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RESEARCH

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Inflammation and oxidative stress markers in type 2 diabetes patients with Advanced Carotid atherosclerosis

Louise Ménégaud^{1,2,3,4}, Aline Laubriet⁵, Valentin Crespy⁵, Damien Leleu^{1,2,3,4}, Thomas Pilot^{1,2,3}, Kevin Van Dongen^{1,2,3}, Jean-Paul Pais de Barros^{1,2,3,6}, Thomas Gautier^{1,2,3}, Jean-Michel Petit^{1,2,3,7}, Charles Thomas^{1,2,3}, Maxime Nguyen^{1,2,3,8}, Eric Steinmetz⁵ and David Masson^{1,2,3,4,9*}

Abstract

Background Type 2 diabetes mellitus (T2DM) is a major global health issue and a significant risk factor for atherosclerosis. Atherosclerosis in T2DM patients has been associated with inflammation, insulin resistance, hyperglycemia, dyslipidemia, and oxidative stress. Identifying molecular features of atherosclerotic plaques in T2DM patients could provide valuable insights into the pathogenesis of the disease.

Methods The MASCADI (Arachidonic Acid Metabolism in Carotid Stenosis Plaque in Diabetic Patients) study aimed to investigate the increase of 2-arachidonoyl-lysophatidylcholine (2-AA-LPC) in carotid plaques from T2DM and control patients and to explore its association with plaque vulnerability as well as with blood and intra-plaque biomarkers altered during diabetes.

Results In a population of elderly, polymedicated patients with advanced stage of atherosclerosis, we found that T2DM patients had higher systemic inflammation markers, such as high-sensitivity C-reactive protein (hsCRP) and IL-1 β , higher levels of oxysterols, increased triglyceride levels, and decreased HDL levels as compared to control patients. Furthermore, 2-AA-LPC was significantly enriched in plaques from diabetic patients, suggesting its potential role in diabetic atherosclerosis. Interestingly, 2-AA-LPC was not associated with systemic markers related to diabetes, such as hsCRP, triglycerides, or HDL cholesterol. However, it was significantly correlated with the levels of inflammatory markers within the plaques such as lysophospholipids and 25-hydroxycholesterol, strengthening the link between local inflammation, arachidonic acid metabolism and diabetes.

Conclusion Our study is in line with a key role for inflammation in the pathogenesis of diabetic atherosclerosis and highlights the involvement of 2-AA-LPC. Further research is needed to better understand the local processes involved in the alteration of plaque composition in T2DM and to identify potential therapeutic targets.

Trial registration The MASCADI was registered on ClinicalTrials.gov (clinical registration number: NCT03202823).

Keywords Diabetes, Atherosclerosis, Carotid, Inflammation, Arachidonic acid

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Background

Type 2 diabetes mellitus (T2DM) is a growing global health concern, affecting millions of people worldwide. It has been widely recognized as an independent risk factor for atherosclerosis, a major contributor to cardiovascular diseases (CVDs) which represent the leading cause of morbidity and mortality in patients with T2DM [1]. Despite extensive research on this field, the underlying mechanisms involved in the development and progression of atherosclerosis in patients with T2DM remain elusive due to their multiplicity and intricate relationships. Indeed, many factors have been implicated in the pathogenesis of atherosclerosis in T2DM, including inflammation, insulin resistance, hyperglycemia, dyslipidemia, and oxidative stress [2–4]. Hyperglycemia, for instance, has been associated with increased production of advanced glycation end-products (AGEs), which can contribute to endothelial dysfunction and plaque formation. Dietary AGEs may also promote vascular dysfunction and inflammation, contributing to the development of vascular complications in subjects with type 2 diabetes [5–7]. Additionally, diabetic dyslipidemia, characterized by elevated triglycerides and low-density lipoprotein (LDL) cholesterol levels, as well as reduced high-density lipoprotein (HDL) cholesterol levels, has been linked to increase plaque vulnerability and accelerated atherosclerosis in T2DM patients [8]. Oxidative stress, caused by an imbalance between the production of reactive oxygen species (ROS) and the antioxidant defense mechanisms, has also been implicated in the development of atherosclerosis in T2DM patients [9]. Elevated ROS levels are well known to promote inflammation, endothelial dysfunction, and plaque instability, thereby exacerbating the atherosclerotic process [10].

In parallel to these advances in understanding the factors contributing to atherosclerosis in T2DM, several studies indicate that atheroma plaques in diabetic patients possess unique characteristics in terms of morphology, vulnerability, composition, and calcification [11–14]. Therefore, identifying molecular features of atherosclerotic plaques in T2DM patients could provide valuable insights into the pathogenesis of the disease and lead to the development of more targeted therapeutic strategies. Recent lipidomic analyses of atheroma plaques have revealed specific signatures associated with diabetes, such as increased free cholesterol content and altered levels of specific phospholipids and lysophospholipids (LPLs) [15, 16]. Again, the mechanisms behind these alterations and their implications for atherosclerosis in T2DM patients remain unclear. In a recent cohort study of 79 patients, we observed a specific enrichment of 2-arachidonoyl-lysophatidylcholine (2-AA-LPC) among other LPC in the plaques of diabetic patients, suggesting an important role for this molecule

