Pharmacological Research 166 (2021) 105462

Contents lists available at ScienceDirect

Pharmacological Research

journal homepage: www.elsevier.com/locate/yphrs

Ficolin-2 serum levels predict the occurrence of acute coronary syndrome in patients with severe carotid artery stenosis

Federico Carbone [a,b], Alessia Valente [c], Carlo Perego [c], Maria Bertolotto [a], Bianca Pane [b], Giovanni Spinella [b], Domenico Palombo [b], Maria-Grazia De Simoni [c], Fabrizio Montecucco [b,d,**], Stefano Fumagalli [c,*]

[a] First Clinic of Internal Medicine, Department of Internal Medicine, University of Genoa, 6 viale Benedetto XV, 16132, Genoa, Italy
[b] IRCCS Ospedale Policlinico San Martino Genoa – Italian Cardiovascular Network, 10 Largo Benzi, 16132, Genoa, Italy
[c] Istituto Di Ricerche Farmacologiche Mario Negri IRCCS, Milan, Italy
[d] First Clinic of Internal Medicine, Department of Internal Medicine and Centre of Excellence for Biomedical Research (CEBR), University of Genoa, 6 viale Benedetto XV, 16132, Genoa, Italy

ARTICLE INFO

Keywords:
Atherosclerosis
Complement system
Biomarkers
Plaque vulnerability

ABSTRACT

Background and purpose: erosion of vulnerable atherosclerotic plaques may cause life-threatening thromboembolic complications. There is indeed an urgent need to recognize a clear-cut biomarker able to identify vulnerable plaques. Here, we focused on circulating proteins belonging to the lectin pathway (LP) of complement activation.
Methods: we analyzed mannose-binding lectin (MBL), ficolin-1, -2 and -3 (LP initiators) levels by ELISA in sera from n = 240 of an already published cohort of patients undergoing endarterectomy for severe carotid stenosis and followed-up until 18 months after surgery. Immunofluorescence followed by confocal and polarized light microscopy was used to detect LP initiator intraplaque localization. Spearman's rank test was drawn to investigate correlation between serum LP levels and circulating inflammatory proteins or intraplaque components. Survival analyses were then performed to test the predictive role of LP on long-term adverse outcome.
Results: ficolins, but not MBL, correlated positively with 1) high circulating levels of inflammatory markers, including MPO, MMP-8, MMP-9, ICAM-1, osteopontin, neutrophil elastase, and; 2) immune cell intraplaque recruitment. Immunofluorescence showed ficolins in calcified plaques and ficolin-2 in cholesterol-enriched plaque regions in association with macrophages. In the multivariate survival analysis, ficolin-2 serum levels predicted a major adverse cardiovascular event during the follow-up, independently of symptomatic status and inflammatory markers (hazard ratio 38.6 [95 % CI 3.9–385.2]).
Conclusions: ficolins support intraplaque immune cell recruitment and inflammatory processes ultimately leading to plaque vulnerability. Especially for ficolin-2 a strong predictive value toward adverse cardiovascular events was demonstrated. This evidence offers potentially new pharmacological target to dampen the inflammatory mechanisms leading to plaque vulnerability.

Abbreviations: ACE-I, angiotensin converting enzyme inhibitor; ARBs, angiotensin receptor blockers; BP, blood pressure; CAD, coronary artery disease; CCL, C-C motif chemokine ligand; CC, cholesterol crystals; CD, cluster of differentiation; ch, cholesterol; CI, interval of confidence; CV, cardiovascular; EDTA, ethylenediaminetetraacetic acid; HDL, high-density lipoprotein; HR, hazard ratio; ICAM, intracellular adhesion molecule; IGF, insulin-like growth factor; LDL, low-density lipoprotein; LP, lectin pathway; MACE, major-adverse cardiovascular event; MASP, MBL-associated serine protease; MBL, mannose-binding lectin; MMP, matrix metalloprotease; MPO, myeloperoxidase; NE, neutrophil elastase; NGS, normal goat serum; OPN, osteopontin; PCSK9, proprotein convertase subtilisin/kexin type 9; PMNs, neutrophils; RAAS, renin-angiotensin-aldosterone system; SMCs, smooth muscle cells; TAG, triglyceride; TIMP, tissue inhibitor of metalloprotease; total-c, total cholesterol; VCAM, vascular cell adhesion molecule; WBC, white blood cells.

* Corresponding author at: Istituto di Ricerche Farmacologiche Mario Negri IRCCS, via Mario Negri 2, 20156, Milano, Italy.
** Corresponding author at: IRCCS Ospedale Policlinico San Martino, largo Rosanna Benzi 10, 16132, Genoa, Italy.
E-mail addresses: fabrizio.montecucco@unige.it (F. Montecucco), stefano.fumagalli@marionegri.it (S. Fumagalli).

https://doi.org/10.1016/j.phrs.2021.105462
Received 23 September 2020; Received in revised form 4 December 2020; Accepted 22 January 2021
Available online 26 January 2021
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1. Introduction

Atherosclerosis is a risk factor for life-threatening atherothrombotic events, like myocardial infarction and ischemic stroke. The degree of carotid stenosis remains the only validated criterion for patient stratification and clinical decision making [1–3]. However it fails to reliably detect plaques vulnerability to erosion/rupture, thus underestimating the risk of thromboembolic complications [4–6]. Advance in non-invasive imaging techniques (i.e. magnetic resonance, computed tomography, positron emission tomography and ultrasound) significantly improved the identification of prone to rupture plaques and predicted adverse cardiovascular (CV) events in selected cohorts. However they are still far from being mainstream techniques in clinical practice [7]. Combining imaging markers with circulating or intraplaque biomarkers can strengthen the identification of vulnerable plaque and help clarify the mechanisms underlining their vulnerability.

