about 15% of the variability. Under these assumptions, at least 169 patients were needed to demonstrate a significant effect of RANKL on the calcification score with an alpha risk of 5% (bilateral formulation) and a power of 80%. In order to take into account 15% of patients lost to follow-up, 198 subjects were planned.

# Data and Resource Availability:

All data generated or analyzed during this study are included in the published article. No applicable resources were generated or analyzed during the current study.

## Results

At baseline, a total of 198 patients were included in the DIACART study. During the study (31.2 +/-3.86 months), 18 patients were lost to follow up and 11 patients died before the second determination of calcification score. RANKL measurement was missing in 6 patients. This led to 163 evaluable patients.

# Patients' characteristics:

Participants' characteristics at baseline (n = 163) are presented in Table 1. At baseline, patients were predominantly middle aged (mean age 64+/- 8.3 years) overweight (mean BMI 29+/- 5.3 $Kg/m^2$) male (80%), with a long duration of diabetes (mean duration 14.7 +/- 10.4 years), and a relatively good glycemic control (mean HbA1c 7.6 +/- 1.4% ; 60 +/- 4 mmol/mol). Less than 20% of the patients had laserized retinopathy or neuropathy. Nearly a third of patients had nephropathy but GFR (mean GFR 77+/- 19 mL/mn) was relatively preserved as expected since GFR < 30 mL/mn was an exclusion criterion. Most of the patients had ischemic cardiomyopathy (69%). Most of the patients were smokers (60%) and treated for hypertension (80%) with arterial pressure at target (126 +/- 17 mmHg). They often took statins (90%), antiplatelet therapies (83%), β-blockers (63%) and renin-angiotensin system inhibitors (79%). Diabetes was often treated with metformin, insulin and sulfonylurea (respectively 79%, 57% and 46%).

Table 1
Baseline characteristics of the patients (n = 163)

| ***Characteristics at baseline (n = 163)*** | | |
|---|---|---|
| *Age (year)* | Med [IQR] | 65 [58–70] |
| | Moy (SD) | 64.17 (8.25) |
| *Sex n (%)* | female | 33 (20.25%) |
| | male | 130 (79.75%) |
| *BMI (Kg/m²)* | Med [IQR] | 28.39 [25.44;31.77] |
| | Moy (std) | 29.07 (5.34) |
| *Waist circumference (cm)* | Med [IQR] | 101 [93;110] |
| | Moy (std) | 101.33 (13.58) |
| *Smoking habit n (%)* | Active or past | 97(59.5) |
| | never | 66(40.5) |
| *Diabetes duration (year)* | Med [IQR] | 12 [6–20] |
| | Moy (std) | 14.66 (10.38) |
| *Glycemia (mmol/l)* | Med [IQR] | 7.7 [6.2;9.25] |
| | Moy (std) | 8.06 (2.72) |
| *HbA1c %* (mmol/mol) | Med [IQR] | 7.4 [6.9;8.25]<br>(57 [52;67]) |
| | Moy (std) | 7.64 (1.36)<br>(60 (4.08)) |
| *Ischemic cardiomyopathy n (%)* | *Yes* | 117 (69.23%) |

Data are given as mean ± SD for normally distributed measures with addition of (median) for non-normally distributed values for variables with a non-Gaussian distribution or as the number (percentage) for binary variables.

ARB angiotensin receptor blockers, ACE angiotensin converting enzyme, BMI body mass index, CVD cardiovascular disease, DBP diastolic blood pressure, FGF-23 fibroblast growth factor 23, GFR MDRD glomerular filtration rate calculated with the modification of diet in renal disease formula, HbA1c haemoglobin A1C, HDL high density lipoprotein, hsCRP high sensitivity C-reactive protein, IGF-1 insulin growth factor-1, IL-6 interleukin 6, iPTH intact parathyroid hormone, LDL low density lipoprotein, MG-H1 Methylglyoxal-derived hydroimidazolone1, dp-ucMGP desphospho-uncarboxylated matrix gla protein, t-ucMGP Total uncarboxylated matrix gla protein, NDS neuropathy disability score, OPG osteoprotegerin, SBP systolic blood pressure, sRAGE soluble form of receptor for advanced glycation end products, sRANKL soluble form of Receptor Activator of Nuclear factor kB Ligand, TyG triglyceride glucose index.

