Computational and Structural Biotechnology Journal 23 (2024) 1680–1688

Contents lists available at ScienceDirect

Computational and Structural Biotechnology Journal

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

Research Article

Angiopoietin-2, vascular endothelial growth factor family, and heparin binding endothelial growth factor are associated with subclinical atherosclerosis in rheumatoid arthritis

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ARTICLE INFO

Keywords:
Rheumatoid arthritis
Atherosclerosis
Inflammation
Biomarker
Growth factors
Plaque presence

ABSTRACT

Introduction: Patients with RA are at a higher risk of developing CV diseases than the general population. The precise mechanisms are still unknown. We evaluated the associations between 8 plasma growth factors (GFs) (angiopoietin-2, EGF, HB-EGF, PLGF, TGF-α, VEGFa, VEGFc, and VEGFd) and subclinical arteriosclerosis in RA patients.

Materials and methods: A total of 199 patients with RA treated at the Hospital Universitari Sant Joan de Reus (Spain) between 2011 and 2015 were included in this cross-sectional study. Carotid intima media thickness (cIMT), carotid plaque presence (cPP) and pulse wave velocity (PWV) were measured. GFs were measured with Bio-Plex Pro Human Cancer Biomarker Panel 2 (Bio-Rad). Multivariate models and partial least square discriminant analysis (PLS-DA) were used for analysis (RStudio, version 4.0.1).

Results: Multivariate models showed that angiopoietin-2 was associated with cPP and PWV in the overall cohort (OR = 1.53 and β = 0.20, respectively). VEGFc (β = 0.29), VEGFa (β = 0.26) and HB-EGF (β = 0.22) were also associated with PWV. VEGFa (OR = 2.36), VEGFd (OR = 2.29), EGF (OR = 2.62), PLGF (OR = 2.54), and HB-EGF (OR = 2.24) were associated with cPP in men. According to PLS-DA, GFs were able to distinguish between patients with and without cPP in the overall cohort, male cohort, and female cohort. In women, angiopoietin-2 was associated with PWV (β = 0.18).

Conclusions: The selected GFs were closely related to atherosclerosis in patients with RA and are potential predictors of CV disease in patients with RA.

1. Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease affecting synovial joints. The prevalence of RA is 0.5–1 % in the general population, and it is more common in women. Its complex pathogenesis involves innate and adaptive immunity and angiogenesis [1]. Without proper treatment, RA can lead to joint destruction, reduced physical function, and decreased quality of life [2,3]. Cardiovascular disease (CV) is the primary cause of illness and death in patients with RA [4], as RA patients have a 50 % increased risk of CV disease compared with that of the general population [4–6]. While traditional CV risk factors including age, sex, smoking, diabetes mellitus, and hypertension play a role, systemic inflammation also contributes to an increased risk of CV disease in RA patients [7]. The cause of this increased risk is not entirely understood, but similarities between the inflammatory process occurring in synovial membranes and that of atherosclerotic plaques, along with elevated proinflammatory cytokines, leading to endothelial dysfunction, are thought to be involved [8,9].

A carotid ultrasound study to evaluate carotid intima media thickness (cIMT) is a surrogate indicator of arteriosclerosis and can predict

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https://doi.org/10.1016/j.csbj.2024.04.042
Received 21 December 2023; Received in revised form 16 April 2024; Accepted 16 April 2024
Available online 16 April 2024

CV events in the general population [10,11] and in RA patients [12]. In patients with RA, a cIMT > 0.91 mm has been associated with an increased risk of CV events [13]. Moreover, carotid plaque presence (cPP) predicts CV events in the general population [14]. The rupture of atherosclerotic plaques is one of the most important mechanisms causing acute coronary syndrome, and plaque rupture depends mainly on its composition [15]. The link between carotid plaques in individuals with RA and an increased risk of CV events and mortality at 5 years of follow-up has been established [16]. Finally, arterial stiffness, particularly pulse wave velocity (PWV), has been shown to be predictors of CV events in both the general population [17] and RA patients [18]. PWV measures the speed at which arterial pressure waves travel through the aorta and major arteries and becomes faster in stiffened arteries [19].

Over the years, several plasmatic growth factors (GFs) have been linked to atherosclerosis and investigated as potential targets for immunotherapy in CV disease treatment [20]. Moreover, GFs are highly involved in inflammation, endothelial dysfunction, and angiogenesis, all of which are cellular processes implicated in the development of RA and CV disease. Angiogenesis is also essential for sustaining the inflammatory synovium in individuals with RA and contributes to the development of atherosclerotic plaques [21,22]. Angiopoietin-2 (Ang-2) is involved in endothelial remodelling and has been associated with CV events in individuals with established CV disease [23,24]. There is evidence suggesting that Ang-2 is a predictor of CV mortality in the general population [25,26]. In patients with RA, higher levels of Ang-2 have been observed in those with CV complications [27,28], and Ang-2 levels were correlated with disease activity, acute phase reactants and cIMT and echocardiography abnormalities [29]. Within the vascular endothelial growth factor (VEGF) family, VEGFa and placental growth factor (PLGF) regulate angiogenesis, and VEGFc and VEGFd control lymphangiogenesis. These proteins influence endothelial cells, affecting vascular permeability, lipid metabolism, and inflammation. In the general population, VEGF family proteins play a role in arteriosclerosis development [30,31], and in RA patients, VEGF is an important index of RA activity and a prognostic factor for joint destruction [32]. The epidermal growth factor (EGF) family is involved in vascular remodelling, and together with heparin-binding epidermal growth factor-like (HB-EGF) and transforming growth factor-α (TGF-α), it has been associated with CV disease through the stimulation of cell proliferation, differentiation, and survival [33,34].