Among them, shear stress is a well-known danger signal linked to complement activation [8,9], a proteolytic cascade belonging to the humoral response of innate immunity. Within this, the lectin pathway (LP) has a well-documented role in humans [10–15] and experimental models of vascular disease [10,11,16,17]. The LP recognition molecules, or initiators, are mannose-binding lectin (MBL), ficolins and collectins complexed with the key proteolytic enzymes MASPs. LP initiators were reported as contributors to plaque progression and vulnerability [18]. We demonstrated that circulating levels of LP proteins correlate with plaque morphology and the occurrence of transient ischemic attack in a cohort of patients undergone endarterectomy [19].

This work then grounds on the hypothesis that the LP of complement activation actively participates in atherosclerotic plaque erosion/rupture. By focusing on potential predictive role of circulating LP proteins, we investigated their potential use to foster diagnosis and stratification of atherosclerotic patients. To do this, we analyzed a cohort of 240 patients undergoing endarterectomy and then followed-up to 18 months.SA

2. Methods

Data are available on reasonable request to the authors. Detailed methods are in Supplemental material.

2.1. Patients

Patients (n = 240) of an already published cohort of patients with severe carotid stenosis were recruited at “IRCCS Ospedale Policlinico San Martino” (Genoa, Italy) with the approval of the local ethical committee Board (ethical approval No. 90/09–29/9/2009, with informed consent). Patients with stenosis ≥70 % (ultrasound Doppler) underwent surgery were included in the study. All patients who developed spontaneous cerebral embolism during 30 min preoperatively and during the dissection phase of the operation (detected by transcranial Doppler insonation of the middle cerebral artery) were excluded from the study. Other exclusion criteria were malignant hypertension, acute coronary artery disease, any cardiac arrhythmias, congestive heart failure (New York Heart Association II, III, and IV classes), liver or renal disorders or function abnormalities, acute and chronic infectious disease, autoimmune and rheumatic diseases, cancer, endocrine diseases, inflammatory bowel diseases and anti-inflammatory (other than aspirin) medications, oral anticoagulant treatments, hormone, cytokine, or growth factor therapies [20].

Primary outcome: to establish whether LP proteins predict the occurrence of major adverse CV events ([MACEs] defined as a composite outcome of fatal/non-fatal myocardial infarction or ischemic stroke) over a follow-up of 18 months.

2.2. Sample processing

Plaque specimens were snap-frozen immediately after surgery and processed as previously published [19,20]. Blood samples were collected in 10 mM of ethylenediaminetetraacetic acid (EDTA) 24 h before surgery and stored −80 °C before being analyzed with routine auto-analyzers to assay hematological.

2.3. Intraplaque marker detection

Intraplaque histological analysis was done on sixteen sections per plaque separated by 105 μm from each other. Stainings and quantifications were done as described previously [21].

2.4. Immunofluorescence

Immunofluorescence was done on 20-μm-thick coronal plaque sections according to the protocol described in [21]. Immunofluorescence was acquired using a scanning sequential mode to avoid bleed-through effects by an A1 confocal system by Nikon or by an Olympus Virtual stage microscope allowing to have a whole plaque overview. The same plaque was acquired using a polarized microscope to detect cholesterol crystals. Images were processed and elaborated by ImageJ and GIMP.

2.5. ELISA

ELISAs were based on standard sandwich enzyme-linked immunosorbent assay technology. Ficolin-2 and Ficolin-3 were determined by sandwich ELISA using specific in-house assays [19]. Serum levels of inflammatory markers were measured as previously described [20–22]. Mean intra- and inter-assay coefficients of variation were 8% for all markers.

2.6. Statistical analysis

Analyses were performed with IBM SPSS Statistics for Windows, Version 21.0 (IBM CO., Armonk, NY). Categorical variables are present as absolute (relative) frequencies. Continuous variables were expressed as median and interquartile range [IQR] since the normality assumption was not demonstrated. Intergroup comparisons were drawn by Fischer exact test or Mann–Whitney U test, as appropriate. Ranked Spearman correlation coefficients were used to establish correlations between continuous variables. Cox proportional hazards models were used to estimate the effect of ficolins on risk of composite outcome (MACE) and the single events fatal/non-fatal ACS or IS. Results were expressed as hazard ratios (HR) and 95 % CI. In the multivariate model, we adjusted for the presence of coronary artery disease and levels of MMP-9 and circulating monocytes. For all statistical analysis a 2-sided p-value of 0.05 was considered as statistically significant.

3. Results

The demographic, biochemical and medical record data of the analyzed cohort are presented in Table 1.

3.1. Correlation of circulating LP protein levels with circulating and intraplaque markers

Mannose-binding lectin correlated positively with hs-IL-6 and negatively with E-selectin (both $p < 0.01$, Fig. 1A, Table 2 and 3). Conversely, a positive correlation with intraplaque neutrophil infiltrate was observed, but limited to the upstream portion of the plaque ($p < 0.01$, Fig. 1A, Table 4).

Ficolin-1 correlated positively ($p < 0.01$) with MMP-8 and -9, myeloperoxidase (MPO) and neutrophil elastase (NE), thus suggesting a role neutrophil recruitment and activation. A negative correlation (p =

Table 1
Clinical characteristics of the overall cohort [n = 240].

