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Plasma proteins associate with carotid plaques and predict incident atherosclerotic cardiovascular events

Andrea Baragetti [a,*], Liliana Grigore [b], Elena Olmastroni [a], Elisa Mattavelli [a,c], Alberico Luigi Catapano [a,b]

[a] Department of Pharmacological and Biomolecular Sciences "Rodolfo Paoletti", University of Milan, Milan, Italy
[b] IRCCS Multimedica Hospital, Milan, Italy
[c] Bassini Hospital, Cinisello Balsamo, Milan, Italy

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ABSTRACT

Purpose: Performing non-invasive carotid imaging is challenging, owing inter-operator variability and organizational barriers, but plasma proteomics can offer an alternative. We sought plasma proteins that associate with the presence of carotid plaques, their number and predict the incidence of clinically overt atherosclerotic cardiovascular events (ASCVD) above currently recognized risk factors in "apparently healthy" subjects.

Methods: We studied the plasma levels of 368 proteins in 664 subjects from the PLIC study, who underwent an ultrasound imaging screening of the carotids to check for the presence of plaques. We clustered, by artificial intelligence (A.I.), the proteins that associate with the presence, the number of plaques and that predict incident ASCVDs over 22 years (198 events were registered).

Findings: 299/664 subjects had at least 1 carotid plaque (1+) (77 with only one plaque, 101 with 2 plaques, 121 with ≥3 plaques (3+)). The remaining 365 subjects with no plaques acted as controls. 106 proteins were associated with 1+ plaques, but 97 proteins significantly predicted 3+ plaques only (AUC = 0.683 (0.601–0.785), $p < 0.001$), when considered alone.

A.I. underscored 87 proteins that improved the performance of the classical risk factors both in detecting 3+ plaques (AUC = 0.918 (0.887–0.943) versus risk factors alone, AUC = 0.760 (0.716–0.801), $p < 0.001$) and in predicting the incident ASCVD (AUC = 0.739 (0.704–0.773) vs risk factors alone AUC = 0.559 (0.521–0.598), $p < 0.001$). The chemotaxis/migration of leukocytes and interleukins/cytokines signaling were biological pathways mostly represented by these proteins.

Discussion and conclusions: Plasma proteomics marks the number of carotid plaques and improve the prediction of incidence ASCVDs in apparently healthy subjects.

1. Introduction

Atherosclerotic Cardiovascular Disease (ASCVD) is the leading cause of death worldwide [1]. In populations exposed to low number of classical risk factors and free from previous ASCVD, a subset of subjects with subclinical ASCVD can be detected [2,3]. However, applying noninvasive vascular imaging procedures to detect subclinical ASCVD, such as those identifying carotid plaques by ultrasound imaging, is challenging due to organizational, economical barriers, and given the substantial inter-operator variability [4,5]. Therefore, if an examination with vascular imaging is not performed, these subjects are still classified as at low risk unlikely to develop an ASCVD over ten years according to the contemporary cardiovascular guidelines [6].

New omics techniques can inform on the activation of vasculotropic pathways related to atherosclerosis [7,8] and there is evidence that plasma proteomics outperforms the clinical algorithms of risk assessment to predict the occurrence or the re-occurrence of a clinically overt ASCVD [9,10]. Nevertheless, the evidence regarding the ability of plasma proteomics to inform the presence of vascular plaques is still limited to few numbers of subjects and by addressing a maximum of 82 plasma proteins linked to cardiovascular disease [11,12]. Also, in other few studies the number of plasma proteins that have been investigated for association with carotid atherosclerosis is limited to 19 in one study [13], 86 [14,15] or 92 in others [16] We previously demonstrated that

* Corresponding author at: Department of Pharmacological and Biomolecular Sciences "Rodolfo Paoletti", University of Milan, Via G. Balzaretti, 9, 20133, Milan, Italy.

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seven inflammatory proteins (PIgR2 (polymeric immunoglobulin receptor), chemokine (C—C motif) ligand 18 (CCL18), CA1 (carbonic anhydrase 1), Fc gamma receptor IIa (CD32), MMP10 (matrix metallopeptidase 10), Fatty acid-binding protein 6 (FABP6), IL7R (interleukin 7 receptor)), out of an unprecedented spectrum of 368 proteins measured in plasma by high-throughput proteomics technique (Olink), can predict the development of at least a plaque in a population of subjects in primary prevention for ASCVD and who were free from carotid atherosclerosis when screened by ultrasound at basal visit. Indeed, people who developed a carotid plaque when ultrasound was performed again over a decennial follow-up, displayed higher levels of PigR2, CCL18, CA1, CD32 and reduced MMP10, FABP6, IL7R versus subjects who did not developed plaques, and the performance of such proteins to predict this development outperformed the currently available algorithms [17]. Anyhow, inflammation is known to play an important role in the onset and progression of plaques [18], and many other proteins/pathways could be involved still during the earliest phases of the atherosclerotic process. In that occasion, we did not consider the possibility that multifocal plaques are surrogate markers of inflammatory conditions in apparently healthy individuals. Moreover, we employed canonical statistical strategies to test the predictive power of plasma proteomics (logistic regression models) and we therefore cannot rule out that the use of statistical tools of machine learning, which can guarantee a higher sensitivity [19], could unravel the existence of additional proteomics signatures able to independently predict ASCVD disease.

We therefore sought to address the question whether we could identify "apparently healthy individuals" carrying asymptomatic carotid plaques among 664 subjects from a sample of the general population of the northern area of Milan, who were in primary cardiovascular prevention at the first evaluation of the study, and whether could also predict occurring ASCVD at 22 years follow up, outperforming the ten years' perspective of the current guidelines. To this end we studied, by Normalized Protein eXpression (NPX) (an index of relative abundance [20]), the plasma levels of 368 proteins from the targeted Cardiometabolic, Inflammation, Cardiovascular II, and III panels (Olink™) and we searched for the most significant proteins whose levels in plasma, recapitulating biologically relevant pathways, can improve the accuracy to predict the occurrence of clinically overt atherosclerotic disease in apparently healthy subjects with asymptomatic atherosclerotic disease at the carotids.

2. Materials and methods

2.1. Study population

The population was from a subgroup of subjects from the northern area of Milan, randomly selected during the basal clinical evaluation of the Progressione delle Lesioni Intimali Carotidee ("PLIC") Study, which initially included 2.606 participants from 2001 to 2003 [21]. Participants in this study were screened at the Center for the Study of Atherosclerosis at E. Bassini Hospital (Cinisello Balsamo, Milan, Italy) for the personal, familial, pathological, and pharmacological clinical history. Targeted plasma proteomics was performed in plasma samples from for 664 subjects, who did not have ASCVD before the blood draw, (Supplementary Fig. S1). Out of them, 365 had no carotid plaques as detected by ultrasound and acted as controls. The characteristics of the subjects were compliant with the Sex and Gender Equity in Research (SAGER) guidelines [22]. This work is an observational study, and it was conducted following the standards of the Strengthening the Reporting of Observational studies in Epidemiology (STROBE) initiative [23].

The PLIC Study has been approved by the Ethics Committee of the University of Milan (SEFAP/Pr.0003). Written informed consent was obtained from all the subjects included in the study (all over 18 years-old), for the use of their biological samples and health data subsequently to their collection during the first evaluation of the study, their anonymized information to be published in this article. This consent was obtained in accordance with the Declaration of Helsinki.

