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Edsfeldt, Andreas; Dias, Nuno; Elmståhl, Barbara; Müller, Markus; Berg, Katarina; Nitulescu, Mihaela; Persson, Ana; Ekberg, Olle; Goncalves, Isabel

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Low carotid calcium score is associated with higher levels of glycosaminoglycans, TNF-alpha and PTH in human carotid plaques

Andreas Edsfeldt, MD,[#,1]; Nuno Dias, MD, PhD[2]; Barbara Elmståhl, MD, PhD[3]; Markus F. Müller, MD, PhD[3]; Katarina Berg, PhD[1]; Mihaela Nitulescu[1]; Ana Persson[4]; Olle Ekberg, MD, PhD[3]; Isabel Gonçalves, MD, PhD[1,4]

[1]Experimental Cardiovascular Research Unit, Clinical Research Center, Clinical Sciences, Lund University, [2] Vascular Center Malmö/Lund,[3] Dept. of Radiology, [4] Dept. of Cardiology Skåne University Hospital, Malmö, Sweden

[#)]Corresponding Author: Andreas Edsfeldt, Clinical Research Center, Entrance 72, House 91:12, Malmö University Hospital, SE-20502 Malmö, Sweden

Phone:+46-40-391207; Fax:+46-40-391212, E-mail:Andreas.Edsfeldt@med.lu.se

Carotid calcium score and inflammation (running title)

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Abstract

Background and Purpose- Computed tomography (CT) is used to study coronary artery plaques, but little is known about its potential to characterize plaque composition.This study assesses the relation between carotid calcium score (CCS) by CT and plaque composition, namely extracellular matrix, inflammatory mediators and calcium metabolites.

Methods– Thirty patients with significant carotid stenosis underwent preoperative CT. CCS was quantified by Agaston calcium score. Plaque components were studied histologically and biochemically (collagen, elastin and glycosaminoglycans(GAG)). Fraktalkine, interferon-γ, interleukine (IL)-10, IL-12 p70, IL-1β, IL-6, MCP-1, PDGF-AB/BB, RANTES and TNF-α and parathyroid hormone (PTH) were measured using Luminex technology.

Results- Plaques with CCS≥400 had more calcium (*P*=0.012), less GAG (*P*=0.002), TNF-α (*P*=0.013) and PTH (*P*=0.028), than those with CCS<400. CCS correlated with plaque content of calcium (r=0.62; *P*<0.001) and inversely with GAG (r=-0.49; *P*=0.006) and TNF-α (r=-0.56; *P*=0.001).

Conclusions– Human carotid plaques with high CCS are richer in calcium and have lower amounts of GAG, PTH and TNF-α, which is one of the main pro-inflammatory cytokines involved in atherosclerosis. This suggests that CCS not only reflects the degree of calcification but also other important biological components relevant for stability, such as inflammation.

Introduction

Advanced atherosclerosis is often associated with dystrophic calcification. A coronary calcium score assessed by computed tomography (CT) has been developed to evaluate calcification in coronary artery plaques.[1] However, little is known about the composition of plaques with high calcium score concerning other components besides calcium.

The aim of this study is to evaluate if CT-based carotid calcium score (CCS) is associated, not only to calcium in the plaque, but also with other extracellular matrix components, inflammation and calcification metabolites.

Methods

For an expanded method section, please see supplemental methods.

Patients

Thirty patients underwent carotid endarterectomy. The indications for surgery were plaques associated with ipsilateral symptoms and stenosis, measured by duplex, >70% or plaques not associated with symptoms and stenosis >80%.

CT

Patients were examined the day before surgery with ECG triggered multidetector CT (Sensation 64, Siemens Medical Solutions, Erlangen, Germany) without intravenous contrast. In accordance with other studies,[2] a cut-off of CCS of 400 was used to classify plaques into high CCS (≥400) or low CCS (<400).

Sample preparation & analysis of extracellular matrix

Plaques were snap-frozen in liquid nitrogen upon surgical removal. Fragments of one-mm, from the most stenotic region, were taken for histology. Plaques were weighed, homogenised and elastin, collagen and sulphated glycosaminoglycans (GAG) were determined as described previously.[3]

Cytokines and PTH assessment

Luminex technology was used to measure cytokines (fraktalkine, interferon-γ, interleukine (IL)-10, IL-12 p70, IL-1β, IL-6, MCP-1, PDGF-AB/BB, RANTES, TNF-α) and parathyroid hormone (PTH).

Histology

Transversal sections from the one-mm-thick fragment were stained with CD68, Oil Red O and Masson. Calcified areas were measured.

Statistics

Results were normalized to plaque wet weights. Variables are presented as mean (standard deviation, SD). Comparisons were performed with unpaired Student's *t* or Mann-Whitney tests depending on variable distribution. Spearman's rho was used. Significance was considered at $P<0.05$.