Demographic	Overall cohort [n = 240]
Age, yr. [IQR]	73 [67–78]
Males, no. [%]	135 (65.5)
Systolic BP, mmHg [IQR]	130 [125–140]
Diastolic BP, mmHg [IQR]	80 (80–90)
Waist circumference [cm]	91 (87–96)
Hypertension, no. [%]	175 (77.4)
Active smokers, no. [%]	52 (27.2)
Previous smokers, no. [%]	106 (51.7)
Type 2 diabetes, no. [%]	55 (27.4)
Dyslipidaemia, no. [%]	123 (61.2)
Chronic CAD†, no. [%]	54 (26.9)
Medications	
RAAS inhibitors	
ACE-I, no. [%]	12 (6.6)
ARBs, no. [%]	114 (53.5)
β-blockers, no. [%]	67 (34.2)
Calcium antagonists, no. [%]	76 (38.4)
Statins, no. [%]	131 (59.5)
Aspirin, no. [%]	140 (65.7)
Thienopyridine, no. [%]	55 (28.5)
Oral antidiabetics, no. [%]	34 (17.9)
Insulin, no. [%]	12 (6.4)
Laboratory findings	
Haematology	
Total WBC, no. × 10^9/l [IQR]	7.0 [6.1–8.1]
Neutrophil, no. × 10^9/l [IQR]	4.5 [3.5–5.4]
Lymphocyte, no. × 10^9/l [IQR]	1.7 [1.4–2.1]
Monocyte, no. × 10^9/l [IQR]	0.4 [0.4–0.5]
Platelet, no. × 10^9/l [IQR]	221 [183–266]
Red blood cell, no. x10^{12}/l [IQR]	4.6 [4.3–4.9]
Chemistry	
Serum total-c mg/dl [IQR]	194 [166–218]
Serum LDL-c mg/dl [IQR]	110 [90–140]
Serum HDL-c mg/dl [IQR]	48 [41–60]
Serum TAG mg/dl [IQR]	122 [91–167]
Fasting glycaemia, mg/dl [IQR]	103 [93–120]
Fasting insulin, mU/L	8.6 [5.9–13.0]
Fibrinogen, mg/dl [IQR]	3.7 [3.2–4.2]
Complement activation biomarkers	
Ficolin-1, ng/mL [IQR]	84.9 [44.8–131.5]
Ficolin-2, OD [IQR]	0.47 [0.35–0.59]
Ficolin-3, OD [IQR]	0.75 [0.66–1.03]
MBL, ng/mL [IQR]	911 [423–1474]

Continuous data are expressed as median/interquartile range [IQR]; categorical data are expressed as number (no) and (%).

0.001) was seen with CCL-2 (Fig. 1B, Table 2). Ficolin-1 had positive correlation with the number of circulating monocytes and platelets (Fig. 1B, Table 3). Analyzing intraplaque components, ficolin-1 correlated with mast cells and lymphocytes ($p \leq 0.01$) both in the upstream and downstream plaque portions to blood flow (Fig. 1B, Table 4).

Ficolin-2 correlated positively with circulating levels of MMP-8 and CCL-4 and negatively with CCL-2, VCAM-1, resistin and E-selectin (all $p < 0.01$, Fig. 1C, Table 2). Ficolin-2 also correlated positively with osteopontin (OPN, $p = 0.001$), an inflammatory protein predictive of major adverse cardiovascular events (MACEs) in atherosclerotic patients [21]. Ficolin-2 showed a positive correlation with circulating monocytes and platelets ($p < 0.01$, Fig. 1C, Table 3), two cell populations reported to interact with LP [19,23,24] and important contributors to plaque growth and morphological evolution [25,26]. Ficolin-2 also correlated negatively with high-density lipoprotein cholesterol (HDL, $p = 0.01$), acknowledged as a protective factor for atherosclerosis [27,28], thus suggesting its detrimental role. In support of this, analyzing intraplaque components, circulating ficolin-2 levels correlated positively ($p \leq 0.01$) with macrophages and neutrophils in the downstream portion of the plaque (Fig. 1C, Table 4). At variance, ficolin-2 correlated negatively with smooth muscle cells (SMCs, $p < 0.05$), a cell population whose growth on the fibrous cap contributes to the formation of a stable atheroma [29].

Ficolin-3 had similar correlations to those of ficolin-2, showing positive correlation with circulating inflammatory markers such as OPN and CCL-4 ($p \leq 0.01$), a stronger positive correlation with NE and MPO ($p < 0.01$) than ficolin-2 and a negative correlation with CCL-2, VCAM-1, resistin and E-selectin (Fig. 1D, Table 2). Ficolin-3 circulating levels correlated positively with monocytes and platelets found in circulation ($p < 0.001$, Fig. 1D, Table 3) and macrophages ($p < 0.001$) and neutrophils ($p \leq 0.01$) found in the downstream portion of the plaque (Fig. 1D, Table 4). Moreover ficolin-3 correlated negatively with SMCs ($p \leq 0.001$), supporting its association with unstable plaque with thin fibrous cap (Fig. 1D, Table 4).

3.2. Analysis of clinical and biochemical parameters and circulating biomarkers after stratification on MBL deficiency

We identified 75 out of 240 MBL-deficient patients (31.3 %), in line with expected frequency [30]. The identification of MBL-deficient patients was based on a cut-off level for circulating MBL of 500 ng/mL. This cut-off was reported to be strongly associated with the genetic variants causing MBL deficiency/low functionality [30]. MBL-sufficient patients (≥ 500 ng/mL) were more likely to take oral anti-diabetics - absolute (relative) frequency was 30 (22.2) vs. 4 (7.3) - and showed lower circulating levels of leptin (median [interquartile range] was 11.7 [5.6–26.3] vs. 20.8 [8.4–37.4]) and TIMP-1 (268 [178–485] vs. 320 [220–627]) than MBL-deficient. On the contrary, MBL-sufficient patients were preferentially older (74 [69–78] vs. 71 [66–76]) and had lower levels of PCSK9 (260 [186–326] vs. 315 [253–443]) and E-selectin (17.4 [11.8–25.9] vs. 19.5 [14.7–30.2], Supplementary Table I).

