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Complement C5 Protein as a Marker of Subclinical Atherosclerosis

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ABSTRACT

BACKGROUND The mechanisms underlying early atherosclerotic plaque formation are not completely understood. Moreover, plasma biomarkers of subclinical atherosclerosis are lacking.

OBJECTIVES The purpose of this study was to analyze the temporal and topologically resolved protein changes taking place in human aortas with early atherosclerosis to find new potential diagnostic and/or therapeutic targets.

METHODS The protein composition of healthy aortas (media layer) or with early atheroma (fatty streak and fibrolipidic, media and intima layers) was analyzed by deep quantitative multiplexed proteomics. Further analysis was performed by Western blot, immunohistochemistry, real-time polymerase chain reaction, and enzyme-linked immunosorbent assay. Plasma levels of complement C5 were analyzed in relation to the presence of generalized (>2 plaques) or incipient (0 to 2 plaques) subclinical atherosclerosis in 2 independent clinical cohorts (PESA [Progression of Early Subclinical Atherosclerosis] [n = 360] and NEFRONA [National Observatory of Atherosclerosis in Nephrology] [n = 394]).

RESULTS Proteins involved in lipid transport, complement system, immunoglobulin superfamily, and hemostasis are increased in early plaques. Components from the complement activation pathway were predominantly increased in the intima of fibrolipidic plaques. Among them, increased C5 protein levels were further confirmed by Western blot, enzyme-linked immunosorbent assay and immunohistochemistry, and associated with in situ complement activation. Plasma C5 was significantly increased in individuals with generalized subclinical atherosclerosis in both PESA and NEFRONA cohorts, independently of risk factors. Moreover, in the PESA study, C5 plasma levels positively correlated with global plaque volume and coronary calcification.

CONCLUSIONS Activation of the complement system is a major alteration in early atherosclerotic plaques and is reflected by increased C5 plasma levels, which have promising value as a novel circulating biomarker of subclinical atherosclerosis. (J Am Coll Cardiol 2020;75:1926–41) © 2020 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

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Atherosclerosis is a complex disease that develops over decades, but is usually detected only at advanced symptomatic stages or after a cardiovascular (CV) event. Recent data reveal that subclinical atherosclerosis is highly prevalent in middle-aged, asymptomatic individuals (1). Detection of subclinical atherosclerosis improves risk stratification (2), highlighting the need for new tools (e.g., imaging or molecular biomarkers) to identify asymptomatic patients at risk. Moreover, current treatments targeting conventional risk factors are of limited value, because atherosclerosis and CV events are known to occur in individuals without risk factors (3). This suggests that beyond risk factors, atherogenesis involves mechanisms and mediators, which could be important in the initial stages of the wall pathology before its clinical expression. The accepted paradigm is that atherosclerosis initiation takes place by a 2-step process. First, the translation of circulating lipids to the vascular wall and their retention in the subendothelium (4), forming fatty streaks (FS). This lipid retention then triggers a process of smooth muscle cell migration and proliferation to form the neointimal fibrocellular cap, which encapsulates the lipid core to form a fibrolipidic (FL) plaque (5). A more comprehensive analysis of the molecular mechanisms underlying this initial pathophysiology in human atherosclerosis could help to identify new diagnostic and/or therapeutic targets.

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Different proteomics approaches have been used to identify new biomarkers or therapeutic targets in humans (6). At early disease stages, Van Eycḱ group recently analyzed whole plaques from coronary and aortic arterial territories detecting an increase in mitochondrial proteins in coronary atherosclerotic plaques; that study also revealed a subset of tissue protein biomarkers found in plasma indicative of early disease in a small cohort (7). Proteomics has also been used to analyze extracellular matrix components of advanced atherosclerotic plaques, identifying a panel of 4 biomarkers that predict CV risk in 2 large patient cohorts (8). These studies reveal that tissue-proteomics may be a promising approach in the search for biomarkers able to predict atherosclerosis.

Given the multiplicity of cell types and molecules involved in atherosclerosis and their variation between compartments and stages, we designed a proteomics analysis of specific regions (media and intima) in 2 consecutive stages of early atherosclerotic lesions (FS and FL plaques). The protein composition of each of these compartments and the pattern of protein alterations that take place along early plaque development have never been analyzed before from a proteome-wide approach. Moreover, due to the systemic nature of the disease, this proteomics analysis of vascular tissue has the potential to identify circulating biomarkers that could be useful in the diagnosis of patients with asymptomatic atherosclerosis.

ABBREVIATIONS AND ACRONYMS

C5 = complement C5

C9 = complement component C9

CACS = coronary artery calcium score

CD59 = CD59 glycoprotein

FL = fibrolipidic

FS = fatty streak(s)

VUS = vascular ultrasound

METHODS

A detailed description of the methods can be found in the Supplemental Appendix.

HUMAN TISSUE SAMPLES. Abdominal aortic samples (healthy [H], FS, or FL plaques [Supplemental Figure 1]) were obtained from brain-deceased organ donors (artificially ventilated, with a beating heart or cold-perfused body) during organ removal for therapeutic transplantation (kidney or liver transplantation) with the authorization of the French Biomedicine Agency (PFS 09-007, BRIF BB-0033-00029; AoS BBMRI-EU/infrastructure BIOBANQUE; No. Access: 2, last: April 15, 2014. [BIORESOURCE]). The aortic tissue was washed and preserved in ringer lactate at 4°C until use. The whole aortic sample was macroscopically processed and dissected, with removal of the adventitia, followed by separation of the intima and media only in FS and FL plaques (as in the healthy aorta, the intima was too thin to be separated from the media).

Aortic samples (100 mg) from media (H [$n = 9$], FS_m [$n = 7$], and FL_m [$n = 11$]) and intima (FS_i [$n = 6$] and FL_i [$n = 12$]) were homogenized within liquid N_2 and protein/mRNA lysates were extracted, quantified, and kept at -80° C until further analysis.

Specific tissue-conditioned medium was obtained by cutting tissue samples (intima and media separately) into small pieces (5 mm^2) and incubated them

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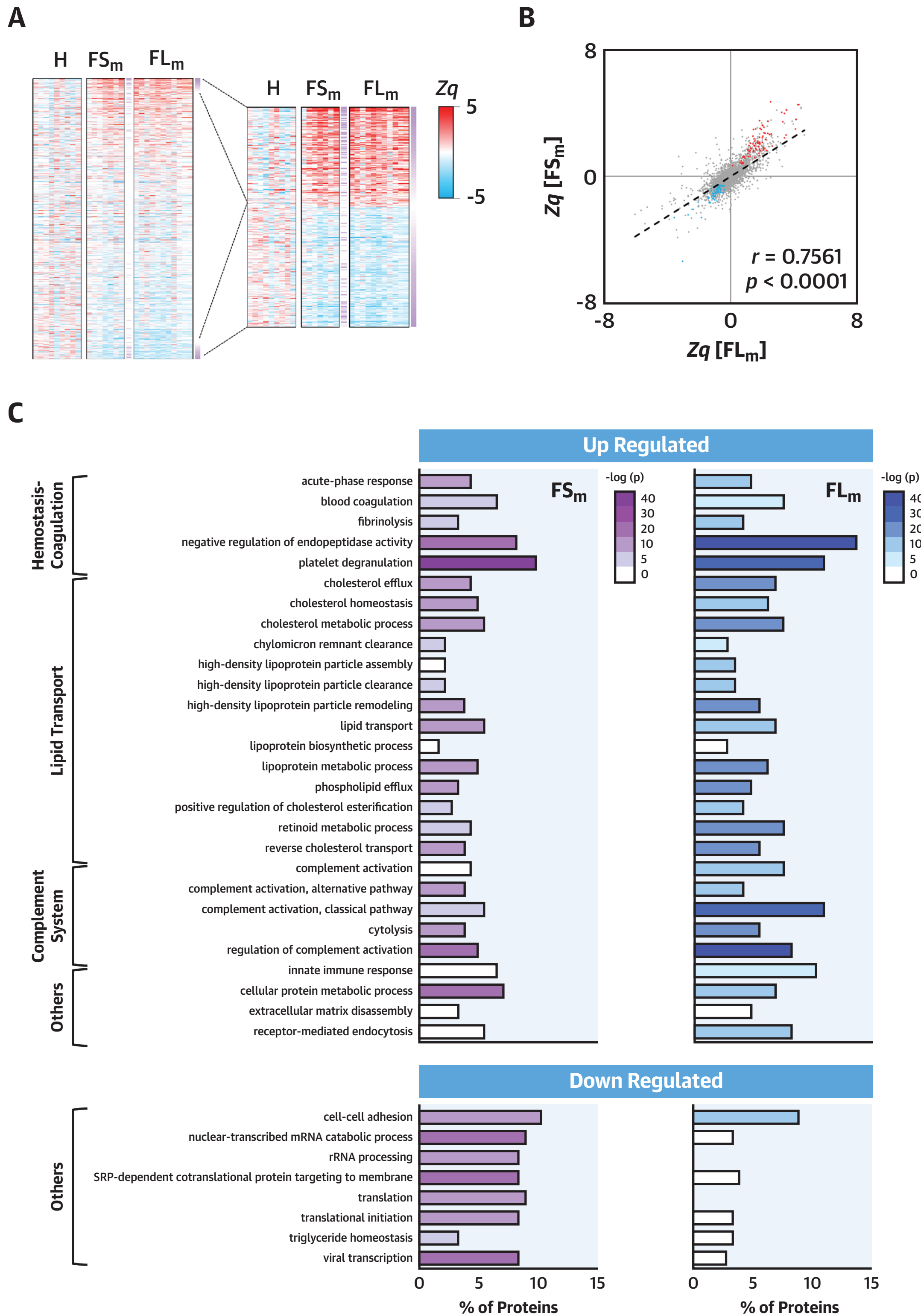
FIGURE 1 Comparison of Differentially Regulated Proteins in the Media of Early Atherosclerotic Tissue