Detailed information regarding the clinical and biochemical parameters of the participants in the study are available as Supplemental Material.

2.2. Carotid ultrasound

Carotid ultrasound analysis was performed by a single expert sonographer, blinded on subject's identity and clinical history, with a linear ultrasound probe (4.0e13.0 MHz frequency, 14 × 48 mm footprint, 38 mm field of view, Vivid S5 GE Healthcare®, Wauwatosa, WI, USA). A detailed description of the ultrasound methodology, based on validated protocol for the PLIC study, has been published elsewhere [17].

Carotid plaques were defined if (i) focal loci were identified and/or (ii) if the measured carotid Intima Media Thickness (IMT) was equal or above than 1.3 mm in any vascular tract (common, bulb, bifurcation, internal and external tracts) of one or both carotid arteries [17].

2.3. Plasma proteomics

368 proteins were measured in plasma by Proximity Extension Assay (PEA) technology. The proteins are included in the Cardiovascular II, Cardiovascular III, Inflammation and Cardio-metabolic panels of Olink™ platform. The complete list of the proteins that are included in the panels, their Uniprot ID and the Olink ID, are provided by the company (http://www.olink.com). Proteins were measured from 200 uL of plasma samples by Proximity Extension Assay (PEA). Plasma samples from each subject was separated from whole blood by centrifugation (4.481g, 12 min at 4 degrees (Eppendorf 5810R, Hamburg, Germany)), aliquoted in 96-wells plates during the first clinical evaluation of each subject (between 1998 and 2000) and immediately stored at −80 degrees up to 2018, when they were sent to Olink™ (Uppsala, Sweden) for proteomic characterization. The final assay read-out of each determination is given in Normalized Protein eXpression (NPX), which is an arbitrary unit on log2-scale where a high value corresponds to a higher protein plasma level. Each PEA measurement has a lower detection limit (LOD) calculated versus negative controls that are included in each run, and measurements of individual proteins below LOD for each panel were removed from further analysis (2 samples from Cardiometabolic panel, 29 samples from Cardiovascular II; 31 samples from the Inflammation panel; all the samples were with measurement>LOD in the Cardiovascular III panel). All assay characteristics including detection limits, the measurements of assay performance, and validations are available from the manufacturer webpage (http://www.olink.com). All raw data can be provided upon reasonable written request to ALC.

2.4. Statistical analysis

2.4.1. Descriptive statistics

Clinical and biochemical data are presented as median with interquartile ranges (the lower 25th percentile and the upper 75th percentile). Before comparing linear variables among two groups, we firstly checked for the type of distribution (Shapiro-Wilk Test). We then used Kolmogorov-Smirnov test to compare normally distributed variables while we Kruskal-Wallis test for non-normally distributed variables. To compare linear variables among four groups (controls, subjects with 3+, with 2 and with 1 carotid plaque), we used Analysis of Variances (ANOVA) with Bonferroni post-hoc analysis. Dichotomous variables were compared by Fisher's chi-square test. Grubb's test for the detection of the outliers was performed for all the linear variables before running tests.

The analysis of proteomics data was performed by using Xgboost prediction model, as previously published in PLIC cohort [19], for details see Supplemental Material.

P values <0.05 were considered significant for all the statistical

Table 1
Clinical and biochemical parameters between controls and the groups of subjects with increasing number of carotid plaques.

	Controls (n = 365)	1 carotid plaque (n = 77)	2 carotid plaques (n = 101)	3+ carotid plaques (n = 121)	P_{trend}	*P*-value (controls vs 1 carotid plaque)	P-value (Controls vs 2 carotid plaques)	P-value (Controls vs 3+ carotid plaques)
Age (years); (median (IQR)	55 (49–60)	54 (48–52)	56 (51–61)	58 (52–62)	0.002	0.246	0.267	0.001
Women; n (%)	249 (68.2)	51 (66.2)	62 (61.4)	75 (61.9)	0.442	0.788	0.185	0.194
Former smokers; n(%)	81 (22.2)	25 (32.5)	22 (21.8)	29 (23.9)	0.276	0.057	0.920	0.696
Active smokers; n(%)	59 (16.6)	14 (18.2)	18 (17.8)	39 (32.2)	0.001	0.672	0.700	<0.001
Regular physical activity; n(%)	164 (44.9)	29 (37.6)	34 (33.6)	41 (33.9)	0.056	0.243	0.043	0.033
BMI (Kg/m^2); (median (IQR)	25.99 (23.59–28.68)	25.95 (23.58–28.85)	26.40 (24.24–29.04)	26.80 (24.13–29.40)	0.349	0.820	0.315	0.139
Waist circumference (cm); (median (IQR)	89 (80–96)	88 (78–95)	90 (83–97)	92 (84–98)	0.065	0.569	0.196	0.023
Hip circumference (cm); (median (IQR)	103 (97–110)	100 (96–108)	102 (97–109)	106 (98–113)	0.146	0.257	0.642	0.102
Systolic blood pressure (mmHg); (median (IQR)	130 (120–140)	130 (120–140)	130 (120–140)	135 (120–150)	0.001	0.932	0.241	<0.001
Diastolic blood pressure (mmHg); (median (IQR)	80 (80–90)	80 (80–90)	80 (80–90)	80 (80–90)	0.028	0.118	0.179	0.005
Anti-hypertensive; n (%)	78 (21.3)	15 (19.5)	17 (16.9)	40 (33.1)	0.016	0.646	0.291	0.010
Glucose (mg/dL); (median (IQR)	88 (82–96)	86 (80–93)	87 (81–96)	89 (82–97)	0.207	0.131	0.590	0.257
Glucose lowering treatments; n(%)	2 (0.5)	0 (0.0)	0 (0.0)	3 (2.5)	0.095	0.510	0.453	0.069
Total cholesterol (mg/dL); median (IQR)	219.0 (196.0–245.0)	223.0 (192.5–255.0)	221.0 (201.0–240.5)	230.0 (199.5–235.5)	0.227	0.512	0.542	0.041
Triglycerides; (mg/dL); median (IQR)	87.0 (63.0–119.50)	89.0 (65.5–140.0)	96.0 (63.5–140.5)	110.0 (80.0–149.0)	0.001	0.327	0.225	<0.001
HDL-C (mg/dL); median (IQR)	57.0 (47.0–68.0)	52.0 (45.0–67.5)	55.0 (46.0–65.5)	49.0 (42.0–58.5)	<0.001	0.339	0.321	<0.001
LDL-C (mg/dL); median (IQR)	140.8 (116.8–163.9)	142.5 (119.3–163.4)	142.2 (121.0–163.3)	155.20 (124.30–178.0)	0.038	0.497	0.755	0.005
ApoB (mg/dL); median (IQR)	92.0 (109.0–125.0)	111.0 (95.5–132.0)	113.0 (95.5–124.5)	119.0 (97.5–136.5)	0.013	0.278	0.286	0.001
ApoA1 (mg/dL); median (IQR)	152.0 (132.5–173.5)	148.0 (129.0–172.5)	146.0 (132.5–168.0)	142.0 (126.0–161.5)	0.006	0.392	0.220	0.001
Lp(a) (mg/dL); median (IQR)	21.1 (4.5–46.1)	17.90 (5.20–43.40)	20.40 (3.15–40.30)	16.45 (3.45–53.22)	0.975	0.960	0.858	0.628
Lipid lowering treatments; n(%)	22 (6.0)	6 (5.2)	14 (13.9)	13 (10.7)	0.062	0.598	0.010	0.085
CRP (mg/L); median (IQR)	1.78 (0.66–3.37)	1.80 (0.86–3.64)	1.51 (0.59–3.85)	1.93 (0.88–4.23)	0.450	0.510	0.768	0.133
Previous ASCVD; n(%)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1.000	1.000	1.000	1.000