3.3. Ficolins' intraplaque distribution

We observed that all ficolins were present within necrotic core, especially in areas enriched with cholesterol crystals (CC, Fig. 2 A, B, C). Cholesterol crystals were identified by polarized microscopy exploiting their optical anisotropy. In order to distinguish cholesterol crystals from other optically anisotropic structures, like collagens, we focused on objects showing the typical needle-like shape of cholesterol crystals [31]. Ficolins were also in the calcified portions of the plaques (Fig. 2 A', B', C'). Being ficolin-2 involved in the CC clearance through macrophage-mediated phagocytosis [18,23], we co-immunolabeled ficolin-2 and CD11b, a surface marker of macrophages. In plaque necrotic core, ficolin-2 surrounded infiltrated CD11b + macrophages, likely providing chemotactic stimuli (Fig. 2D). Neutrophils are another immune cell population executing phagocytosis within the plaques' necrotic core. We therefore co-immunolabeled ficolin-2 and neutrophils' elastase. The necrotic core had many infiltrated neutrophils, with few of them showing positivity for ficolin-2 (Fig. 2E).

3.4. Ficolins' predictive value for major adverse cardiovascular event over the follow-up

A 18-month clinical follow-up was available for 194 patients (80.5 %). The composite outcome of MACEs occurred in 11 patients (5.7 %). Supplementary Table II shows the demographic, biochemical and medical record data after stratification for patients according with the occurrence or not of MACEs during the follow-up. The choice for the 18-month follow-up was based on the high rate of drop out from the study after this period.

Patients with MACEs had chronic artery disease more frequently (36.4 vs. 18.6 %, $p = 0.02$) and higher levels of circulating monocytes (0.5 [0.4–0.7] · 10^9 vs. 0.4 [0.3–0.5] · 10^9 monocytes per blood liter, $p = 0.024$) than non-MACEs. Moreover, patients experiencing MACEs had increased serum levels of ficolin-1 (136.4 [96.1–201.6] vs. 76.9 [34.3–120.0] ng/mL, $p = 0.004$) and ficolin-2 (0.70 [0.48–1.07] vs. 0.47 [0.35–0.57] OD, $p = 0.009$) than non-MACEs.

Cox proportional hazard regression analyses showed that risk of

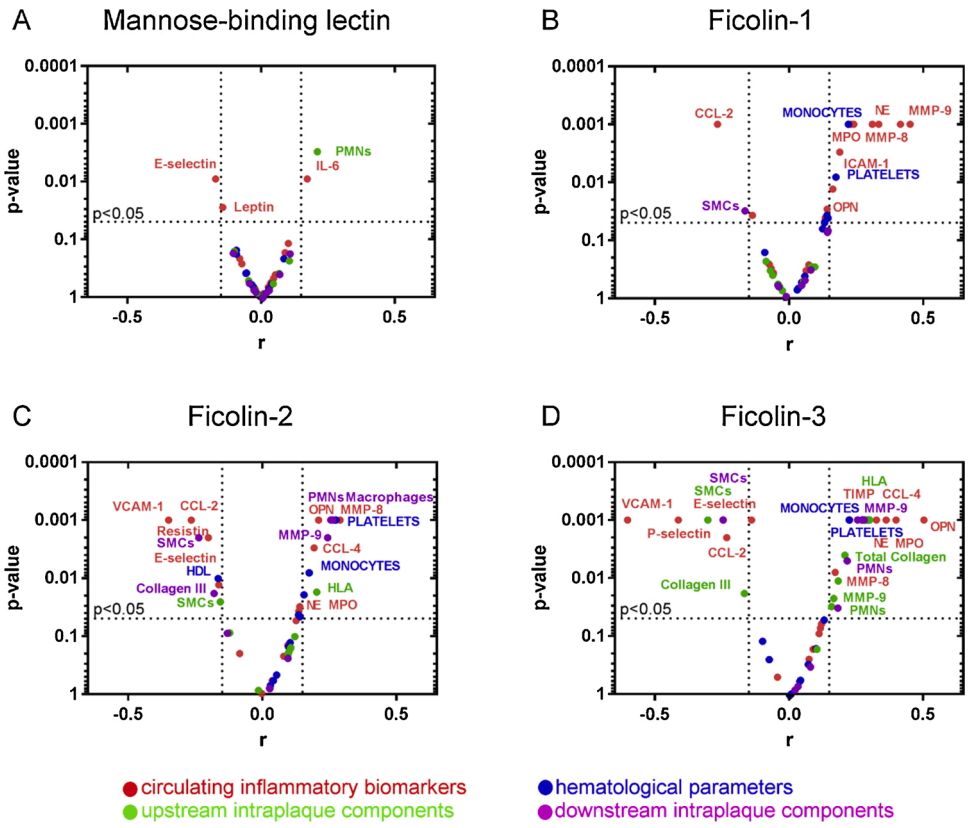

Fig. 1. Volcano plots showing correlations of LP protein serum levels with circulating and intraplaque markers. Correlation r on the x-axis (linear), p-value on the y-axis (logarithmic). MBL (A), Ficolin-1 (B), Ficolin-2 (C) and Ficolin-3 (D) circulating levels were correlated with circulating inflammatory markers (red dots), hematological parameters (blue), upstream (green) and downstream (purple) intraplaque components. Top left quadrant indicates statistically significant inverse correlations, top right quadrant indicates statistically significant direct correlations. Comparisons were drawn by Mann-Whitney *U* test.

Table 2
Ficolins (-1, -2, -3), mannose-binding lectin (MBL) and correlations with circulating inflammatory biomarkers.