| *Characteristics at baseline (n = 163)* | | |
|---|---|---|
| | *No* | 52 (30.77%) |
| *Calcification score* | Med [IQR] | 526.73 [55.25-2253.41] |
| | Moy (std) | 2363.87 (5612.52) |
| *NDS ≥ 6* | *Yes* | 25 (15.34%) |
| | *No* | 138 (84.66%) |
| *Laserised retinopathy n (%)* | *Yes* | 26 (15.95%) |
| | *No* | 137 (84.05%) |
| *GFR MDRD (mL/mn)* | Med [IQR] | 77 [63.5;89] |
| | Moy (std) | 76.79 (18.84) |
| *Microalbuminuria (mg/l)* | Med [IQR] | 20 [8.55;66.95] |
| | Moy (std) | 169.46 (917.75) |
| *urinary albumin-creatinine ratio > 3 mg/mmol n(%)* | *Yes* | 53(32.52) |
| | *No* | 110(67.48) |
| *Hypertension n (%)* | *Yes* | 131(80.4) |
| | *No* | 32(19.6) |
| *Triglycerides (mmol/l)* | Med [IQR] | 1.25 [0.91;2] |
| | Moy (std) | 1.59 (1.07) |
| *Total Cholesterol (mmol/l)* | Med [IQR] | 3.52 [2.7;4.31] |
| | Moy (std) | 3.68 (1.23) |
| *HDL Cholesterol (mmol/l)* | Med [IQR] | 1.06 [0.88;1.27] |
| | Moy (std) | 1.11 (0.34) |

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| *Characteristics at baseline (n = 163)* | | |
|---|---|---|
| *LDL Cholesterol (mmol/l)* | Med [IQR] | 1.84 [1.46;2.35] |
| | Moy (std) | 1.99 (0.75) |
| *SBP (mmHg)* | Med [IQR] | 124 [115;135] |
| | Moy (std) | 125.78 (16.46) |
| *DBP (mmHg)* | Med [IQR] | 72 [67;78] |
| | Moy (std) | 72.98 (8.69) |
| hsCRP (mg/l) | Med [IQR] | 1.2 [0.5;2.6] |
| | Moy (std) | 2.18 (2.5) |
| *IL-6 (pg/ml)* | Med [IQR] | 2.8 [2.1;4] |
| | Moy (std) | 3.64 (3.75) |
| *Adiponectine* | Med [IQR] | 3.5 [2.7;5.05] |
| | Moy (std) | 4.19 (2.61) |
| *TyG index* | Med [IQR] | 8.99 [8.53;9.49] |
| | Moy (std) | 9.01 (0.72) |
| *IGF-1(ng/ml)* | Med [IQR] | 139[105;170] |
| | Moy (std) | 139.13 (46.85) |
| *Standardized IGF-1(ng/ml)* | Med [IQR] | -0.12 (-1.21-0.8) |
| | Moy (std) | -0.35 (1.54) |
| *MG-H1 (µmol/g prot)* | Med [IQR] | 2.79 [2.48;3.06] |
| | Moy (std) | 2.93 (1.32) |
| *Pentosidine (nmol/g prot)* | Med [IQR] | 1.1 [0.84;1.42] |

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| *Characteristics at baseline (n = 163)* | | |
|---|---|---|
| | Moy (std) | 1.21 (0.59) |
| *Carboxyméthyllysine* | Med [IQR] | 0.14 [0.13;0.16] |
| | Moy (std) | 0.15 (0.03) |
| *(µmol/g prot)* | | |
| *sRAGE (pg/ml)* | Med [IQR] | 828.77 [582.61;1181.96] |
| | Moy (std) | 948.17 (535.68) |
| *Corrected calcium (mmol/l)* | Med [IQR] | 2.31 [2.24;2.38] |
| | Moy (std) | 2.32 (0.11) |
| *Phosphorus (mmol/l)* | Med [IQR] | 1.01 [0.91;1.13] |
| | Moy (std) | 1.01 (0.15) |
| *iPTH (pg/ml)* | Med [IQR] | 47.05 [36.75;64.97] |
| | Moy (std) | 54.54 (26.55) |
| *sRankl (pmol/l)* | Med [IQR] | 8.06 [2.59;16.8] |