The table reports the clinical characteristics and the biochemical parameters of the subjects with increasing number of plaques (n = 77 subjects with 1 plaque, 101 subjects with 2 plaques and 121 subjects with 3+ plaques). Linear variables are reported as median and interquartile ranges (IQR) after verifying non-normal distribution (Shapiro-Wilk test). *P* values derive from by Analysis of Variances (ANOVA, P_{trend}) and Bonferroni post-hoc analysis (between the groups). Dichotomous variables are reported as absolute numbers (n) and percentage out of total sample within both groups and they were compared by Fisher's chi-square test.

analyses. SPSS™ v.24 for Windows (IBM corporation®) was used for all statistical analyses. Graphs were prepared using Graphpad® (v.9.0) and Excel (Microsoft®).

2.4.2. *Artificial Intelligence (A.I.)*

Proteomics data were analyzed by artificial intelligence (A.I.) model, previously published for proteomics data in PLIC cohort [9,19]. A detailed description is reported in Supplemental Material.

2.4.3. *Bioinformatics and Gene Ontology (GO) enrichment analyses*

A detailed description of the method used for the mining of biological processes represented by the plasma proteins is reported in Supplemental Material.

3. Results

3.1. *Plasma proteins predict the presence of carotid plaques*

Out of 664 apparently healthy subjects, 299 presented with at least one plaque in one of the carotids, while 365 did not present any plaque and acted as "controls" (the clinical and biochemical parameters are reported in Table 1). Out of the 299 subjects presenting at least one plaque, 77 had one, 101 subjects had 2 and 121 subjects presented with 3 or more plaques (3 up to 7 plaques; from now on defined as "3+). The ultrasonographic results are reported in Supplemental Table 2. The analysis of the prevalence of the main ASCVD risk factors showed a significant trend for active smoking, hypertension, dyslipidemia, increased waist circumference and reduced physically activity to associate with the presence of a carotid plaque (Supplemental Table 1). However, these risk factors, together with increased age, were more prevalent in subjects with 3+ plaques (Supplemental Table 3). Of note, the plasma levels of a widely used marker of chronic low-grade inflammation, CRP, did not differ in subjects with at least one plaque vs controls (P_{trend} = 0.071; Supplemental Table 1), and it was also not increased as a function of the number of plaques (P_{trend} = 0.450; Table 1).

By contrast, 106 proteins displayed significantly different NPX (7 reduced and 99 increased) in the plasma of subjects with at least one

Table 2
Proteins whose change in plasmatic levels marks an increased number of plaques.

Uniprot ID	Gene name	1 plaque	2 plaques	3+ plaques	Plasma levels
O00220	TNFRSF10A	✓	✓	✓	↑
P27930	IL1R2	✓	✓	✓	↑
Q14005	IL16	✓	✓	✓	↑
P15144	ANPEP	✓	✓	✓	↑
P15085	CPA1	✓	✓	✓	↑
P15086	CPB1	✓	✓	✓	↑
Q99988	GDF15	X	✓	✓	↑
P05231	IL6	X	✓	✓	↑
Q96D42	HAVCR1	X	✓	✓	↑
Q16651	PRSS8	X	✓	✓	↑
P04080	CSTB	X	✓	✓	↑
P14210	HGF	X	✓	✓	↑
P36222	CHI3L1	X	✓	✓	↑
Q14116	IL18	X	✓	✓	↑
Q16270	IGFBP7	X	✓	✓	↑
P13686	ACP5	X	✓	✓	↑
Q03405	PLAUR	X	✓	✓	↑
P07339	CTSD	X	✓	✓	↑
P49763	PGF	X	✓	✓	↑
P16871	IL7R	X	✓	✓	↓
P19438	TNFRSF1A	X	✓	✓	↑
P14780	MMP9	X	✓	✓	↑
P31997	CEACAM8	X	✓	✓	↑
O15467	CCL16	X	✓	✓	↑
P78380	OLR1	X	✓	✓	↑
Q8NBP7	PCSK9	X	✓	✓	↑
P05121	SERPINE1	X	✓	✓	↑
P78556	CCL20	X	✓	✓	↑
Q15113	PCOLCE	X	✓	✓	↑
Q15166	PON3	X	✓	✓	↓
Q9Y275	TNFSF13B	✓	X	✓	↑
P10646	TFPI	✓	X	✓	↑

In the table "✓" indicates if the protein is associated with the presence of that number of plaques. Proteins are sorted in descending order if they are associated with all the number of plaques (6 proteins), if only with 2 or with 3+ plaques and if they are associated with 1 plaque (24 proteins) and with 3+ plaques (2 proteins). "↑" and "↓" indicate that the plasma levels of the protein are increased or reduced (respectively) in presence of the number of plaques.

carotid plaque versus controls (Supplementary Fig. S2A, Supplemental Table 4 A). Furthermore, the number of proteins with different plasmatic NPX increased as a function of the number of plaques. In fact, the plasma levels of 17 proteins were significantly different in subjects with 1 carotid plaque as compared to controls (1 reduced and 16 increased; Supplementary Fig. S2B, Supplemental Table 4B), those of 47 were different in subjects with 2 plaques (4 proteins reduced and 43 increased; Supplementary Fig. S2C, Supplemental Table 4C) and those of 97 were different in subjects with 3+ plaques versus controls (9 reduced and 88 increased; Supplementary Fig. S2D, Supplemental Table 4D). The plasma levels of 6 proteins were increased with all the number of plaques, while the levels of 24 proteins were associated with the presence of 2 and of 3+ plaques (of them, 22 were with increased levels, while 2, IL7R and PON3, with reduced levels). The complete list of proteins, their UniProt IDs is available at Table 2 and the indication of their increased or reduced plasma levels refers to the raw data available a Supplemental Table 3B,C,D). This observation indicates that a larger set of proteins, compared to the commonly used CRP, can better identify the increase of carotid atherosclerosis.

The 106 proteins provided significant sensitivity to identify subjects with at least one plaque from controls (AUC = 0.584 (0.513–0.654), $p = 0.037$) and the performance of the 97 proteins associated with the presence of 3+ plaques was even superior (AUC = 0.683 (0.601–0.785), $p < 0.001$). By contrast, neither the 17 nor the 47 proteins, displayed significant sensitivity in predicting the presence of 1 or with 2 carotid plaques (AUC = 0.504 (0.461–0.591), $p = 0.948$ and AUC = 0.589 (0.503–0.671), $p = 0.083$, respectively).

Table 3
Proteins classified by the machine learning model as the most important to identify subjects with at least 1 plaque compared to controls.

