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Lipids associated with atherosclerotic plaque instability revealed by mass spectrometry imaging of human carotid arteries

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ABSTRACT

Background and aims: Lipids constitute one of the main components of atherosclerosis lesions and are the mediators of many mechanisms involved in plaque progression and stability. Here we tested the hypothesis that lipids known to be involved in plaque development exhibited associations with plaque vulnerability. We used spatial lipidomics to overcome plaque heterogeneity and to compare lipids from specific regions of symptomatic and asymptomatic human carotid atherosclerotic plaques.

Methods: Carotid atherosclerotic plaques were collected from symptomatic and asymptomatic patients. Plaque lipids were analyzed with the spatial lipidomics technique matrix-assisted laser desorption/ionization mass spectrometry imaging, and histology and immunofluorescence were used to segment the plaques into histomolecularly distinct regions.

Results: Macrophage-rich regions from symptomatic lesions were found to be enriched in phosphatidylcholines (synthesized to counteract excess free cholesterol), while the same region from asymptomatic plaques were enriched in polyunsaturated cholesteryl esters and triglycerides, characteristic of functional lipid droplets. Vascular smooth muscle cells (VSMCs) of the fibrous cap of asymptomatic plaques were enriched in lysophosphatidylcholines and cholesteryl esters, know to promote VSMC proliferation and migration, crucial for the buildup of the fibrous cap stabilizing the plaque.

Conclusions: The investigation of the region-specific lipid composition of symptomatic and asymptomatic human atherosclerotic plaques revealed specific lipid markers of plaque outcome, which could be linked to known biological characteristics of stable plaques.

1. Introduction

Atherosclerosis is the main cause of ischemic diseases, accounting for about three quarters of cardiovascular-related deaths [1]. Processes contributing to plaque onset and progression evolve simultaneously, involving multiple cell types, making the plaque extremely heterogeneous [2]. The worst evolution of an atherosclerotic plaque is its rupture with consequent thrombosis [2]. Several characteristics are considered typical of a vulnerable plaque, a thin fibrous cap and a large lipid core, sustained inflammation, hemorrhage, neovascularization, and little or no calcification [3]. While these characteristics are well established, much less is known about the events that lead to plaque destabilization.

Lipids play an important role in atherosclerosis, triggering its genesis and forming the plaque core. They are involved in many mechanisms of plaque progression and stability, including inflammation, vascular smooth muscle cell (VSMC) proliferation, and immune cell activation [4].

The role of lipids in plaque destabilization is dependent on the cell

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type expressing them. Lysophosphatidylcholines (LPC) have been shown to stimulate the migration and proliferation of VSMCs [5,6], processes essential to the formation of the fibrous cap that confers stability to the plaque. Sphingomyelins (SM) have been associated with plaque size and development in rodent models [7–9], though the extent of this association in human patients remains uncertain. Despite insights gained from *in vitro* and animal studies, the characterization of specific lipid species within distinct regions of human plaques and their correlation with clinical outcomes pose significant technical challenges. Atherosclerotic plaques are characterized by their high cellular heterogeneity; to assess the role of lipids in plaque vulnerability it is important to separate changes in lipid content associated with clinical outcome from those due to cellular heterogeneity. Spatial lipidomics based on matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) allows for lipid characterization of such heterogeneous tissue samples [10], and has been of increasing interest for cardiovascular research [11–13]. MALDI-MSI has been used to investigate the plaque composition of mouse [14,15], rabbit [16] and human [17–19] atherosclerotic lesions. More recently, a protocol developed to characterize the lipid composition of human carotid atherosclerotic plaques [20] was used to identify region-specific differences [21]. Importantly, these studies did not investigate a correlation between lipid composition and plaque vulnerability.

The goal of this study was to identify lipid markers of plaque vulnerability. This work builds on a previous study in which we reported the development of a spatial lipidomics approach that combined MALDI-MSI with histological and immunofluorescence staining to enable the lipid profiles of histologically specific regions within the heterogenous plaques to be determined [22]. Here, we applied the method to the largest dataset of human atherosclerotic plaques analyzed by spatial lipidomics, identifying lipids associated with plaque destabilization in multiple plaque regions.

2. Materials and methods

An extended version of these methods section can be found in Supplementary Material 1.

2.1. Tissue collection

Atherosclerosis tissue samples were collected at the Department of Vascular Surgery of the Azienda Ospedaliero Universitaria Pisana. Clinical data were acquired from medical records, according to the declaration of Helsinki. All subjects gave written informed consent to participate in the study. The ethical approval was granted by the Ethics Commission of Tuscany Region (Protocol 37,200, Comitato Etico Regionale per la Sperimentazione Clinica della Regione Toscana, Area Vasta Nord Ovest, approved 26/06/2019). Patients were classified as symptomatic if they suffered from TIA (Transient Ischemic Attack) during the last six months before surgery [23]. Patients underwent carotid endarterectomy if eligible according to the European Society of Vascular Surgery 2017 guidelines [23]. Symptomatic patients with stenosis between 50 % and 99 % were eligible for surgery. Asymptomatic patients were eligible if the stenosis was between 70 % and 99 %, the patient's life expectancy was more than 5 years, and one or more imaging characteristics associated with an increased risk of ipsilateral stroke were present [23]. The plaques were evaluated and histologically classified according to the American Heart Association scheme [24,25]. A summary of the histopathological evaluation is provided in Supplementary Material 3. The homogeneity of atherosclerosis risk factors, comorbidities and drug treatment between patient groups were evaluated using a *t*-test and the independent proportions test using GraphPad Prism v5 (GraphPad Software, Inc., San Diego, CA, USA) and Microsoft Excel. Carotid plaques were collected after scheduled or emergency endarterectomy surgery, immediately rinsed in physiological solution and frozen on dry ice at −78.5 °C. Samples were then stored at −80 °C.