and more generally for arachidonic acid metabolism in atherosclerosis associated with diabetes [17]. However, in a subsequent study, we did not observe any differences in AA derived mediators such as leukotriene B4 (LTB4), thromboxane B2 (TxB2) or prostaglandin E2 (PGE2) in carotid plaques from control or T2DM patients [18]. The aim of the MASCADI (Arachidonic Acid Metabolism in Carotid Stenosis Plaque in Diabetic Patients) study was not only to confirm the increase in 2-AA-LPC in a larger cohort (101 T2DM and 99 control patients) but also to explore the association of this molecule with plaque vulnerability as well as with systemic and local biomarkers that are altered during the course of diabetes. Notably, we examined the relationships between 2-AA-LPC levels and inflammatory markers, triglycerides, glycated hemoglobin (HbA1c), and oxidative stress markers such as oxysterols.

Materials and methods

The MASCADI protocol was reviewed and approved by the regional Ethics Committee (Comité de Protection des Personnes Est, Dijon, France CPP Est III, CHRU Nancy, N° 2017-A00022-51) and recorded on ClinicalTrials.gov (clinical registration number: NCT03202823). Two-hundred-and-one patients were enrolled. As described previously, patients enrolled in the present study were admitted to the Department of Cardiovascular Surgery at the University Hospital of Dijon (Burgundy, France) for the surgical treatment of an atheromatous internal carotid artery (ICA) stenosis whether symptomatic or not [18]. A stenosis was considered symptomatic if stroke or transient ischemic attack (TIA) attributed to the ICA lesion occurred within the previous 6 months before intervention. According to the trial category (Interventional research that does not involve drugs or non-CE-marked medical devices and that involves only minimal risks and constraints for the patient) and the ethics committee, all patients received written information note on the study. Oral consent was obtained from the patient and an attestation of the patient's oral consent was signed by the investigator physician and countersigned by the patient, according to the french law in this type of trial. Blood serum samples and EDTA total blood samples were collected in parallel on the day of surgery. For all patients, carotid endarterectomy (CEA) was performed within the carotid bulb, with en-bloc removal of the entire plaque, and resulting samples were frozen at -80 °C prior to lipidomic analysis.

Clinical data, imaging plaque characteristics, and biological analysis

Demographics, vascular risk factors (hypertension, active smoking, type 2 diabetes, dyslipidemia with detailed treatment), chronic renal insufficiency and symptomatic

stenosis were collected. Patients were divided into groups according to their diabetic status: control group and diabetic group. Preoperative plaque imaging assessment included for all patients doppler ultrasound evaluating plaque echolucency and degree of stenosis according to NASCET measures, and CT angiographies (CTA) describing the presence of plaque ulceration and calcification. Glycated hemoglobin A1c (HbA1c) was measured by high pressure liquid chromatography on a Tosoh G8 analyzer (Tosoh Bioscience, Tokyo, Japan), C reactive protein (CRP), troponin I, creatinine, total cholesterol, HDL cholesterol and triglycerides were determined on a Dimension Vista analyzer using dedicated reagents (Siemens). Perioperative hematology parameters were determined on a XN-9000 analyzer (Sysmex). Serum IL-1 β levels were determined using a high-sensitivity enzyme-linked immunosorbent assay (ThermoFisher Scientific) with a limit of quantification (LOQ) of 0.05 pg/mL. IL-6 and IL-18 were determined by commercial ELISA Kit (Human IL-6 and Human Total IL-18 DuoSet ELISA Ref DY318 and DY206, R&D System, Minneapolis, MN, USA).

Lysophospholipid quantitation by LC-MS2

For lipidomic analysis, carotid plaques were opened longitudinally and core samples of atheroma plaques were harvested by using 3 mm biopsy punches (Kai Medical, Solingen, Germany). Sites of harvesting were normalized between the samples by selecting the plaque area corresponding to the maximal stenosis. Plaque biopsies (2–10 mg) were crushed in an Omni Bead Ruptor 24 apparatus (Omni International, Kennesaw, USA) with 1000 μ L of phosphate buffer saline and circa twenty 1.4 mm OD zirconium oxide beads ($S=6.95$ m/s, $T=30$ s, $C=3$; $D=10$ s). Lipids corresponding to 1 mg of tissue were spiked with a mix containing 1 mg of di14:0-PC, 1 mg of di14:0-PE, 1 mg of d18:1/17:0-SM and 0.1 mg of 14:0-LPC used as internal standards. Phospholipids and lysophospholipids were analyzed by LC-MS/MS using a 6460 mass spectrometer (Agilent Technologies) equipped with an electrospray ionization source and MassHunter data system. The liquid chromatography was performed on a ZorBAX Eclipse SB-C18 (2.1 mm \times 50 mm, 1.8 mm) (Agilent Technologies, Santa Clara, CA, USA).

