

Protein Profiling in Presymptomatic Individuals Separates Myeloperoxidase–Antineutrophil Cytoplasmic Antibody and Proteinase 3–Antineutrophil Cytoplasmic Antibody Vasculitides

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Objective. Antineutrophil cytoplasmic antibody (ANCA)–associated vasculitis (AAV) is a chronic relapsing condition with unknown etiology. To gain insight into the molecular processes underlying the disease, we examined biomarkers in blood samples collected prior to symptom onset.

Methods. The National Patient Register and Cause of Death register were searched for AAV-related International Classification of Diseases, Ninth Revision and Tenth Revision codes and linked to the registers from 5 biobanks. Eighty-five AAV patients with samples predating symptom onset of AAV were identified. For each case of AAV, 2 matched controls were included. Proteinase 3 (PR3)–ANCA and myeloperoxidase (MPO)–ANCA expression levels were analyzed using enzyme-linked immunosorbent assays. Using an Olink Inflammation panel, 73 of 92 proteins were included after quality control. Data were replicated in a second cohort of 48 presymptomatic individuals and 96 controls.

Results. Of the 20 proteins with the lowest *P* values in the original cohort, 7 were replicated in the second cohort and 5 proteins were found to be significant between the groups in a meta-analysis. Eleven different pathways were identified in network enrichment analyses and were found to be significant in both cohorts. Stratification of samples obtained ≤5 years before symptom onset showed significant levels of CCL23, vascular endothelial growth factor A, and hepatocyte growth factor, which were also increased at borderline significant levels in the replication cohort (interleukin-6 was found to be significantly increased in the replication cohort). In presymptomatic AAV patients, 6 proteins were associated with MPO–ANCA positivity, and 7 proteins were associated with PR3–ANCA positivity.

Conclusion. To our knowledge, this is the first study to identify protein markers preceding symptom onset in AAV patients. These findings set the stage for further research into the underlying cellular and molecular mechanisms in the pathogenesis of AAV and the diversification of patients into PR3–ANCA+ and MPO–ANCA+ subphenotypes.

INTRODUCTION

The antineutrophil cytoplasmic antibody (ANCA)–associated vasculitides (AAVs) comprise a group of rare, potentially life-threatening diseases characterized by necrotizing inflammation of small blood vessels and the presence of ANCAs (1). ANCAs

targeting proteinase 3 (PR3) and myeloperoxidase (MPO) expressed by innate immune cells (neutrophils and monocytes) are salient pathogenic features of the small vessel vasculitides granulomatosis with polyangiitis (GPA) and microscopic polyangiitis (MPA). Although GPA and MPA share common immunologic and pathologic features, such as ANCAs and a high

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degree of renal involvement, they are genetically distinct subsets (1,2).

The pathogenic processes leading to AAV are poorly understood. The biomarkers used in clinical practice to date are MPO-ANCA and PR3-ANCA, in addition to C-reactive protein, erythrocyte sedimentation rate, and analyses of the urine. In GPA and MPA, it has been suggested that some serologic biomarkers are related to disease activity, such as tissue inhibitor of metalloproteinases 1, matrix metalloproteinase 3 (MMP-3), CXCL13, interleukin-6 (IL-6), pentraxin 3, semaphorin 4D, neutrophil extracellular trap remnant levels, and the alternative complement pathway (3). During active disease in both PR3-positive AAV (PR3-AAV) and MPO-positive AAV (MPO-AAV), T cells and B cells are activated, and several overlapping biomarkers have been identified between the two (4). However, these 2 serotypes are associated with different protein expression profiles, as summarized by Kronbichler et al (4), and studies on the biomarkers preceding the onset of AAV are lacking.

In this study, we sought to gain insight into the early molecular processes involved in the immunopathogenesis of AAV prior to the onset of symptoms, and to identify potential early biomarkers differentiating the MPO-AAV and PR3-AAV serotypes. Furthermore, we aimed to replicate the results in a second cohort.

PRE-PATIENTS AND METHODS

Participants. For this case-control study, using the Swedish National Patient Register of inpatient care and the Cause of Death Register, we identified individuals diagnosed as having AAV or polyarteritis nodosa (PAN) using the recorded International Classification of Diseases, Ninth Revision (ICD-9) codes (1987–1997; codes 446A, 446.4, and 446E) and ICD-10 codes (1998–2011; codes M30.0–30.1, M31.3, and M31.7). The personal identification numbers of individuals with an AAV diagnosis (age ≥ 18 years; samples collected before symptom onset) were linked to the registers of 5 Swedish biobanks. Medical records were reviewed and the European Medicines Agency algorithm (excluding PAN) was used to identify the timing of symptom onset and to confirm AAV diagnoses after onset (5). The criteria for recruitment and for collection and storage of blood samples have been described in detail previously (6). In total, 85 presymptomatic individuals were identified with available samples. After disease onset in these 85 patients, 65 were classified as having GPA, 16 as having MPA, and 4 as having eosinophilic GPA (5). For each presymptomatic individual, 2 controls unaffected by AAV were identified within the same biobank and matched for age, sex, and sampling date ($n = 163$ controls). Of the originally matched case-control trios, 78 pre-patients had 2 matched controls and 7 pre-patients had 1 matched control (Supplementary Table 1, available on the *Arthritis & Rheumatology* website at <http://onlinelibrary.wiley.com/doi/10.1002/art.42425>).