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in RPMI 1640 medium without FBS for 24 h at 37°C (6 ml/g of wet tissue). The tissue-conditioned medium was collected after centrifugation and frozen at −80° C.

PROTEOMICS. Briefly, protein extracts were trypsin-digested and the resulting peptides were labeled with 10-plex TMT isobaric labelling and fractionated. Fractions were subjected to LC-MS/MS analysis using an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts). Identification, quantification, and systems-biology analysis were performed as described (9–13).

WESTERN BLOT. Equal protein amounts (10 ug) from homogenized tissues were resolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Membranes were incubated with antibodies against C5 (A396, Quidel, San Diego, California) and glyceraldehyde 3-phosphate dehydrogenase (GADPH) (MAB374, Millipore, Billerica, Massachusetts) and processed as described (14).

IMMUNOHISTOCHEMISTRY. Paraffin aortic samples were incubated with primary antibodies against C5 (A306, Quidel) or an anti C9 (a generous gift of Prof. P. Morgan, Cardiff University, United Kingdom) as described (14).

REAL-TIME POLYMERASE CHAIN REACTION. Human pathological and control tissues were snap frozen in liquid N_2 and homogenates (0.2 g) were divided and resuspended for mRNA analysis of C5 (Hs01004342_m1), CD59 (Hs00174141_m1), complement decay-accelerating factor (Hs00892618_m1), and GADPH (Hs02786624_g1) as described (14).

ENZYME-LINKED IMMUNOSORBENT ASSAY. Plates (Costar #33669) were coated with capture antihuman C5 antibody eculizumab overnight. A calibration curve was constructed with known concentrations of purified C5 (Complement Technology, Tyler, Texas). Detecting goat antihuman C5 (Quidel, A306) and HRP conjugated rabbit anti-goat (Cat P0160, DAKO, Troy, Michigan) were used. The enzyme-linked immunosorbent assay (ELISA) was developed with OPD substrate (Cat. P9187, Sigma, St. Louis, Missouri). Intraprecision and interprecision coefficients of variation were 6.7% and 9%, respectively.

For sC5b-9, we used the following reagents: coating antibody (Goat α-Mouse IgG2a, Human ads-UNLB, Cat #1080-01 Southern Biotech, Birmingham, Alabama); first antibody (Anti-Human TCC, Cat #HM2167, Hycult Biotech, Wayne Pennsylvania), detecting antibody (1:1 mix of the antiC5 mouse monoclonals SIM378.11.2.1 and SIM378.55.1.1); and goat antimouse IgG1 (human ads-HRP Southern Biotech, Cat #1070-05).

C5a and CD59 were detected with commercial ELISAs (DY2037, R&D, Minneapolis, Minnesota; and RAB1027, Sigma, respectively) following manufacturerś instructions. Samples with a code were randomly distributed between plates, and the personnel who performed the ELISA were blinded to the clinical data associated to the codes of samples.

HUMAN PLASMA SAMPLES. Test cohort. The study population consisted of 360 male participants enrolled in the PESA study (1). The PESA study is aimed at detecting early atherosclerosis in a middle age (age 40 to 54 years) low-risk population by noninvasive imaging, including 2-dimensional (2D) and 3-dimensional (3D) vascular ultrasound (VUS) for peripheral atherosclerosis evaluation and noncontrast cardiac computed tomography (CT) for coronary artery calcium score (CACS) quantification. All participants were free of clinically apparent CV disease and had a normal renal function. The institutional ethics committee approved the study protocol, and all participants provided written informed consent.

Validation cohort. The study population consisted of 394 control participants (54% men, 46% women) from the NEFRONA study (15). Participants were enrolled at 9 Spanish primary care centers distributed across 7 Spanish regions. They were free of overt CV disease, active infections, or kidney impairment, and were examined for the presence of subclinical atherosclerosis with 2DVUS. Each local ethics

FIGURE 1 Continued

(A) Heat map showing protein abundance changes (*Zq*) in the media layer of fatty streak (FS_m) and fibrolipidic (FL_m) plaques in relation to the average value in healthy aorta (H). Increased **(red)** or decreased **(blue)** abundances are expressed according to the indicated *Zq* scale. The **inset at the right** show the statistically significant abundance changes. **(B)** Correlation between identified proteins from FS and FL media. **(C)** Enrichment analysis of proteins differentially expressed in the media layer of atherosclerotic tissue. The **bars** show the relative proportion of changing proteins that belong to each one of the main biological processes (GOBP categories) that are significantly enriched. Bars are color-coded according to $-\log_2(p)$, where *p* refers to the p value obtained for Bonferroni enrichment. Only the significantly enriched GOBPs are shown [$-\log_2(p) = 5$ correspond to a p value <0.05]. The **color of the bars** represents the logarithm of Bonferroni-corrected p values of category enrichment. The complete list of identified GOBPs is displayed in Supplemental Table 2.

committee approved the study, and participants gave informed consent.

STATISTICAL ANALYSIS. Results are expressed as mean ± SE or median (interquartile ranges). Normality of data was analyzed by the Kolmogorov-Smirnov test. For multiple comparisons, we used analysis of variance followed by Tukey post hoc test or Kruskal-Wallis test followed by Dunn analysis. C5 in participants with incipient versus generalized atherosclerosis was analyzed by Mann-Whitney *U* test. The association between C5 and generalized atherosclerosis, adjusting for the identified confounders, was assessed by multivariate logistic regression analysis. The association between C5 and plaque burden or calcification adjusting for the identified confounders was assessed by multivariate linear regression analysis. Statistical analysis were performed using SPSS software version 23.0 (SPSS, Inc., Chicago, Illinois). Statistical significance was assigned at $p < 0.05$.