Plasma and plaque oxysterol quantitation by gas chromatography mass spectrometry (GC-MS)

Homogenates from plaques were crushed with NaCl 0.9% to obtain a final concentration of 0.33 mg tissue per ml. A volume of sample corresponding to 15 mg of tissue was saponified for 45 min at 56 $^{\circ}$ C with 60 μ L of potassium hydroxide 10 mol \cdot L $^{-1}$ and 1.2 ml of ethanol-BHT (50 mg \cdot L $^{-1}$) containing 20 μ L of standard-intern mix (μ g per sample: 0.2 (25-OH-cholesterol-d6); 0.4

(27-OH-cholesterol-d6); 0.5 (7 α -OH-cholesterol-d7); 0.5 (7 β -OH-cholesterol-d7); 1 (7-keto-cholesterol-d7)). Sterols were extracted with 5 ml of hexane and 1 ml of water. After evaporation of the organic phase, sterols were derivatized with 100 μ L of a mixture of bis(trimethylsilyl)trifluoroacetamide/trimethylchlorosilane 4/1 v/v for 1 h at 80 $^{\circ}$ C. After evaporation of the silylating reagent, 100 μ L of hexane were added. Analysis of trimethylsilyl ethers of sterols was performed by GC-MS in a 7890 A gas chromatograph coupled with a 5975 C Mass Detector (Agilent Technologies). Separation was achieved on a HP-5MS 30 m \times 250 μ m column (Agilent Technologies, Santa-Clara, USA) using helium as carrier gas and the following GC conditions: injection at 250 $^{\circ}$ C using the pulsed split, oven temperature programme: initial temperature 150 $^{\circ}$ C up to 280 $^{\circ}$ C at a rate of 15 $^{\circ}$ C \cdot min $^{-1}$, up to 290 $^{\circ}$ C at a rate of 1 $^{\circ}$ C \cdot min $^{-1}$ for 2 min. The MSD was set up as follow: EI at 70 eV mode, source temperature at 230 $^{\circ}$ C. Data were acquired in SIM mode using following quantitation ions (m/z): 131.1 for 25-OH-cholesterol; 137.1 for 25-OH-cholesterol d6; 417.4 for 27-OH-cholesterol; 423.4 for 27-OH cholesterol-d6; 456.4 for 7 α -7 β cholesterol; 463.5 for 7 α -7 β cholesterol-d7; 472.4 for 7-keto-cholesterol; 479.4 for 7-keto-cholesterol d7.

Statistical analysis

We compared patients with diabetes to patient without diabetes. Normality was assessed using the Shapiro-Wilk test. Accordingly, quantitative data were presented as medians [interquartile range] or means (standard deviation) if normally distributed. Groups were compared with a Student's t-test or the Kruskal-Wallis nonparametric test. When indicated, significance was corrected for multiple comparison by the Benjamini-Hochberg procedure. Qualitative data are presented as absolute value and percentage and compared by chi-squared or Fisher's exact tests, as appropriate. Correlations were evaluated with the Spearman correlation method. Missing data were considered to be at random and were omitted in the analysis. R studio was used for statistical analysis.

Results

Patient characteristics and biological parameters in type 2 diabetic and control patients

The baseline characteristics are presented in Table 1. T2DM and control patients displayed similar demographic characteristics. Diabetic patients had a higher prevalence of hypertension and a higher BMI. No differences were found regarding the frequency of symptomatic plaques or major plaque characteristics (echogenicity, ulceration). However, the frequency of plaques with high levels of calcifications was higher in T2DM patients. The degree of stenosis was also significantly increased in diabetic patients. Regarding the biological parameters,

Table 1 Baseline characteristics. Data are expressed as mean \pm S.D. (for normal distribution), medians [25th -75th percentiles] or % of the population. Groups were compared with a student's test or Kruskal-Wallis non-parametric test; qualitative data were compared using chi-squared or Fischer's test