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| *Characteristics at baseline (n = 163)* | | |
|---|---|---|
| | Moy (std) | 15.04 (21.94) |
| *OPG (pmol/l)* | Med [IQR] | 6.06 [5.26;7.13] |
| | Moy (std) | 6.49 (1.89) |
| *OPG/RANKL* | Med [IQR] | 0.73 [0.33–2.25] |
| | Moy (std) | 9.97 (23.89) |
| *Fetuin A (g/l)* | Med [IQR] | 0.65 [0.51;1.13] |
| | Moy (std) | 0.83 (0.45) |
| *FGF-23 (UI/ml)* | Med [IQR] | 20.87 [11.65;34.07] |
| | Moy (std) | 27.57 (30.38) |
| *t-ucMGP* | Med [IQR] | 4733 [3680–5490] |
| | Moy (std) | 4801.2 (1634.04) |
| *Dp-ucMGP* | Med [IQR] | 561 [339–761] |
| | Moy (std) | 643.73 (487.16) |
| *Insulin use n (%)* | *Yes* | 75(46) |
| | *No* | 88(54) |
| *Metformin use n (%)* | *Yes* | 130(79.75) |
| | *No* | 33(20.25) |
| *Sulfonylurea use n (%)* | *Yes* | 71 (43.6) |
| | *No* | 92 (56.4) |
| *Statin use n (%)* | *Yes* | 146(89.6) |
| | *No* | 17(10.4) |

Data are given as mean ± SD for normally distributed measures with addition of (median) for non-normally distributed values for variables with a non-Gaussian distribution or as the number (percentage) for binary variables.

ARB angiotensin receptor blockers, ACE angiotensin converting enzyme, BMI body mass index, CVD cardiovascular disease, DBP diastolic blood pressure, FGF-23 fibroblast growth factor 23, GFR MDRD glomerular filtration rate calculated with the modification of diet in renal disease formula, HbA1c haemoglobin A1C, HDL high density lipoprotein, hsCRP high sensitivity C-reactive protein, IGF-1 insulin growth factor-1, IL-6 interleukin 6, iPTH intact parathyroid hormone, LDL low density lipoprotein, MG-H1 Methylglyoxal-derived hydroimidazolone1, dp-ucMGP desphospho-uncarboxylated matrix gla protein, t-ucMGP Total uncarboxylated matrix gla protein, NDS neuropathy disability score, OPG osteoprotegerin, SBP systolic blood pressure, sRAGE soluble form of receptor for advanced glycation end products, sRANKL soluble form of Receptor Activator of Nuclear factor kB Ligand, TyG triglyceride glucose index.

| *Characteristics at baseline (n = 163)* | | |
|---|---|---|
| *Antiplatelet use n (%)* | *Yes* | 136(83.4) |
| | *No* | 27(16.6) |
| *ARB and ACE inhibitors use n (%)* | *Yes* | 128(78.5) |
| | *No* | 35(21.5) |
| *Beta-blocker use n (%)* | *Yes* | 102(62.6) |
| | *No* | 61(37.4) |

Data are given as mean ± SD for normally distributed measures with addition of (median) for non-normally distributed values for variables with a non-Gaussian distribution or as the number (percentage) for binary variables.

ARB angiotensin receptor blockers, ACE angiotensin converting enzyme, BMI body mass index, CVD cardiovascular disease, DBP diastolic blood pressure, FGF-23 fibroblast growth factor 23, GFR MDRD glomerular filtration rate calculated with the modification of diet in renal disease formula, HbA1c haemoglobin A1C, HDL high density lipoprotein, hsCRP high sensitivity C-reactive protein, IGF-1 insulin growth factor-1, IL-6 interleukin 6, iPTH intact parathyroid hormone, LDL low density lipoprotein, MG-H1 Methylglyoxal-derived hydroimidazolone1, dp-ucMGP desphospho-uncarboxylated matrix gla protein, t-ucMGP Total uncarboxylated matrix gla protein, NDS neuropathy disability score, OPG osteoprotegerin, SBP systolic blood pressure, sRAGE soluble form of receptor for advanced glycation end products, sRANKL soluble form of Receptor Activator of Nuclear factor kB Ligand, TyG triglyceride glucose index.