RESULTS

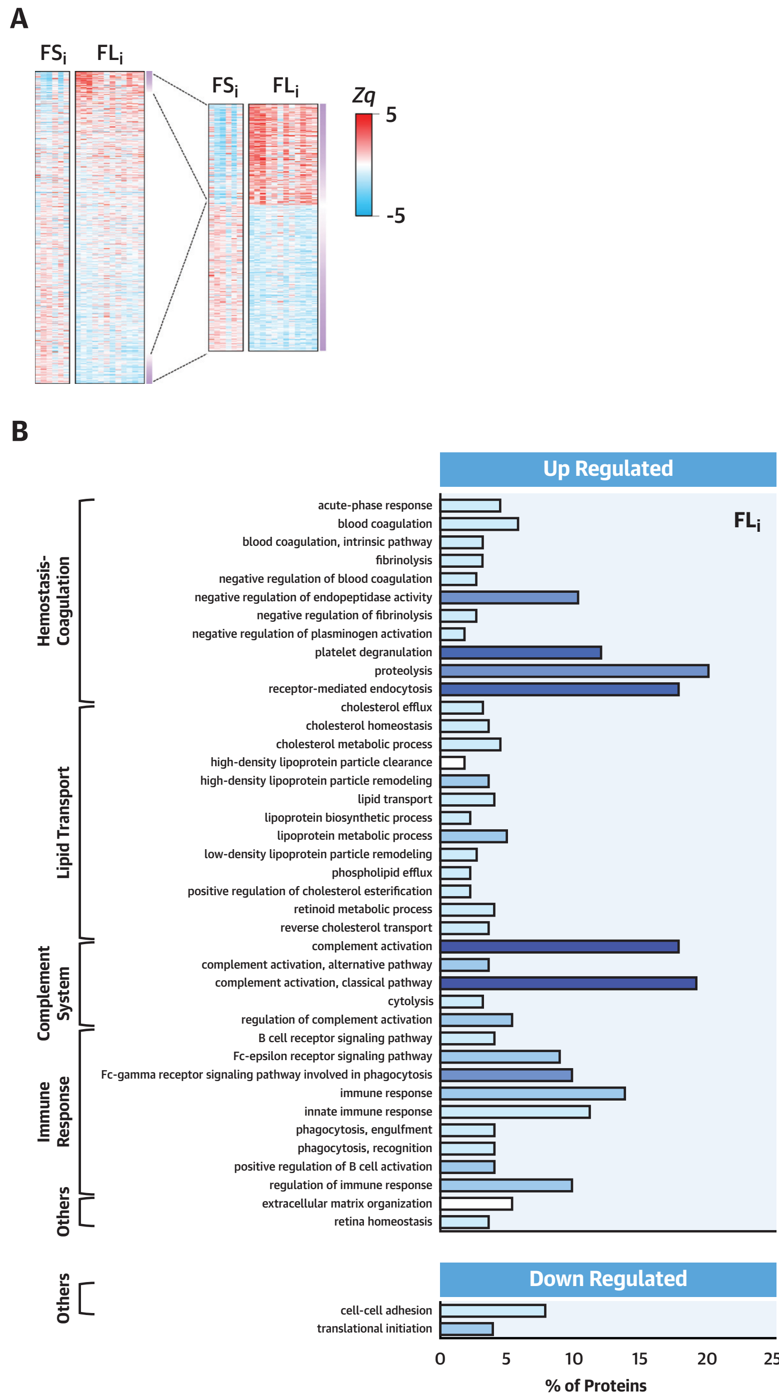
QUANTITATIVE PROTEOMICS REVEALS INCREASED ABUNDANCE OF LIPID TRANSPORT, HEMOSTASIS, AND COMPLEMENT PROTEINS IN THE MEDIA AND INTIMA LAYERS OF EARLY ATHEROSCLEROSIS PLAQUES. We analyzed separately the intima and media layers of aortas with FS and FL plaques as well as the media of healthy aortas (Supplemental Figure 2). More than 6,500 proteins were identified in the media and in the intima of atherosclerotic plaques. After discarding proteins identified with a single peptide, proteins present in <60% of either media or intima samples, and protein isoforms, 3,079 medial and 3,332 intimal proteins remained (henceforth, “identified proteins”) (Supplemental Table 1). Of those, 2,660 proteins were common to media and intima layers, with total abundance similar in the different compartments and tissues (Supplemental Figure 3). However, the media layer had higher amounts of proteins belonging to the vascular system, whereas the intima layer had higher amounts of proteins known to be present in liver and plasma (Supplemental Figure 3).

In total, 340 proteins in FS media (184 up and 156 down) and 328 in FL media (149 up and 179 down) significantly changed their relative abundance when compared with healthy media (**Figure 1A**). The changes in FS and FL media were quite similar, showing a high correlation (**Figure 1B**). The up-regulated proteins were significantly enriched in biological categories mostly related to hemostasis/coagulation, lipid transport/metabolism, and complement system. The relatively few down-regulated processes were mostly related to protein synthesis and regulation (rRNA processing and translation) (**Figure 1C**). In the intima layer, 557 proteins were significantly altered in FL (229 up and 328 down) when compared with FS plaques (**Figure 2A**). The biological processes increased in FL intima were quite similar to those found in the media, but proteins related to the immune response (including immunoglobulin superfamily) were also increased in the intima. Down-regulated processes were even less common in the intima than in the media (**Figure 2B**).

PROTEINS FROM THE COMPLEMENT ACTIVATION PATHWAY GRADUALLY ACCUMULATE WITH DISEASE PROGRESSION IN EARLY ATHEROMA. We further inspected the biological process altered by analyzing abundance changes in functional subcategories produced by coordinated protein alterations (Systems Biology Triangle model) (12) as well as the individual behavior of their components. Inspection of the proteins related to lipid transport revealed a generalized increase in apolipoproteins and associated proteins, and in proteins related to lipid transfer and receptors, in both the media and intima (Supplemental Figures 4A, 5A, and 5B). These proteins gradually accumulated in the plaques, reaching up to 6% of the total protein amount in FL intima (Supplemental Figure 5C), due mainly to apolipoproteins (Supplemental Figure 5D). A generalized increase of proteins was also observed in the group of hemostasis/coagulation (Supplemental Figures 4B, 6A, and 6B). Absolute accumulation of these proteins in the plaques occurred mainly in the intima, mostly due to the common coagulation pathway and hemostasis regulators (Supplemental Figures 6C and 6D). Among proteins involved in the immune response, antigen and Ig receptors showed a generalized relative abundance increase in the intima (Supplemental Figures 4C, 7A, and 7B). These proteins strongly accumulated in the intima, reaching up to 4% of total protein composition (Supplemental Figures 7C and 7D).

Inspection of proteins of the complement system revealed a generalized increase in relative abundance of proteins involved in complement activation in both media and intima (**Figures 3A and 3B**, Supplemental Figure 4D). Regulators showing increased expression were mostly of the alternative pathway, while CD59 (a membrane attack complex [MAC] inhibitor) was decreased (**Figure 3B**). The total absolute abundance of complement proteins increased in parallel with disease progression, reaching up to 2.5% of total composition (**Figure 3C**). The absolute increase in the

FIGURE 2 **Comparison of Differentially Regulated Proteins in the Intima of Early Atherosclerotic Tissue**



(A) Heat map of the Zq values representing identified proteins that increase **(red)** or decrease **(blue)** in the intima layer of fibrolipidic (FL_i) versus fatty streak (FS_i) plaques. The **inset at the right** shows the statistically significant abundance changes in samples of FLi versus FSi. **(B)** Enrichment analysis of significant altered proteins identified in the intimal layer of atherosclerotic tissue. The **bars** show the relative proportion of changing proteins that belong to each one of the main biological processes (GOBP categories) that are significantly enriched. Bars are color coded according to $-\log_2(p)$, where p refers to the p value obtained for Bonferroni enrichment. Only the significantly enriched GOBPs are shown [$-\log_2(p) = 5$ correspond to a p value <0.05]. The complete list of identified GOBPs are listed in Supplemental Table 3.