	Controls (n = 98)	Diabetics (n = 101)	p-value
Age (years)	74.0 [62.2;79.0]	72.0 [65.0;78.0]	0.766
Gender (M/F)	72/26	77/24	0.653
BMI (kg/m ²)	26.0 [23.0;28.0]	27.4 [25.0;30.0]	0.001
Active Smoking	28 (28.6%)	24 (23.8%)	0.440
Hypertension/antihypertensive drugs	80 (81.6%)	95 (94.1%)	0.007
Renal insufficiency	11 (11.2%)	12 (11.9%)	0.885
Diabetic treatment			
No treatment or MHD		9 (10%)	-
Biguanid		41 (45%)	-
Sulfonylurea		12 (13%)	-
Insulin		16 (17%)	-
DDP-4 inhibitor		5 (5.4%)	-
other		4 (4.4%)	
Hypolipidemic treatment			
Statin	53 (57%)	58 (63%)	
Symptomatic lesions	38 (38.8%)	42 (41.6%)	0.686
Hyperechoic	29 (29.6%)	36 (35.6%)	0.632
Hypoechoic	18 (18.4%)	15 (14.9%)	0.505
NASCET	75.0 [65.0;85.0]	80.0[70.0;87.0]	0.005
Plaque Ulceration	13 (13.3%)	15 (14.9%)	0.748
Calcifications			0.104
1	14 (14.3%)	17 (16.8%)	
2	75 (76.5%)	65 (64.4%)	
3	9 (9.2%)	19 (18.8%)	
HbA1c	5.74 [5.48;5.97]	6.79 [6.34;7.48]	<0.001
Creatinine.μmol/L	74.9 [60.9;89.0]	71.8 [57.4;85.4]	0.305
Total cholesterol mmol/L	3.88 [3.24;4.44]	3.56 [2.83;4.21]	0.043
Triglycerides mmol/L	1.36 [0.94;1.82]	1.67 [1.14;2.36]	0.017
LDLc mmol/L	2.04 [1.49;2.66]	1.98 [1.46;2.54]	0.407
HDLc mmol/L	1.13 (0.34)	0.96 (0.28)	<0.001
Hemoglobin (g/dl) (M)	12.8 [11.6;14.4]	13.2 [11.2;13.9]	0.540
Hemoglobin (g/dl) (F)	13.1 [11.7;14.4]	12.3 [10.6;15.2]	0.582
Erythrocytes (10 ¹² /L) (M)	4.28 [3.86;4.70]	4.27 [3.68;4.55]	0.582
Erythrocytes (10 ¹² /L) (F)	4.21 [3.79;4.78]	4.16 [3.60;4.63]	0.644
Platelets (10 ⁹ /L)	220 [181;253]	229 [188;284]	0.261
Neutrophils(10 ⁹ /L)	5.54 [4.52;7.51]	6.61 [4.83;8.72]	0.058
Lymphocytes (10 ⁹ /L)	1.76 [1.34;2.43]	1.69 [1.23;2.15]	0.482
Monocytes (10 ⁹ /L)	0.76 [0.59;0.94]	0.82 [0.64;0.99]	0.225

T2DM patients presented relatively balanced glyce-mic parameters (HbA1c: 5.74% Vs 6.79% $p < 0.0001$). As expected, there was a significant increase in plasma tri-glyceride levels associated with a decrease in HDL levels in diabetic patients. There were minor differences in total cholesterol and LDL cholesterol levels, but as expected the majority of the patients in both groups were under statin treatment. No differences were observed for most of the hematology parameters, however there was a nearly significant increase in absolute neutrophil counts

in T2DM patients (6.61 10⁹/L [4.83–8.72] Vs 5.54 10⁹/L [4.83–8.72] $p = 0.058$)

Systemic inflammation and oxidative stress markers in the study population

In order to explore systemic inflammation and oxida-tive stress markers, levels of high-sensitivity C-reactive protein (hs-CRP), IL-1 β , IL-6, IL-18 and serum con-centrations of oxysterols were measured in our popula-tion. The 7-oxidized forms are primarily formed during nonenzymatic oxidative processes and are markers of

Table 2 Plasma oxysterols levels and inflammation biomarkers in controls and diabetic patients. Data are expressed as medians [25th-75th percentiles]. Groups were compared with a Mann-Whitney non-parametric test

	Controls	Type 2 diabetics	p-overall
Oxysterols (mmol/mol cholesterol)			
7 α -Hydroxycholesterol	0.31 [0.21;0.42]	0.36 [0.26;0.46]	0.029
7 β -Hydroxycholesterol	0.39 [0.26;0.52]	0.47 [0.33;0.66]	0.040
7-ketocholesterol	0.85 [0.59;1.37]	0.90 [0.70;1.38]	0.166
25-hydroxycholesterol	0.14 [0.11;0.19]	0.14 [0.10;0.22]	0.854
27-hydroxycholesterol	0.04 [0.03;0.05]	0.04 [0.03;0.06]	0.652
Hs CRP (mg/L)	1.85 [0.96;4.75]	3.24 [1.17;8.75]	0.028
IL-1 β (ng/l)	0.05 [0.05;0.08]	0.06 [0.05;0.12]	0.017
IL-18 (μ g/l)	0.61 [0.45;0.85]	0.70 [0.50;0.96]	0.098
IL-6 (ng/l)	5.14 [4.02;10.1]	4.97 [3.73;7.43]	0.273

oxidative stress in diabetes [19, 20], whereas 27-hydroxycholesterol and 25-hydroxycholesterol are produced enzymatically, with 25-hydroxycholesterol being actively produced upon inflammatory response. Interestingly, although the population was mainly beyond 70 years old and affected by advanced stage atherosclerosis, a significant difference was found regarding inflammatory markers, with increased levels of hs-CRP and IL-1 β in T2DM patients (Table 2; Fig. 1A-B). Surprisingly, while IL-6 plasma levels were positively correlated with hs-CRP ($r=0.347$, $p=4.7 \cdot 10^{-6}$) they were similar in both groups. Interestingly, there was a strong tendency toward an increase in IL-18 levels in type T2DM patients (Table 2).

7 β -hydroxycholesterol and 7 α -hydroxycholesterol levels were significantly increased in T2DM while there was no difference for other oxysterols (Table 2). Interestingly, hsCRP levels were positively associated with plasma triglyceride levels, both in the general population and among diabetic patients ($r=0.2746$, $p=0.0057$ in T2DM and $r=0.348$, $p=0.0004$ in controls) (Fig. 1C).