	Ficolin-1		Ficolin-2		Ficolin-3		MBL	
	r	*p*-value	*r*	*p*-value	*r*	*p*-value	*r*	*p*-value
CCL-2	**−0.267**	**<0.001**	**−0.265**	**<0.001**	**−0.232**	**<0.002**	−0.072	0.267
CCL-3	−0.067	0.305	−0.084	0.199	0.121	0.062	−0.080	0.217
CCL-4	0.063	0.332	**0.193**	**0.003**	**0.399**	**<0.001**	0.036	0.580
CCL-5	−0.058	0.371	−0.002	0.970	−0.043	0.512	0.047	0.474
P-selectin	−0.059	0.361	**−0.201**	**0.002**	**−0.413**	**<0.001**	0.008	0.901
E-selectin	**−0.137**	**0.037**	**−0.162**	**0.013**	**−0.245**	**<0.001**	**−0.170**	**0.009**
L-selectin	**0.232**	**<0.001**	−0.001	0.993	**0.326**	**<0.001**	0.010	0.875
ICAM-1	**0.189**	**0.003**	0.080	0.221	0.075	0.251	0.090	0.170
VCAM-1	−0.074	0.259	**−0.349**	**<0.001**	**−0.602**	**<0.001**	0.027	0.675
MMP-9	**0.415**	**<0.001**	0.095	0.144	0.117	0.073	0.038	0.557
pro-MMP-9	**0.451**	**<0.001**	**0.290**	**<0.001**	**0.362**	**<0.001**	0.031	0.701
MMP-8	**0.310**	**<0.001**	**0.270**	**<0.001**	**0.172**	**0.008**	0.010	0.873
MPO	**0.241**	**<0.001**	**0.140**	**0.031**	**0.226**	**<0.001**	0.054	0.413
NE	**0.334**	**<0.001**	**0.140**	**0.031**	**0.226**	**<0.001**	0.054	0.413
Resistin	**0.140**	**0.040**	**−0.201**	**0.002**	**−0.140**	**<0.001**	−0.022	0.731
Leptin	**0.163**	**0.013**	**0.136**	**0.037**	0.090	0.168	**−0.143**	**0.028**
TIMP-1	0.073	0.264	0.126	0.054	**0.286**	**<0.001**	0.005	0.936
OPN	**0.142**	**0.029**	**0.210**	**0.001**	**0.503**	**<0.001**	0.102	0.118
hs-IL-6	**0.135**	**0.041**	0.037	0.580	0.112	0.091	**0.173**	**0.009**

Spearman rank correlation coefficient. Comparisons were drawn by Mann-Whitney *U* test

MACE increased with increasing serum levels of ficolin-2 and ficolin-3 (HR 50.6 [95 % CI 5.8–440.9], p < 0.001 and HR 15.9 [95 % CI 1.0–242.8], p = 0.047, respectively) but not ficolin-1 and MBL. For ficolin-2, the predictive value for MACE was confirmed after adjustment for previous coronary artery disease, levels of MMP-9 and of circulating monocytes (HR 38.6 [95 % CI 3.9–385.2]; p = 0.002). Data shown in Table 5 and Fig. 3.

4. Discussion

This work originally shows that ficolins – recognition molecules activating the LP of complement – support inflammatory events leading to plaque instability in atherosclerotic patients. Specifically, serum levels of ficolin-2 can be a sensitive systemic biomarker of vulnerable plaques, with a predictive value of cardiovascular disease symptoms.

At present atherosclerotic patients are diagnosed based on the degree of stenosis (vessel narrowing) assessed with ultrasound Doppler, to decide the best therapeutic option, i.e. surgical removal of the plaque

Table 3
Ficolins (-1, -2, -3) and mannose-binding lectin (MBL) correlation with hematological and biochemical parameters.

	Ficolin-1		Ficolin-2		Ficolin-3		MBL	
	r	*p*-value	*r*	*p*-value	*r*	*p*-value	*r*	*p*-value
Age	0.020	0.764	−0.176	0.006	−0.083	0.202	0.103	0.111
sBP	0.031	0.637	0.094	0.146	**0.290**	**<0.001**	0.082	0.207
Total WBC	**0.128**	**0.048**	**0.151**	**0.019**	0.039	0.549	−0.059	0.366
PMNs	**0.128**	**0.047**	0.100	0.124	0.000	0.999	−0.057	0.362
Monocytes	**0.216**	**0.001**	**0.170**	**0.008**	**0.219**	**0.001**	0.037	0.565
Lymphocytes	0.027	0.674	0.131	0.042	0.068	0.293	−0.094	0.147
PLT	**0.17**	**0.008**	**0.269**	**<0.001**	**0.267**	**<0.001**	0.016	0.810
Total-Ch	0.044	0.501	0.038	0.558	0.005	0.942	0.001	0.987
HDL	−0.093	0.154	**−0.166**	**0.010**	−0.101	0.118	−0.031	0.631
LDL	0.055	0.394	0.050	0.445	0.037	0.569	−0.009	0.893
TAG	**0.137**	**0.036**	**0.171**	**0.008**	0.127	0.051	−0.037	0.575
Glycaemia	0.121	0.061	0.027	0.675	−0.076	0.242	0.026	0.684
Insulinemia	**0.141**	**0.040**	**0.137**	**0.045**	0.097	0.158	−0.094	0.172

Spearman rank correlation coefficient.

Table 4
Ficolins (-1, -2, -3), mannose-binding lectin (MBL) and correlations with intraplaque components.