# Progression of lower limb calcification and independent predictors:

At baseline, mean +/- SD and median lower limb arterial calcification scores were 2364+/- 5613 and 527 respectively. At the end of the study, mean +/- SD and median lower limb calcification scores were 3739 +/- 6886 and 1355 respectively.

In univariate analysis (cf Table 2), lower limb log calcification score at the end of follow-up is associated with (β coefficient [slope], 95% CI, p-value) baseline log calcification score (0.88, 0.81–0.95, $p < 0.001$), age (0.15, 0.06–0.25, $p < 0.001$), male sex (3.51, 1.64–5.38, $p < 0.001$), tobacco use (for patients without tobacco consumption, -1.69; -3.27– -0.12, $p = 0.0035$), ischemic cardiomyopathy (3.73, 2.15–5.32, $p < 0.001$), MDRD (-0.06, -0.1– -0.02, $p = 0.005$), t-ucMGP (-2.74, -4.85– -0.64, $p = 0.011$), dp-ucMGP (1.61, 0.53–2.7, $p = 0.004$), Fetuin A (-1.82, -3.36– -0.29, $p = 0.02$), pentosidine (2.16, 0.35–3.96, $p = 0.02$), CML (1.61, 0.53–2.7, $p = 0.004$), MG-H1 (-3.15, -5.79– -0.51, $p = 0.020$) and iPTH (0.04, 0.01–0.07, $p = 0.012$). Peripheral neuropathy (defined by NDS ≥ 6) is not associated with lower limb arterial calcification progression (0.15, -0.86–1.17, $p = 0.765$). HbA1c, calcemia, phosphoremia, RAGE, Fetuin A, FGF-23, IGF-1, LDL-cholesterol, HDL-cholesterol, total cholesterol, blood pressure, BMI, hsCRP and IL-6 are not either associated with lower limb arterial calcification progression.

Table 2
Univariate linear regression analysis: variables associated with calcification score at the end of follow-up (n = 163 patients; mean follow-up duration = 31.2 +/-3.86 months )

| | β coefficient [slope] | IC95 | p.value |
|---|---|---|---|
| Log(calcium score at baseline) | 0.88 | [0.81;0.95] | **< 0.001** |
| Follow-up duration | -0.07 | [-0.27;0.13] | 0.486 |
| Age | 0.15 | [0.06;0.25] | **0.001** |
| Sex (male) | 3.51 | [1.64–5.38] | **< 0.001** |
| Smoking habits (never) | -1.69 | [-3.27- -0.12] | **0.035** |
| Hypertension (absence) | -0.75 | [-2.72-1.22] | 0.453 |
| BMI | -0.05 | [-0.2-0.09] | 0.480 |
| Waist circumference | 0 | [-0.02-0.03] | 0.753 |
| HbA1c | 0.05 | [-0.53-0.62] | 0.874 |
| NDS ≥ 6 | 0.15 | [-0.86-1.17] | 0.765 |
| Ischemic cardiomyopathy | 3.73 | [2.15–5.32] | **< 0.001** |
| hsCRP | -0.16 | [-0.47-0.16] | 0.322 |
| Log IL-6 | 1.05 | [-0.22-2.32] | 0.104 |
| Triglycerides | 0.43 | [-0.3-1.16] | 0.25 |
| Cholesterol total | -0.51- | [-1.39-0.37] | 0.25 |
| HDL | -2.01 | [-4.27-0.25] | 0.08 |
| LDL | 0.56 | [-1.6-0.48] | 0.29 |
| Log(sRANKL) | 0.04 | [-0.39-0.46] | 0.857 |
| Log (OPG) | 1.84 | [-1.14-4.83] | 0.225 |
| Log( OPG/sRANKL) | 0 | [-0.41-0.41] | 0.990 |
| iPTH | 0.04 | [0.01–0.07] | **0.012** |

P-values indicating significance are shown in bold type.