A replication cohort was created which included individuals with samples predating disease onset identified from 2 separate biobanks in Sweden. The patients had been diagnosed according to ICD-9 (446E) and ICD-10 (M30.1, M31.3, M31.7) codes. The review of medical records was conducted as it was for the original cohort. Fifty individuals with samples predating disease onset and 98 controls were identified, and 48 pre-patients and 96 controls from this group passed the quality control (QC). Compared with the original cohort, these individuals were significantly older at sampling and at diagnosis, their time before diagnosis had been significantly longer, and the storage of the samples differed significantly (Supplementary Table 1, <http://onlinelibrary.wiley.com/doi/10.1002/art.42425>). Distribution of the AAV diagnoses after disease onset did not differ between the 2 cohorts. This study complied with the Declaration of Helsinki and was approved by the Regional Ethics Committees at Umeå University (Dnr 2012-52-31 M, 2012-386-32 M).

Protein measurements. To analyze the relative levels of 92 immune system-related protein markers, we used the Proseek Multiplex Inflammation panel I with multiplex extension assay (Olink Proteomics), according to the manufacturer's instructions. The limit of detection was determined for each biomarker based on the mean value of triplicate negative controls analyzed in each run. The protein levels are expressed as relative quantification (Normalized Protein eXpression), which is logarithmically (\log_2) related to protein concentration. With a threshold of 75% of samples having values higher than the limit of detection, 73 of 92 proteins (79%) were used for further statistical analyses. There was a strong correlation (P values ranging from $P = 0.0087$ to $P < 0.0001$) between 21 samples analyzed both in the original cohort and in the replication cohort. Seventy-two protein markers passed the QC in the replication cohort. ANCA analyses (i.e., PR3-ANCA, MPO-ANCA, and ANCA-screen) are described in the Supplementary Methods and Supplementary Table 1 (<http://onlinelibrary.wiley.com/doi/10.1002/art.42425>).

Statistical analyses. Statistical calculations were performed using SPSS version 27.0 (IBM Corp) and R software (7). Descriptive data for pre-patients and controls are presented as proportions, means with SDs, and medians with interquartile ranges (IQRs), as appropriate. Nonparametric tests were used for comparisons between continuous data for the subgroups, and Student's t -tests were used for comparisons between the cohorts. Frequencies were compared using chi-square tests or Fisher's exact tests, as appropriate. Conditional logistic regression analyses adjusted for cohort, age, sex, and sampling time were used, and results were presented as odds ratios (ORs) with 95% confidence intervals (95% CIs). Correlation analyses were performed using Spearman's rank correlation. The association between sample storage time until analysis and the predating time (time between sampling and onset of symptoms) was

significant ($r_s = 0.39$, $P < 0.001$), but after subtraction of the pre-dating time from the total sample storage time (“postsymptom storage time”), no correlation with pre-dating time was observed ($r_s = 0.05$, $P = 0.66$). In the logistic regression analyses, when analyzing stratified data among the pre-patients, we adjusted for sex, plasma versus serum, age at sampling, and postsymptom storage time until analysis. To explore associations between pre-dating time and cytokine levels, we used a generalized additive model, with the adjustments mentioned above, with the 95% CIs of the curves representing the separate smooth terms. To compare curves between presymptomatic AAV patients and controls, F test was used to compare nested models, with the controls curve as reference. P values are presented as P_{corr} after Benjamini-Hochberg correction for multiple comparisons, and P values less than or equal to 0.05 were considered significant.

To classify presymptomatic individuals as compared to controls for the original and replication cohorts, we used the random forests (RF) method (8) with out-of-bag estimation for both class probabilities and error rates used in estimating the area under the curve (AUC). The out-of-bag estimates closely approximate external validation (9). A fixed-effect meta-analysis was performed using the `rma.mv` function in the `metafor` library in R, with default settings using the estimates and standard errors from the adjusted conditional regression for the original cohort and the adjusted logistic model for the replication cohort, respectively (10).

Network enrichment analysis. To characterize differences at the pathway level, we employed the network enrichment analysis (NEA) method (11,12). NEA detects pathway enrichment of differentially expressed protein lists, designated as an “altered gene set” (AGS), by accounting for and evaluating network edges between any AGS proteins and a pathway list (the “functional gene set” [FGS]). In this way, NEA can incorporate pathway proteins that may not show altered expression or for which experimental data are lacking. This approach was particularly valuable in the analysis of the compact protein panel. For the global network in NEA, we used functional links from several curated databases collected in the Pathway Commons project version 9, with 846,631 links among 20,063 unique human proteins (see the Supplementary Methods, <http://onlinelibrary.wiley.com/doi/10.1002/art.42425>) (13).

Differential enrichment. When enrichment is analyzed on a reduced, predefined set of proteins (i.e., protein universe) with a certain functional bias (inflammation in this case), then any enrichment method risks spuriously identifying multiple FGSs relevant to just this functional focus. Therefore, in addition to the standard NEA, we implemented a control permutation test, as previously described (see the Supplementary Methods, <http://onlinelibrary.wiley.com/doi/10.1002/art.42425>) (12).