However, there are limited investigations on these GFs and their associations with CV disease in RA patients. Early detection of CV disease in RA patients is essential to avoid its consequences and to implement possible therapeutic strategies to reduce future CV risk. Therefore, the discovery of new biomarkers that can facilitate the early detection of CV disease in patients with RA is highly important.

In our current investigation, we sought to evaluate the relationships between 8 plasma GFs (Ang-2, EGF, HB-EGF, PLGF, TGF-α, VEGFa, VEGFc, and VEGFd) and surrogate markers of subclinical arteriosclerosis in a cohort of RA patients. Additionally, due to the strong relationship between inflammation and the atherosclerotic process in RA, we also studied the relationships between the abovementioned GFs and inflammatory markers of the disease (DAS28-ESR, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) and fibrinogen). Moreover, analyses are reported globally and stratified by sex to assess potential physiological differences between men and women [35].

2. Materials and methods

2.1. Patients

The RA population included in this cross-sectional study has been previously described in other publications [36,37]. Patients who were consecutively treated at the University Hospital Sant Joan de Reus in Spain between 2011 and 2015 through external consultations, and were aged between 18 and 80 years without concurrent illnesses, had a diagnosis of RA based on history, clinical examination, laboratory results, and imaging, and met the classification criteria outlined by the American College of Rheumatology in 1987, were included in this study. Individuals who were either older than 80 years or younger than 18 years, and those with acute intercurrent illnesses were excluded from the study. Additionally, participants whose disease diagnosis had changed were also excluded. The determination of the sample size was based on standard calculations using G*Power software [38]. We enroled a total of 199 patients and carried out blood collection and carotid ultrasonography on the same day of their medical visit without altering their usual clinical therapy. The determination of patients' sex was defined based on external examination of body characteristics. Information on the presence of classic CV risk factors (smoking, hypertension, diabetes, and dyslipidaemia), as well as the history of CV events and drug consumption, was collected. Clinical measurements, which comprise of body mass index (BMI), waist circumference (WC), and systolic and diastolic blood pressure (SBP, DBP), were evaluated. The disease activity score (DAS28) was derived from the ESR, while pain was measured using a visual analogue scale ranging from 0 to 10. Patients reported any disability using the health assessment questionnaire (HAQ) index. The DAS28 variable was classified into four categories: remission (DAS28 < 2.6), low activity (2.6 ≤ DAS28 < 3.2), moderate activity (3.2 ≤ DAS28 ≤ 5), or high activity (DAS28 > 5). Rheumatoid factor (RF) positivity (RF+) was defined as RF values greater than 20, and anti-citrullinated protein antibody (ACPA) positivity (ACPA+) was defined as ACPA values greater than 3.

The study was approved by the Clinical Research Ethics Committee of our hospital (11–04-28/4proj5), and written informed consent was obtained for all the participants. Adhering to our institution's guidelines and the Helsinki Declaration, we conducted the investigation. Patients did not contribute to the development of the study.

2.2. Laboratory measurements

Blood samples were collected from 199 fasting patients who had abstained from food for at least 12 h. The plasma was separated from the blood by centrifugation at 3000 rpm for 10 min, and the resulting plasma samples were stored at −80 °C for analysis. The plasma samples were analyzed using both enzymatic and conventional methods.

2.3. Ultrasound evaluation of carotid intima–media thickness and arterial stiffness

cIMT measures the thickness of the innermost two layers of the carotid artery wall, with higher cIMT indicating an increased presence of atherosclerosis. To measure cIMT, we used a My Lab 60 X-Vision sonographer (Esaote SpA, Genova, Italy) with a linear array ultrasound probe small parts broadband transducer (5–12 MHz). We identified and digitally recorded the far wall of the common carotid artery (1 cm proximal to the bifurcation), the bifurcation, and the internal carotid artery (1 cm distal to the bifurcation) of the left and right carotid arteries. In vivo measurements of cIMT were performed at common carotid arteries using QIMT© radiofrequency image processing software (Esaote SpA, Genova, Italy). To reduce observer variability, a single operator obtained and measured the images. We averaged the measurements of the left and right common carotid arteries to obtain the mean cIMT. Following the Mannheim consensus, we defined cPP as a focal structure encroaching into the arterial lumen by a minimum of 0.5 mm, or 50 % of the surrounding IMT value, or displaying a thickness greater than 1.5 mm measured from the intima–lumen interface to the media–adventitia interface. The presence and number of carotid plaques are clinically relevant, as they serve as significant predictors of CV events, thereby helping in the risk stratification of CV disease [39].

Arterial stiffness can be accurately assessed by measurements of the PWV. PWV was measured directly at both common carotid arteries using an ultrasound linear probe (5–12 MHz) as a tonometer and analysed in

vivo with Quality Arterial Stiffness (QAS©) radiofrequency software (Esaote SpA, Genova, Italy). Signal-based vascular ultrasound from Esaote employs signal-based technology and includes QAS measurements. The signal is a reflected ultrasound signal that is captured by the transducer and converted into an electric signal preserving all the characteristics of the acoustic wave in terms of amplitude and phase. Local arterial stiffness is estimated as systo-diastolic changes in arterial diameter/area over systo-diastolic changes in distending pressure (pulse pressure). Maximum and minimum carotid diameters were acquired using the attained distension curves, and vascular stiffness parameters were calculated after calibration for blood pressure. The final values were the median measurements of the right and left common carotid arteries. The examination was performed according to standardised measurements. PWV measures the speed at which the arterial pressure wave travels through the arteries and is obtained from brachial blood pressure and accurate measurements of the diameter and change in diameter of carotid arteries. Higher PWV values indicate greater arterial stiffness [40].