2.2. Sample preparation

Carotid plaques were transversally cut on dry ice and embedded in a solution of 2 % low melting point agarose. Tissue sections were cut using a Leica CM1950 cryostat (Leica, Wetzlar, Germany) and consecutive sections were mounted onto glass slides for histological and immunofluorescence assessment. Two consecutive tissue sections of 12 μm thickness were mounted onto ITO conductive slides for MALDI-MSI.

2.3. MALDI-MSI data acquisition

Consecutive sections were sprayed with norharmane (7 mg/mL, $CHCl_3$:MeOH 70:30) and 2,5-dihydroxybenzoic acid (DHB, 30 mg/mL in MeOH:H_2O 70:30, 0.2 % TFA) using a SunCollect (SunChrom, Friedrichsdorf, Germany) spraying system. MALDI-MSI was performed using an EP-MALDI source [26] (Spectroglyph LLC., Kennewick, WA, USA) equipped with a 349 nm laser (Spectra-Physics, Santa Clara, CA, USA) and coupled to an Orbitrap Q-Exactive Plus (Thermo Scientific, Bremen, Germany). All experiments were performed using 30 × 30 μm pixel size and positive ion mode.

2.4. Histological analysis

The MALDI-MSI analyzed tissue sections were stained with hematoxylin and eosin (H&E) after data acquisition. Residual MALDI matrix was removed using ethanol washes followed by rehydration and H&E staining. Consecutive tissue sections were stained with Masson's Trichrome. High resolution optical images of the histologically stained tissues were recorded using an Aperio CS2 scanner at 20 × magnification (Aperio Technologies Inc., Vista, CA, USA) and processed using Aperio ImageScope (v 12.2.2.5015, Aperio Technologies Inc.).

2.5. Immunofluorescence

Monoclonal anti-α-SMA and anti-human CD68 antibodies were used for immunofluorescence microscopy to identify vascular smooth muscle cells (VSMCs) and macrophages, respectively (Supplementary Fig. 1). Tissue sections were incubated with the primary antibodies for 2h at room temperature in the dark. AlexaFluor 568 goat anti-mouse secondary antibody was then added and incubated for 1 h at room temperature in the dark.

2.6. Histological annotation

Tissue annotation, co-registration with MALDI-MSI data, and transfer of annotations were performed as previously described [22]. In brief, histological and immunofluorescence images were used to annotate the histological tissue sections. Five regions were annotated: collagen, lipid-necrotic core, CD68 positive macrophages, α-SMA positive VSMCs of the media layer (outer VSMCs) and α-SMA positive VSMCs that have migrated into the intima layer (inner VSMCs). VSMCs of the media layer and of the fibrous cap were treated as separate regions (Supplementary Fig. 2). All histological annotations were confirmed by a pathologist (A. P.) specialized in cardiovascular disease. The H&E image was then co-registered to the MALDI MSI dataset [22].

2.7. MSI data preprocessing

MSI data were imported into Matlab R2019b (MathWorks, Natick, MA, USA) for processing and analysis. After pre-processing, peak-picking, TIC normalization, and bright spot removal (>99.5th percentile) the datasets from all tissue sections were combined into a merged datacube, using spatial offsets to separate the different datasets.

Table 1
Summary of plaque classification of symptomatic and asymptomatic patients. TIA: Transient ischemic attack.

	Patient	Symptoms	Plaque classification
Symptomatic	Sympt. 1	TIA	Type VIb
	Sympt. 2	TIA	Type VIb
	Sympt. 3	Repeated TIA	Type VIb
	Sympt. 4	TIA	Type VIc
	Sympt. 5	TIA	Type VIb
	Sympt. 6	TIA	Type VIb
	Sympt. 7	TIA	Type VIb
	Sympt. 8	TIA	Type VIb
	Sympt. 9	TIA	Type Va
Asymptomatic	Asympt. 1	None	Type VI
	Asympt. 2	None	Type VIb
	Asympt. 3	None	Type IVa
	Asympt. 4	None	Type Va
	Asympt. 5	None	Type III
	Asympt. 6	None	Type Va
	Asympt. 7	None	Type Vb
	Asympt. 8	None	Type VIb
	Asympt. 9	None	Type VIb
	Asympt. 10	None	Type Vc
	Asympt. 11	None	Type VIc
	Asympt. 12	None	Type Vc
	Asympt. 13	None	Type VIb
	Asympt. 14	None	Type VIb
	Asympt. 15	None	Type VIb
	Asympt. 16	None	Type VIb or VIc
	Asympt. 17	None	Type VIc
	Asympt. 18	None	Type Vc
	Asympt. 19	None	Type VIb
	Asympt. 20	None	Type VIb

2.8. MSI data analysis

The lipid profiles from histomolecularly specific regions were extracted from each tissue section's MALDI-MSI dataset, and compared using hierarchical cluster analysis (Euclidean distance, average linkage).

Partial least squares (PLS) regression was performed to identify lipids characteristic of symptomatic plaques. PLS was performed in Matlab. The number of components used for each PLS model was determined using the weight randomization test [27]; in each case the first PLS axis was used because it maximizes the covariance between the response and the predictors [28]. To avoid bias arising from the different sizes of the regions in different patient samples, PLS regression was performed by randomly extracting a defined number of pixels from each patient's region-of-interest (ROI); the number of pixels corresponded to 50 % of the pixels contained in the smallest ROI.

Lipid features that discriminated between symptomatic and asymptomatic plaques were defined as those with a variable importance in projection (VIP) greater than one [29], and with a loading on the first PLS axis exceeding the 90th percentile of the positive (increased in symptomatic) or negative (increased in asymptomatic) values. The entire process was repeated three times, only those lipids consistently identified as dysregulated in all replicates were considered as candidate features.

Lipids were assigned using the lipid database LIPID MAPS [30] (retrieved on the 1st October 2020) by searching for $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$ and $[M + H\text{-}H_2O]^+$ adducts with 0.005 Da tolerance. Assignments were further refined using a database of lipids identified by MS/MS in human adipose tissue [31] to limit assignments to lipids that have previously been identified in human samples.