1+ plaques versus controls					
Protein	Uniprot ID	Importance factor	Mean NPX (±S.E.) in subjects with 1+ plaque	Mean NPX (±S.E.) in controls	P value
EN-RAGE	P80511	0,134	2.74 ± 0.04	2.59 ± 0.04	1.92×10^{-2}
SCF	P21583	0,084	9.95 ± 0.02	10.01 ± 0.02	1.66×10^{-1}
IGFBP-3	P17936	0,073	4.17 ± 0.02	4.15 ± 0.02	5.24×10^{-1}
PLXNB2	O15031	0,066	0.82 ± 0.01	0.80 ± 0.01	1.58×10^{-1}
CD4	P01730	0,066	3.83 ± 0.02	3.80 ± 0.02	3.26×10^{-1}
ARTN	Q5T4W7	0,062	1.27 ± 0.02	1.30 ± 0.02	4.11×10^{-1}
FABP4	P15090	0,053	5.56 ± 0.04	5.48 ± 0.03	1.45×10^{-1}
GIF	P27352	0,052	6.15 ± 0.05	6.06 ± 0.04	1.73×10^{-1}
CEACAM8	P31997	0,048	3.24 ± 0.04	3.06 ± 0.03	9.74×10^{-4}
STAMBP	O95630	0,043	4.11 ± 0.06	4.04 ± 0.05	3.61×10^{-1}
F11	P03951	0,038	6.66 ± 0.02	6.63 ± 0.01	2.71×10^{-1}
TF	P13726	0,036	5.59 ± 0.02	5.54 ± 0.02	4.85×10^{-2}
MMP-1	P03956	0,036	10.94 ± 0.08	11.01 ± 0.07	5.39×10^{-1}
CSF-1	P09603	0,036	8.71 ± 0.01	8.70 ± 0.01	7.86×10^{-1}
TNF-R2	P20333	0,035	4.94 ± 0.02	4.88 ± 0.01	3.34×10^{-2}
OPN	P10451	0,035	4.61 ± 0.04	4.49 ± 0.04	3.19×10^{-2}
IL6	P05231	0,035	2.73 ± 0.04	2.54 ± 0.03	4.56×10^{-4}
CA4	P22748	0,034	1.16 ± 0.01	1.15 ± 0.02	6.95×10^{-1}
MMP-10	P09238	0,032	5.71 ± 0.04	5.75 ± 0.03	4.89×10^{-1}

The table reports the proteins identified as top contributors to identify subjects with 1+ plaques vs controls by the gradient boosting model of artificial intelligence. The Uniprot-ID of each protein is reported, together with the importance factor derived from the model (see methods in the main manuscript for more information) and the mean NPX values (with the relative standard error (S.E.)) in the group of subjects with 3+ plaques and in the controls. The *p* value (in linear scale and scientific representation) of the difference between the mean NPX values is also reported.

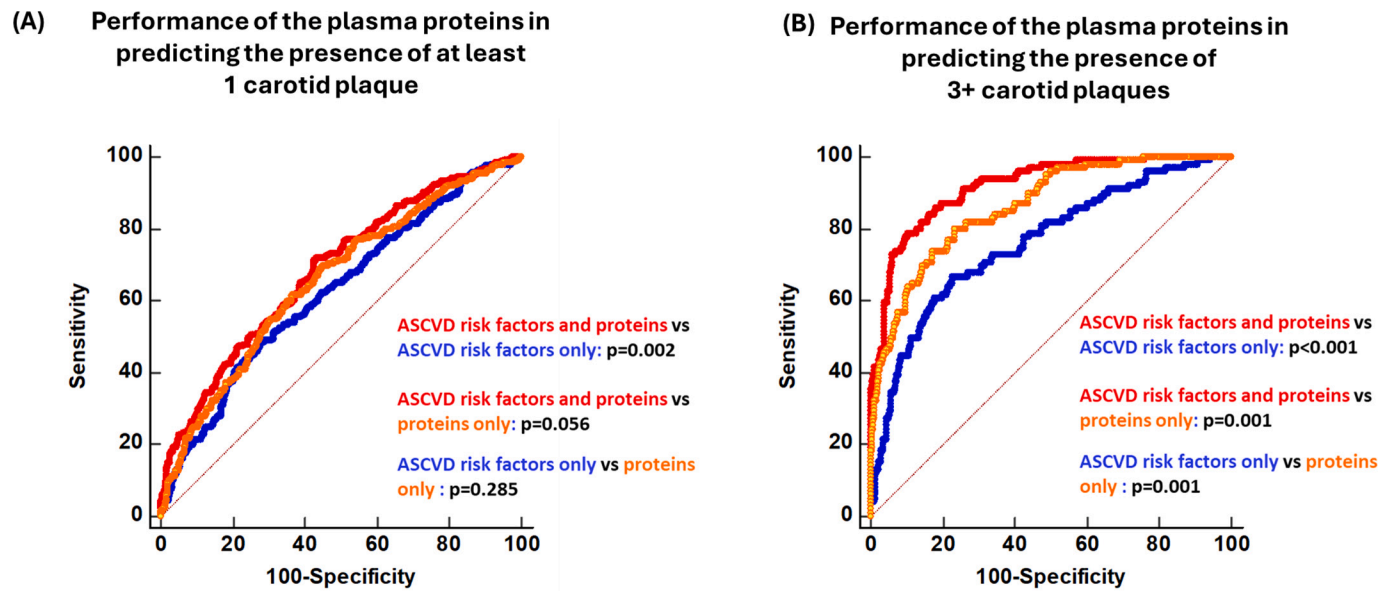

Fig. 1. Performance of the plasma proteins to predict the presence of carotid plaques.
Comparison of the accuracy (Receiver Operating Curve "ROC") of the model to discriminate: (A) subjects with at least one carotid plaque ("1+") from the controls, when considering the ASCVD risk factors together with the 19 hit protein predictors (red curve) versus the accuracy of the model that included the ASCVD risk factors only (blue curve) or of the model that included the proteins only (yellow curve); (B) subjects with three or more carotid plaques ("3+") from the controls, when considering the ASCVD risk factors together with the 87 hit protein predictors (red curve) versus the accuracy of the model that included the ASCVD risk factors only (blue curve). *P* values are derived from Hanley-McNeil test.

3.2. Plasma proteins improve the performance of ASCVD risk factors to predict the presence of carotid plaques

Hypertension, active smoking habit, increased waist circumference, dyslipidemia and reduced physical activity contributed for a ROC with AUC = 0.624 (0.586–0.662) in identifying subjects with at least 1 plaque. We were next prompted to therefore address whether plasma proteins predict the presence of carotid plaques over and above these currently recognized ASCVD risk factors.

19 proteins were identified by the A.I. model to significantly associate with the presence of at least 1 carotid plaque. Table 3 reports these proteins by descending importance factor derived from random forest classifier and their mean NPX (±standard error), being reduced for 4 and increased for 15 proteins in subjects with carotid plaques vs controls.

These proteins performed as well as the ASCVD risk factors, when considered separately in the model (AUC = 0.655 (0.617–0.692), p = 0.285), and significantly increased the performance, when included together with the same ASCVD risk factors in the model ($p = 0.002$ vs the AUC of the risk factors alone) (Fig. 1A). 87 proteins, out the 97 associated with the presence of 3+ plaques, were identified by A.I. as the more significant predictors of the presence of 3+ plaques. Table 4 reports these proteins by descending importance factor derived from random forest classifier and their mean NPX (±standard error).