2.4. Growth factor quantification

The levels of the different GFs (Ang-2, HG-EGF, EGF, PLGF, TGFa, VEGFa, VEGFc and VEGFd) in blood plasma were measured using the Bio-Plex Pro Human Cancer Biomarker Panel 2 Standards (Bio-Rad, USA).

2.5. Statistical analysis

For the purpose of descriptive analysis, the means and standard deviations (SDs) are used for continuous normally distributed variables, while the medians and interquartile ranges (IQRs) are used for continuous non-normally distributed variables. Categorical variables are presented as the percentage and number of individuals. T tests and Mann—Whitney U tests were used to evaluate differences between normally and nonnormally distributed variables, respectively. Chi-squared tests were used to evaluate differences between categorical variables. Statistical models and machine learning algorithms were applied to the overall cohort and stratified by sex. To evaluate the associations between GF and continuous dependent variables (cIMT, PWV and distensibility), multivariate linear models were adjusted. Multivariate logistic models were used to estimate the individual associations of each GF with cPP. The areas under the curve (AUCs) were calculated and plotted on receiver operating characteristic (ROC) curves. The AUCs quantify the overall performance of the classification power of a model, and the ROC curves are graphical representations of binary classification accuracy. To assess whether the effect of the GFs on cPP and PWV was independent of disease activity level, we conducted interaction analyses between each GF and DAS28 variable, adjusting for various confounders. Partial least square discriminant analysis (PLS-DA), which is a machine learning supervised algorithm, was used to discriminate between patients with or without cPP using all GFs simultaneously. PLS-DA relates the X matrix (independent variables) and the Y matrix (dependent variable) to find the maximum discrimination between the two groups. Through PLS-DA, we generated latent variables (LVs), which are linear combinations of the included variables aimed at maximising covariance between predictor variables (X) and the response variable (Y). Each LV provides insight into the contribution of individual variables towards classifying the response variable. We generated two LVs for each model to facilitate graphical representation. PLS-DA models were cross-validated using the 5-fold cross-validation method. Models were adjusted for traditional and previously known confounders [37], including age, sex, BMI, disease duration, DAS28 score and treatment. Treatments included disease-modifying antirheumatic drugs (DMARDs), nonsteroidal anti-inflammatory drugs (NSAIDs), corticosteroids and biologic therapies. To evaluate the performance of the models as well as the benefits of including the selected GFs, R-squared (R^2), ΔR^2 and the Akaike information criterion (AIC) were used for each model as goodness-of-fit parameters. R^2 is a measure of the variability explained by the model, indicating the proportion of variance captured by the independent variables. ΔR^2 calculates the difference in variability explained by models with and without a GF, allowing us to assess the additional contribution of that GF to the explanatory power of the model. AIC is a nested-model goodness of fit measure that provides a way to balance the model with its complexity, aiming to select the model that best describes the data while avoiding overfitting. A lower AIC is a better model indicator. Finally, we computed a global concentration score considering the plasma concentrations of all the GFs studied. To calculate this score, the levels of each GF were divided into tertiles, and we assigned a score of 3, 2 or 1 to the highest, middle and lowest expression tertiles, respectively. The scores of the different GFs were summed to obtain the global score for each patient. This global score was analysed as tertiles, with the third tertile having the highest score and the first having the lowest score. Statistical analyses were performed in R Studio, version 4.0.1. P values < 0.05 were considered to indicate statistical significance.

3. Results

3.1. Characteristics of patients with RA

Table 1 illustrates the general traits of the Spanish RA cohort that were part of the study (n = 199), both for the entire cohort and divided by sex. The mean age of the overall cohort was 57.8 (12.4) years, the mean disease duration was 8 years (3–13), and 66 % of the patients were female. A total of 25.12 % of the patients were in remission, 18.59 % presented low disease activity, 45.72 % presented moderate disease activity, and 10.55 % presented high disease activity. Seventy-five percent of the patients were administered conventional synthetic disease-modifying antirheumatic drugs (csDMARDs), 21.6% received biological treatment, 57.28 % were given nonsteroidal anti-inflammatory drugs (NSAIDs), 51 % were prescribed corticosteroids, 24.62 % received renin-angiotensin-aldosterone system (RAAS) inhibitors, 26.13 % received other antihypertensive treatments, 16.08 % received statins, and 0.15 % received other lipid-lowering drugs. No differences were observed between men and women regarding the concentrations of the studied GFs. The waist circumference, systolic blood pressure (SBP), diastolic blood pressure (DBP) and cIMT were increased in men, and the HDL-C level was decreased. The prevalences of hypertension, cPP and previous CV disease were greater in men. Finally, the DAS28 and HAQ scores were greater in women.

3.2. Associations of growth factors with carotid plaque presence (cPP) and cIMT

Multivariate logistic regression models adjusted for known confounders, including age, sex, DAS28, disease duration, and treatment (Table 2 legend), revealed that elevated levels of Ang-2 significantly increased the OR of cPP by 1.53-fold in the overall population (OR = 1.53, p = 0.02, ΔR^2 = 2 %). No other GFs showed significant associations with cPP in the overall cohort (Table 2).

Stratified analyses indicated that, in male patients, elevated levels of VEGFa increased the OR of presenting carotid plaque by 2.36 units (p = 0.04, ΔR^2 = 6 %). Similarly, in male patients, elevated levels of VEGFd (p = 0.04, ΔR^2 = 5 %), EGF (p = 0.02, ΔR^2 = 7 %), PLGF (p = 0.03, ΔR^2 = 6 %), and HB-EGF (p = 0.04, ΔR^2 = 5 %) were associated with increased odds of cPP, with ORs of 2.29, 2.62, 2.54, and 2.24, respectively. Furthermore, male patients with the highest global concentration score had 14.58 times greater odds of having cPP than male patients with the lowest global concentration score (OR = 14.58, p = 0.01, ΔR^2 = 10.37 %). No associations were found between GF and cPP in women (Table 2). According to the interaction analysis of cPP, no significant differences were found between the different GFs and DAS28

Table 1
Description of the general characteristics, the disease features and the treatments of the overall RA cohort and stratified by sex.