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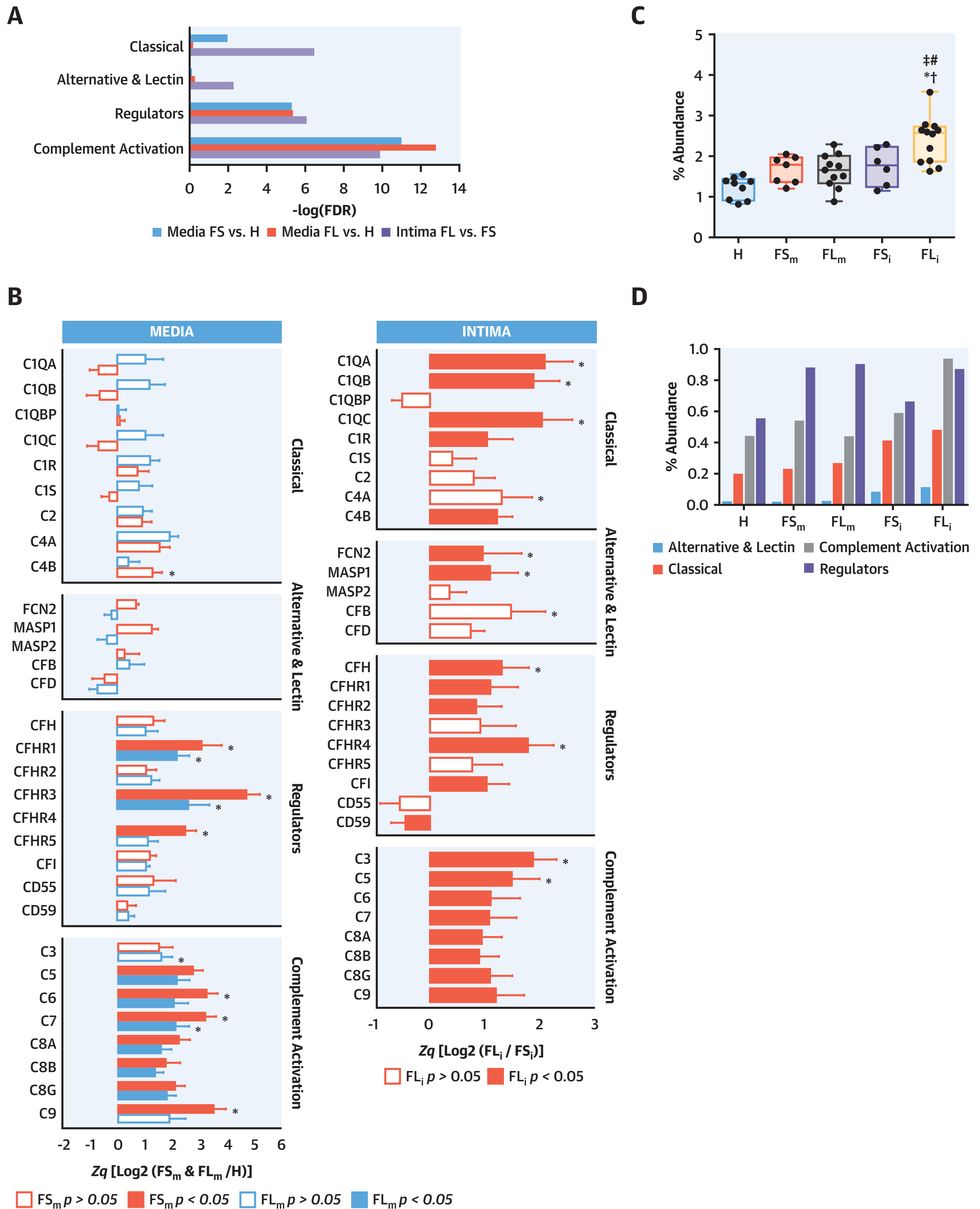
FIGURE 3 Changes in the Proteins Related to the Complement System in Early Atherosclerotic Tissue



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media was mainly due to proteins from the complement activation, while in the intima both classical and complement activation proteins accumulated (**Figure 3D**).

Protein-protein association network analysis revealed strong interactions with high confidence associations between proteins belonging to the 4 different functional groups (Supplemental Figure 8). Finally, we observed a strong positive correlation of the abundance of the complement system with the other 3 functional groups in the intima, and also with the lipid transport/metabolism group in the media (**Figure 4**).

To further study the prominent changes in complement activation observed in FS and FL plaques, we selected C5 as a representative MAC molecule. The gradual increase in C5 protein levels in the media and intima with increasing plaque complexity was confirmed by Western blot (**Figure 5A**). FS and FL plaques also showed increased C5-positive staining, colocalizing with a marker of complement activation (C5b-9 or MAC) (**Figure 5B**). Increased complement activation in media and intima of FL plaques was also confirmed by measuring the abundance of C5a and C5b-9 complex in early atherosclerotic tissue-conditioned medium by ELISA (**Figure 5C**). Moreover, C5 showed a strong positive correlation with C5a and C5b-9 protein levels in tissue-conditioned medium (**Figure 5C**), while, consistently, the MAC inhibitor CD59 showed a negative correlation (Supplemental Figure 9). Finally, no significant differences in mRNA levels of C5, complement decay-accelerating factor or CD59 were observed between H, FS, and FL aortic samples (Supplemental Figure 10), indicating that complement accumulation was not due to increased protein synthesis.

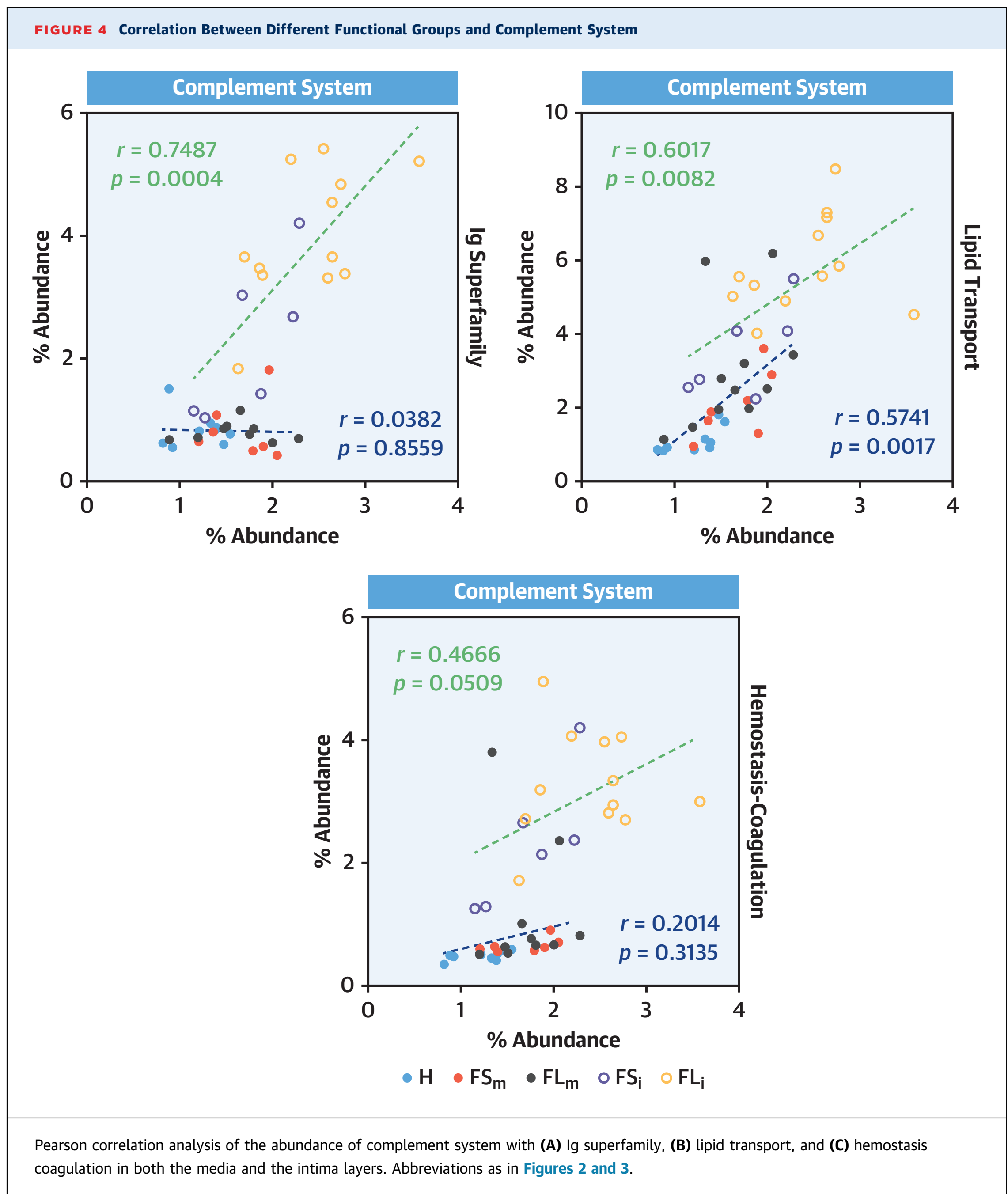
COMPLEMENT C5 IN PLASMA IS AN INDEPENDENT PREDICTOR OF GENERALIZED SUBCLINICAL ATHEROSCLEROSIS IN 2 DIFFERENT POPULATIONS. To study whether complement activation in early atherosclerosis lesions was reflected in plasma, we measured C5 plasma levels in 360 male participants in the PESA study. Overall, they were young to middle-aged low-risk individuals, categorized by 2DVUS imaging into 2 groups having incipient (0 to 2 plaques) or generalized (>2 plaques) subclinical atherosclerosis (**Table 1**). We found that C5 plasma levels were higher in the individuals with generalized subclinical disease than in those with incipient atherosclerosis (**Table 1**). Logistic regression analysis identified C5 as independent predictor of the presence of generalized atherosclerosis in addition to other CV risk factors (**Table 2**). Furthermore, multivariate linear regression analysis showed independent associations between volumetric plaque burden, measured by 3DVUS (16), and C5 levels (**Table 3**). Finally, coronary artery calcification, measured by CT, was also independently associated with C5 levels (**Table 3**).