Results

CCS correlated positively with the plaque area of calcium measured histologically (r=0.62; *P*<0.001, Fig 1A) and negatively with GAG content (r=-0.49; *P*=0.006, Fig 1B). TNF-α measured in the plaques correlated negatively with CCS (r=-0.56; *P*=0.001, Fig 1C).

Plaques with high CCS (CCS≥400, n=14) had higher histological plaque areas of calcium (% of area) compared to plaques with low CCS (11.4 (SD 11.4) vs 6.6 (SD 11.8), *P*=0.012, Fig 2A). Plaques with high CCS had lower contents of GAG (mg/g) (5.4 (SD 3) vs 9 (SD 3.2), *P*=0.002, Fig 2B), TNF-α (pg/g) (125.3 (SD 101.6) vs 286.6 (SD 217.4), *P*=0.013, Fig 2C) and PTH (pg/g) than those with low CCS (6.8 (SD 8.8) vs 15.53 (SD 9.2), *P*=0.028, Fig 2D).

No other significant results were found**,** please see supplemental tables .

Discussion

The novelty of this study was the assessment of other plaque components, beside calcium, in relation to CCS. GAG correlated negatively with CCS i.e. plaques with low CCS had more GAG than plaques with high CCS. GAG are essential for the retention of LDL in the vessel wall.[4, 5] Injury to the arterial wall increases the production of proteoglycan variants with enhanced LDL binding,[6] and thereby increase retention in the arterial wall, leading to inflammation.

Plaques with lower CCS had more TNF-α. TNF-α inhibits osteogenesis and bone collagen synthesis under inflammatory conditions and causes osteoclastic bone resorption.[7, 8] Vascular endothelial cells activated by TNF-α contribute to bone loss by regulated production of osteoprotegerin and of the receptor activator of NF-kappaB ligand (RANKL), a signal for full osteoclast development and activation.[9] In human osteoblastic cell lines, TNF-α inhibits formation and mineralization of calcification nodules.[10] Similar processes might occur in plaques and therefore support our findings.

In bone PTH, the major calcium-regulating hormone, stimulates osteoblasts to increase RANKL expression, which binds to RANK, its receptor in osteoclasts, stimulating osteoclast fusion and increasing bone resorption. Vascular calcification, earlier considered as a passive endstage of atherosclerosis, is today considered an active process similar to bone calcification,[11] expressing bone matrix protein and regulated through calcium-regulating hormones. Higher PTH in plaques with low CCS supports these similarities, suggesting that PTH might lead to the "decalcification" of plaques.

Finally, CCS correlated with calcium, showing that CCS is a valid way to evaluate calcium content of plaques.

Summary

This study shows that plaques with lower CCS have less calcium and most importantly more GAG, TNF-α and PTH. This suggests that studying carotid plaques with CT measuring CCS, not only reflects the degree of calcification but also other important biological components relevant for stability, such as inflammation.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

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Figure Legends

Fig. 1: Correlation between the carotid calcium score (CCS) and A) calcium, B) glycosaminoglycans (GAG), C) TNF-α.

Fig. 2: Plaques with carotid calcium score (CCS)≥400 have A) more calcium, B) less glycosaminoglycans (GAG), C) TNF-α and D) parathyroid hormone (PTH) than plaques with CCS<400.

Figure 1.