Lipid species characterizing the macrophage-rich region and inner VSMCs, obtained by PLS regression at the pixel level, were further investigated at the patient level. The average lipid profiles of the macrophage-rich region and inner VSMC region were obtained from each patient, and the lipids compared between patients with asymptomatic and symptomatic plaques using a two-tailed *t*-test (GraphPad, v. 8.0.1, La Jolla, CA, USA).

3. Results

3.1. Patient characteristics and histological plaque classification

Carotid atherosclerotic plaques were collected from 29 patients undergoing surgical endarterectomy. According to the guidelines of the European Society of Vascular Surgery [23], they were divided into two groups: 1) asymptomatic and 2) suffering from TIA symptoms less than 6 months prior to surgery. Nine patients were classified as symptomatic, and twenty as asymptomatic. Further data about the 29 atherosclerosis patients was collected from their clinical records, including risk factors such as sex, age, tobacco consumption, cholesterol and triglycerides blood levels, and obesity (considered as BMI>30) [32], comorbidities like diabetes, hypercholesterolemia, (>240 mg/dL) and hypertension (>140/90 mmHg), and medical treatment (statins, anti-hypertension, beta-blockers and anti-platelet drugs). The distribution of these factors in the symptomatic and asymptomatic patient groups was then compared; risk factors, comorbidities and treatments were compared using an independent proportion test and age, cholesterol and triglycerides levels were compared using a *t*-test. This analysis demonstrated that the symptomatic and asymptomatic patients were not significantly different for age, cholesterol and triglycerides blood levels, risk factors, comorbidities, and treatment (Supplementary Fig. 3).

Paques were evaluated and classified according to American Heart Association guidelines [24,25]. A summary of these classifications is shown in Table 1; an extended description of the plaques is provided in Supplementary Material 3. All the plaques, except for one type IVa and one type III lesion, were classified as type V or type VI, corresponding to the latest stages of plaque development.

3.2. Lipid profiles of plaque regions

Carotid plaques were embedded, sectioned, and mounted on glass slides, and then analyzed using a multimodal spatial lipidomics approach based on MALDI-MSI of lipids, histology, and immunofluorescence. MALDI-MSI was employed to measure the lipid composition of

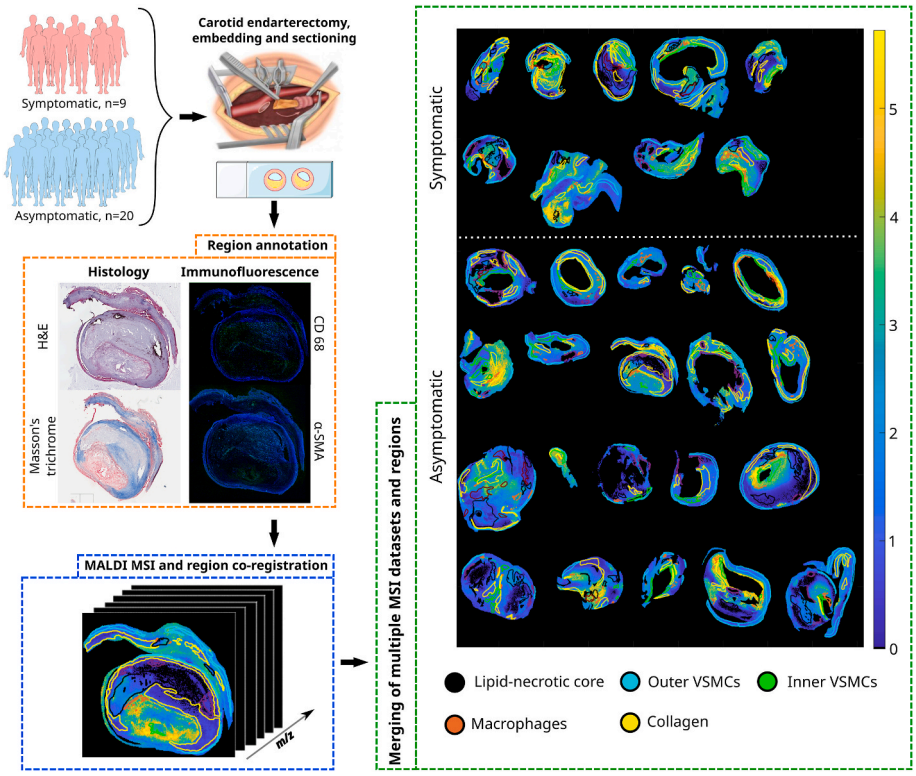

Fig. 1. Summary of the spatial lipidomics approach used for the comparison of symptomatic and asymptomatic carotid atherosclerotic plaques.