RESULTS

Biomarker levels in plasma and serum. Of the 73 proteins analyzed using conditional logistic regression analyses across the 85 presymptomatic AAV individuals and 163 matched controls, 8 of the 20 proteins with the lowest P values were significantly altered between the 2 groups (Table 1). Two of these proteins (CCL23 and CXCL5) were found at higher levels in individuals with presymptomatic AAV. The remaining 6 proteins—Flt-3L, STAM-binding protein (STAMPB), adenosine deaminase (ADA), tumor necrosis factor β (TNF β), CX3CL1, and IL-15 receptor subunit α (IL-15R α)—were at lower levels in pre-patients than in controls (Table 1). After correction for multiple testing, none of the 20 protein markers were significant.

Of the 72 biomarkers passing QC, 7 of the 20 proteins that had the lowest P values in the original cohort also had significant P values in the replication cohort (Supplementary Table 2, <http://onlinelibrary.wiley.com/doi/10.1002/art.42425>). In the replication cohort, levels of monocyte chemotactic protein 1 (MCP-1), fibroblast growth factor 19 (FGF-19), and CCL19 were significantly different in AAV pre-patients compared to controls, while these proteins did not reach significance in the original cohort. Expression levels of 2 proteins were not significant in either cohort (CCL11 and extracellular newly identified receptor for advanced glycation end products [ENRAGE]-binding protein), and TNF β (LTA) expression was not significant in the replication cohort. Flt-3L expression levels were significantly different in both cohorts, although in the opposite direction (i.e., being decreased versus increased) (Supplementary Table 2, <http://onlinelibrary.wiley.com/doi/10.1002/art.42425>). After correcting the P value for multiple testing, no significant difference remained. The meta-analysis combined the results from the original and replication cohorts and confirmed that levels of 4 of the 20 proteins with the lowest P values in the original cohort (CCL23, CXCL5, ENRAGE, and CCL19) and levels of 4 proteins from the replication cohort (vascular endothelial growth factor A [VEGF-A], transforming growth factor β 1 [TGF β 1] proprotein, ENRAGE, and CCL19) were significantly different between presymptomatic AAV individuals and controls. The point estimates from the meta-analysis only differed marginally from the results of the original and replication cohorts (Supplementary Table 3, <http://onlinelibrary.wiley.com/doi/10.1002/art.42425>).

Analysis of pre-patient gene sets. For further analysis using NEA, we used the 20 proteins with the lowest P values from unadjusted conditional logistic regression analysis as a single AGS (Figure 1), with pre-patients and their matched controls as the dependent variable, and compared for enrichment against gene sets from publicly available pathway databases. Because the Olink protein panel already consisted of proteins related to inflammation, differential enrichment was evaluated using a random permutation test, which reported cases enriched specifically

Table 1. Protein markers with the lowest *P* values in conditional logistic regression models in pre-patients before the onset of antineutrophil cytoplasmic antibody-associated vasculitis and matched controls*

Protein	Pre-patient protein levels, median (IQR)	Control protein levels, median (IQR)	OR (95% CI)	<i>P</i> †	<i>P</i> ‡
CCL23	9.63 (0.9)	9.46 (0.6)	2.02 (1.23–3.32)	0.006	0.31
CXCL5	12.05 (1.33)	11.53 (0.92)	1.36 (1.01–1.82)	0.041	0.43
Flt-3LG	8.99 (0.76)	9.18 (0.7)	0.63 (0.42–0.94)	0.025	0.36
STAMBP	4.39 (1.21)	4.52 (1.16)	0.62 (0.43–0.88)	0.008	0.31
ADA	2.81 (1.54)	3.23 (1.52)	0.62 (0.42–0.93)	0.021	0.36
TNFβ (LTA)	4.55 (0.78)	4.72 (0.62)	0.61 (0.4–0.93)	0.020	0.36
CX3CL1	5.64 (0.77)	5.69 (0.55)	0.57 (0.34–0.95)	0.030	0.37
IL-15Rα	1.39 (0.55)	1.47 (0.57)	0.52 (0.27–1.00)	0.050	0.45
SLAMF1	1.27 (0.52)	1.19 (0.51)	1.46 (0.84–2.55)	0.176	0.67
ENRAGE (S100A12)	6.78 (2.19)	6.45 (2.46)	1.23 (0.97–1.56)	0.094	0.57
MMP-1	10.99 (1.8)	10.52 (1.08)	1.18 (0.95–1.46)	0.138	0.63
CCL19	8.04 (2.26)	7.71 (1.5)	1.12 (0.95–1.32)	0.161	0.65
ST1A1 (SULT1A1)	2.91 (1.42)	3.08 (1.54)	0.83 (0.66–1.05)	0.122	0.63
SIRT-2	3.17 (1.52)	3.29 (1.5)	0.82 (0.64–1.06)	0.132	0.63
CCL25	5.14 (1.23)	5.25 (1.22)	0.82 (0.62–1.08)	0.151	0.65
CCL11	5.97 (0.95)	5.91 (0.92)	0.81 (0.58–1.12)	0.202	0.67
FGF-19	7.68 (1.35)	7.79 (0.86)	0.79 (0.62–1.02)	0.066	0.51
SCF (KITLG)	8.67 (0.96)	8.67 (0.56)	0.75 (0.52–1.07)	0.111	0.63
MCP-1 (CCL2)	11.9 (1.12)	11.99 (1.24)	0.71 (0.48–1.06)	0.092	0.57
DNER	8.47 (0.51)	8.52 (0.37)	0.45 (0.19–1.07)	0.070	0.51