Because our PESA subcohort included only men, we further analyzed C5 plasma levels in 394 asymptomatic patients included in the NEFRONA study as a control group (15) (**Table 1**). C5 levels were also higher in individuals with generalized subclinical atherosclerosis than in those with incipient atherosclerosis in this population (**Table 1**). Furthermore, multivariable logistic regression analysis identified C5 as an independent predictor of the

FIGURE 3 Continued

(A) Analysis of coordinated protein changes for the different complement system subcategories calculated using the SBT model; statistical significance was expressed using the FDR. **(B)** Quantitative data for all identified proteins related to the complement system and subcategorized according to their specific characteristics. The **bars** indicate Zq values expressed as mean ± SD from 6 to 12 independent samples per group. Statistically significant changes were calculated using either analysis of variance followed by Tukey's post hoc test (media layer) or Student's *t*-test (intima layer) (**filled bars**: $p < 0.05$) or the WSPP model, as explained in the Supplemental Methods (*false discovery rate < 0.05). **(C)** Summed absolute abundance of the proteins involved in the different subcategories related to the complement system. Values are mean ± SD from 6 to 12 independent samples per group (*$p \leq 0.05$ FL_i vs. H; †$p \leq 0.05$ FL_i vs. FS_m; ‡$p \leq 0.05$ FL_i vs. FL_m; #$p \leq 0.05$ FL_i vs. FS_i) calculated using analysis of variance test followed by Tukey post hoc test. **(D)** Distribution of absolute abundances of proteins from the complement system in each subcategory. **Box plots** represent the median and 25 and 75 percentiles, and **error bars** 10 and 90 percentiles. C1QA = complement C1q subcomponent subunit A; C1QB = complement C1q subcomponent subunit B; C1QBP = complement component 1 Q subcomponent-binding protein, mitochondrial; C1QC = complement C1q subcomponent subunit C; C1R = complement C1r subcomponent; C1S = complement C1s subcomponent; C2 = complement C2; C3 = complement C3; C4A = complement C4-A; C4B = complement C4-B; C5 = complement C5; C6 = complement component C6; C7 = complement component C7; C8A = complement component C8 alpha chain; C8B = complement component C8 beta chain; C8G = complement component C8 gamma chain; C9 = complement component C9; CD55 = complement decay-accelerating factor; CD59 = CD59 glycoprotein; CFB = complement factor B; CFD = complement factor D; CFH = complement factor H; CFHR1 = complement factor H-related protein 1; CFHR2 = complement factor H-related protein 2; CFHR3 = complement factor H-related protein 3; CFHR4 = complement factor H-related protein 4; CFHR5 = complement factor H-related protein 5; CFI = complement factor I; FCN2 = ficolin-2; FLi = fibrolipidic intima; FLm = fibrolipidic media; FSi = fatty streak intima; FSm= fatty streak media; H = healthy; MASP1 = mannan-binding lectin serine protease 1; MASP2 = mannan-binding lectin serine protease 2; other abbreviations as in **Figure 2**.

FIGURE 4 Correlation Between Different Functional Groups and Complement System



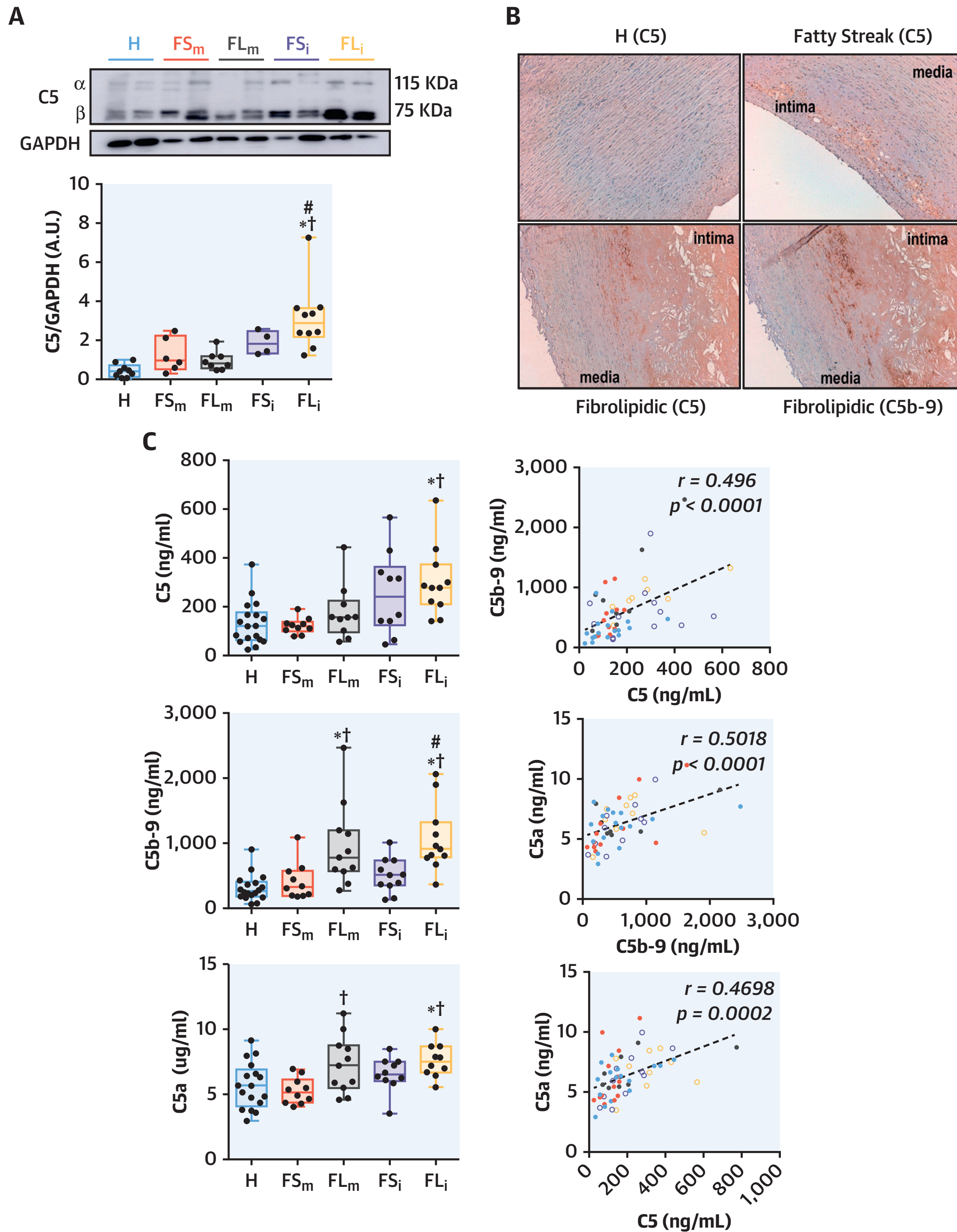
Pearson correlation analysis of the abundance of complement system with **(A)** Ig superfamily, **(B)** lipid transport, and **(C)** hemostasis coagulation in both the media and the intima layers. Abbreviations as in Figures 2 and 3.

presence of generalized atherosclerosis in addition to other CV risk factors in the NEFRONA population (Table 2). In contrast, C5a levels were not different between individuals with generalized subclinical disease than in those with incipient atherosclerosis either in PESA (3.89 ng/ml [2.10 to 7.81 ng/ml] vs. 4.18 ng/ml [2.34 to 9.23 ng/ml]; p = NS) or NEFRONA (4.52 ng/ml [2.43 to 8.21 ng/ml] vs. 3.81 ng/ml [2.41 to 6.47 ng/ml]; p = NS) studies.