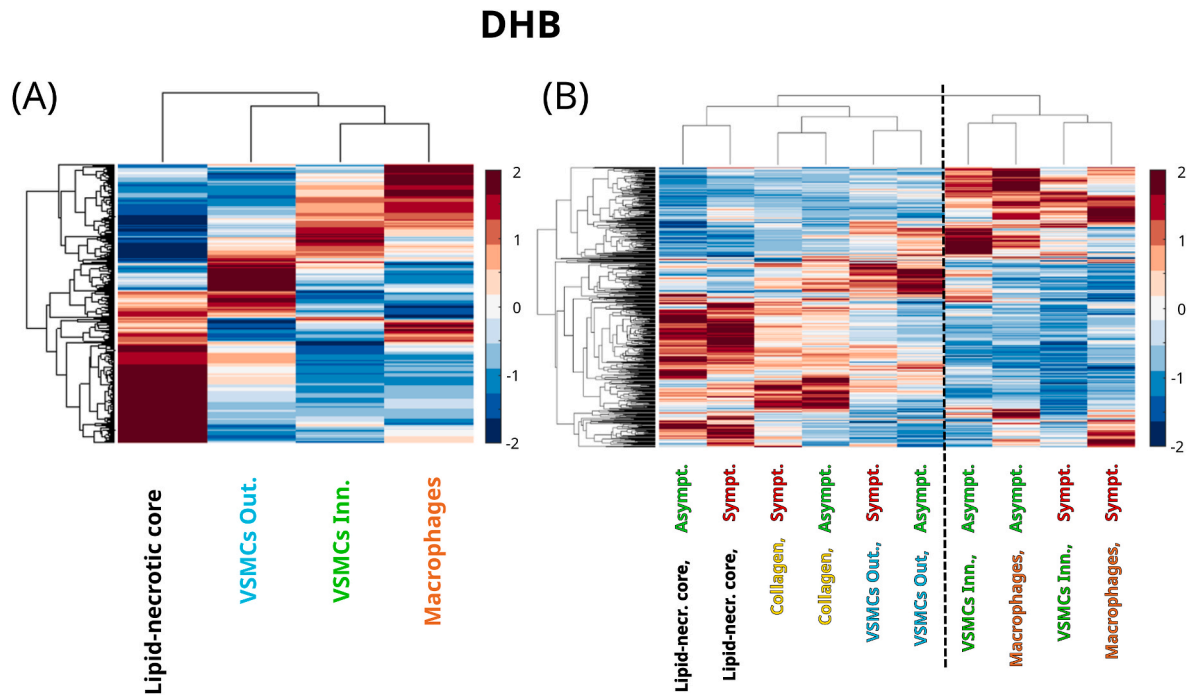

Fig. 2. Hierarchical cluster analysis for DHB matrix.
(A) Hierarchical cluster analysis of lipid profiles of the lipid-necrotic core, VSMCs of the outer and inner plaque layers, and macrophage-rich region. (B) Hierarchical cluster analysis when lipid profiles from symptomatic and asymptomatic plaques were input as separate profiles. The dotted line separates plaque regions that cluster according to histology from plaque regions that cluster based on the patient's clinical phenotype. Analysis was performed on the lipids detected by MALDI-MSI using the matrix DHB. The results of the same analyses for the norharmane datasets are available in Supplementary Fig. 5.

the tissue section in a spatially resolved manner, while histology and immunofluorescence were used to define specific regions based on tissue morphology and protein expression (Fig. 1). The combination of CD68 and α-SMA immunofluorescence microscopy with histopathological analysis using H&E and Masson's Trichrome, was used to define the following regions-of-interest (ROI) in each plaque: necrotic core,

macrophage-rich region, collagen-rich region, and VSMCs of the inner and outer layers of the plaque. These annotations were then coregistered to the MALDI-MSI datasets the lipid profiles of each ROI, from each patient's plaque, extracted for subsequent statistical comparison.

Hierarchical cluster analysis was performed to compare the lipid profiles of the necrotic core region, macrophage rich region, and VSMCs of the inner and outer layers of the plaque (Fig. 2A and Supplementary Fig. 5A for matrices DHB and norharmane, respectively). The analysis revealed that VSMCs of the inner layer of the plaque exhibited a lipid profile closer to the macrophage rich region than to VSMCs of the outer plaque region. These results indicate that VSMCs of the fibrous cap have a different lipid composition than VSMCs of the outer plaque region (the media layer of the artery). It is known that VSMCs of the arterial media layer can lose their contractile phenotype, migrate towards the vessel lumen and acquire a synthetic phenotype [33]. In addition, VSMCs can acquire macrophage-like characteristics and contribute to the foam cell population [34,35]. These results suggest that such cytological alterations are mirrored by their lipid profile. These findings were confirmed when the analysis was performed separately on the ROIs from symptomatic and asymptomatic plaques (Supplementary Material 4 and Supplementary Fig. 4).

A hierarchical cluster analysis was then performed to compare the regions from symptomatic and asymptomatic plaques. The results are shown in Fig. 2B and Supplementary Fig. 5B for the matrices DHB and norharmane, respectively. It revealed that the lipid profiles from symptomatic and asymptomatic patients split into two groups. The necrotic lipid core, collagen-rich regions, and VSMCs of the outer layer had similar profiles regardless of the patient's clinical phenotype, whereas the lipid profiles of VSMCs of the inner layer and macrophage-rich ROIs clustered according to phenotype. This result confirmed the lipid profile similarities of these two regions, and an association between the lipid composition of these regions and plaque vulnerability.

3.3. Lipid features of plaque vulnerability

PLS analysis was then performed on each region, to identify specific lipids that differentiated symptomatic from asymptomatic plaques, here used as a proxy of plaque vulnerability. To guard against bias introduced during the random sampling of pixels the PLS analysis was repeated three times, considering as dysregulated only those lipid ions identified as dysregulated in all replicates (Supplementary Material 2). Fig. 3A and Supplementary Table 1 report the lipids from the macrophage-rich regions that discriminated between the symptomatic and asymptomatic plaques. Macrophage-rich regions of symptomatic plaques were found to be enriched in phosphatidylcholine (PC) (Supplementary Fig. 6) and sphingomyelins (SM). Closer examination of the lipids revealed that whereas long chain SM were associated with symptomatic plaques, very-long chain SM were found to be characteristic of asymptomatic plaques.

Lysophosphatidylcholines (LPC), in particular LPC 16:0 and LPC 18:0/LPC 20:3, were detected at higher levels in the macrophage-rich regions of asymptomatic plaques. LPC 16:0 and LPC 18:0 have previously been detected in the necrotic core of human carotid atherosclerotic plaques [36]. LPC act on macrophages and VSMCs and can have both pro- or anti-atherogenic effects [37]. Here, the upregulation of these species in the macrophage-rich region of asymptomatic plaques suggests a prevailing stimulation of anti-atherogenic processes. Macrophages of asymptomatic plaques were also found to be enriched in polyunsaturated cholesteryl esters (CE), in diglycerides (DG 32:0 and DG 34:1) and triglycerides (TG 54:5 or TG 52:2).

3.4. VSMCs of the inner layer

The hierarchical cluster analysis demonstrated that the lipid profile of the VSMCs of the inner layer (fibrous cap) of the plaque resembled that of the macrophage-rich region. Similarly, inner VSMCs of symptomatic plaques were also enriched in PC (Supplementary Fig. 6) and long-chain SM, while inner VSMCs of asymptomatic plaques were characterized by increased amounts of LPC, very-long chain SM, and polyunsaturated CE (Fig. 3B and Supplementary Table 2).