* Levels of the protein markers in pre-patients and controls were compared pairwise, with pre-patients and controls as the dependent variables. Conditional logistic regression was used to compare each pre-patient to their control, so that significant differences could be detected even when the groups as a whole had very similar medians. *P* values less than or equal to 0.05 were considered significant. IQR = interquartile range; OR = odds ratio; 95% CI = 95% confidence interval; ADA = adenosine deaminase; TNFβ = tumor necrosis factor β; IL-15Rα = interleukin-15 receptor α; SLAMF1 = signaling lymphocytic activation molecule; ENRAGE = extracellular newly identified receptor for advanced glycation end products; MMP-1 = matrix metalloproteinase 1; SIRT-2 = sirtuin 2; FGF-19 = fibroblast growth factor 19; SCF = stem cell factor; MCP-1 = monocyte chemotactic protein 1; DNER = Delta/Notch-like epidermal growth factor-related receptor.

† Uncorrected *P* value.

‡ Benjamini-Hochberg corrected *P* value.

(i.e., more than would be expected from a random set of 20 proteins from the Olink panel). Taking the results of these analyses into account, we identified 11 different pathways as both significant (Bonferroni-adjusted NEA, $P < 10^{-5}$) and specific (differential enrichment, $P < 0.01$). Although NEA accounted for network links connecting AGS to pathway genes so that their membership in the latter was not strictly required, we also found that 18 of the 20 proteins were members of at least 1 of the 11 pathways (Figure 1).

Again using the 20 proteins with the lowest *P* values for the original cohort, all 11 pathways identified in the original cohort were found to be significant in the replication cohort. In the replication cohort, 16 of the 20 proteins were members of at least 1 of the pathways, of which 8 also occurred in the 20-gene list of the original cohort (MCP-1, CCL11, CCL19, Flt-3L, ENRAGE, FGF-19, TNFβ, and STAMBP). The same pathways that were found to be significant in the original cohort were also significant in the replication cohort due to the enrichment of a different set of proteins, apart from the 8 that were significant in both cohorts. In other words, the pathogenic process appeared to be more robust at the pathway level than at the level of individual proteins. Furthermore, we integrated results from the 2 cohorts by calculating combined *P* values for each of the 11 pathways using Fisher's

exact test (14), yielding high significance for all pathways ($P < 0.0011$ by Fisher's exact test) (Figure 1).

RF as a predictive model for future AAV. To evaluate the 72 proteins as potential predictors of future AAV, we estimated separate RF models for the original and replication cohorts. Among the 30 markers with the highest variable importance in each cohort, we identified 12 overlapping markers (Supplementary Table 4, <http://onlinelibrary.wiley.com/doi/10.1002/art.42425>). Next, we used the RF model estimated using the original cohort for prediction in the replication cohort and obtained an AUC of 0.62 (95% CI 0.52–0.72), 19.1% sensitivity, 90.5% specificity, a positive predictive value of 0.5, and a negative predictive value of 0.694.

Levels of biomarkers stratified for predating time.

To investigate the relationship between time before symptom onset and biomarker level, we applied an adjusted generalized additive model. In the model, we found that the following 9 proteins were significantly related to the time before onset of symptoms: CXCL9, CD244, VEGF-A, CXCL1, tumor necrosis factor receptor superfamily 9 (TNFRSF9), osteoprotegerin (OPG), colony-stimulating factor 1 (CSF1), interferon-γ (IFNγ), and CD40