These proteins significantly improved the AUC of the ASCVD risk factors to predict the presence of the 3+ plaques ((0.760 (0.716–0.801)) both alone (AUC = 0.862 (0.825–0.894); $P = 0.001$) and when they were considered on top of the ASCVD risk factors in the same model (AUC = 0.918 (0.887–0.943), $p < 0.001$) (Fig. 1B). Altogether these findings support the concept that plasma proteomics independently predict the presence of a higher number of carotid plaques and improve our capability to identify apparently healthy subjects with diffuse carotid atherosclerosis (three or more plaques).

3.3. Plasma proteins associated with carotid plaques recapitulate biological pathways involved in atherosclerotic disease

Next, we studied by gene ontology (GO) enrichment analysis whether the proteins that associated with the presence of carotid plaques inform on the expression of biological processes ("GO_bp") in plasma involved in atherosclerosis.

The 19 proteins predicting the presence of at least 1 carotid plaque clustered into 25 GO_bp, while a larger number of processes, 165, were represented by the 87 proteins predicting the presence of 3+ plaques (Supplemental Tables 4,5). Only 14 GO_bp were shared (Fig. 2A), including monocyte chemotaxis (GO:0002548), inflammatory response (GO:0006954), immune response (GO:0006955), proteolysis (GO:0006508) and other pathways involved in cell adhesion to vasculature, response to interleukins, T cell proliferation (Supplemental Table 6), supporting the concept that plasma proteins recapitulate the onset of pathophysiological mechanisms during the establishment of advanced atherosclerotic stages.

Moreover, the folds of enrichment (that is the percentage of proteins belonging to one pathway out of the total number of proteins belonging to all the annotated pathways) of the 25 GO_bp associated with 1+ carotid plaques were far lower compared to the ones of the 165 GO_bp associated with the presence of 3+ carotid plaques (Fig. 2B,C). Similarly, fewer GO_bps, with lower folds of enrichments, were also found when performing gene ontology analysis with the proteins associated with only 1 or with only 2 plaques (Supplementary Fig. S3 A,B).

Together these observations indicate that the enrichment of biological processes in plasma is informative for the progression toward more diffuse carotid atherosclerosis and for a more profound engagement of some processes common to atherosclerotic disease.

3.4. Plasma proteins associated with number of carotid plaques predict the incidence of clinically overt ASCVDs

Next to study whether the proteins that identify subjects with carotid plaques could also improve the performance of the commonly used risk factors to predict occurring ASCVDs, we followed the population over 22 (21−23) years.

198 ASCVD events occurred over time (29.81% in the population). Overall, the performance of classical risk factors in predicting the occurrence of the events was modest in this cohort of apparently healthy

Table 4
Proteins classified by the machine learning model as the most important to identify subjects with 3+ plaques compared to controls.

3+ plaques versus controls					
Protein	Uniprot ID	Importance factor	Mean NPX (±S.E.) in subjects with 3+ plaques	Mean NPX (±S.E.) in controls	P value
GDF-15	Q99988	0,046	5.19 ± 0.04	4.96 ± 0.02	1.17×10^{-7}
SCGB3A2	Q96PL1	0,029	3.08 ± 0.10	2.79 ± 0.04	3.40×10^{-3}
TNFRSF14	Q92956	0,027	4.29 ± 0.03	4.21 ± 0.02	1.86×10^{-2}
ALCAM	Q13740	0,023	5.11 ± 0.03	5.03 ± 0.01	6.66×10^{-3}
PCSK9	Q8NBP7	0,022	3.24 ± 0.03	3.15 ± 0.02	9.92×10^{-3}
PLTP	P55058	0,018	1.57 ± 0.03	1.68 ± 0.03	3.32×10^{-2}
TIMP1	P01033	0,018	4.93 ± 0.03	4.85 ± 0.02	3.51×10^{-2}
GAS6	Q14393	0,018	3.29 ± 0.04	3.39 ± 0.03	4.47×10^{-2}
MMP-12	P39900	0,018	5.63 ± 0.06	5.22 ± 0.03	1.51×10^{-9}
EpCAM	P16422	0,018	5.99 ± 0.10	5.71 ± 0.05	1.25×10^{-2}
CCL18	P55774	0,017	6.26 ± 0.06	5.94 ± 0.03	1.18×10^{-5}
CCL15	Q16663	0,017	7.12 ± 0.04	6.99 ± 0.02	3.11×10^{-3}
Flt3L	P49771	0,017	9.27 ± 0.04	9.17 ± 0.02	2.33×10^{-2}
CCL25	O15444	0,017	5.33 ± 0.05	5.10 ± 0.04	3.11×10^{-3}
CCL20	P78556	0,017	5.67 ± 0.08	5.43 ± 0.05	1.93×10^{-2}
IL1RN	P18510	0,016	4.31 ± 0.08	3.98 ± 0.04	1.51×10^{-4}
LGALS9	O00182	0,016	8.22 ± 0.03	8.09 ± 0.02	4.13×10^{-4}
HAVCR1	Q96D42	0,016	7.13 ± 0.06	6.78 ± 0.03	2.37×10^{-6}
TR-AP	P13686	0,016	1.83 ± 0.03	1.66 ± 0.02	2.08×10^{-4}
FAS	P25445	0,016	5.08 ± 0.03	4.98 ± 0.01	4.38×10^{-3}
CFHR5	Q9BXR6	0,015	7.67 ± 0.04	7.54 ± 0.02	7.06×10^{-3}
ACE2	Q9BYF1	0,015	2.91 ± 0.05	2.73 ± 0.03	3.61×10^{-3}
TNFRSF10C	O14798	0,015	5.82 ± 0.04	5.69 ± 0.02	1.09×10^{-2}
CPB1	P15086	0,015	5.13 ± 0.06	4.99 ± 0.03	2.66×10^{-2}
CCL16	O15467	0,015	6.20 ± 0.04	6.05 ± 0.02	7.97×10^{-3}
CCL11	P51671	0,015	6.88 ± 0.05	6.74 ± 0.03	3.50×10^{-2}
TNF-R2	P20333	0,014	4.97 ± 0.03	4.88 ± 0.01	8.20×10^{-3}
MCP-1	P13500	0,014	3.51 ± 0.07	3.38 ± 0.02	4.20×10^{-2}
tPA	P00750	0,014	6.95 ± 0.04	6.73 ± 0.03	3.38×10^{-4}
RARRES2	Q99969	0,014	11.11 ± 0.02	11.03 ± 0.01	4.62×10^{-3}
CST3	P01034	0,013	6.66 ± 0.03	6.58 ± 0.02	4.19×10^{-2}
SPP1	P10451	0,013	4.66 ± 0.06	4.49 ± 0.04	2.41×10^{-2}
IL18BP	O95998	0,013	5.76 ± 0.03	5.65 ± 0.02	4.15×10^{-3}
IGFBP7	Q16270	0,012	6.83 ± 0.03	6.69 ± 0.02	1.02×10^{-4}
PRSS8	Q16651	0,011	8.73 ± 0.03	8.54 ± 0.02	2.98×10^{-6}
hOSCAR	Q8IYS5	0,011	9.71 ± 0.03	9.63 ± 0.01	1.04×10^{-2}
PGLYRP1	O75594	0,011	7.00 ± 0.06	6.85 ± 0.03	3.50×10^{-2}
IL1R2	P27930	0,011	5.87 ± 0.02	5.77 ± 0.01	1.34×10^{-3}
CDCP1	Q9H5V8	0,011	3.26 ± 0.05	3.01 ± 0.03	8.56×10^{-5}
OPG	O00300	0,011	3.60 ± 0.03	3.46 ± 0.01	5.27×10^{-5}
PLAU	P00749	0,011	9.09 ± 0.03	9.01 ± 0.02	4.26×10^{-2}
IL6	P05231	0,011	2.86 ± 0.06	2.54 ± 0.03	1.40×10^{-5}
SCF	P21583	0,011	9.89 ± 0.04	10.01 ± 0.02	2.21×10^{-2}
CD5	P06127	0,011	4.25 ± 0.03	4.16 ± 0.02	2.01×10^{-2}
CXCL16	Q9H2A7	0,010	4.63 ± 0.02	4.56 ± 0.01	1.92×10^{-2}
TNFSF13B	Q9Y275	0,010	7.01 ± 0.03	6.85 ± 0.01	7.35×10^{-6}