DISCUSSION

TOPOLOGICALLY RESOLVED ANALYSIS OF THE PLAQUE PROTEOME. In the present study, we have performed a deep quantitative proteomics analysis of the intimal and medial layers of early human atherosclerotic plaques. We found that although a considerable number of the identified proteins are shared between media and intima, their actual

FIGURE 5 Complement Activation in Early Atherosclerotic Tissue and Tissue-Conditioned Medium



(A) Validation of complement C5 in the tissue samples used for the proteomic study by Western blot. Relative band intensity values are mean ± SD of between 6 and 12 independent samples per group; results are normalized to GADPH. **(B)** Immunohistochemistry of complement C5 in fatty streak (FS) and fibrolipid (FL) lesions in subclinical atherosclerotic tissues showing colocalization with MAC (B7C9 staining). **(C)** Enzyme-linked immunosorbent assay analysis of soluble levels of complement C5, C5b-9 and C5a in tissue-conditioned medium; the charts show Pearson correlation between the soluble levels of complement C5, C5b-9 and C5a. *p ≤ 0.05 vs. H, †p ≤ 0.05 vs. FS_m, ‡p ≤ 0.05 vs. FL_m, #p ≤ 0.05 vs. FS_i, calculated using analysis of variance test followed by Tukey post hoc test. **Box plots** represent the median and 25th and 75th percentiles, and **error bars** 10 and 90 percentiles. Abbreviations as in Figures 2 and 3.

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TABLE 1 Clinical Characteristics of the 2 Cohorts

	All (PESA Study) (n = 360)	Incipient Subclinical Atherosclerosis (n = 186)	Generalized Subclinical Atherosclerosis (n = 174)	p Value	All (NEFRONA Study) (n = 394)	Incipient Subclinical Atherosclerosis (n = 303)	Generalized Subclinical Atherosclerosis (n = 91)	p Value
Age, yrs	49 (46–52)	48 (46–51)	50 (47–52)	<0.001	55 (46–63)	53 (43–60)	61 (57–67)	<0.001
Male	100.0	100.0	100.0	NS	53.5	47.2	74.7	<0.001
Smoking	26.9	17.7	36.8	<0.001	60.6	57.1	72.5	<0.05
Diabetes	2.5	0.5	4.6	<0.01	11.2	8.2	20.9	<0.001
SBP, mm Hg	120 ± 12.01	119 ± 11.9	123 ± 12	<0.01	132 ± 17	130 ± 17	141 ± 16	<0.001
DBP, mm Hg	76 ± 9	74 ± 9	77 ± 9	<0.01	80 ± 10	79 ± 10	83 ± 9	<0.005
Total cholesterol, mg/dl	203 (182–226)	199 (177–220)	205 (188–234)	<0.01	203 ± 34	201 ± 34	208 ± 35	NS
HDL cholesterol, mg/dl	43 ± 10	44 ± 10	42 ± 10	NS	51 (42–62)	51 (42–64)	50 (43–60)	NS
LDL cholesterol, mg/dl	140 ± 31	141 ± 29	139 ± 33	NS	127 ± 32	126 ± 32	130 ± 31	NS
Triglycerides, mg/dl	95 (74–132)	91 (68–126)	105 (77–144)	<0.001	99 (71–140)	92 (67–133)	115 (83–150)	<0.001
Glucose, mg/dl	92 (87–99)	92 (87–97)	92 (87–101)	NS	97 (89–106)	96 (88–104)	105 (95–117)	<0.001
hsCRP, mg/l	0.12 (0.06–0.21)	0.11 (0.06–0.19)	0.12 (0.06–0.22)	NS	1.5 (0.8–3.0)	1.4 (0.8–2.9)	1.9 (0.9–3.7)	NS
C5, ug/ml	53.7 (17.7–137.4)	50.8 (17.7–137.4)	58.0 (19.9–119.6)	<0.001	50.4 (0.6–227.4)	49.3 (0.6–184.9)	57.9 (11.6–227.4)	0.005

Values are median (interquartile range), %, or mean ± SE.
DBP = diastolic blood pressure; HDL = high-density lipoprotein; LDL = low-density lipoprotein; NS = nonsignificant; PESA = Progression of Early Subclinical Atherosclerosis; SBP = systolic blood pressure.

proportions are quite different in each compartment. Besides, a large proportion of these proteins are known to be synthesized in extravascular tissues (e.g., albumin or apolipoproteins in the liver), suggesting that an important amount of the proteins identified in atherosclerotic plaques could be derived either from direct interaction (e.g., with receptors) or by a passive retention from the blood. The percolation of soluble mediators from the blood to the intima and media layers could be favored by radial mass transport, conveyed by advective hydraulic conductance (17). In fact, a previous study showed a correlation between the abundance of 4 major plasma proteins in the intima (albumin, apolipoprotein B, immunoglobulin [Ig] G, and fibrinogen) and the presence and growth of early coronary atherosclerosis (18). Another interesting result is that the molecular changes that take place during atherogenesis in the media of FL plaques are already present in the media layer of FS plaques. In asymptomatic patients with atherosclerotic plaques of the PESA study, ^{18}F-FDG uptake was mainly detected in plaque-free arterial segments, suggesting that a high metabolic activity could be associated with the medial layer in early atherosclerosis (19). On the whole, our data highlight the contribution of both the media and intima layers during the different stages of atherogenesis.

Our proteomics study has identified more than 6,000 proteins from which more than 2,500 well-validated proteins were found in both the media and intima layers. These proteins were enriched in 4 main categories. One of the categories related to lipid advective radial transport and metabolism, which fits with the view that initial vessel wall injury derives from plasma lipid transport and intimal retention within the arterial wall (4). However, our compartment-resolved proteomic studies indicated a previously unappreciated contribution of the media layer to lipid and lipoprotein deposition and metabolism within the arterial wall. Another main category was hemostasis, a tightly regulated process involving platelet activation, blood clotting, and vascular repair. A previous study detected significantly higher activities of tissue factor, FII, FX, and FXII in early atherosclerotic lesions than in stable advanced atherosclerotic lesions (20), suggesting an enhanced procoagulant state in early atherosclerosis. In agreement, we have shown an increase of proteins of different coagulation pathways (e.g., FX, thrombin, and fibrinogen) and inhibitors (e.g., SERPINs) both in the media and intima layers. Finally, we also showed increased presence of Igs in atherosclerotic plaques, which has been previously documented (21). Ig receptors could favor the accumulation of Igs; among them, increased significant levels of Igll5 and PIGR are observed in the intima of early atherosclerotic plaques for the first time, whose role deserves further studies.

THE ROLE OF COMPLEMENT ACTIVATION IN EARLY ATHEROSCLEROSIS. Our data highlighted an increase of proteins related to complement activation mainly associated with the classical (or so called “Ig-

dependent") pathway. In addition to Igs, a variety of potential triggers has been described in the atherosclerotic lesion that could activate the complement system, such as modified LDLs (22), cholesterol crystals (23), and hemostatic proteins (24). Thus, we observed a positive correlation between lipid metabolism/transport and complement both in the intima and media layers, and also with Ig and hemostasis groups in the intima layer, supporting the functional relationship between the main categories detected in our study. Atherosclerotic plaques thus seem to be a privileged surface for complement activation with several triggers potentially involved in complement deposition and activation.