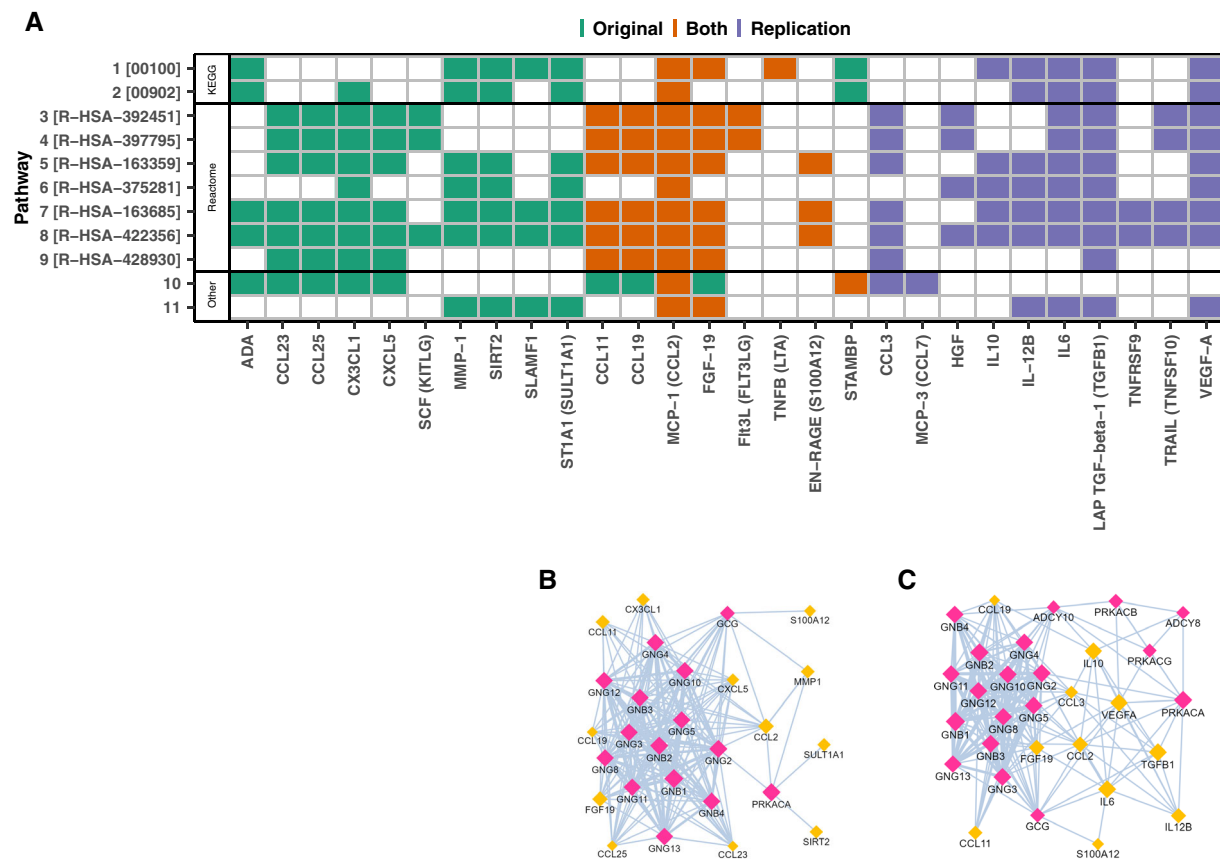


Figure 1. Analysis of protein expression in individuals with presymptomatic antineutrophil cytoplasmic antibody-associated vasculitis and matched controls in the original cohort and the replication cohort. **A**, The 11 pathways identified using network enrichment analysis (NEA). Pathway collection for the functional gene set (FGS) included all entries from BioCarta, KEGG, Reactome, and WikiPathways, as well as a Molecular Signatures Database collection of hallmarks. The analysis was run in the R package NEArender version 1.4, and Z-scores and *P* values for the network enrichment associated with each altered gene set (AGS)–FGS pair were calculated. In order to integrate the results per FGS from 2 independent studies, Fisher's exact test was used to calculate combined *P* values for the following items: 1) steroid biosynthesis, 2) monoterpenoid biosynthesis, 3) G $\beta\gamma$ signaling through PI3K γ , 4) G-protein $\beta\gamma$ signaling, 5) glucagon signaling in metabolic regulation, 6) hormone ligand-binding receptors, 7) integration of energy metabolism, 8) regulation of insulin secretion, 9) thromboxane signaling through T prostanoid receptor, 10) visual signal transduction (cones), and 11) cholesterol biosynthesis. **B** and **C**, Example of subnetworks from the “glucagon signaling in metabolic regulation” pathway with links connecting AGS proteins (yellow) with FGS proteins (pink) from the original cohort (**B**) and the replication cohort (**C**). ADA = adenosine deaminase; SCF = stem cell factor; MMP-1 = matrix metalloproteinase 1; SIRT2 = sirtuin 2; SLAMF1 = signaling lymphocytic activation molecule family member 1; MCP-1 = monocyte chemotactic protein 1; FGF-19 = fibroblast growth factor 19; TNFB = tumor necrosis factor β ; EN-RAGE = extracellular newly identified receptor for advanced glycation end products; HGF = hepatocyte growth factor; IL10 = interleukin-10; TNFRSF9 = tumor necrosis factor receptor superfamily 9; VEGF-A = vascular endothelial growth factor A.

(Figure 2). After correction for multiple testing, however, none of these results remained statistically significant. For 3 of the 9 proteins (VEGF-A, CXCL1, and OPG), the curves were significantly different between presymptomatic AAV individuals and controls ($P < 0.05$). The differences between pre-patients and controls were most evident closer to symptom onset (e.g., ≤ 5 years). Due to longer predating time in the replication cohort, this difference was not apparent.