BMI body mass index, DBP diastolic blood pressure, FGF-23 fibroblast growth factor 23, GFR MDRD glomerular filtration rate calculated with the modification of diet in renal disease formula, HbA1c haemoglobin A1C, hsCRP high sensitivity C-reactive protein, IGF-1 insulin growth factor-1, IL-6 interleukin 6, iPTH intact parathyroid hormone, MG-H1 Methylglyoxal-derived hydroimidazolone1, dp-ucMGP desphospho-uncarboxylated matrix gla protein, t-ucMGP Total uncarboxylated matrix gla protein, NDS neuropathy disability score, OPG osteoprotegerin, sRAGE soluble form of receptor for advanced glycation end products, sRANKL soluble form of Receptor Activator of Nuclear factor kB Ligand, TyG triglyceride glucose index.

| | β coefficient [slope] | IC95 | p.value |
|---|---|---|---|
| Log (Corrected calcium) | -9.99 | [-26-6.02] | 0.220 |
| Log (t-ucMGP) | -2.74 | [-4.85- -0.64] | **0.011** |
| Log (dp-ucMGP) | 1.61 | [0.53–2.7] | **0.004** |
| Log( FGF-23) | 0.56 | [-0.03-1.16] | 0.064 |
| Log( Fetuine A) | -1.82 | [-3.36- -0.29] | **0.020** |
| Log (Pentosidine) | 2.16 | [0.35–3.96] | **0.020** |
| Log(Carboxymethylysine) | 1.3 | [-2.67-5.27] | 0.519 |
| Log (MG-H1) | -3.15 | [-5.79 - -0.51] | **0.020** |
| Log (sRAGE) | 0.69 | [-0.79-2.16] | 0.361 |
| Log (IGF-1) | -2.06 | [-4.15-0.02] | 0.052 |
| Standardized IGF-1 | -0.24 | [-0.75-0.27] | 0.363 |
| Log adiponectin | 1.59 | [0.12–3.06] | 0.034 |
| TyG INDEX | 0.02 | [-0.02-0.06] | 0.251 |

P-values indicating significance are shown in bold type.

BMI body mass index, DBP diastolic blood pressure, FGF-23 fibroblast growth factor 23, GFR MDRD glomerular filtration rate calculated with the modification of diet in renal disease formula, HbA1c haemoglobin A1C, hsCRP high sensitivity C-reactive protein, IGF-1 insulin growth factor-1, IL-6 interleukin 6, iPTH intact parathyroid hormone, MG-H1 Methylglyoxal-derived hydroimidazolone1, dp-ucMGP desphospho-uncarboxylated matrix gla protein, t-ucMGP Total uncarboxylated matrix gla protein, NDS neuropathy disability score, OPG osteoprotegerin, sRAGE soluble form of receptor for advanced glycation end products, sRANKL soluble form of Receptor Activator of Nuclear factor kB Ligand, TyG triglyceride glucose index.

In multivariate analysis (cf Table 3), and after exclusion of highly influential observations, progression of lower limb arterial calcification is associated with (β coefficient [slope], 95% CI, p-value) baseline log calcification score (1.02, 1.00–1.04, $p < 0.001$), duration of follow up (0.04, 0.01–0.06, $p = 0.004$), ischemic cardiomyopathy (0.36, 0.15–0.57, $p = 0.001$), triglycerides (0.11, 0.03–0.2, $p = 0.007$) and log RANKL (0.07, 0.02–0.11, $p = 0.016$). Similarly, we analyzed the predictive power of the others biomarkers. Log(OPG/RANKL ratio) is also associated with lower limb arterial calcification progression (-0.06, -0.12–0, $p = 0.03$). However, OPG alone (-0.15, -0.52–0.23, $p = 0.409$) is not associated with lower limb arterial calcification progression. Other markers potentially involved in arterial calcification pathophysiology are not associated with lower limb arterial calcification progression in the DIACART study.