The complement activation observed by proteomics was validated by Western blot and immunohistochemistry studies confirming the increase in C5 protein levels in atherosclerotic plaques. Moreover, C5 colocalized with C5b-9/MAC in tissue and C5 levels showed a positive correlation with C5a and C5b-9 in tissue-conditioned medium, confirming tissular complement activation. Interestingly, this was accompanied with a decrease on the MAC regulator CD59 in FL tissue and tissue-conditioned media. The functional consequences of complement activation may be related to the proliferation of smooth muscle and endothelial cells, accompanied by the release of various chemotactic, proadhesion, and procoagulant cytokines from these cells (25). Atherosclerosis can be decreased by increasing CD59 (CD59 transgenic mice) and administering either antiC5 antibody (26) or siRNAC5 in the liver (confirmed by reduced C5 plasma levels) (27) in ApoE KO mice. However, the results of previous interventional studies mainly targeting C5 in CV patients were unconvincing (28). Several explanations could account for the lack of effect of these C5 therapies (e.g., pexelizumab), such as the delay on the initiation of therapy or the sustained MAC activation in plasma of CV patients contrasting with the complete MAC inhibition observed in cell culture studies (29). On the whole, strategies to halt complement activation could be of therapeutic value, although their implementation in the clinical setting deserves further studies.

COMPLEMENT C5 AS A BIOMARKER OF SUBCLINICAL ATHEROSCLEROSIS. The increased protein levels of C5 observed in atherosclerotic plaques was not accompanied by increased mRNA levels; this, along with the high levels of extravascular proteins in early atherosclerotic plaques, led us to hypothesize a plasmatic origin of C5. Then, we tested the levels of C5 in plasma of subjects with subclinical atherosclerosis. In our PESA subcohort, plasma C5 was higher in

TABLE 2 Logistic Regression Analysis of the Association With the Presence of Subclinical Generalized Atherosclerosis

	Beta	p Value	OR (95% CI)
Univariate analysis			
PESA			
C5	**0.347**	**0.001**	**1.41 (1.16-1.72)**
Age	**0.255**	**0.008**	**1.29 (1.07-1.56)**
Smoking	**0.49**	**<0.0001**	**1.63 (1.38-1.94)**
SBP	**0.335**	**0.001**	**1.40 (1.14-1.70)**
DBP	**0.276**	**0.005**	**1.32 (1.09-1.60)**
Diabetes	**0.33**	**0.014**	**1.39 (1.07-1.81)**
Total cholesterol	**0.238**	**0.015**	**1.27 (1.05-1.54)**
Triglycerides	**0.273**	**0.008**	**1.31 (1.07-1.61)**
LDL cholesterol	−0.101	0.29	0.90 (0.75-1.09)
Glucose	0.158	0.111	1.17 (0.96-1.42)
NEFRONA			
C5	**0.38**	**0.001**	**1.46 (1.17-1.82)**
Age	**1.044**	**<0.0001**	**2.84 (2.05-3.93)**
Sex	**0.597**	**<0.0001**	**1.82 (1.40-2.36)**
Smoking	**0.335**	**0.009**	**1.40 (1.09-1.80)**
SBP	**0.649**	**<0.0001**	**1.91 (1.49-2.45)**
DBP	**0.355**	**0.003**	**1.43 (1.12-1.81)**
Diabetes	**0.339**	**0.001**	**1.40 (1.14-1.72)**
Total cholesterol	0.191	0.113	1.21 (0.96-1.53)
Triglycerides	**0.272**	**0.018**	**1.31 (1.05-1.65)**
LDL cholesterol	0.113	0.378	1.20 (0.87-1.44)
Glucose	**0.422**	**0.0003**	**1.52 (1.22-1.91)**
Multivariate analysis			
PESA			
C5	**0.263**	**0.016**	**1.30 (1.05-1.61)**
Age	**0.211**	**0.046**	**1.23 (1.00-1.52)**
Smoking	**0.573**	**<0.0001**	**1.77 (1.43-2.19)**
SBP	**0.421**	**0.02**	**1.52 (1.07-2.17)**
DBP	−0.037	0.835	0.96 (0.68-1.36)
Diabetes	**0.351**	**0.024**	**1.42 (1.05-1.93)**
Total cholesterol	**0.34**	**0.004**	**1.40 (1.12-1.79)**
Triglycerides	0.116	0.312	1.123 (0.90-1.41)
LDL cholesterol	−0.135	0.221	0.87 (0.70-1.08)
Glucose	−0.082	0.533	0.92 (0.71-1.19)
NEFRONA			
C5	**0.408**	**0.005**	**1.50 (1.13-2.00)**
Age	**1.01**	**0.016**	**2.74 (1.74-4.32)**
Sex	**0.433**	**0.016**	**1.54 (1.08-2.19)**
Smoking	0.342	0.053	1.41 (0.99-1.99)
SBP	0.296	0.178	1.34 (0.87-2.07)
DBP	−0.036	0.868	0.96 (0.63-1.47)
Diabetes	0.105	0.536	1.11 (0.80-1.55)
Total cholesterol	−0.017	0.97	0.98 (0.40-2.39)
Triglycerides	0.025	0.905	1.02 (0.68-1.59)
LDL cholesterol	0.253	0.57	1.29 (0.54-3.08)
Glucose	0.309	0.12	1.36 (0.92-2.01)

Values in **bold** indicate statistical significance. To calculate odds ratios continuous variables are expressed in units of SD. In PESA, SD values are: C5: 19.31 μg/ml; age: 3.73 years; SBP: 12.09 mm Hg; DBP: 9.42 mm Hg; total cholesterol: 35.01 mg/dl; triglycerides: 59.81 mg/dl; LDL cholesterol: 31.05 mg/dl; glucose: 12.44 mg/dl. In NEFRONA, C5: 34.24 μg/ml; age: 11.82 years; SBP: 16.95 mm Hg; DBP: 9.77 mm Hg; total cholesterol: 34.20 mg/dl; triglycerides: 68.85 mg/dl; LDL cholesterol: 32.15 mg/dl; glucose: 23.63 mg/dl.

CI = confidence interval; OR = odds ratio; other abbreviations as in Table 1.

TABLE 3 Linear Regression Analysis of the Association With Plaque Burden and CAC Score in the PESA study

	Plaque Burden		CAC Score	
	Beta	p Value	Beta	p Value
Univariate analysis				
C5	**0.154**	**0.001**	**0.176**	**0.0002**
Age	**0.155**	**0.001**	**0.13**	**0.006**
Smoking	**0.28**	**<0.0001**	**0.113**	**0.017**
SBP	0.088	0.065	**0.13**	**0.006**
DBP	**0.095**	**0.046**	**0.147**	**0.002**
Diabetes	**0.165**	**<0.001**	**0.124**	**0.009**
Total–Cholesterol	0.048	0.313	0.02	0.679
Triglycerides	**0.153**	**0.001**	0.084	0.075
LDL–Cholesterol	−0.254	0.528	−0.469	0.956
HDL–Cholesterol	**−0.097**	**0.041**	−0.000457	0.992
Glucose	0.067	0.161	0.088	0.063
Multivariate analysis				
C5	**0.101**	**0.02**	**0.144**	**0.002**
Age	**0.229**	**<0.0001**	0.092	0.052
Smoking	**0.29**	**<0.0001**	**0.094**	**0.047**
SBP	**0.144**	**0.044**	0.033	0.674
DBP	−0.037	0.605	0.109	0.162
Diabetes	**0.149**	**0.005**	0.1	0.078
Total cholesterol	**0.139**	**0.005**	0.003	0.95
Triglycerides	0.036	0.444	0.051	0.321
LDL cholesterol	−0.029	0.526	0.008	0.865
HDL cholesterol	−0.021	0.658	0.045	0.376
Glucose	−0.048	0.361	−0.006	0.916

Values in **bold** indicate statistical significance.
CAC = coronary artery calcification; other abbreviations as in Table 1.

asymptomatic individuals with generalized atherosclerosis (>2 plaques) than in those with incipient atherosclerosis, independently of risk factors. This finding was validated in NEFRONA study participants free from clinical CV disease (**Central Illustration**). In contrast, C5a was similar between subjects with generalized versus incipient atherosclerosis in both cohorts, in agreement with a previous study where C5a was not associated to surrogate markers of subclinical atherosclerosis such as IMT (30).