Furthermore, pre-patients were stratified into 2 groups based on the time of sample collection: ≤ 5 years and ≤ 1 year before symptom onset. Within the last year preceding symptom onset (≤ 1 year), 9 protein markers (CXCL1, hepatocyte growth factor [HGF], TGFB1, CCL23, VEGF-A, Axin-1, CCL20, MMP10,

and IL-6) showed increased levels in presymptomatic individuals compared to controls in the original cohort ($P < 0.05$, $P_{\text{corr}} > 0.05$) (Table 2 and Supplementary Table 5, <http://onlinelibrary.wiley.com/doi/10.1002/art.42425>). In the ≤ 5 -years group, expression levels of 4 of the proteins (CCL20, CCL23, HGF, and VEGF-A) were also significantly higher. The ORs for associations of the protein markers were increased between the ≤ 5 -years group and the ≤ 1 -year group. In the replication cohort, only 15 samples were available for the ≤ 5 -years group. IL-6 was confirmed to be significantly increased in presymptomatic individuals compared to controls (OR 4.68 [95% CI 1.17–18.66]; $P = 0.029$), and borderline significances were found for VEGF-A ($P = 0.054$, $P_{\text{corr}} > 0.05$), CCL23 ($P = 0.066$, $P_{\text{corr}} > 0.05$), and

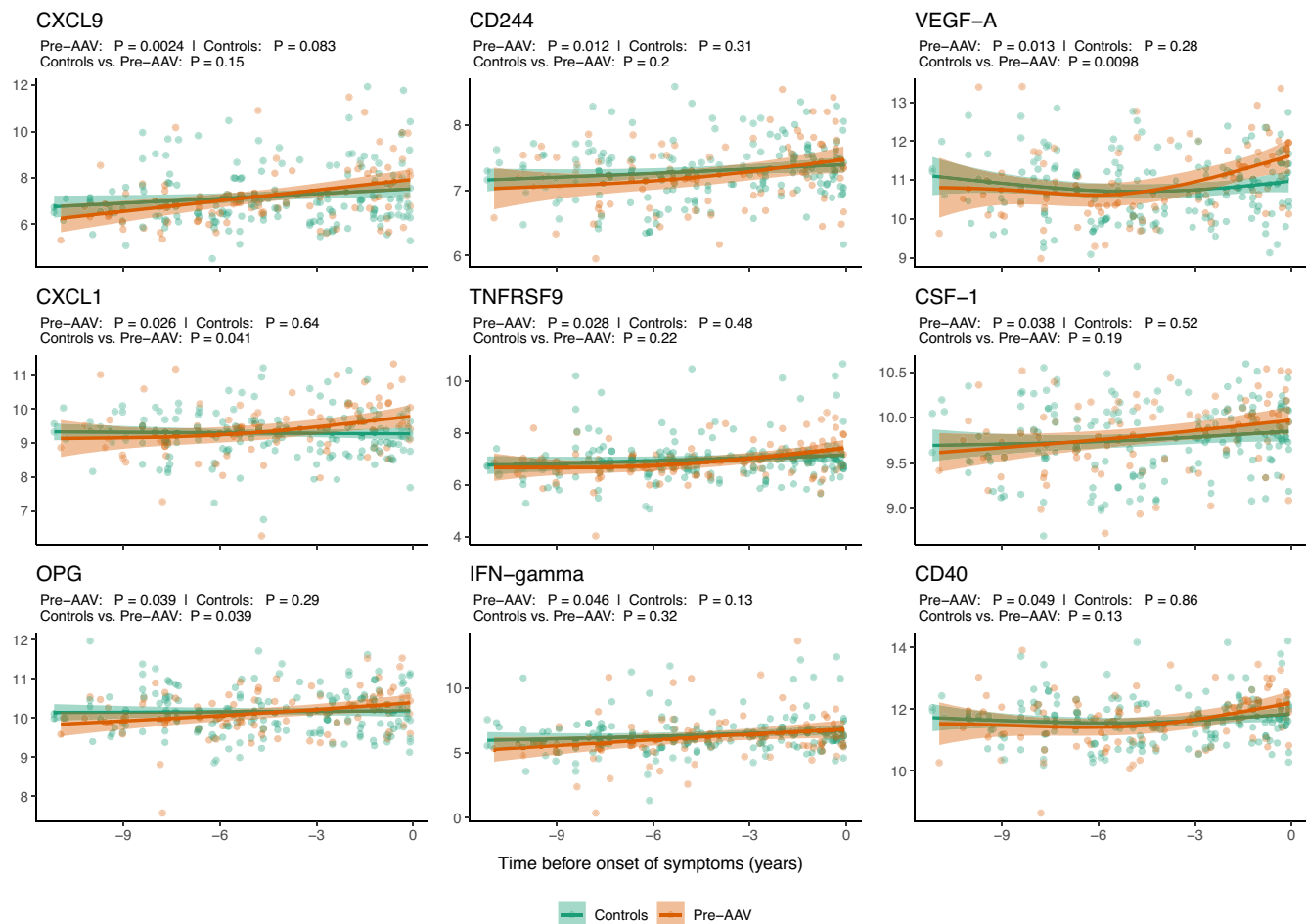


Figure 2. The 9 inflammatory cytokines showing a significant association between protein expression level and time before symptom onset in individuals with presymptomatic antineutrophil cytoplasmic antibody-associated vasculitis (pre-AAV). Controls are shown at the corresponding time of their matched presymptomatic AAV individual. Circles represent individual participants. Lines represent fitting by the smooth generalized additive model, adjusted for age, excess storage time, plasma/serum, and sex. $P < 0.05$ for comparisons of time before symptom onset between individuals with presymptomatic AAV and controls, and for the comparison of presymptomatic AAV and control curves. CSF-1 = colony-stimulating factor 1; OPG = osteoprotegerin; IFN-gamma = interferon- γ (see Figure 1 for other definitions).