General characteristics of the cohort				
	RA (n = 199)	Female (n = 132)	Male (n = 67)	p
Characteristics of the groups				
Sex – female (%, n)	66 %, 132			
Age (years, SD)	57.8 (12.4)	57.3 (12.53)	58.65 (12.17)	0.47
Body mass index (kg/m3, IQR)	26.80 (23.24 – 31.19)	26.47 (22.70 – 31.63)	27.85 (25.68 – 30.67)	0.13
Waist circumference (cm, SD)	91.88 (15.11)	88.04 (15.11)	99.45 (12.17)	< 0.001
SBP (mmHg, IQR)	135 (120 – 150)	133.5 (120 – 148.5)	139 (128 – 156.5)	0.04
DBP (mmHg, IQR)	80 (71.50 – 89)	80 (70.75 – 88)	85 (75 – 90)	0.02
LDL cholesterol (mg/dL, IQR)	115 (99 – 135)	115 (97 – 135.5)	118 (100 – 134.5)	0.64
HDL cholesterol (mg/dL, IQR)	66 (53.50 – 75)	69 (61 – 80.25)	54 (43 – 66)	< 0.001
Tryglycerides (mg/dL, IQR)	92 (69 – 127.5)	88.50 (65.75 – 125.25)	94 (75 – 131)	0.31
Glucose (mg/dL, IQR)	89 (82 – 99)	88 (81 – 97)	93 (84 – 102)	0.07
Current smoker (%, n)	27 %, 54	28 %, 37	25.37 %, 17	0.82
Hypertension (%, n)	59.29 %, 118	53.03 %, 70	71.64 %, 48	0.01
Diabetes mellitus (%, n)	11.55 %, 23	10.6 %, 14	13.43 %, 9	0.72
Dyslipidaemia (%, n)	40.70 %, 81	39.39 %, 52	43.28 %, 29	0.71
Disease features				
Disease duration (years, IQR)	8 (3–13)	8.5 (3 – 13.25)	6 (2 – 11.50)	0.33
DAS28 (median, IQR)	3.43 (2.6 – 4.26)	3.59 (2.77 – 4.62)	3 (2.41 – 3.70)	< 0.001
Remission (%, n)	25.12 %, 50	27	23	0.77
Low activity (%, n)	18.59 %, 37	19	18	1
Moderate activity (%, n)	45.72 %, 91	68	23	< 0.001
High activity (%, n)	10.55 %, 21	18	3	0.01
HAQ (median, IQR)	0.25 (0 – 0.75)	0.5 (0.125 – 0.875)	0 (0 – 0.25)	< 0.001
Rheumatoid factor + (%, n)	74.37 %, 148	72.72 %, 96	77.61 %, 52	0.57
ACPA + (%, n)	73.86 %, 147	74.24 %, 98	73.13 %, 49	1
ESR (mm/h, IQR)	31 (18.50 – 50.50)	31 (18.75 – 54)	29 (18.50 – 46.50)	0.30
CRP (mg/dL, IQR)	0.5 (0.2 – 0.9)	0.4 (0.2–0.9)	0.4 (0.2 – 0.95)	0.68
Fibrinogen (mg/dL, SD)	445.64 (96.53)	442.21 (95.50)	452.40 (98.91)	0.49
Treatments (%, n)				
RA treatment				
csDMARDs	74.87 %, 149	71.21 %, 94	82.09 %, 55	0.13
Biological agent	21.6 %, 43	24.24 %, 32	16.41 %, 11	0.27
NSAIDs	57.28 %, 114	58.33 %, 77	55.22 %, 37	0.79
Corticosteroids (mean oral dose: 2.91 mg/day)	51 %, 102	52.27 %, 69	49.25 %, 33	0.80
Hypertension treatment				
RAAS inhibitors	24.62 %, 49	24.24 %, 32	25.37 %, 17	0.98
Other hypotensive treatments	26.13 %, 52	22.72 %, 30	32.83 %, 22	0.17
Lipid lowering treatment				
Statins	16.08 %, 32	15.15 %, 20	17.91 %, 12	0.77
Other lipid lowering treatments	0.15 %, 3 %	0.15 %, 2	0.14 %, 1	1
GROWTH FACTORS				
Angiopoietin-2 (pg/ml)	1034.4 (633.6 – 1664.5)	1039.4 (641.4 – 1691.1)	1027 (600.3 – 1471.7)	0.42
HB EGF (pg/ml)	83.17 (56.16 – 132.78)	85.52 (54.28 – 137.35)	81.53 (61.95 – 124.80)	0.72
EGF (pg/ml)	62 (33.26 – 112.19)	63.69 (33.88 – 118.67)	60.20 (28.42 – 106.95)	0.45
PLGF (pg/ml)	130.76 (81.68 – 215.28)	127.90 (81.03 – 218.57)	138.78 (82.98 – 206.77)	0.99
TGFa (pg/ml)	105.31 (56.81 – 184.60)	102.84 (56.91 – 190.00)	110.25 (56.81 – 171.37)	0.69
VEGFa (pg/ml)	607.44 (402.63 – 968.56)	597.81 (414.74 – 1006.84)	636 (397.1 – 843.4)	0.67
VEGFc (pg/ml)	991.09 (589.48 – 1558.21)	1003.7 (622.6 – 1616.5)	961.04 (555.65–1334.99)	0.33
VEGFd (pg/ml)	1028.7 (730.9 – 1436.7)	1050.9 (759.3 – 1397.2)	998.6 (682.2 – 1488.2)	0.85
Ultrasound measurements and CV events				
cIMT (μm)	636 (571.8 – 709.8)	610.2 (565.5–694.2)	667.5 (608.5 – 744.5)	0.003
PWV (m/s)	7.92 (6.84 – 9.63)	7.93 (6.7 – 9.7)	7.94 (6.7 – 9.7)	0.73
Plaque presence (%, n)	43.71 %, 87	35.6 %, 47	59.7 %, 40	0.002
Previous CV events (%, n)	9.5 %, 19	3.7 %, 5	20.8 %, 14	< 0.001