Enrichment of 2-AA-LPC in carotid plaques of diabetic patients

We had previously found an increase in the relative proportion of 2-AA-LPC within other lysophosphatidylcholines in carotid plaques of type 2 diabetic patients [17]. In the present study, we could confirm these alterations in a larger cohort of patients. LPC levels were measured as previously described. As shown in Table 3 while total LPC levels were not significantly different in the two populations, we confirmed the relative increase of 2-AA-LPC in diabetic patients (0.37% in control Vs 0.45% in T2DM patients $p=0.013$), thus confirming the interest of this molecule in the context of diabetic atheroma. As with plasma, we also measured oxysterols in the plaques, focusing on the most abundant oxysterols (25-hydroxycholesterol, 27-hydroxycholesterol, 7 α -hydroxycholesterol, 7 β -hydroxycholesterol, and 7-ketocholesterol). Levels of oxysterols within the plaque have also been associated with inflammation, oxidative stress and plaque evolution [21–24]. As indicated in Table 3, a trend toward an increase in 7- β hydroxycholesterol was found in diabetic patients, while other species were not significantly altered.

Independent association of 2-AA-LPC with T2D risk

After confirming the increase of 2-AA-LPC within the plaques of diabetic patients, we wanted to ensure that these molecules were independently associated with the risk of type 2 diabetes through a multivariate logistic

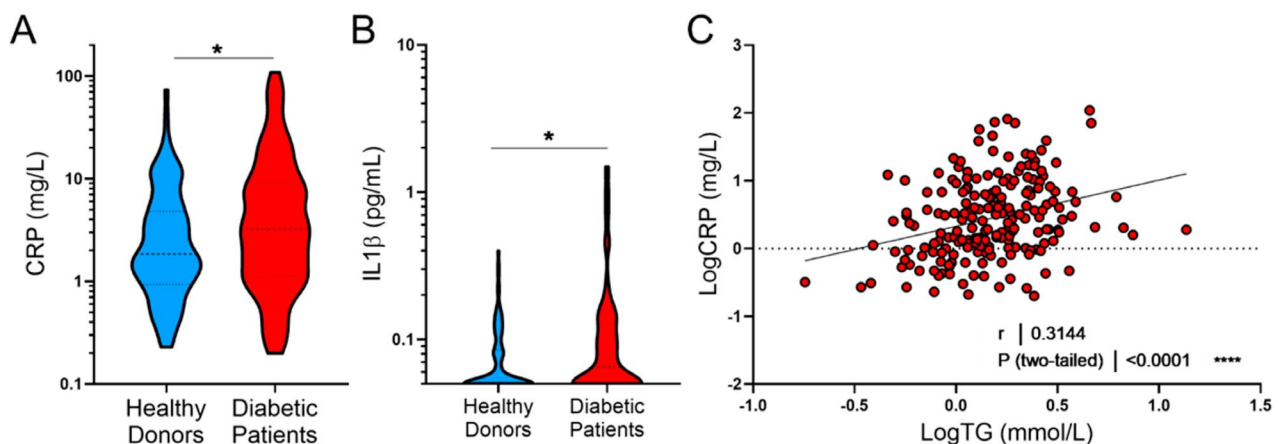


Fig. 1 **A:** Violin plot of C-reactive protein (CRP) levels in control and T2DM patients. **B:** Violin plot of IL-1 β levels in control and T2DM patients. **C:** Correlation between plasma HsCRP levels and triglycerides (R and P values were obtained by the Spearman rank test)

Table 3 Lysophosphatidylcholine and oxysterols in carotid plaques from control and type 2 diabetic patients. Data are expressed as mean \pm S.D. (for normal distribution), medians [25th-75th percentiles]. Groups were compared with a Student's test or Kruskal-Wallis non-parametric test

	Controls	Type 2 Diabetics	p.overall
	<i>N</i> = 98	<i>N</i> = 101	
Total LPC (mmol/mol cholesterol)	5.23 [3.05;6.93]	4.84 [3.36;8.10]	0.941
	LPC relative levels		
16.0.LPC.sn1	40.5 (2.81)	40.8 (3.10)	0.543
16.0.LPC.sn2	6.84 [6.07;7.46]	6.82 [6.12;7.55]	0.977
18.2.LPC.sn1	5.38 [4.08;6.84]	5.26 [3.89;6.89]	0.829
18.2.LPC.sn2	1.06 [0.74;1.37]	1.02 [0.80;1.44]	0.946
18.1.LPC.sn1	10.0 (2.04)	9.83 (1.81)	0.505
18.1.LPC.sn2	1.83 [1.54;2.23]	1.88 [1.52;2.12]	0.870
18.0.LPC.sn1	26.2 (4.18)	25.5 (4.28)	0.294
18.0.LPC.sn2	4.52 [3.92;5.44]	4.49 [3.65;5.29]	0.333
20.4.LPC.sn1	1.50 [1.13;1.98]	1.55 [1.19;2.26]	0.132
20.4.LPC.sn2	0.37 [0.26;0.62]	0.45 [0.34;0.75]	0.013
20.3.LPC.sn1	0.84 [0.65;1.07]	0.87 [0.68;1.21]	0.201
20.3.LPC.sn2	0.20 [0.14;0.26]	0.19 [0.15;0.30]	0.337
Oxysterols (mmol/mol cholesterol)			
27-Hydroxycholesterol	3.33 [2.02;7.25]	3.35 [1.84;6.74]	0.945
25-Hydroxycholesterol	0.39 [0.28;0.62]	0.43 [0.30;0.75]	0.273
7 α -Hydroxycholesterol	0.75 [0.44;1.11]	0.78 [0.49;1.47]	0.246
7 β -Hydroxycholesterol	0.94 [0.64;1.27]	1.11 [0.70;1.76]	0.063
7-ketocholesterol	2.03 [1.16;3.88]	1.93 [1.11;3.94]	0.940