3.5. VSMCs of the outer layer

The hierarchical cluster analysis shown in Fig. 2 demonstrated that the lipid composition of the VSMCs of the outer part of the arterial media layer differed from the VSMCs of the plaque's fibrous cap (inner VSMCs). Nonetheless, the inner and outer layer VSMCs shared some characteristics regarding which lipids were found to be characteristic of symptomatic and asymptomatic patients (Supplementary Table 3).

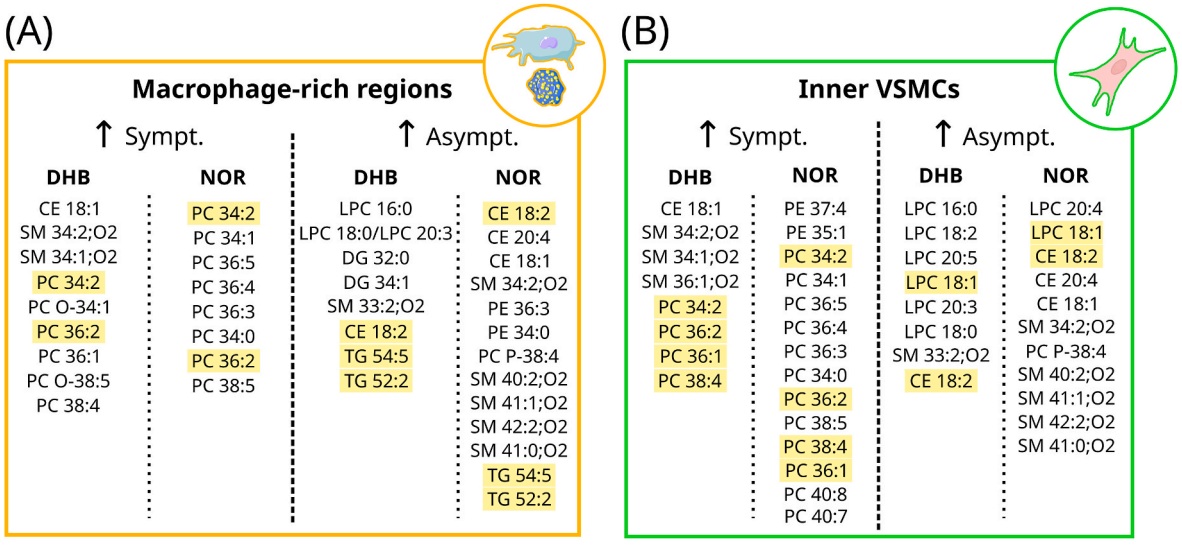

Fig. 3. Lipids in the macrophage region (A) and in the VSMCs of the inner layer (B) of plaques found to be associated with symptomatic and asymptomatic status. Species with a variable importance in the projection (VIP) score >1 and with a loading on the first partial least squares (PLS) axis exceeding the 90th percentile of the positive or negative values are reported. DHB: Lipids identified from the MALDI-MSI dataset recorded using 2,5-dihydroxybenzoic acid matrix; NOR: Lipids identified using norharmane matrix; Lipids dysregulated in both DHB and NOR datasets are highlighted in yellow. CE: Cholesteryl ester; SM: Sphingomyelin; PC: Phosphatidylcholine; LPC: Lysophosphatidylcholine; DG: Diglyceride; TG: Triglyceride; PE: Phosphatidylethanolamine; PC P: Plasmalogen-PC; PC O: Alkyl-PC. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