n = number of individuals, SBP = systolic blood pressure, DBP=diastolic blood pressure, LDL = low density lipoprotein, HDL = high density lipoprotein, HAQ = health assessment questionnaire index, ACPA = citrullinated anti-cyclic peptide antibodies, ESR = erythrocyte sedimentation rate, CRP = C-reactive protein, DAS28 = disease activity score, DMARDs = disease-modifying antirheumatic drugs, NSAIDs = non-steroidal anti-inflammatory drugs, cIMT = carotid intima media thickness, PWV = pulse wave velocity, CV = cardiovascular, p = p value.

(Supplementary Table S1.1).

When model performance was evaluated, we observed that the inclusion of the different GFs in the models, both in the overall cohort and in male patients, increased the R^2 and AUC (Fig. 1) and decreased the AIC. This improvement enhanced the predictive power, the explanation of cPP variability, and the overall quality of the model. Notably, the association of the global concentration score with cPP in male patients resulted in the most significant increase in R^2 and AUC and the most substantial decrease in AIC (Table 2).

Additionally, we employed PLS-DA models to jointly evaluate the power of GFs in classifying the overall cohort and men and women with or without cPP. For the overall cohort, the optimal number of latent variables (LVs) chosen was 2, explaining 48 % of the cPP variability (LV1 = 11 %, LV2 = 37 %). The two-dimensional plot in Fig. 2A demonstrates a good tendency to classify patients with and without cPP, with a significant contribution from GFs in LV2, which explains most of the

Table 2
Summaries of the multivariate logistic regression models to estimate the associations between growth factors and cPP. Basal models are adjusted for age, sex, BMI, disease duration, csDMARDs, NSDAIDs, biological drugs, corticoids and DAS28.

	OR	p	R^2 (%)	AUC	AIC
Overall cohort					
Basal Model			24	0.81	226.95
Basal Model					
+ angiopoietin-2	1.53	0.02	26	0.82	223.63
Men patient					
Basal Model			33	0.85	78.61
Basal Model					
+ VEGFa	2.36	0.04	39	0.88	75.43
Basal Model					
+ VEGFd	2.29	0.04	38	0.87	75.88
Basal Model					
+ EGF	2.62	0.02	40	0.89	74.05
Basal Model					
+ PLGF	2.54	0.03	39	0.88	74.99
Basal Model					
+ HB-EGF	2.24	0.04	38	0.87	75.85
Basal Model					
+ score (T3)	14.58	0.01	43.28	0.90	73.24

OR = odds ratio, p = p-value, AUC = area under the curve, AIC = Akaike information criteria, cPP = carotid plaque presence.

total variability (Supplementary Fig. S1). Similarly, in male patients, the optimal number of LVs chosen was 2, explaining 54 % of the cPP variability (LV1 = 37 %, LV2 = 17 %). The two-dimensional representation in Fig. 2B shows good classification of men with and without cPP. GFs such as EGF, PLGF, and VEGFc play key roles in this classification (Supplementary Fig. S2). Finally, in women, the optimal number of LVs was also 2 (LV1 = 12 %, LV2 = 39 %), explaining 52 % of the cPP variability (Fig. 2C). The two-dimensional representation exhibited a good tendency to classify women with and without cPP, with significant contributions from several GFs in LV2 (Supplementary Fig. S3).

No significant associations were found between the GFs studied and cIMT either in the overall cohort or when stratified by sex.

3.3. Associations of growth factors with arterial stiffness

Multivariate linear regression analyses adjusted for confounders, including DAS28, disease duration, hypertension status, and treatment (Table 3 legend), revealed positive associations between Ang-2 (β = 0.44, p = 0.002) and PWV in the overall population.

After stratification of the population by sex, analyses revealed positive associations between the levels of Ang-2 (β = 0.57, p = 0.02), VEGFa (β = 0.50, p = 0.03), VEGFc (β = 0.59, p = 0.01), and HB-EGF (β = 0.45, p = 0.048) and the PWV in male patients and between the level of Ang-2 in female patients (β = 0.41, p = 0.03). The inclusion of these GFs in the models improved the overall model quality for the entire cohort, male patients, and female patients, increasing the explained variability (R^2) and decreasing the AIC (Table 3). The interaction of GFs and DAS28 was not significantly associated with PWV (Supplementary Table S1.2). No associations were found between GF and distensibility.

3.4. Associations of growth factors with inflammatory markers of the disease

VEGFd was significantly associated with ESR (β = 5.36, p = 0.003) and fibrinogen level (β = 19.03, p = 0.006) in the overall cohort, as observed in the multivariate linear regression analyses adjusted for age, sex, BMI, disease duration, and treatments.

Furthermore, sex-stratified analyses revealed that VEGFd was associated with DAS28 (β = 0.27, p = 0.02), ESR (β = 7.54, p = 0.002) and fibrinogen (β = 24.68, p = 0.004) in women. However, no significant associations were found in the male group of patients (Supplementary Table S2).