regression, including the altered lipid markers within the plaques (7 β -hydroxycholesterol, 2-AA-LPC) and the plasma markers modified during diabetes (HsCRP, triglycerides, HbA1c, and 7 β -hydroxycholesterol). In all cases, 2-AA-LPC was positively and independently associated with the risk of diabetes, whether HbA1c was incorporated or not in the model (Fig. 2 AB).

Exploring the mechanisms behind increased 2-AA-LPC in T2D plaques

Finally, in order to understand the mechanisms associated with the increase of 2-AA-LPC in the plaques of diabetic patients, we examined the association, through a correlation matrix, between 2AA-LPC, the biomarkers measured within the plaque (LPC and oxysterols) with plasma markers related to hyperglycemia (HbA1c), dyslipidemia (triglycerides), inflammation (CRP), and oxidative stress (7- β -OH-cholesterol). Interestingly, no correlation was found between the plasma markers and the markers measured within the plaque, suggesting that the alterations in the plaque composition are mainly driven by local metabolic and inflammatory processes (Fig. 3A). As for the plaque markers, 2-AA-LPC appeared to be primarily associated with markers related to inflammation (total levels of LPC, 27-hydroxycholesterol, 25-hydroxycholesterol) and negatively with oxidative stress markers (Fig. 3A). Interestingly, a strong correlation was found between the levels of

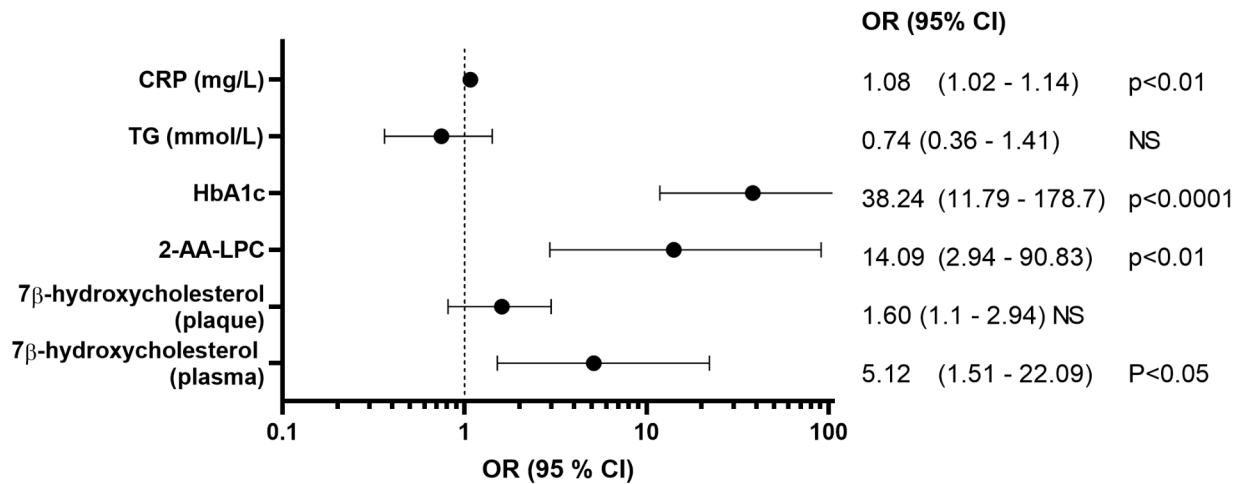
25-hydroxycholesterol and the levels of 2-AA-LPC, suggesting related production pathways (Fig. 3 AB).

Discussion

In this study, we examined the differences between T2DM and matched atherosclerotic patients in terms of plaque characteristics, lipid composition, biological parameters, systemic inflammation, and oxidative stress markers. Even within our specific cohort of elderly, poly-medicated patients with advanced atherosclerosis, we identified a characteristic diabetic signature. This included hyperglycemia, dyslipidemia (elevated TG and reduced HDL), oxidative stress (increased plasma 7 α - and 7 β -hydroxycholesterol), and heightened inflammation markers (hs-CRP and IL-1 β) in the T2DM group. While no differences in plaque vulnerability or echogenicity between the groups was observed, we did find a significant enrichment of 2-AA-LPC in the plaques of diabetic patients, underlining the relevance of this molecule in the context of diabetic atheroma.