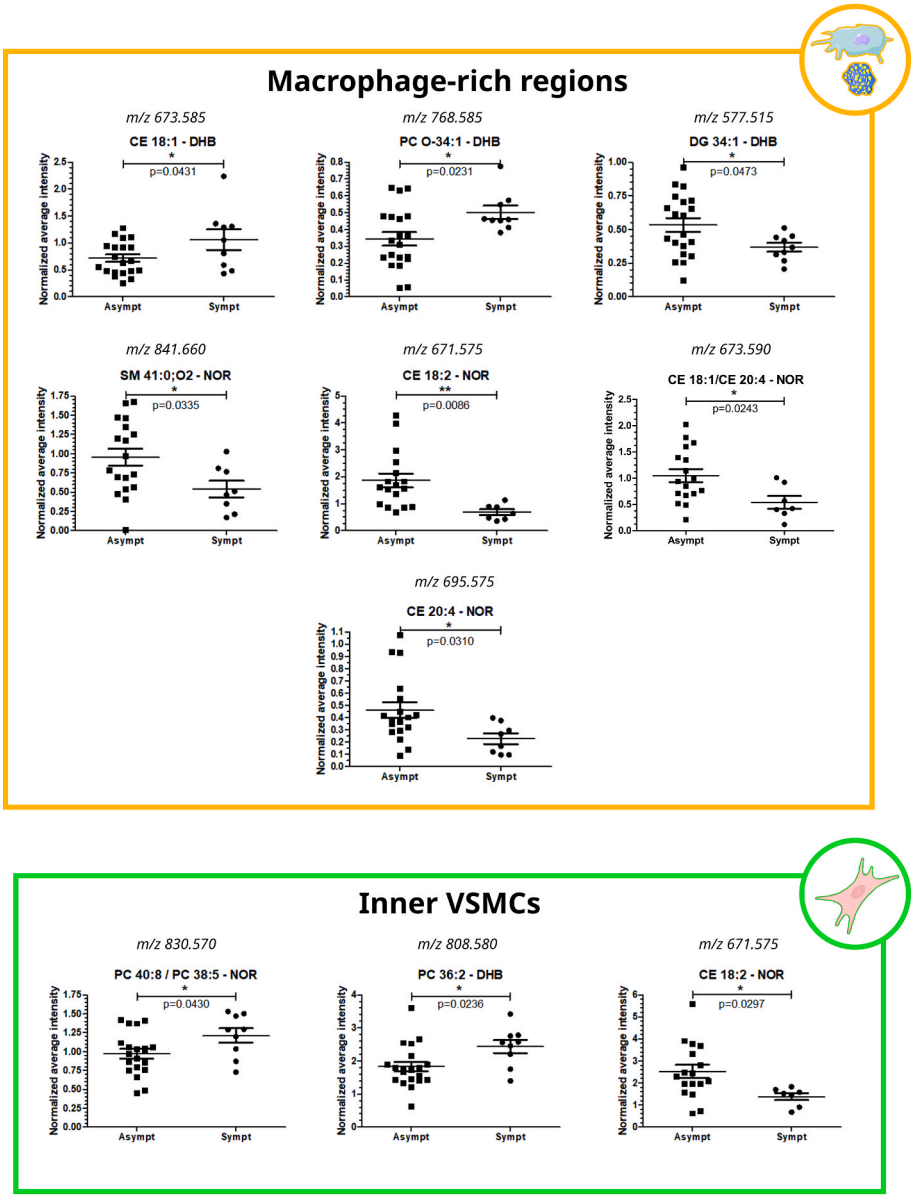

Fig. 4. Comparison of the average lipid intensity of lipids discriminating from symptomatic and asymptomatic plaques in the macrophage-rich region and in the inner VSMCs region.
Average spectra was calculated for each patient and for each region. Intensities of the lipid of interest was compared between symptomatic and asymptomatic patients with a two-tailed *t*-test (*: $p < 0.05$). Lipids that exhibited a significant difference are reported. DHB: DHB dataset, NOR: Norharmane dataset. CE 20:4 was significant in macrophage-rich regions for both the $[M+H]^+$ (*m/z* 673.590) and the $[M+Na]^+$ (*m/z* 695.575) adducts.

VSMCs of the outer layer of symptomatic plaques were enriched in PC, and the asymptomatic plaques were also enriched in LPC and SM. In contrast to that observed for inner VSMCs, both long chain and very-long chain SM were increased in the outer VSMCs of asymptomatic plaques.

3.6. Necrotic lipid core and collagen

Supplementary Tables 4 and 5 show the lipid species dysregulated in the necrotic lipid core and in the collagen-rich region respectively. The necrotic core of symptomatic plaques was enriched in LPC and very-long chain SM; asymptomatic plaques was found to be enriched in long-chain SM. LPC were found more markedly associated with the collagen-rich region of asymptomatic plaques, together with CE.

3.7. Patient-wise comparison

The lipid profiles of the macrophage-rich regions and VSMCs of the inner plaque exhibited the greatest dependence on plaque symptomaticity (Fig. 2). The lipid species determined by PLS analysis of the spatial lipidomics data (Fig. 3) were then tested at the patient level. The average lipid profile of the regions was calculated for each patient and the lipid intensities between symptomatic and asymptomatic patients compared using a two-tailed *t*-test. This analysis confirmed the results obtained at the pixel level, Fig. 4, namely that CE 18:1, PC O-34-1 were detected at significantly increased levels in the macrophage-rich regions of symptomatic plaques, while DG 34:1, CE 20:4, and SM 40:0; O2 were detected at lower levels. Similarly, several long chain lipids were detected at significantly higher levels in the inner VSMCs of symptomatic plaques.

4. Discussion

This spatial lipidomics study revealed the accumulation of specific lipids in symptomatic *versus* asymptomatic atherosclerotic plaques. This is the first report of lipid changes, in specific human plaque compartments, that are associated with plaque vulnerability; the analysis of real patient samples is essential because animal models cannot satisfactorily reproduce the biochemical composition and evolution of human plaques.

Lipids play a crucial role in atherosclerosis since they are the responsible of the disease onset and constitute the core of the plaque. Lipids are biologically active and can stimulate cells by acting as signal molecules, by influencing the energetic metabolism or by modulating cell membrane stiffness [4,38]. Lipids are also important mediators of many cellular processes that can be crucial for plaque destabilization, such as inflammation [39], cell migration [40,41] and epithelial-to-mesenchymal transition [42].

Atherosclerotic plaques are complex, heterogeneous lesions formed by different and differently distributed cell populations. This cellular heterogeneity leads to a heterogeneous distribution of lipids. Circumventing this heterogeneity is necessary to identify molecules that contribute to plaque formation [4,38] and which may drive the lesion towards stability or instability. By combining MALDI MSI with histology and immunofluorescence, we analyzed the lipid composition of histomolecularly distinct regions to identify markers of plaque vulnerability/clinical risk.