Table 2. Protein markers from the inflammatory panel differing between presymptomatic AAV individuals and their matched controls compared using conditional logistic regression, stratified by time to symptom onset*

Protein	Original cohort				Replication cohort	
	≤ 5 years before onset (n = 47)		≤ 1 year before onset (n = 15)		≤ 5 years before onset (n = 15)	
	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
CCL23	3.79 (1.69–8.53)	0.001	4.18 (1.01–17.34)	0.049	8.43 (0.87–81.72)	0.066
VEGF-A	2.47 (1.3–4.69)	0.006	3.48 (1.09–11.09)	0.035	4.28 (0.976–18.77)	0.054
CCL20	1.47 (1.06–2.05)	0.02	2.14 (1.09–4.19)	0.026	0.97 (0.45–2.07)	0.935
HGF	1.89 (1.08–3.33)	0.027	6.01 (1.44–25.18)	0.014	3.81 (0.82–17.68)	0.088
Flt-3L (Flt-3LG)	0.55 (0.32–0.94)	0.03	0.55 (0.23–1.29)	0.17	4.97 (0.84–29.24)	0.076
IL-6	1.19 (0.98–1.43)	0.074	1.66 (1.01–2.75)	0.047	4.68 (1.17–18.66)	0.029
CXCL1	1.52 (0.95–2.43)	0.081	6.11 (1.37–27.16)	0.017	1.59 (0.60–4.57)	0.376
TGF β 1	1.66 (0.84–3.28)	0.14	4.78 (1.06–21.68)	0.042	3.07 (0.49–19.31)	0.232
Axin-1	1.25 (0.83–1.87)	0.28	2.9 (1.04–8.1)	0.042	1.48 (0.47–4.94)	0.521
MMP-10	1.47 (0.92–2.35)	0.11	2.56 (1.00–6.53)	0.050	0.59 (0.19–1.82)	0.360

* P values less than or equal to 0.05 were considered significant. VEGF-A = vascular endothelial growth factor A; HGF = hepatocyte growth factor (see Table 1 for other definitions).

HGF ($P = 0.088$, $P_{\text{corr}} > 0.05$). The expression level of Flt-3L ($P = 0.076$, $P_{\text{corr}} > 0.05$) was borderline significantly increased in presymptomatic individuals compared to controls in the replication cohort (Table 2).

Differing of biomarker levels by ANCA status.

Of 73 proteins analyzed, we found 31 proteins that differed significantly between MPO-ANCA+ or PR3-ANCA+ individuals compared to MPO-ANCA- or PR3-ANCA- individuals; 28 proteins were significant for MPO-ANCA positivity ($P_{\text{corr}} < 0.05$ for 9 proteins) and 5 proteins were significant for PR3-ANCA positivity ($P_{\text{corr}} > 0.05$) (Table 3, Supplementary Table 6, <http://onlinelibrary.wiley.com/doi/10.1002/art.42425>). In the conditional analyses, after the adjustments described above, 24 proteins were associated with ANCA-screen (above cutoff), MPO-ANCA, or PR3-ANCA (Table 3).

The expression levels of 20 of these proteins are presented with unadjusted P values in Figure 3, stratified by MPO-ANCA and PR3-ANCA positivity and negativity. Fifteen of the proteins were significantly increased in MPO-ANCA+ individuals ($P < 0.05$ to 0.001), and 2 of these proteins (ENRAGE and tumor necrosis factor ligand superfamily 14 [TNFSF14]) were increased

in both serotypes (Figure 3). In PR3-ANCA+ individuals, another 2 of these proteins were increased: TGF α and oncostatin M (OSM; $P < 0.01$ for both proteins). FGF-19 expression was decreased in PR3-ANCA+ individuals in contrast to individuals who were MPO-ANCA+ (Figure 3).

In a separate analysis of MPO-ANCA, 6 of the proteins were still associated with MPO-ANCA positivity after adjustments: CSF-1, TNFSF14, MCP-1, urokinase (uPA), FGF-19, and CD244 (Table 3). In the adjusted model, ENRAGE, TGF α , and OSM expression levels remained significantly increased in PR3-ANCA+ individuals. Inverse associations were found between PR3-ANCA and expression levels of FGF-19, CXCL5, TRANCE, and Delta/Notch-like epidermal growth factor-related receptor (DNER) (Table 3). Further analyses separating PR3-ANCA+ individuals from MPO-ANCA- individuals or vice versa strengthened the significance for most proteins, with an additional 18 proteins found to be significantly related to MPO-ANCA after correction for multiple testing ($P_{\text{corr}} < 0.05$ for both PR3-ANCA and MPO-ANCA). Apart from the 28 proteins related to MPO-ANCA, 7 additional proteins were significantly altered (TRAIL, TRANCE, IL-8, OSM, TGFB1, CASP-8, and TGF α ; $P < 0.05$ for all) (Supplementary Table 6, <http://onlinelibrary.wiley.com/doi/10.1002/art.42425>).