Our findings align with the existing literature that underscore the positive association of inflammation markers with the risk of diabetes [25–28]. The association of hs-CRP and IL-1 β with plasma triglyceride levels and BMI in both the general population and diabetic patients suggests that systemic inflammation plays a crucial role in the pathogenesis of diabetic atherosclerosis and is related to diabetic dyslipidemia. It is noteworthy

A



B

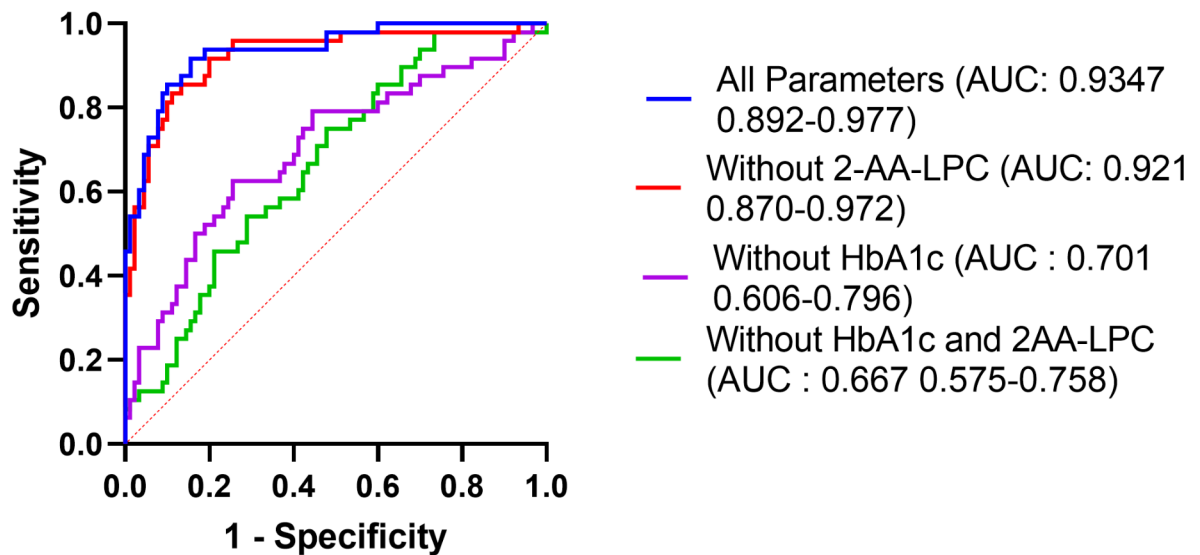


Fig. 2 A: Forest plots showing odds ratio values and 95% confidence intervals for multivariable logistic regression analysis of diabetes and biological variables. **B:** Receiver operating characteristic curve (ROC) for the prediction of type 2 diabetes based on the selected biological parameters. (AUC: Area under the Curve)

that this heightened inflammation is apparent even in our study population, which comprises elderly patients with advanced atherosclerosis. While IL-1β was previously linked with the risk of diabetes, evidence for increased IL-1β levels is limited due to the typically low circulating levels of IL-1β [25, 29, 30]. It is therefore noteworthy

that we could document here significantly higher IL-1β plasma concentrations in T2DM patients with advanced atherosclerosis. While no differences were observed for IL-6, the increase in IL-1β levels and the strong tendency for IL-18 are consistent with an activation of inflammation. Increased activity of NLRP3 inflammasome, which