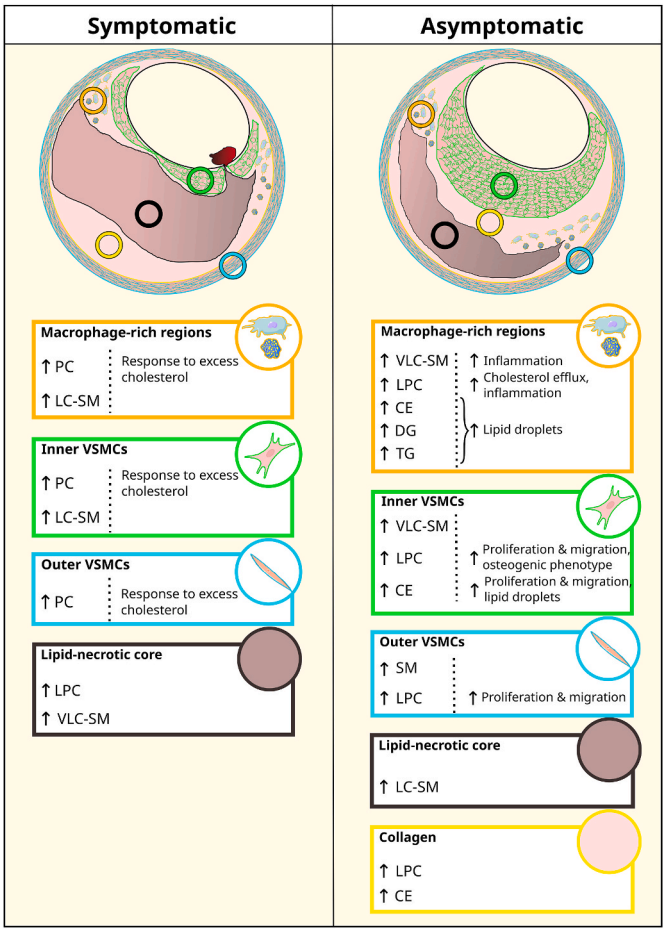

Fig. 5. Summary of lipid alterations in different regions and cell types of symptomatic and asymptomatic human carotid plaques.
Colored circles indicate macrophage-rich regions (orange), VSMCs of the inner layer (green), VSMCs of the outer layer (cyan), necrotic lipid core (black), and collagen (yellow). Known biological functions of the lipid class in the corresponding cell type is also reported. PC: phosphatidylcholines; LPC: lysophosphatidylcholines; LC-SM: long-chain sphingomyelins; VLC-SM: very long-chain sphingomyelins; CE: cholesteryl esters. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

We found that lipid profile of VSMCs in the inner plaque region resembles more closely that of the macrophage-rich region compared to VSMCs of the outer plaque layer. VSMCs migrate from the media layer towards the plaque luminal surface, changing their phenotype and acquiring macrophage-like characteristics. The results presented here suggest that these phenotypic and lipid changes occur in parallel. It was also observed that the lipid profiles of the macrophage-rich regions and VSMCs of the inner plaque layer were the most affected by plaque phenotype, confirming the importance of these regions to plaque stability and fate. Fig. 5 provides a summary of all the lipid classes we found dysregulated in regions of symptomatic and asymptomatic human carotid plaques and their potential involvement in pathogenic mechanisms.

The lipid profile of macrophage-rich regions from symptomatic plaques were enriched in PC and long-chain SM, while the same region of asymptomatic plaques was characterized by LPC, very long-chain SM, polyunsaturated CE, DG and TG. PC were recently reported to be spatially correlated with the macrophage-rich regions of symptomatic human carotid atherosclerotic plaques [19]. This study was performed using the MSI technique desorption electrospray ionization (DESI), an ionization technique distinct from MALDI. The identification of PC dysregulation in macrophage-rich regions of symptomatic plaques, by both MALDI (this study) and DESI (recent study), provides strong evidence of the important role of macrophage dysfunction in determining plaque outcome. PC and cholesterol have opposing effects on the fluidity of cell membranes, thus the free cholesterol to PC ratio is maintained in a narrow range to ensure membrane functionality [43]. Macrophages in atherosclerotic plaques undergo cholesterol overload during disease progression. As a response, it has been reported that macrophages increase PC biosynthesis to keep the free cholesterol to PC ratio below cytotoxic levels [43,44]. We surmise that macrophages in symptomatic plaques are less able to store excess cholesterol in lipid droplets and experience a higher load of free cholesterol, which induces PC synthesis. Macrophage-rich regions from asymptomatic plaques were also enriched in CE and TG, which form the core of lipid droplets in foam cells [45]. These results support the notion that functional and activated macrophages loaded with lipid droplets, but still capable to export cholesterol out of the cell, are beneficial for plaque stability; whereas macrophages loaded instead with free cholesterol accumulate PC and are associated with plaque destabilization and rupture.

PCs and ceramides are converted by sphingomyelin synthase to SMs [46]. We found that macrophage-rich ROIs of symptomatic plaques were enriched in long chain SM, while the same region of asymptomatic plaques was enriched in very-long chain SM. SM have been previously detected in atherosclerotic plaques [47,48] and it has been shown that the inhibition of their synthesis reduces plaque size in rodents [7,49]. In particular, SM are believed to modulate macrophage activity, hence influencing plaque stability. Inhibition of sphingomyelin synthase 2 (SMS2, which mostly affects very-long chain SM [50]) in murine macrophages increases cholesterol efflux [51], reduces inflammation, and stabilizes the plaque by decreasing the lipid core and increasing collagen content [52]. The association of SM content of macrophages with plaque stability has only been investigated in animal models and we now provide the first evidence of this phenomenon in human arteries. Differences between long chain and very-long chain SM are known, *e.g.* the latter are more affected by SMS2 inhibition [50]. In addition, very-long chain SM are more effective in activating macrophages [50,53] likely by influencing the response of membrane receptors like the Toll-like receptor 4 (TLR4) [53]. Our study provides evidence that long and very-long chain SM are differently associated with plaque stability.

Macrophage-rich regions of asymptomatic plaques were enriched in

pro-inflammatory LPC, which are known to induce the expression of scavenger receptors on macrophages and stimulate the production of pro-inflammatory factors [45]. At the same time, LPC attenuate cellular uptake of oxidized low-density lipoproteins and promote cholesterol efflux from cells through high-density lipoproteins [37].

The lipid profiles of VSMCs of the inner plaque were similar to those observed in the macrophage-rich regions (Fig. 2), and lipid changes associated with plaque vulnerability also mirrored those of the macrophage-rich ROI. PC accumulation in symptomatic plaques may represent a homeostatic response to the increased load of free cholesterol. VSMCs from asymptomatic plaques seem more able to manage excess lipids by storing them in droplets, as suggested by the increase in CE. It has been reported that cholesterol can stimulate VSMCs towards a macrophage/fibroblast-like phenotype [54]. Cholesterol esterification, needed to store excess cholesterol, is believed to trigger VSMC proliferation and migration [55,56], two key steps in the production of the fibrous cap that stabilizes the plaque. VSMCs of asymptomatic plaques were found to be enriched in LPC, which stimulate cell proliferation and migration [5,6] favoring the buildup of the fibrous cap and stabilizing the plaque. LPC also stimulate VSMCs towards an osteogenic phenotype by supporting calcification, another characteristic of stable plaques [57,58].