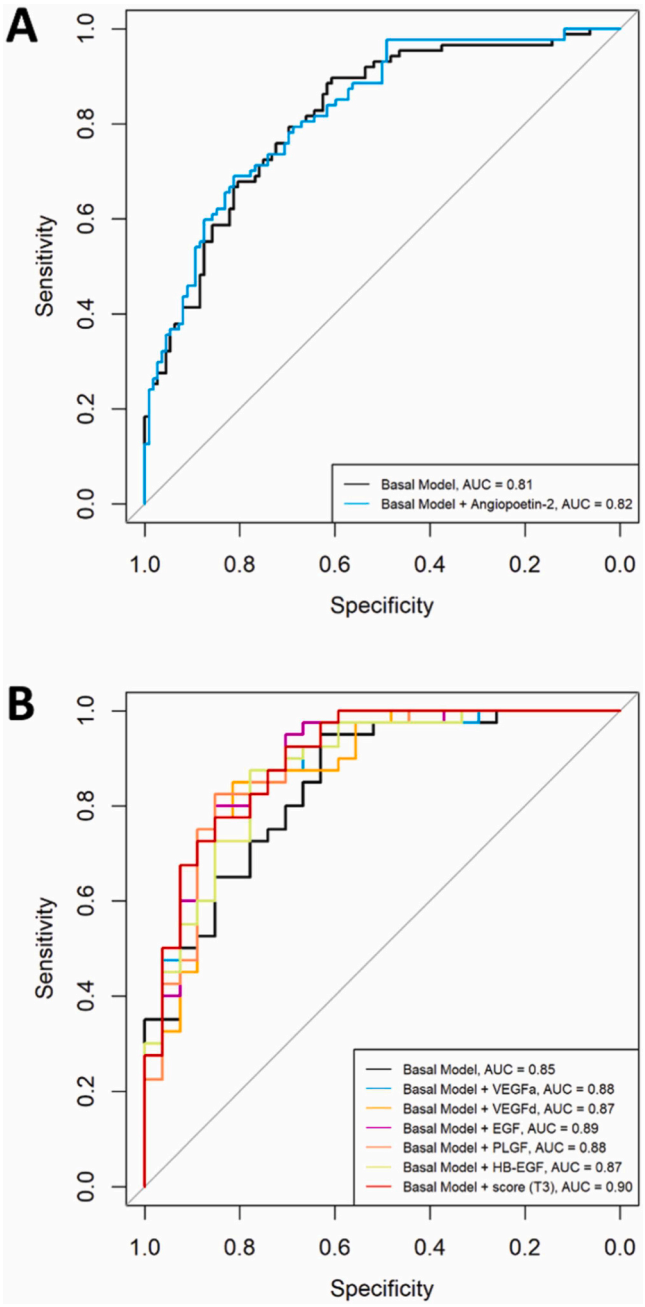

Fig. 1. ROC curves of the multivariate logistic models. A Classification of plaque presence or absence in the overall cohort B Classification of plaque presence or absence in men patients. Basal models are adjusted for age, sex, BMI, disease duration, csDMARDs, NSDAIDs, biological drugs, corticoids and DAS28. AUC = area under the curve.

4. Discussion

In this study, we investigated the associations between eight plasma GFs (Ang-2, EGF, HB-EGF, PLGF, TGF-α, VEGFa, VEGFc, and VEGFd) and different surrogate markers of subclinical arteriosclerosis in a cohort of patients with RA. Our results revealed that increased levels of Ang-2 were associated with increased odds of cPP in the overall cohort. Moreover, PLS-DA models indicated that Ang-2 was a notable classifier when GFs were jointly assessed to distinguish between patients with or