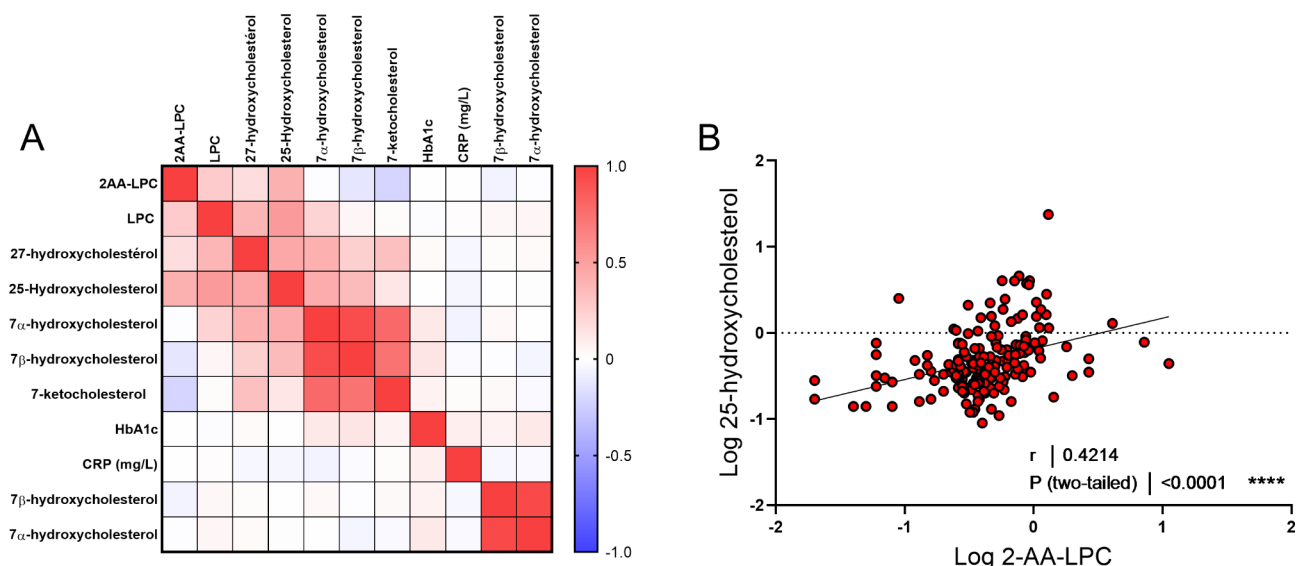


Fig. 3 **A:** Correlation matrix of 2-AA-LPC with systemic and carotid plaque biomarkers. Correlations were determined by Spearman's rank correlation. Intersections between lines and columns indicate the Spearman's rank correlation coefficient (ρ) between the two parameters. Spearman ρ value is visualized by blue or red intensities. **B:** Correlation between relative levels of 2-AA-LPC and 25-hydroxycholesterol levels in carotid plaques (R and P values) were obtained by the Spearman rank test

acts as a molecular platform for IL-1 β and IL-18 secretion, has been previously documented in T2DM. It may play an important role to increase the cardiovascular risk in these patients [31, 32].

We found mild differences in circulating oxysterol levels between diabetic and control patients, with only a moderate increase in 7 α and 7 β -hydroxycholesterol levels. Despite previous studies reporting increased plasma oxysterol levels in T2DM patients, this has not been consistently observed, especially when oxysterol levels were normalized by total cholesterol concentrations [20, 33, 34]. Again, these differences may be attributed to the specificities of our study population, which warrants further investigation. Interestingly, we found no significant correlation between plasma oxysterol levels and their enrichment within the plaque, as observed previously [22]. More generally, there was no relationship between blood biomarkers and the plaque molecular components. This suggests that the alterations in plaque composition are mainly driven by local metabolic and inflammatory processes and are not directly related to systemic markers [35].

The relative increase in 2-AA-LPC in the plaques of diabetic patients observed in the present study is in line with our previous observation [17]. Moreover, multivariate logistic regression analysis further supports the idea that 2-AA-LPC is independently associated with the risk of type 2 diabetes. 2-AA-LPC enrichment was not associated with systemic markers related to diabetes such as hsCRP, triglycerides or HDL cholesterol. To note, we previously observed a significant correlation of 2-AA-LPC with HbA1c [17] which is not retrieved here in a larger

cohort of T2DM patients. Besides, 2-AA-LPC enrichment significantly correlated with inflammatory markers within the plaques, i.e. total LPCs, 27-hydroxycholesterol and 25-hydroxycholesterol, highlighting the potential role of inflammation in the development of diabetic atherosclerosis. Interestingly, the strong correlation between the levels of 25-hydroxycholesterol and the levels of 2-AA-LPC supports the hypothesis of related production pathways for the two molecules. A recent study has shown that 25-hydroxycholesterol accumulates in severe coronary artery lesions and demonstrated a specific role of 25-hydroxycholesterol production by macrophages to promote inflammation and vascular remodeling [23].

Our study has some limitations. The cross-sectional design prevents us from establishing causal relationships between the observed changes and the development of diabetic atherosclerosis. Furthermore, the criteria for symptomatic lesions were based on the occurrence of CV events in the six months preceding surgery, and we lack post-surgery follow-up data on the occurrence of CV events. To confirm our findings and further explore the involved underlying mechanisms, longitudinal studies with larger sample sizes are necessary.

Conclusions

Our study provides evidence for increased inflammation markers and enrichment of 2-AA-LPC in diabetic patients, strengthening the potential role of inflammation in the pathogenesis of diabetic atherosclerosis. While several inflammatory pathways could account for the increase production of 2-AA-LPC, [36–39] further research is needed to better understand the local

processes involved in the alterations of plaque composition in T2DM and to identify potential therapeutic targets for the prevention and treatment of diabetic atherosclerosis.

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Author's contributions

Conceptualization, D.M., A.L. and L.M.; Methodology, D.M., M.N., C.T. and L.M.; Investigation, L.M., A.L., D.L. K. V.D., V.C., M.N., T.P., JPPDB. Writing – Original Draft, D.M.; C.T. and L.M.; Writing – Review & Editing; Resources, E.S., V.C., A.L., M.N., T.G., and J.M.P. Supervision, D.M. All the authors read and approved the final manuscript.

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Data availability

All the data and details of analytical methods are available from the corresponding authors upon request.

Declarations

Ethics approval and consent to participate

The MASCADI protocol was reviewed and approved by the regional Ethics Committee (Comit e de Protection des Personnes Est, Dijon, France CPP Est III, CHRU Nancy, N  2017-A00022-51) and recorded on ClinicalTrials.gov (clinical registration number: NCT03202823). According to the trial category (Interventional research that does not involve drugs or non-CE-marked medical devices and that involves only minimal risks and constraints for the patient) and the ethics committee, all patients received written information note on the study. Oral consent was obtained from the patient and an attestation of the patient's oral consent was signed by the investigator physician and countersigned by the patient, according to the french law in this type of trial.

Consent for publication

N.A.

Competing interests

The authors have no conflict of interest to disclose.

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