Table 3
Final multivariate linear regression model: variables independently associated with the progression of calcification score during follow-up (n = 163 patients ; mean follow up duration = 31.2 +/-3.86 months )

| | β | IC95 | p.value |
|---|---|---|---|
| Log(calcium score at baseline) | 1.02 | [1-1.04] | **< 0.001** |
| Follow-up duration | 0.04 | [0.01;0.06] | **0.004** |
| Ischemic cardiomyopathy | 0.36 | [0.15;0.57] | **0.001** |
| Log(sRANKL) | 0.07 | [0.02;0.11] | **0.016** |
| Log( OPG/sRANKL) | -0.06 | [-0.12-0] | **0.03** |
| Triglycerides | 0.11 | [0.03;0.2] | **0.007** |
| P-values indicating significance are shown in bold type. | | | |
| OPG osteoprotegerin, sRANKL soluble form of Receptor Activator of Nuclear factor kB Ligand. | | | |

# Discussions

To our knowledge, the DIACART study is the first prospective study conducted in patients with Type 2 Diabetes, designed to better understand the clinical and biological factors involved in lower limb arterial calcification progression. We show in this study that the prevalence of arterial calcification of the lower limbs in type 2 diabetic patients with high cardiovascular risk is high but also that it can progress significantly. Our results highlight the putative role of RANK/RANKL pathway and circulating triglycerides in this phenomenon. We confirm also that calcification progression is correlated with other well-known risk factors such as the severity of pre-existing calcification, duration of follow-up and history of ischemic cardiomyopathy. In a previous cross-sectional study from the DIACART cohort, we don't found association between serum RANKL and circulating triglycerides with lower limb calcification score(*5*). The longitudinal design of the present study could explain the differences observed with cross-sectionnal analysis performed at baseline. Indeed, vascular calcification is a dynamic disease, which depends in part on calcium score at baseline and on the duration of follow-up, but other factors take also an important place.

Our results suggest that one emerging pathway in arterial calcification pathophysiology is the RANKL/RANK/OPG system. RANKL, which is produced by osteoblasts, vascular cells, stromal cells, T-cells, macrophages and monocytes, binds to its receptor RANK which is expressed by osteoclasts but also by vascular smooth muscular cells (VSMCs). This binding activates several downstream targets such as the Nuclear Factor-kappa B. In contrast, OPG acts as a decoy receptor blocking the RANKL-RANK interaction(*25*). RANKL actively promotes vascular calcification by inducing, via its receptor RANK, differentiation of VSMCs into osteoblast-like cells(*12, 26*). OPG is also suspected to be involved in the regulation of vascular calcification process. Indeed, OPG-deficient mice develop accelerated arterial

calcification whereas inactivation of RANKL signaling in these mice counteracts this effect(*27*). This suggests that RANKL plays a central role in the regulation of vascular calcification, whereas OPG could play rather a modulatory function. In human, both tissue and serum RANKL have been associated with lower limb artery and carotid calcification(*12, 28*). The RANKL/OPG ratio has been also correlated with coronary artery calcium score(*29*). In the DIACART study, both circulating RANKL and RANKL/OPG ratio, but not serum OPG, are correlated with progression of lower limb arterial calcification. Thus when circulating RANKL concentration increases, it would be associated with an acceleration of the arterial calcification process.

This association suggests that it could be interesting to develop strategies targeting RANKL to prevent lower limb arterial calcification in diabetic patients. Denosumab is a human monoclonal antibody which inhibits RANKL pathway. It is one of the latest therapeutic options for osteoporosis(*30*). It has been shown that Denosumab attenuates aortic calcification in a murine model(*31*). In human, one study has explored the effect of Denosumab on aortic calcification in a large cohort of postmenopausal women followed for 3 years. There was no influence of this treatment on aortic calcification and cardiovascular events, but the choice of the population (at very low risk of arterial calcification) could explain these negative results(*32*). Another study has prospectively explored the effect of 12 months of Denosumab treatment on coronary artery calcium scores in 48 patients on hemodialysis(*33*). Interestingly, the coronary artery calcium score of patients treated with Denosumab did not increase, although the patients had a very high coronary artery calcium score at baseline. These data suggest that Denosumab could stop the progression of vascular calcification in patients highly exposed to this risk. Therefore, interventional studies are needed to demonstrate that targeting RANKL may be beneficial on peripheral arterial calcification in Type 2 Diabetes.