	Ficolin-1		Ficolin-2		Ficolin-3		MBL	
	r	*p*-value	*r*	*p*-value	*r*	*p*-value	*r*	*p*-value
Upstream								
Red-oil	0.079	0.270	0.092	0.199	**0.179**	**0.011**	0.064	0.369
Macrophages	−0.038	0.597	−0.017	0.817	0.100	0.162	0.006	0.933
PMNs	−0.063	0.380	0.117	0.098	**0.154**	**0.030**	**0.206**	**0.003**
Total collagen	−0.044	0.538	0.095	0.181	**0.203**	**0.004**	−0.018	0.806
Collagen I	−0.028	0.693	0.101	0.159	**0.278**	**<0.001**	−0.102	0.154
Collagen III	−0.072	0.316	−0.123	0.085	**−0.168**	**0.018**	−0.028	0.698
SMCs	0.050	0.481	**−0.158**	**0.025**	**−0.303**	**<0.001**	−0.049	0.492
HLA	0.092	0.273	**0.198**	**0.017**	**0.295**	**<0.001**	0.102	0.224
MMP-9	−0.088	0.219	0.102	0.151	**0.162**	**0.022**	0.042	0.556
Downstream								
Red-oil	0.078	0.309	0.092	0.231	0.030	0.700	−0.106	0.167
Macrophages	0.141	0.066	**0.258**	**0.001**	**0.269**	**<0.001**	−0.046	0.549
PMNs	0.139	0.070	**0.250**	**0.001**	**0.212**	**0.005**	−0.029	0.708
Total collagen	0.044	0.564	0.024	0.751	**0.250**	**0.001**	0.028	0.721
Collagen I	−0.041	0.597	−0.132	0.086	0.076	0.326	−0.022	0.773
Collagen III	−0.044	0.569	**−0.181**	**0.018**	0.019	0.803	0.018	0.812
SMCs	**−0.166**	**0.030**	**−0.237**	**0.002**	**−0.247**	**0.001**	0.105	0.171
HLA	−0.013	0.875	0.025	0.760	**0.177**	**0.032**	0.003	0.968
MMP-9	0.056	0.464	**0.239**	**0.002**	**0.274**	**<0.001**	0.067	0.385

Spearman rank correlation coefficient.

(endarterectomy if stenosis > 70 %) or pharmacological treatment [1,3,32]. However more than stenosis, plaque vulnerability, associated with thromboembolic complications [4], raises the risk of adverse cardiovascular events like ischemic stroke [5,6]. Standard diagnosis underestimates the mechanisms of plaque erosion and fails to detect vulnerable plaques. Moreover endarterectomy is associated with surgical complications and risks, especially for elderly patients, who, in case of stable plaques may benefit more from pharmacological treatment than endarterectomy (CANTOS trial [33]). Endarterectomy indeed does not eliminate the risk of acute cardiovascular events, i.e. the risk for stroke re-occurrence within 8 years after surgery remains elevated (20 % of >80 % stenotic patients) [2]. Thus the identification of biomarkers associated with plaque detrimental evolution is critically needed [34,35]. Moreover, knowing the precise mechanisms occurring at the plaque and associated with a specific biomarker would widen its use into a theranostic fashion.

Our work started from previous studies reporting the involvement of the complement system in the progression of atherosclerosis. The complement system is a physiologic circulating component of innate immunity which, in diseased conditions, is activated upon recognition of damage-associated molecular patterns (DAMPs). DAMPs include glycoproteins and proteins from the extracellular matrix, intracellular proteins, DNA or RNA fragments, heat-shock proteins, all present within the atherosclerotic plaque, thus offering an inflammatory milieu for complement activation. On activation, the complement system coordinates inflammation, phagocytosis and cell death, thus critically contributing to pathophysiological processes [36]. Among the pathways of complement activation, the lectin pathway (LP) has a prominent role in atherosclerosis [19,23]. This is likely due to the multiple roles of LP over the well-known complement cascade activation, placing it at a cross road of the interplay between inflammation and coagulation, referred to as thromboinflammation, occurring within the vascular bed [37].

Mannose-binding lectin (MBL) was the first discovered and thus more widely studied initiator of the LP. A few studies reported MBL involvement in atherosclerosis supporting either an anti-atherogenic [38–42] or a pro-atherogenic [43–45] role, possibly depending on disease process and specific clinical setting [43,46]. In the present work we observed negligible correlations of MBL serum levels with circulating, hematologic and intraplaque components. It is well known that in humans genetic variants cause MBL deficiency/low levels in about 20–30 % of the general population [14,15,30,47], thus we may expect that genetically determined MBL levels hampered proper correlation analysis. Interestingly, stroke patients with MBL deficiency have smaller infarctions and better outcomes [10,13], posing the rationale to stratify the cohort in MBL-sufficient and MBL-deficient patients using a cut-off at