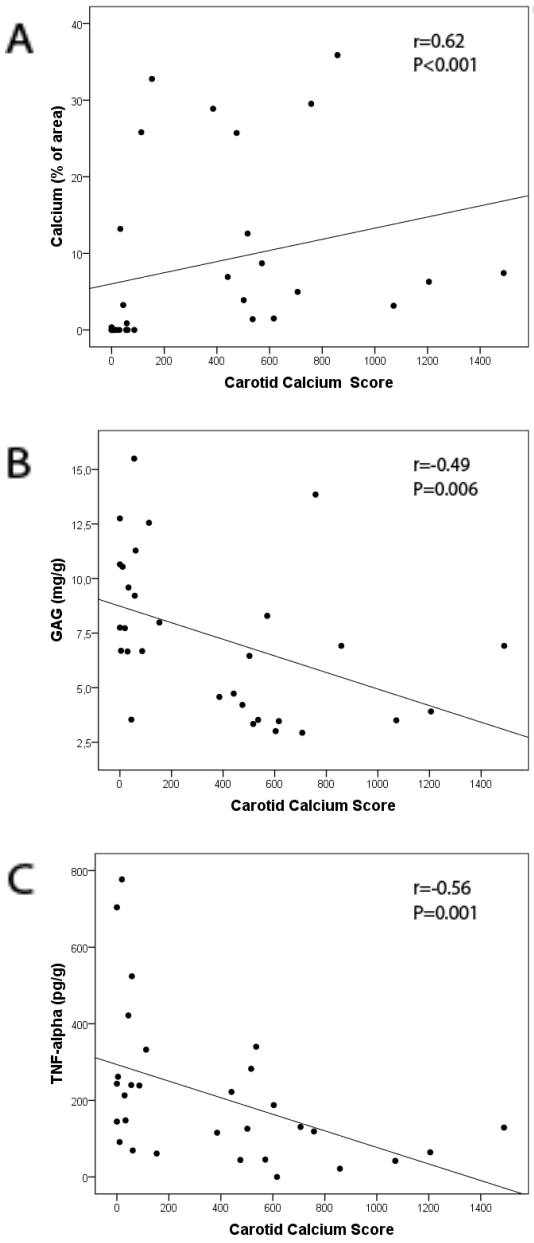
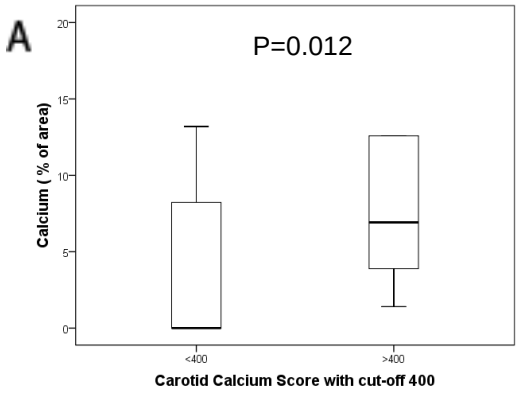
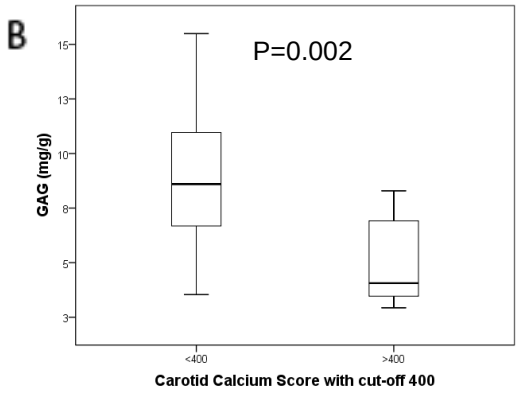
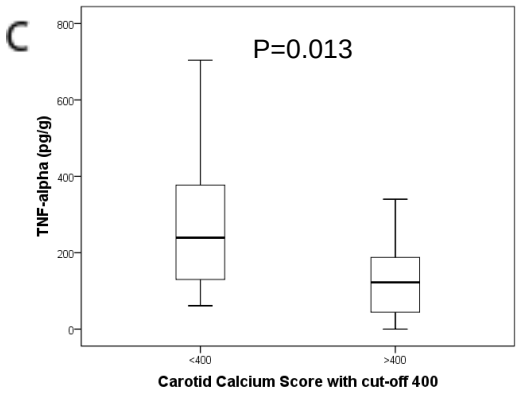
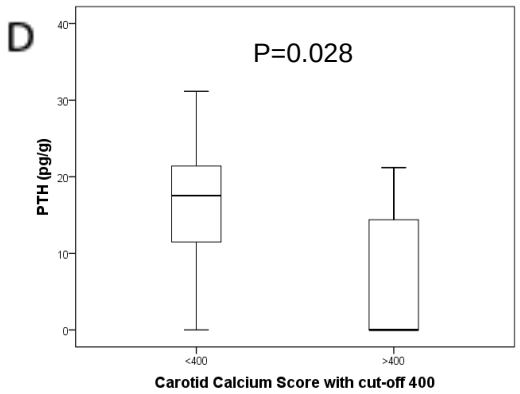

A
r=0.62
P<0.001
Calcium (% of area)
Carotid Calcium Score
B
r=-0.49
P=0.006
GAG (mg/g)
Carotid Calcium Score
C
r=-0.56
P=0.001
TNF-alpha (pg/g)
Carotid Calcium Score

Figure 2.


A
P=0.012
Calcium (% of area)
20
15
10
5
0
<400
>400
Carotid Calcium Score with cut-off 400


B
P=0.002
GAG (mg/g)
15
13
10
8
5
3
<400
>400
Carotid Calcium Score with cut-off 400


C
P=0.013
TNF-alpha (pg/g)
800
600
400
200
0
<400
>400
Carotid Calcium Score with cut-off 400


D
P=0.028
PTH (pg/g)
40
30
20
10
0
<400
>400
Carotid Calcium Score with cut-off 400

SUPPLEMENTAL MATERIAL

Low carotid calcium score is associated with higher levels of glycosaminoglycans, TNF-α and PTH in human carotid plaques

Methods:

Patients

Patients with atrial fibrillation, aortic valve disease, mechanical heart valves, chronic inflammatory disease, ipsilateral carotid artery occlusion or restenosis after previous CEA were excluded from this study. Cardiovascular risk factors and medications were recorded. Allpatients were preoperatively assessed by a neurologist. Informed consent was given by each patient. The study was approved by the local ethical committee.