The outer layer VSMCs lipid profiles of symptomatic plaques were also found to be enriched in PC, which might suggest a partial cholesterol load also in these cells far from the vessel lumen. Similarly, LPC enrichment of the outer VSMCs of asymptomatic plaques might have a role in cell proliferation and migration, and thereby recruitment of VSMCs to the plaque lumen.

Patient-wise comparison of lipid intensities averaged across macrophage-rich and inner VSMC regions confirmed the differential expression of some of these lipids in symptomatic and asymptomatic plaques.

The differences in region-specific lipid content between symptomatic and asymptomatic plaques indicated that macrophages in symptomatic plaques are less able to store cholesterol in lipid droplets and instead synthesize PCs to counterbalance the excess free cholesterol; Macrophages from asymptomatic plaques are more able to store cholesterol in functional lipid droplets (increased CEs, DGs and TGs), sustain the inflammation and are involved in cholesterol efflux (increased VLC-SM and LPC). The results also indicate that VSMCs of the inner layer of symptomatic plaques may experience free cholesterol load as well, that induces PC synthesis. VSMCs of the inner layer of asymptomatic plaques were also associated with lipids connected with proliferation, migration and the development of an osteogenic phenotype (increased LPCs and CE), processes that are crucial for the formation of a plaque-stabilizing fibrous cap.

The association of the lipid plaque composition with plaque symptomaticity provides a snapshot of the processes occurring in the plaque. This region-specific information is crucial to understand the role of lipids – one of the major components of the plaque – in the processes leading to atherosclerotic plaque destabilization, and thereby to develop plaque-stabilizing drugs [59].

4.1. Limitations

Ex-vivo tissue samples can only provide a single snapshot of the dynamics behind the transition from a stable to a vulnerable plaque. However, the clinical evaluation is a powerful criterion for the identification of patients with increased risk of plaque-related thrombosis and consequent stroke [23]. Histological indicators of plaque vulnerability, such as the thickness of the fibrous cap and the presence of a large lipid pool and hemorrhage, have been reported as predictors of plaque rupture [60] but were not used here as we chose to focus the analysis on lipid changes associated with clinical phenotype. It was reasoned that a comparison of lipid changes associated with i) clinical phenotype, ii) fibrous cap thickness, and iii) lipid pool size, would become overly descriptive. To enable retrospective analysis, we have made the datasets available (see data availability statement for details where the datasets can be accessed).

Atherosclerosis is known to be associated with multiple factors such as age, sex, comorbidities, medical treatment and lifestyle [61–63]. Here patients were selected to balance for age, sex, risk factors, comorbidities, and medical treatments between the asymptomatic and the symptomatic patient groups. Nonetheless, we cannot exclude an effect of the confounding factors on the plaque lipid composition of a single patient. The difficulties involved in patient enrollment and the large number of confounding factors involved in atherosclerosis make complete patient matching very challenging.

Lipid assignment in MALDI MSI is typically performed by matching the exact mass of the detected ions with a database. Here we used the LIPID MAPS database [30] and all assignments were then refined using a database of lipids identified by MS/MS of a human adipose tissue lipid extract [31].

The MALDI-MSI experiments reported here were recorded with high mass resolution and accurate mass, nevertheless some of the peaks could be assigned to multiple species. For example, m/z 673.590 in the norharmane dataset could be assigned to the sodiated ion of CE 18:1 or the protonated ion of CE 20:4 (Fig. 4). Close examination of the data indicated that the protonated ion of CE 20:4 contributed most of the intensity because this peak was detected at lower levels in the symptomatic plaques, in accordance with the lower level of the sodiated ion of CE 20:4 in symptomatic plaques but contrary to the higher levels of CE 18:1 in symptomatic plaques (DHB dataset, Fig. 4). MALDI-MS/MS-MSI and MALDI-ion-mobility-MSI have been performed to distinguish lipids directly from the tissue, but would require separate databases of reference MS/MS spectra/collision-cross-sections to separate the contributions from the isobaric ions detected here [64].

Atherosclerotic plaques are 3D structures with a variable composition along the artery (longitudinal variation) as well as across the artery. The tissue sections analyzed here were taken from the longitudinal center of the plaque. A spatial lipidomics analysis of the longitudinal structure could be performed by 3D MALDI-MSI [15,18] or by analyzing tissue sections sampled at different longitudinal positions along the plaque [21]. However, such an approach was not practically feasible: a 3D analysis at 30 μm voxel size, spanning just 3 mm along the artery, would require 100 tissue sections to be analyzed (circa 100 days measurement time), histology ($\times$ 2), and immunofluorescence ($\times$ 2) from each patient. Moreover, a previous 3D MALDI-MSI study of human carotid atherosclerotic plaques demonstrated that lipid composition correlated with histological features [18], suggesting that the lipid composition of histological regions changes little across the longitudinal dimension.

4.2. Concluding remarks

Here we demonstrated an association between the levels of lipids and plaque vulnerability for histomolecularly specific regions of atherosclerotic plaques, thus providing a basis for future investigations of these molecules as drivers of plaque stability/instability. In particular, PC and LC-SM were found to be associated with macrophage-rich regions and VSMCs of the inner layer of symptomatic plaques, while LPC, CE and VLC-SM were associated with the same regions of asymptomatic plaques. Our results might also aid pharmacological/biological therapies aimed at modulating the turnover of selected lipid species as a mean to stabilize atherosclerotic plaques.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The MALDI-MSI data have been deposited to the ProteomeXchange Consortium via the PRIDE [65] partner repository with the dataset identifier PXD039822.

CRediT authorship contribution statement

Francesco Greco: Conceptualization, Methodology, Investigation, Data curation, Software, Formal analysis, Writing – original draft. **Giulia Bertagna:** Resources. **Laura Quercioli:** Resources. **Angela Pucci:** Data curation, Validation. **Silvia Rocchiccioli:** Resources, Project administration. **Mauro Ferrari:** Resources, Conceptualization, Writing – review & editing. **Fabio A. Recchia:** Supervision, Writing – review & editing. **Liam A. McDonnell:** Conceptualization, Methodology, Resources, Project administration, Supervision, Writing – review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2024.118555.

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