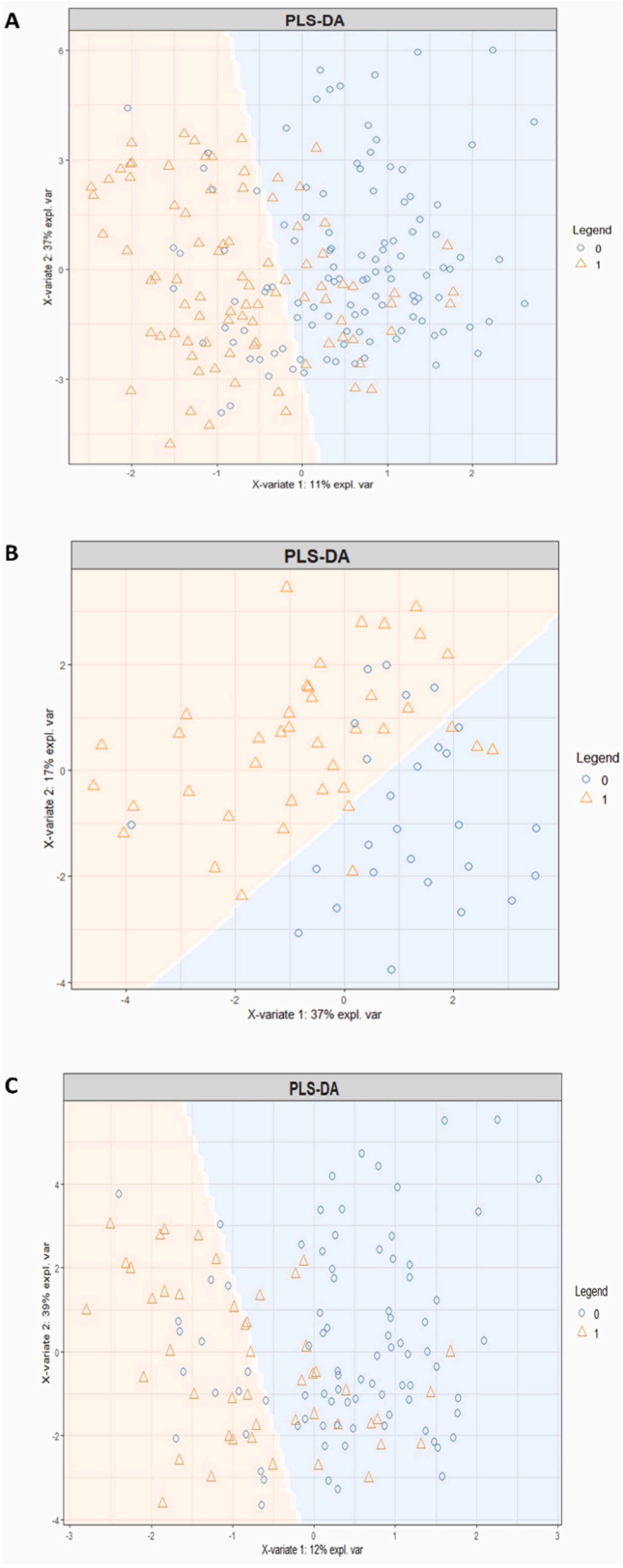

Fig. 2. PLS-DA two-dimensional distribution of patients with and without plaque presence. A overall cohort; B men patients; C women patients. X matrix contains the following variables: angiopoetin-2, HB-EGF, EGF, PLGF, TGFa, VEGFa, VEGFc, VEGFd, age, sex, BMI, disease duration, csDMARDs, NSDAIDs, biological drugs, corticoids and DAS28. 0 = no plaque presence, 1 = plaque presence. X-variate 1 = latent variable 1 representation with its variability explained. X-variate 2 = latent variable 2 representation with its variability explained.

without cPP in both men and women. We also observed that increased Ang-2 levels were associated with increased arterial stiffness (PWV) in the overall cohort and in both male and female patients. Our results are in line with those of previously published studies, as Ang-2 is known to be a proinflammatory destabilising factor and has been previously associated with both RA and CV disease. In this regard, several single nucleotide polymorphisms in the Ang-2 gene are related to increased odds of developing RA [41], and patients with early RA exhibit increased levels of Ang-2 compared with healthy individuals [27,29]. In addition, Ang-2 is correlated with markers of inflammation, endothelial activation, vascular dysfunction and disease activity, such as CRP, ESR and DAS28 [27,32,42]. Interestingly, patients with RA who eventually developed CV disease exhibited higher levels of Ang-2 than did patients with RA who did not develop CV disease [27]. Our results reinforce and complement this finding, as our associations are based on the subclinical atherosclerotic processes that precede CV events. Moreover, serum Ang-2 levels were greater in RA patients with CV disease and patients with high CV than in those at low risk [28] Similarly, Omar et al. reported that Ang-2 levels were greater in patients with RA with cIMT values > 0.6 mm presented than in patients with cIMT values ≤ 0.6 mm [29]. Our research provided new insights into the connection between Ang-2 and CVD by demonstrating a significant correlation between Ang-2 levels and cPP in our RA patient cohort. This finding suggests that Ang-2 plays a pivotal role in the atherosclerotic process of RA. Moreover, our results were adjusted for multiple confounders, which enhances the robustness and accuracy of our findings and increases the likelihood of reflecting true relationships. When GFs were evaluated together, we also observed that Ang-2 was a potential classifier of patients with or without cPP in the overall cohort and in both male and female patients. Finally, we also observed that Ang-2 levels were associated with vascular stiffness in RA patients, regardless of sex. The role of Ang-2 in increasing arterial stiffness has been observed in other diseases, such as HIV and chronic kidney disease [43,44], but this is the first time Ang-2 has been observed in RA, strengthening the potential role of this GF in the atherosclerotic process of RA.

Our sex-specific analyses showed that elevated levels of VEGFa, VEGFd, EGF, PLGF and HB-EGF were individually associated with increased odds of cPP in male patients. No associations were found between GFs and cPP in women. Additionally, we also observed that VEGFc was associated with PWV in the overall cohort and that VEGFa, VEGFc and HB-EGF were associated with PWV in male patients. These results highlight the physiological differences between males and females, providing insight into the underlying atherosclerotic process in both sexes. However, much less data have been published about the role of the VEGF family, which is known to be involved in angiogenesis and inflammation [45]. A meta-analysis including 2508 RA patients and 2489 control individuals revealed a positive correlation between circulating VEGF family GF levels and DAS28 [46]. Another study examined the role of the VEGF family in ischaemic heart disease in patients with RA, and although a correlation was observed between disease duration and VEGF levels, no differences were observed between RA patients with and without CV disease [47]. These findings suggest that VEGF family GFs may play a role in the structure and biology of carotid plaques and could be therapeutic targets for CV disease in RA [31,48]. Even less evidence has been published on the role of PLGF in CV disease in patients with RA. However, several studies have linked the expression levels of PLGF with inflammation in RA, as it has been observed that PLGF triggers the production of proinflammatory cytokines [49]. Moreover, PLGF has been reported to play a proatherogenic role in the general population and in populations with other diseases, such as T1DM [50,51].

We also evaluated the classification power of these GFs in

Table 3
Summaries of the multivariate lineal regression models to estimate the associations between growth factors and PWV. Basal models are adjusted for age, sex, BMI, disease duration, csDMARDs, NSDAIDs, biological drugs, corticoids, DAS28 and hypertension.