CT

The image acquisition protocol was as follows: spiral mode, 0.37-second gantry rotation, slice collimation 64 x 0.6 mm, tube settings 120 kVp/110 mAs. A caudocranial scanning direction was selected, covering from the aortic arch to the vertex. Axial reconstruction of 0.75 mm were obtained and transferred into a vascular dedicated digital workstation for CT-scan image analysis (TeraRecon Aquarius Workstation, TeraRecon, CA, USA). A specific calcium scoring module was used. Calcification was marked on each axial image using a threshold of 130 Hounsfield units (HU). The software provided thereafter isotropically interpolated Agatston score and calcium volume. The measurement of CT images was done by the same observer and blinded to clinical information, histological and biochemical analysis.

Cytokines

Aliquots of plaque homogenate (50 μL) were centrifuged 13000 g (10 minutes). Supernatant (25 μL) was analysed. The rest of the procedure was performed according to the manufacturersinstructions (Milliplex Kit - MPXHCYTO-60K Lot # 1691735 Millipore, USA Milliplex Kit -HBN1B-51K-04 Lot # 1674160 Millipore, USA) and analyzed with Luminex 100 IS 2.3(Austin, Texas, USA).

Histology

Sections were fixed with Histochoice (Amresco, Ohio, USA), dipped in 60% isopropanol and then in 0.4% Oil Red O in 60% isopropanol (for 20 min) to stain lipids. Masson's trichrome using Ponceau-acid fuchsin (Chroma-Gesellschaft, Schimdt GmbH, Germany) and aniline blue (BDH, Dorset, England) was used to assess plaque collagen content. For macrophage assessment primary antibody monoclonal mouse anti-human CD68 (DakoCytomation, Glostrup, Denmark), diluted in 10% rabbit serum 1:100, and secondary antibody polyclonal rabbit anti-mouse (DakoCytomation, Glostrup, Denmark), dilution 1:200 in 10% of rabbit serum, were used. To assess the calcified areas, the area of the holes where calcium had been present was measured and to assess collagen stained area of plaque (% area) was quantified blindly using Biopix iQ 2.1.8 (Gothenburg, Sweden) after scanning with ScanScope Console Version 8.2 (LRI imaging AB, Vista Californien, USA) and photograhed with Aperio image scope v.8.0 (Aperio, Vista Californien, USA).

Supplemental Tables

TABLE I. Correlations between carotid calcium score and cytokines, histology and extracellular matrix.

Variable (n=30)	r	*P*
Fraktalkine (pg/g)	-0.105	0.580
Interferon-γ (pg/g)	0.051	0.790
IL-10 (pg/g)	-0.138	0.468
IL-12p(70) (pg/g)	0.016	0.932
IL-1β (pg/g)	-0.067	0.726
IL-6 (pg/g)	-0.075	0.695
MCP-1 (pg/g)	-0.144	0.446
PDGF- AB/BB (pg/g)	-0.039	0.838
RANTES (pg/g)	-0.194	0.304
TNF-α (pg/g)	-0.555	0.001
PTH (pg/g)	-0.269	0.150
Glycosaminoglycans (mg/g)	-0.493	0.006
Elastin (mg/g)	-0.015	0.936
Collagen (mg/g)	-0.003	0.988
Masson (% of area)	0.146	0.449
CD68 (% of area)	0.000	1.000
Oil Red O (% of area)	0.003	0.988
Calcium (% of area)	0.619	<0.001

TABLE II. Associations between high and low calcified plaques (cut off carotid calcium score 400), cytokines, histology and extracellular matrix.

Variable (n=30)	*P*
Fraktalkine (pg/g)	0.400
Interferon-γ (pg/g)	0.759
IL-10 (pg/g)	0.313
IL-12p(70) (pg/g)	0.822
IL-1β (pg/g)	0.377
IL-6 (pg/g)	0.608
MCP-1 (pg/g)	0.448
PDGF- AB/BB (pg/g)	0.377
RANTES (pg/g)	0.473
TNF-α (pg/g)	0.013
PTH (pg/g)	0.028
Glycosaminoglycans (mg/g)	0.002
Elastin (mg/g)	0.951
Collagen (mg/g)	0.423
Masson (% of area)	0.559
CD68 (% of area)	0.880
Oil Red O (% of area)	0.589
Calcium (% of area)	0.012