Our study also highlights, for the first time, the association between plasma triglycerides and the progression of lower limb arterial calcification. Some studies have shown a relationship between circulating triglycerides and coronary artery calcium score in patients without diabetes(*34*). In Type 2 Diabetes, plasma triglycerides have been independently associated with coronary calcification score and, in Type 1 Diabetes, it is a biomarker of coronary artery calcification score progression(*35, 36*). Genetic predisposition to elevated triglyceride levels is also associated with the presence of mitral annular calcification(*37*). Triglycerides are molecules composed of glycerol and fatty acids. Once accumulated in tissue, triglycerides can generate species such as free fatty acids, sphingolipids and particularly ceramides, which could induce pathophysiological pathways involved in vascular calcification. Saturated fatty acids are associated with vascular disease and with the development of vascular smooth muscular cells calcification via NF-kB pathway induction(*38*). Among sphingolipids species, ceramides are known to induce human vascular smooth muscle cell calcification via p38 mitogen-activated protein kinase signaling(*39*). It would be interesting to study the role of lipid derivatives, such as sphingolipids, on lower limb vascular calcification development occurring in Type 2 Diabetes.

Even though circulating triglycerides are a strong marker of insulin resistance, it seems that, in our study, the association of circulating triglycerides and vascular calcification progression could not be explained

by the association between insulin resistance with arterial calcification. Indeed all others biomarkers of insulin resistance (TyG index (Triglyceride-Glucose index), waist circumference and serum adiponectin) tested in the DIACART study were not associated with lower limb vascular calcification.

To our knowledge, no study has studied the effect of fibrates on arterial calcification. Studies are therefore needed to explore if targeting triglycerides alone could reduce lower limb arterial calcification progression.

The strengths of the present study are its prospective design, the objective quantitative assessment of arterial calcification by CT, the measurement of numerous original markers and the use of a high sensitive kit to measure RANKL. The limitations are the relative small sample size and the absence of a non-diabetic control population.

# Conclusions

We show that lower limb arterial calcification is a pathological process frequent in patients with Type 2 Diabetes and which can progress rapidly. Mechanisms involved in arterial calcification observed in diabetic peripheral arteriopathy are still incompletely understood. Our study highlights the association of RANKL and triglycerides with the progression of lower limb arterial calcification in patients with Type 2 Diabetes. Although we show only association between biomarkers and peripheral arterial calcification, these results open new therapeutic perspectives and interventional studies are required to confirm this hypothesis.

# Abbreviations

AGEs
advanced glycation end-products
BMI
Body Mass Index
CML
Carboxymethyllysine
DIACART
acronym of « DIAbète et Calcification ARTerielle »
dp-ucMGP
desphospho–uncarboxylated Matrix Gla Protein
GFR
Glomerular Filtration Rate
HbA1c
glycated hemoglobin
hsCRP
high-sensitivity C-Reactive Protein

IGF-1
Insulin like Growth Factor 1
IL-6
Intelukine-6
iPTH
intact parathyroid hormone
MG-H1
Methylglyoxal-derived hydroimidazolone1
NDS
Neuropathy Disability Score
OPG
osteoprotegerin
RANK
Receptor Activator of Nuclear factor κB
RANKL
Receptor Activator of Nuclear factor κB ligand
sRAGE
soluble receptor of advanced glycation end-products
sRANKL
soluble RANKL
t-ucMGP
total-uncarboxylated Matrix Gla Protein
TyG index
triglycerides glucose index

# Declarations

**Ethics approval and consent to participate:** The study was approved by the local ethics committee (PARIS VI CPP) and registered in ClinicalTrials.gov (Identifier: NCT02431234). All patients were informed about the study objectives and procedure. Participants gave their written informed consent to participation prior to inclusion.

**Consent for publication:** Not applicable

**Availability of data and materials:** Data are available on request from the corresponding author.

**Competing interests:** No conflict of interest in the area of this study. DIACART study was supported by a fund from the Lilly Company. The company was neither involved in the design of the study nor in data collection.

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