(*continued on next page*)

Table 4 (*continued*)

3+ plaques versus controls					
Protein	Uniprot ID	Importance factor	Mean NPX (±S.E.) in subjects with 3+ plaques	Mean NPX (±S.E.) in controls	P value
CPA1	P15085	0,010	4.56 ± 0.06	4.41 ± 0.03	2.33×10^{-2}
SHPS1	P78324	0,010	2.98 ± 0.03	2.88 ± 0.02	1.01×10^{-2}
PCOLCE	Q15113	0,009	5.32 ± 0.04	5.22 ± 0.02	2.34×10^{-2}
FAP	Q12884	0,009	1.17 ± 0.02	1.23 ± 0.01	4.10×10^{-2}
LDLr	P01130	0,009	4.48 ± 0.05	4.31 ± 0.03	2.86×10^{-3}
ANGPTL3	Q9Y5C1	0,008	2.82 ± 0.04	2.71 ± 0.02	3.24×10^{-2}
PGF	P49763	0,008	7.17 ± 0.02	7.06 ± 0.01	7.76×10^{-4}
FABP6	P51161	0,008	1.50 ± 0.05	1.64 ± 0.03	5.10×10^{-2}
PLAUR	Q03405	0,008	4.51 ± 0.04	4.36 ± 0.02	5.65×10^{-4}
CCL3	P10147	0,008	5.70 ± 0.07	5.45 ± 0.04	4.10×10^{-3}
THBS2	P35442	0,007	5.16 ± 0.02	5.11 ± 0.01	4.43×10^{-2}
CCL17	Q92583	0,007	8.36 ± 0.08	8.12 ± 0.05	1.70×10^{-2}
IL2RA	P01589	0,007	3.83 ± 0.04	3.72 ± 0.02	1.58×10^{-2}
CTSD	P07339	0,007	4.36 ± 0.03	4.21 ± 0.02	5.72×10^{-4}
LGALS4	P56470	0,007	2.85 ± 0.04	2.69 ± 0.02	4.06×10^{-4}
PON3	Q15166	0,007	4.82 ± 0.07	5.00 ± 0.04	4.35×10^{-2}
TNF-R1	P19438	0,007	5.83 ± 0.03	5.74 ± 0.01	5.06×10^{-3}
TGF-alpha	P01135	0,007	3.91 ± 0.07	3.74 ± 0.03	2.22×10^{-2}
CCL13	Q99616	0,007	14.09 ± 0.06	13.89 ± 0.03	8.90×10^{-3}
CSTB	P04080	0,006	4.91 ± 0.05	4.65 ± 0.03	5.12×10^{-6}
RETN	Q9HD89	0,006	6.38 ± 0.04	6.23 ± 0.02	3.66×10^{-3}
CCL4	P13236	0,006	5.96 ± 0.06	5.77 ± 0.04	1.77×10^{-2}
ICAM1	P05362	0,005	5.72 ± 0.03	5.61 ± 0.02	7.04×10^{-3}
FCGR2A	P12318	0,005	1.93 ± 0.05	1.73 ± 0.04	1.20×10^{-2}
REN	P00797	0,005	7.16 ± 0.07	6.97 ± 0.04	2.13×10^{-2}
IL16	Q14005	0,005	5.49 ± 0.06	5.27 ± 0.04	3.54×10^{-3}
SELPLG	Q14242	0,005	4.08 ± 0.03	4.01 ± 0.01	1.46×10^{-2}
FABP2	P12104	0,005	7.67 ± 0.07	7.43 ± 0.03	2.02×10^{-2}
LGALS3	P17931	0,005	5.66 ± 0.03	5.53 ± 0.02	3.98×10^{-4}
CHI3L1	P36222	0,005	6.60 ± 0.07	6.24 ± 0.04	3.43×10^{-5}
TNFRSF10A	O00220	0,004	2.43 ± 0.03	2.32 ± 0.01	5.47×10^{-4}
CA5A	P35218	0,004	2.36 ± 0.06	2.20 ± 0.04	3.91×10^{-2}
VEGFD	O43915	0,004	6.40 ± 0.04	6.29 ± 0.02	1.69×10^{-2}
TFPI	P10646	0,004	9.26 ± 0.03	9.15 ± 0.01	1.92×10^{-3}
CCL7	P80098	0,004	1.84 ± 0.03	1.70 ± 0.02	8.94×10^{-3}
CEACAM8	P31997	0,003	3.26 ± 0.07	3.05 ± 0.03	5.87×10^{-3}
MMP-9	P14780	0,003	4.86 ± 0.07	4.62 ± 0.04	5.18×10^{-3}
SERPINE1	P05121	0,002	5.90 ± 0.07	5.68 ± 0.04	1.33×10^{-2}
IL18	Q14116	0,002	7.89 ± 0.08	7.55 ± 0.04	5.41×10^{-5}
IL7R	P16871	0,001	1.23 ± 0.04	1.36 ± 0.02	3.41×10^{-3}
TRAIL-R2	O14763	0,001	4.62 ± 0.03	4.47 ± 0.02	3.50×10^{-3}

The table reports the proteins identified as top contributors to identify subjects with 3+ plaques vs controls by the gradient boosting model of artificial intelligence. The Uniprot-ID of each protein is reported, together with the importance factor derived from the model (see methods in the main manuscript for more information) and the mean NPX values (with the relative standard error (S.E.)) in the group of subjects with 3+ plaques and in the controls. The *p* value (in linear scale and scientific representation) of the difference between the mean NPX values is also reported.

individuals (AUC = 0.559 (0.521–0.598)).