	β	p	R^2 (%)	AIC
Overall cohort				
Basal Model			35.24	832.80
Basal Model				
+ angiopoietin-2	0.44	0.002	38.4	834.79
Male patients				
Basal Model			33.06	279.20
Basal Model				
+ angiopoietin-2	0.57	0.02	43.14	274.05
Basal Model				
+ VEGFa	0.50	0.03	41.81	275.57
Basal Model				
+ VEGFc	0.59	0.01	43.38	273.78
Basal Model				
+ HB-EGF	0.47	0.04	41.04	276.44
Female patients				
Basal Model			38.21	573.39
Basal Model				
+ angiopoietin-2	0.41	0.01	40.58	570.28

PWV = pulse wave velocity, β = beta coefficient, p = p-value, AIC = Akaike information criteria.

categorising patients with and without cPP collectively using a global concentration score, which considered the concentration of each of the GFs studied, and a machine learning algorithm (PLS-DA) that avoided multicollinearity problems and allowed the incorporation of all the GFs studied into the same model. Evaluating biomarkers collectively provides a more comprehensive and accurate assessment of disease status, improving predictive models and enhancing research validation. In this regard, we observed that the global concentration score was significantly associated with cPP in male patients. Interestingly, the association of this score with cPP was the most relevant compared with any of the individual GFs, as it was the variable with the highest ΔR^2. When PLS-DA models were used, we observed good classification not only in the overall cohort but also in male and female patients. Moreover, latent variable plots show that GFs play a pivotal role in classifying patients with and without cPP. These results suggest the combined use of the GFs as a potential tool for detecting patients with cPP.

No significant associations were found between cIMT and the studied GFs, either in the entire cohort or among males and females. These results are in accordance with the findings of prior research, where no associations were found between VEGFa or Ang-2 and cIMT [26,52]. The lack of an association between GF levels and cIMT might be attributed to several factors. cIMT and cPP represent different atherosclerotic stages. cIMT reflects earlier changes, and cPP indicates more advanced pathology. GFs may exert a more significant impact on later atherosclerotic stages or severe vascular changes not captured by cIMT. Furthermore, the complexity of atherosclerosis and cIMT development, which involves other factors, such as lipid accumulation and mechanical stress, may also explain these differences. Finally, the specificity of GF actions across different vascular beds coupled with their interactions with other essential molecules may also explain the differences observed in our study. Nevertheless, in the present study, several GFs were associated with cPP, which is considered a more powerful predictor of cardiovascular risk than cIMT. Thus, the role of cIMT as a CV risk predictor is increasingly questioned due to its reflection of an earlier atherosclerotic status, its intrinsic increase with age, and its high variability depending on the measurement method [53].

Finally, our investigation of the connection between the selected GFs and the inflammatory markers of the disease revealed that VEGFd displayed a significant association with ESR and fibrinogen levels in the overall population and with the DAS28, ESR, and fibrinogen in female patients. No other statistically significant associations found. VEGFd might activate specific signalling pathways that are not activated by other VEGF family GFs, suggesting a need for more targeted research to fully understand the role of VEGFd in the inflammatory pattern of RA.

Lee & Bae (2018) reported a positive correlation between VEGF family member levels and disease activity in patients with RA [46]. Moreover, the VEGF family has also been linked to inflammation, even in healthy individuals [54]. However, another recent publication did not find any significant correlation between VEGF and disease activity, leading to uncertain conclusions [55]. Nevertheless, our group of patients underwent extensive treatment at the time of inclusion, which may have influenced our results.

Our research has several limitations. Firstly, we cannot determine causality for any of the associations we discovered due to the cross-sectional nature of our study. Secondly, our selection of RA patients was limited to a specific region, which may result in findings being population-specific and not applicable to other populations. Lastly, further validation and follow-up studies are required to confirm the clinical relevance of the GFs we studied. However, the robustness of our statistical analyses suggests that the GFs we studied have the potential to play a role in assessing the atherosclerotic state of RA patients. Moreover, the observed differences in the sex-stratified analyses, which may be caused by hormonal, genetic, or environmental effects, underscore the importance of considering sex as a pivotal variable in RA research. Future research avenues of interest would involve examining the predictive capacity of the selected GFs to anticipate the development of carotid plaques and CV episodes. Exploring whether the levels of GFs fluctuate in response to disease activity changes and the impact of therapies on them would further elucidate the potential roles that we have identified.

In conclusion, our study revealed differential associations between several plasma GFs and surrogate markers of atherosclerosis and revealed that evaluating GFs conjunctively may be a powerful tool for assessing the atherosclerotic state of patients with RA. Importantly, our findings have potential clinical implications. These biomarkers are promising for predicting CV risk in RA patients and underscore the potential utility of these biomarkers in CV risk stratification. Thus, they could be valuable in identifying patients who may benefit from more proactive CV risk management strategies. Additionally, our results highlight the importance of tailoring interventions based on both sex and individual GF profiles, offering a potential strategy to enhance the effectiveness of CV risk management in the RA patient population.

Funding

This research was funded by Instituto de Salud Carlos III through the project “FIS PI20/00443” and co-funded by the European Union.

CRediT authorship contribution statement

Daiana Ibarretxe: Project administration, Methodology. **Roser Rosales:** Project administration, Methodology. **Luis Masana:** Writing – review & editing, Writing – original draft, Validation, Conceptualization. **Joan-Carles Vallvé:** Writing – review & editing, Writing – original draft, Validation, Supervision, Investigation, Data curation, Conceptualization. **Silvia Paredes:** Writing – review & editing, Writing – original draft, Validation, Supervision, Conceptualization. **Anna Pàmies:** Writing – review & editing, Investigation, Conceptualization. **Dídac Llop:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used PaperPal in order to enhance readability and language. After using this tool, the authors reviewed and edited the content as needed and take full

responsibility for the content of the publication.

Declaration of Competing Interest

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

Acknowledgements

We would like to thank all the patients for their essential collaboration. We thank CERCA Programme (Generalitat de Catalunya).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.csbj.2024.04.042.

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