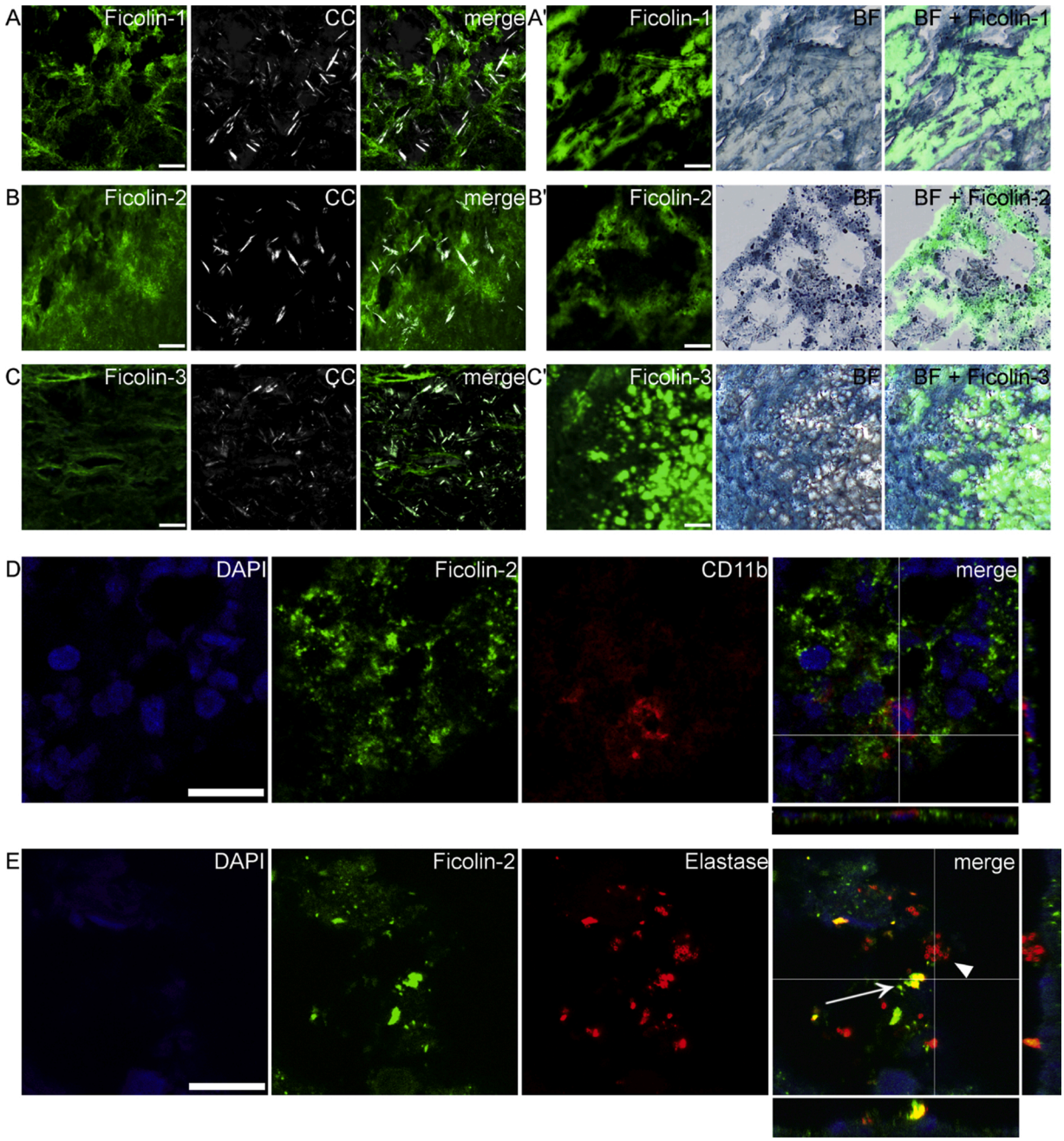

Fig. 2. Intraplaque localization of ficolins. Ficolin-1 (A), -2 (B) and, to a lesser extent, -3 (C, green) were present in necrotic core areas enriched in cholesterol crystals (CC, white). All ficolins were also present in calcified parts of the plaques visible at brightfield microscopy (BF, A', B' and C'). Scale bars 50 μm. Panel D shows ficolin-2 (green) surrounding macrophages (CD11b, red) recruited to the plaques' necrotic core. Panel E shows ficolin-2 (green) and neutrophils' elastase (red) in the necrotic core. Few neutrophils appeared positive for ficolin-2 (arrow), but the majority of them was located in areas negative for ficolin-2 (arrowhead). Scale bars 20 μm.

500 ng/mL of MBL [30]. Eighty out of 240 patients (31 %) resulted as MBL-deficient. MBL-deficient used oral antidiabetics less frequently than MBL-sufficient patients and, likely associated with this, had increased IGF-1 levels. This observation might imply a pro-atherogenic role for MBL, by favoring co-morbidities like diabetes that could increase the risk of a cardiovascular pathology. On the contrary, supporting an anti-atherogenic role for MBL, MBL-deficient were 3 year younger than MBL-sufficient and had increased circulating levels of leptin, a pro-inflammatory mediator associated with cardiovascular risk and PCSK9, a robust predictor of acute coronary syndromes in patients with severe carotid artery stenosis [48]. The fact that MBL-deficient patients had increased TIMP-1 levels may either support MBL's anti-atherogenic role, considering TIMP-1 like an indicator of increased inflammatory stress, or MBL's pro-atherogenic role, due to TIMP-1 ability to antagonize MMPs. Thus MBL's role in atherosclerosis could not be clarified, presently ruling it out from use as a biomarker.

Circulating levels of ficolins, other LP initiators, correlated with inflammatory biomarkers implicated in plaque vulnerability such as MPO, MMP-9, ICAM-1, osteopontin and MMP-8. Moreover, ficolins were present in the necrotic core of the analyzed plaques, showing a strong presence in areas enriched with cholesterol crystals or calcification. We focused on ficolin-2, the initiator seeming pivotal in atherosclerotic plaque evolution [18,19]. We previously reported that ficolin-2 intraplaque presence correlates with the size of the lipid core, suggesting that

Table 5
Cox regression. The risk of a MACE was assessed over 18-month clinical follow up (available for 194 patients, 80.5 %).

Univariate			Multivariate		
	HR (95 % CI)	*p*-value		HR (95 % CI)	*p*-value
Ficolin-1	1.00 (0.99–1.01)	0.168	**Ficolin-2**	38.64 (3.88–385.22)	**0.002**
			CAD	2.95 (0.71–12.30)	0.138
Ficolin-2	50.58 (5.80–440.90)	**<0.001**	MMP-9	1.01 (1.00–1.02)	**0.032**
			Monocytes	5.30 (0.37–75.69)	0.219
Ficolin-3	15.85 (1.04–242.83)	**0.047**			
			Ficolin-3	8.76 (0.41–187.12)	0.165
MBL	1.00 (0.99–1.00)	0.247	CAD	2.52 (0.61–10.46)	0.202
			MMP-9	1.01 (1.00–1.02)	**0.034**
Age	0.96 (0.89–1.03)	0.228	Monocytes	6.00 (0.48–74.36)	0.163
Sex (Male)	0.41 (0.09–1.99)	0.254			
Hypertension	0.94 (0.25–3.54)	0.940			
Dyslipidemia	1.95 (0.52–7.36)	0.323			
Diabetes	1.02 (0.22–4.78)	0.984			
Smokers	0.73 (0.16–3.38)	0.686			
CAD	3.48 (1.17–10.37)	**0.025**			
MMP-9	1.01 (1.00–1.02)	**0.021**			
Monocytes	17.18 (2.34–126.17)	**0.005**			

Data are presented as hazard ratio (HR) and 95 % confidence interval (CI).