The 19 hit predictors of 1+ plaque improved the AUC of the ROC of risk factors considered alone, only if they included in the model together with the risk factors, (AUC = 0.623 (0.585–0.660), $p = 0.023$), but not when considered separately from the ASCVD risk factors (AUC = 0.619 (0.581–0.656), $p = 0.062$; Fig. 3A). By contrast, the contribution of the

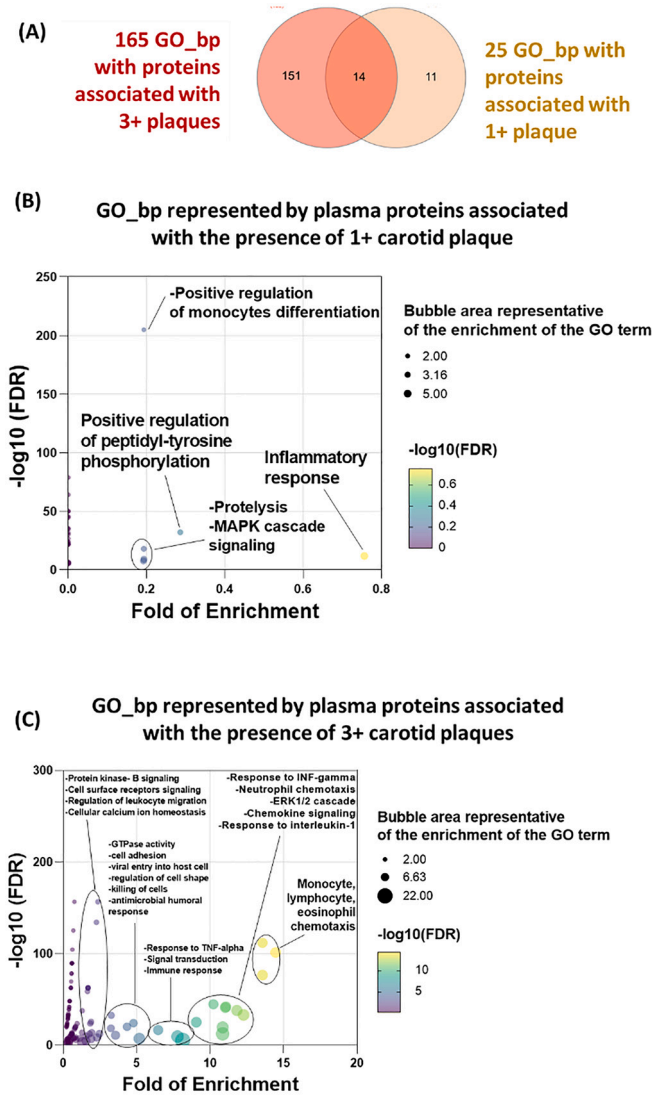

Fig. 2. Plasma proteins associated with carotid plaques recapitulate biological pathways involved in atherosclerotic disease.
(A) Venn diagram reporting the number of shared GO_bp among the ones enriched with the proteins predicting the presence of 1+ or 3+ plaques. Bubble plots showing (B) the 47 biological processes ("GO_bp") enriched in the plasma of subjects with 1 or with 2 carotid plaques and (C) the 38 biological processes enriched in the plasma of subjects with three or more plaques only (3+). The pathways are listed by descending order of folds of enrichment of the signal from proteome analysis (see methods).

87 proteins predicting the presence of 3+ plaques was more robust (AUC of the model of the proteins on top of risk factors to predict the incident event was equal to 0.739 (0.704–0.773) versus the AUC of risk factors only $p < 0.001$; Fig. 3B). Moreover, their performance when considered alone was superior to the one of the risk factors only (AUC = 0.731 (0.696–0.765); $p < 0.001$), and comparable with the AUC of the model combining them with the risk factors ($p = 0.398$).

Finally, when the subjects were not stratified by the presence of carotid plaques, the plasma levels of only 12 proteins were different in subjects who developed an event (1 with reduced NPX and 11 with increased NPX versus the ones of subjects who did not develop an event; Supplementary Fig. S4) and their performance to predict the risk of developing an incident event was not significant (the AUC achieved by the model these proteins was 0.507 (0.435–0.579), $p = 0.870$), substantiating the importance of the proteins associated with the presence of diffuse carotid atherosclerosis as independent predictors for increased ASCVD risk in apparently healthy individuals.

4. Discussion

In this study we searched for proteins, out of a set of 368 measured in plasma by PEA technology, whose plasma levels, recapitulating biologically relevant pathways for atherosclerosis, predict the presence of carotid plaques and the occurrence of clinically overt ASCVD in apparently healthy subjects.

We detected proteins whose plasma levels significantly discriminates subjects who, although being apparently healthy according to the analysis of the classical ASCVD risk factors, present with carotid atherosclerosis versus subjects who do not display atherosclerosis and that, in our study, were considered as "controls". Moreover, our data indicate the quantification of the number of plaques can unmask a more significant degree of association between plasma proteins and carotid atherosclerosis. Indeed, the number of proteins associated with carotid atherosclerosis (either reduced or increased) was more abundant as a function of the number of carotid plaques detectable by ultrasound, and we also provide a specific set of 87 proteins ($N = 5$ with reduced expression while $N = 82$ with increased plasma levels versus controls) that were more predictive of diffuse atherosclerosis (3+ plaques). By contrast, plasma proteomics was not able to significantly discriminate neither the subjects presenting with 2 or with just one carotid plaque. The finding that proteomics did not discriminate the subjects with lower number of plaques could perhaps be due to the smaller number of subjects in these groups. Anyhow, we cannot rule out the effect of the clinical conditions that mostly contribute to hemorheological turbulence and endothelial damage, such as hypertension and smoking habit, increased waist circumference, dyslipidemia and reduced physical activity, which were prevalent in subjects with 3+ plaques but not in the subjects with 2 or with 1 plaque only versus controls and which were also associated with higher NPX values for the majority of proteins analyzed (Supplementary Fig. S5 A-E).

Of note, the added power of plasma proteomics on top of that of classical ASCVD risk, was significantly superior to detect subjects with at least one carotid plaque, but it was markedly higher to detect the presence of 3+ plaques. This significant improvement held true even when we replicated the analysis on subsets of subjects, matched for prevalence of hypertension, active smoking habit, dyslipidemia, frequency of physical activity and similar waist circumference (AUC = 0.840 (0.771–0.895) for the model with proteins and ASCVD risk factors vs AUC = 0.684 (0.602–0.757) with ASCVD risk factors alone, $p < 0.001$; data not shown). This observation further supports that plasma proteomics can improve our capability to classify, with higher accuracy, individuals among the apparently healthy population toward higher degrees of ASCVD risk.

We also reported comparable levels of CRP, a commonly considered marker of low-grade inflammation in clinics, in subjects with 3+ plaques versus controls. We do not rule out the relevance of CRP, which is generally elevated in subjects at higher ASCVD risk and co-morbid patients, but we are more likely to support the possibility that pre-clinical activation of inflammatory pathways in "apparently healthy" individuals could be captured more significantly by alternative sets of proteins in plasma.