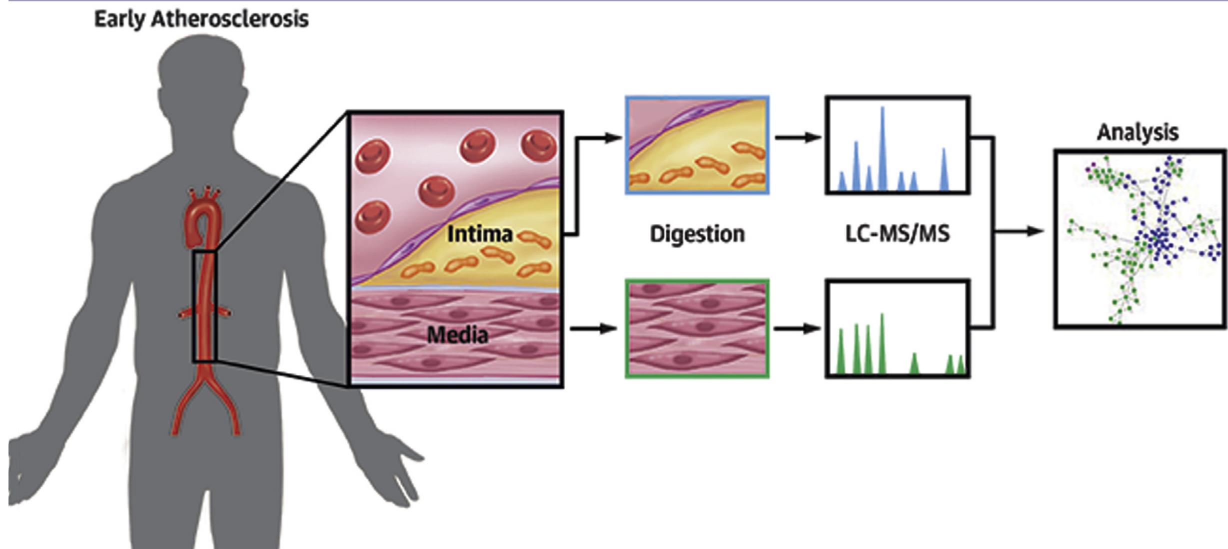
Both the PESA and NEFRONA studies analyzed the presence of atherosclerotic plaques in different territories (carotid and femoral arteries) by 2DVUS. However, the PESA study includes 3DVUS analysis of volumetric plaque burden and CT assessment of coronary calcification. In the PESA study, inclusion of plaque burden quantification in the detection of plaque presence provided a closer match association with global CV risk (16). Moreover, the CACS is an excellent surrogate marker of underlying coronary atherosclerosis burden and is probably the most useful current way to improve risk assessment among individuals at intermediate risk (31). Interestingly, both plaque burden and CACS are associated with CV events (2). Our study shows a significant positive association of C5 with atherosclerosis burden and calcification in asymptomatic PESA study participants, independently of risk factors. However, in such a complex disease such as atherosclerosis, a single biomarker may be insufficient to detect atherosclerotic plaques and/or to predict CV risk. Thus, the potential role of C5 as subclinical atherosclerosis biomarker should be assessed together with other biomarkers of different pathological mechanisms as those observed in our proteomic study of plaques.

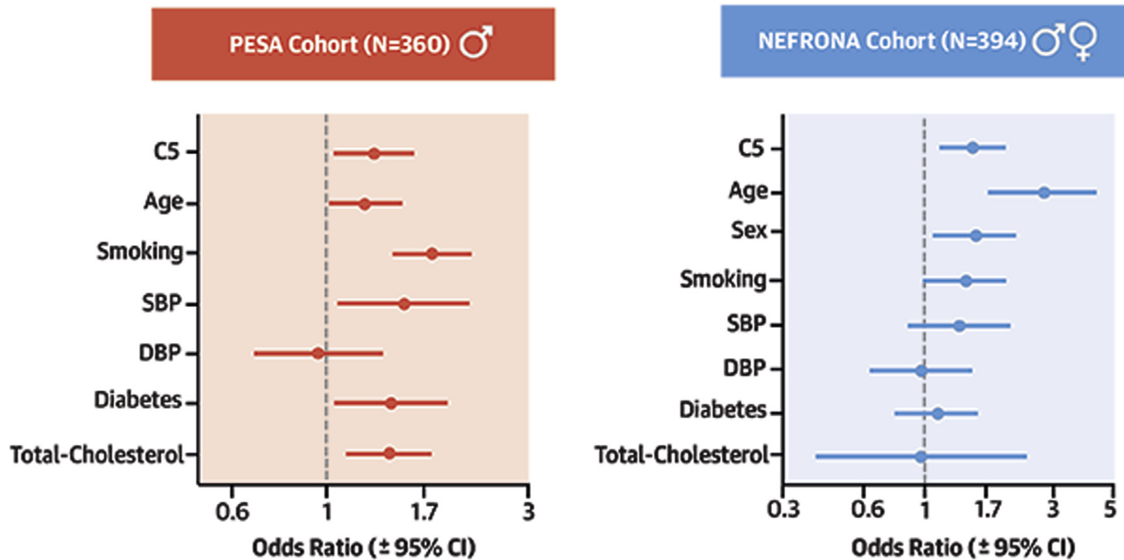
STUDY LIMITATIONS. We have studied aortic tissues due to easier accessibility of samples, but the analysis of coronary vessels would be more relevant for myocardial infarction. However, we cannot macroscopically separate intima and media of coronary arteries, which would preclude the topological analysis performed in the present study. In the PESA study, we did not have information on plaque characterization to define plaque types in a similar way as we did in the in vitro study; moreover, FS lesions are not detected by common noninvasive imaging methods following standard definitions; thus, we used the extent of atherosclerosis disease measured as the number of plaques present in each participant (incipient [0 to 2 plaques] vs. generalized [>2 plaques]) to define progressive stages of atherosclerosis. Ultimately, the clinical validation of the plasma C5 as an early biomarker of CV disease will come from the association with future incident CV events. Nonetheless, the validity of C5 is indirectly supported by the consistent association with other validated markers of disease severity and CV risk (larger plaque volumes and mainly higher CACS). Future follow-up data from the PESA or other prospective cohorts will help confirm this hypothesis by evaluating associations with CV outcomes.

CONCLUSIONS

Proteomic analysis of aortic tissues highlights complement accumulation and activation in the intima of early atherosclerotic plaques. This was paralleled by elevated complement C5 levels in plasma of low-risk asymptomatic individuals, correlating with plaque volume and CACS, 2 markers of disease severity and

CENTRAL ILLUSTRATION Plaque Proteomics Identifies Complement C5 as a Biomarker of Subclinical Atherosclerosis





Martínez-López, D. et al. J Am Coll Cardiol. 2020;75(16):1926–41.

(Top) Schematic of proteomic analysis of media and intima of fatty streaks (FS) and fibrolipidic (FL) plaques. **(Bottom)** Odds ratio (expressed in units of SD) of the association of complement C5 and risk factors with generalized atherosclerosis (>2 plaques), adjusted for potential confounding factors.

CV events. Thus, the identification of C5 as a biomarker of atherosclerotic burden in subclinical atherosclerosis provides an additional potential tool for CV risk prediction.

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PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: Complement C5 protein accumulates in the intima layer of early atheromatous lesions, and plasma levels of complement C5 are elevated in asymptomatic individuals in proportion to the extent of atherosclerosis.

TRANSLATIONAL OUTLOOK: Clinical and epidemiological studies are needed to validate the accuracy and reliability of plasma complement C5 protein levels as a biomarker of the extent of atherosclerosis in various populations and its utility as a screening method.

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KEY WORDS biomarkers, complement system, proteomics, subclinical atherosclerosis

APPENDIX For an expanded Methods section and supplemental figures, please see the online version of this paper.