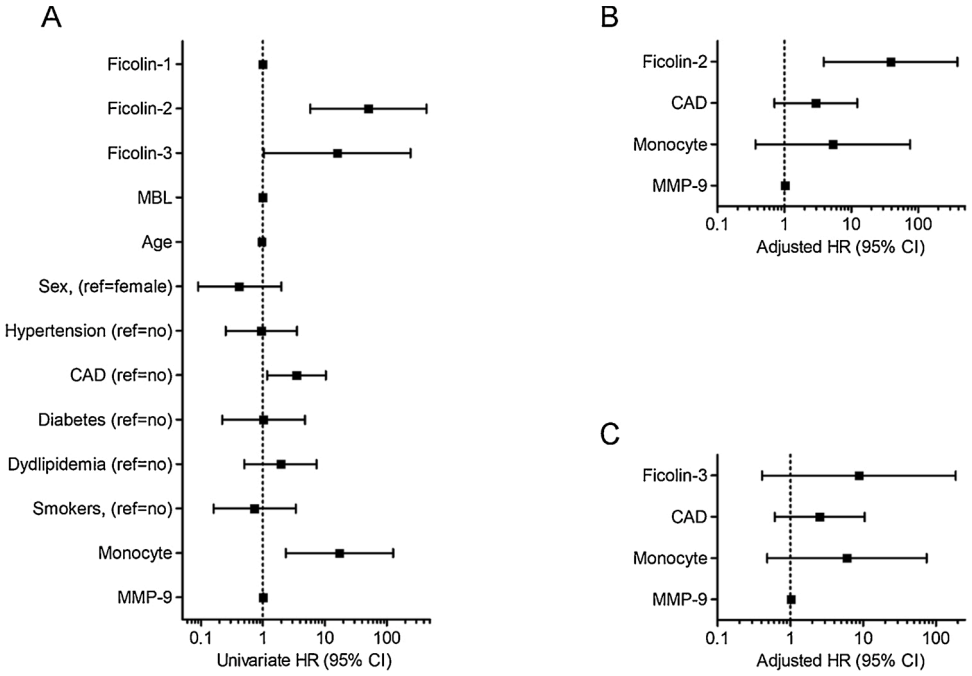

Fig. 3. Forest plots relative to the Cox regression analysis of the risk of a MACE assessed over 18-month clinical follow up (available for 194 patients, 80.5 %). A) Univariate analysis. B, C) Multivariate analysis showing adjusted hazard ratio for ficolin-2 (B) or ficolin-3 (C) after correction for CAD and circulating levels of monocytes and MMP-9. Data are presented as hazard ratio (HR) and 95 % confidence interval (CI).

it is recruited on plaque growing. The plaque lipid core is enriched in cholesterol crystals, a target of ficolin-2 to stimulate phagocytosis by monocytes and granulocytes, as demonstrated in vitro [18]. Our data supports this idea, showing ficolin-2 presence in plaques' core areas enriched in cholesterol crystals, where it attracted macrophages and, to a lesser extent, neutrophils. Then high circulating levels of ficolin-2 were associated with intraplaque recruitment of immune cells, such as macrophages and neutrophils, implicated in the vulnerable evolution of the plaque.

Interpreting the value of ficolin-2 circulating levels as biomarkers of plaque vulnerability is challenging. Ficolin-2 levels in blood are determined by the net balance between consumption due to its use (decrease) and accumulation due to its synthesis in the liver (increase). Consumption is typically observed in acute events that fully activate the cascade, while accumulation is associated with chronic inflammatory states demanding fresh proteins in circulation. Conceivably, ficolin-2 serum levels were reported lower in patients after acute stroke (acute condition) than in asymptomatic patients with severe carotid atherosclerosis (chronic condition) [12]. Moreover, ficolin-2 serum levels were higher in atherosclerotic patients than healthy controls [12]. This likely confirms the involvement of ficolin-2 in the atherosclerotic process, during which it chronically accumulates in circulation, and its use after the acute event – like stroke - leading to consumption [15]. The fact that ficolin-2 circulating levels are low in patients with advanced vulnerable plaques [19] suggests that ficolin-2 is used (consumed) in a sub-acute inflammatory phase such as plaque erosion. On the contrary, as shown here, ficolin-2 accumulates in patients with a growing plaque, especially if the plaque is loading cholesterol crystals and immune cells. High ficolin-2 levels at this stage of plaque morphological evolution may be independent predictors of a future major adverse cardiovascular event,

as shown in our work.

Based on present and previous data, we hypothesize that ficolin-2 circulating levels change over the course of the atherosclerotic process, offering reasons to use them as biomarkers informing on the specific morphological state of the plaque. This hypothesis also reveals the main limitation of this study, i.e. a single time point assessment for LP protein levels (1 day before endarterectomy). Nevertheless ficolin-2 stands as a potential biomarker to anticipate a major cardiovascular event in atherosclerotic patients. The integration of imaging markers with reliable circulating biomarkers would strengthen the identification of vulnerable plaques, thus stratifying patients to select appropriately prophylactic endarterectomy or advanced medical treatment.

Sources of funding

Funded by the Italian Ministry of Health – Rete Cardiologica IRCCS, Project code RCR-2019-23669118_001, cup code D41F19000250001, and by ERA-NET NEURON JTC 2019, 'STATEMENT', project code NEURON-082.

Declaration of Competing Interest

None.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.phrs.2021.105462.

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