Furthermore, by combining a high-sensitive gradient boosting A.I. strategy together with the analysis of pathways, we also provided a biological association between the number of carotid plaques and a larger number of proteins compared to the ones that we associated, in a former analysis, with the development of at least one carotid over time [17]. For instance, Growth Differentiation Factor 15 (GDF-15), whose NPX was increased in subjects with 3+ plaques versus controls, ranged first out of the 87 proteins in the random forest classifier (Table 4), confirming independent epidemiological studies that previously associated its increased plasma levels to ASCVD risk [24], to carotid atherosclerosis, increased carotid IMT [9,14,15] and high-risk coronary

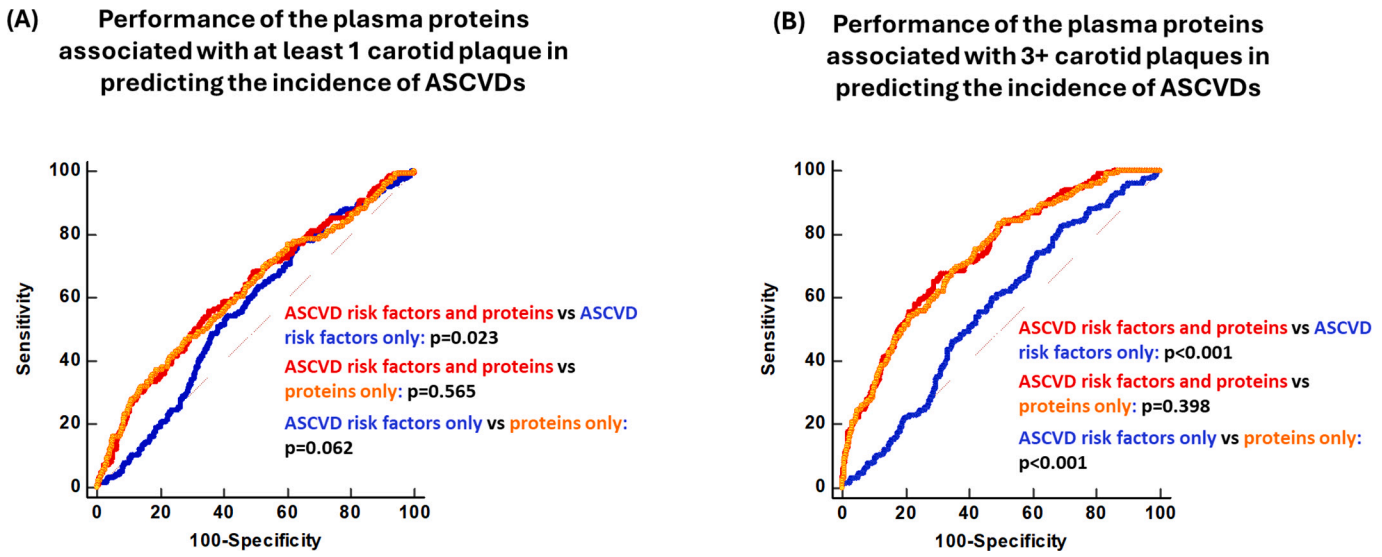

Fig. 3. Performance of the plasma proteins associated with carotid plaques to predict the incidence of clinically overt ASCVDs.
Comparison of the accuracy (Receiver Operating Curve "ROC") of the model to identify: (A) subjects with incident ASCVD, when considering the ASCVD risk factors and the 19 proteins associated with at least 1 carotid plaque ("1+") (red curve), versus the accuracy of the model that included the ASCVD risk factors only (blue curve) or of the model that included the proteins only (yellow curve); (B) subjects with incident ASCVD, when considering the ASCVD risk factors and the 87 proteins associated with three or more carotid plaque ("3+") (red curve), versus the accuracy of the model that included the ASCVD risk factors only (blue curve). P values are derived from Hanley-McNeil test.

plaques defined by computed tomography angiography [25]. However, our additional GO analysis found that alternative set of proteins, including the spectrum of β-chemokines and other interleukins/cytokines, but not GDF-15, significantly clustered into biological pathways enriched both in subjects with at least 1 carotid plaque (Supplemental Table 4) and in subjects with 3+ plaques (Supplemental Table 5). These predictions are in line with the findings of previous independent studies, where higher plasma levels of few chemokines (CXCL1) and markers of T lymphocytes activation (CD40L/CD40R, PDL-2) associated with both a larger area of carotid plaques, estimated by ultrasound in one study that quantified 19 proteins in total [13], and with thicker carotid intima-media layer in another study, where 92 proteins were quantified in the plasma of subjects at higher ASCVD risk [16]. From a translational point of view, these findings outline further mechanisms, involving immune cells chemotaxis in atherosclerotic lesions [26] and lymphocytes activation in the atheroma [27], taking place yet during the subclinical stages of atherosclerosis.

Finally, the robustness of our predictions is also supported by our follow-up analysis. Indeed, both the 19 proteins predicting the presence of at least 1 and the 87 proteins predicting the presence of 3+ plaques also independently predicted the occurring clinically overt ASCVDs over a large twenty-years follow-up. Our findings reinforce a recent study that identified 11 proteins, out of 92 analyzed in total, whose increased plasma levels outperformed the accuracy of ASCVD risk factors in predicting the risk of occurring ASCVDs over a decennial follow-up [16]. Out of those 11 proteins, CCL17 and CEACAM8 only were also confirmed in our set of proteins that predicted the presence of 3+ plaques and the risk of events. The far higher set of proteins that we measured, and the twenty years follow-up supports the future possibility to integrate the clinical algorithms, which often underestimate the risk in apparently healthy subjects due to their limited ten-year perspective, with proteomic fingerprints to build-up a personalized risk prediction. Anyhow, we acknowledge that more studies are needed to simplify the observations from such types of proteomics studies and to propose cost-effective dedicated panels to be used in clinics in the future.

Our study has some limitations, and some technical aspects of our work should be discussed.

Firstly, we did not have information regarding the presence of atherosclerosis coronary arteries or peripheral vessels, which have been found to be also prevalent among apparently healthy individuals [2,3]. Whether the plasma proteomics signatures that we associated with the number of carotid plaques also inform on the presence of atherosclerosis in other vascular territories is an intriguing aspect to be considered in the future given the correlation between the presence of plaques in the carotid arteries and in the coronary bed [28–30].

Second, our A.I. model, although previously demonstrated to provide superior accuracy compared to common statistical tools [19], was trained and applied in internal sets of our population and, consequently, our findings need to be validated in independent cohorts.

Third, the methodological assessment of our proteomics analysis, although providing an optimal sensitivity to target low proteins abundancy in plasma, otherwise not reachable by common techniques of molecular biology [7,8], relies on PCR-based amplification reactions with antibodies-linked oligonucleotides. Thus, a quantitative validation of our results is needed and will add further strength; However, for some of the proteins we have identified, a validation by immunoassays has been provided [31]. All in all, we have provided information from one of the largest panels so far reported by studies using this strategy [11,13–16].

In conclusion, we identified a set of plasma proteins that, informing on pathways that are biologically relevant for the development of atherosclerosis, can identify apparently healthy subjects with subclinical cardiovascular damage and at higher risk of occurring clinically overt ASCVDs.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vph.2024.107394.

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CRediT authorship contribution statement

Andrea Baragetti: Writing – review & editing, Writing – original draft, Visualization, Resources, Funding acquisition, Formal analysis, Conceptualization. **Liliana Grigore:** Writing – review & editing, Investigation. **Elena Olmastroni:** Writing – review & editing, Investigation, Formal analysis. **Elisa Mattavelli:** Visualization, Investigation. **Alberico Luigi Catapano:** Writing – review & editing, Resources, Funding acquisition.

Declaration of competing interest

The authors declare no conflicts of Interest relevant to the submitted work.

Data availability

Data will be made available on request.

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