Table 3. Protein markers significantly associated with ANCA levels above the cutoff, MPO-ANCA positivity or negativity, and/or PR3-ANCA positivity or negativity in presymptomatic AAV individuals*

Protein	ANCA-screen above/below cutoff (n = 30/55)		MPO pos/neg (n = 9/75)		PR3 pos/neg (n = 22/62)	
	OR (95% CI)	P	OR (95% CI)	P	OR (95% CI)	P
CSF-1	11.55 (2.03–88.72)	0.010	96.13 (2.61–9,594.74)	0.026	2.61 (0.46–17.3)	0.293
CCL23	6.32 (2.24–21.74)	0.001	1.6 (0.33–7.32)	0.547	2.77 (1.02–8.73)	0.060
PD-L1 (CD274)	3.47 (1.45–9.94)	0.011	3.4 (0.88–16.12)	0.095	1.26 (0.56–3.09)	0.592
VEGF-A	2.93 (1.48–6.86)	0.006	1.34 (0.5–3.55)	0.549	2.09 (1.03–4.95)	0.063
CD40	2.63 (1.31–6.12)	0.013	2.87 (0.93–11.06)	0.094	1.34 (0.69–2.87)	0.413
IL-18	2.49 (1.26–5.58)	0.015	1.85 (0.62–5.58)	0.259	1.53 (0.74–3.34)	0.262
HGF	2.2 (1.22–4.5)	0.017	2.23 (0.86–6.61)	0.118	1.34 (0.76–2.61)	0.336
TNFSF9	2.19 (1.11–4.75)	0.033	2.58 (0.96–8.36)	0.080	1 (0.46–2.15)	0.999
MCP-4 (CCL13)	1.93 (1.06–3.88)	0.046	1.29 (0.53–3.57)	0.596	1.14 (0.62–2.2)	0.688
CXCL9	1.82 (1.18–3.05)	0.013	1.5 (0.82–2.79)	0.178	1.24 (0.8–1.97)	0.334
ENRAGE (S100A12)	1.76 (1.07–3.07)	0.032	1.51 (0.7–3.62)	0.313	2.75 (1.43–6.12)	0.006
TNFSF14	1.75 (1.12–2.98)	0.023	2.58 (1.11–7.3)	0.046	1.33 (0.83–2.26)	0.263
TGF α (TGFA)	1.7 (1.04–2.95)	0.042	1.08 (0.53–2.23)	0.819	2.22 (1.19–4.56)	0.018
SIRT-2	1.59 (1.03–2.58)	0.046	1.13 (0.54–2.41)	0.741	1.41 (0.87–2.4)	0.179
CCL20	1.5 (1.06–2.2)	0.028	1.59 (0.92–2.87)	0.100	1.12 (0.78–1.63)	0.534
MCP-1 (CCL2)	1.4 (0.7–3.01)	0.356	7.96 (2.08–48.56)	0.007	0.81 (0.38–1.75)	0.583
IL-6	1.39 (1.02–1.97)	0.047	1.4 (0.88–2.26)	0.150	1.32 (0.92–1.91)	0.132
OSM	1.32 (0.93–1.92)	0.128	1.34 (0.77–2.43)	0.315	1.62 (1.05–2.7)	0.042
uPA (PLAU)	1 (0.4–2.54)	0.998	12.6 (1.97–134.89)	0.016	0.4 (0.12–1.11)	0.093
FGF-19	0.9 (0.57–1.4)	0.632	2.62 (1.14–7.32)	0.037	0.49 (0.26–0.85)	0.019
CD244	0.9 (0.33–2.41)	0.827	6.39 (1.23–53.05)	0.047	0.29 (0.08–0.96)	0.053
CXCL5	0.87 (0.59–1.29)	0.483	1.91 (0.88–7.07)	0.202	0.62 (0.39–0.94)	0.029
TRANCE (TNFSF11)	0.57 (0.32–0.97)	0.045	0.67 (0.29–1.41)	0.300	0.5 (0.24–0.94)	0.039
DNER	0.34 (0.07–1.5)	0.164	1.79 (0.19–19.55)	0.611	0.14 (0.02–0.78)	0.030

* P values less than or equal to 0.05 were considered significant. Binary logistic regression analyses adjusted for sex, age, postsymptom storage time (time from symptom onset until sample analysis), and plasma/serum. ANCA = antineutrophil cytoplasmic antibody; MPO = myeloperoxidase; PR3 = proteinase 3; AAV = antineutrophil cytoplasmic antibody-associated vasculitis; CSF-1 = colony-stimulating factor 1; PD-L1 = programmed death ligand 1; VEGF-A = vascular endothelial growth factor A; IL-18 = interleukin-18; HGF = hepatocyte growth factor; TNFSF9 = tumor necrosis factor receptor superfamily 9; MCP-4 = monocyte chemoattractant protein 4; ENRAGE = extracellular newly identified receptor for advanced glycation end products; TNFSF14 = tumor necrosis factor ligand superfamily 14; TGF α = transforming growth factor α ; SIRT-2 = sirtuin 2; OSM = oncostatin M; uPA = urokinase; FGF-19 = FGF-19 = fibroblast growth factor 19; DNER = Delta/Notch-like epidermal growth factor-related receptor.

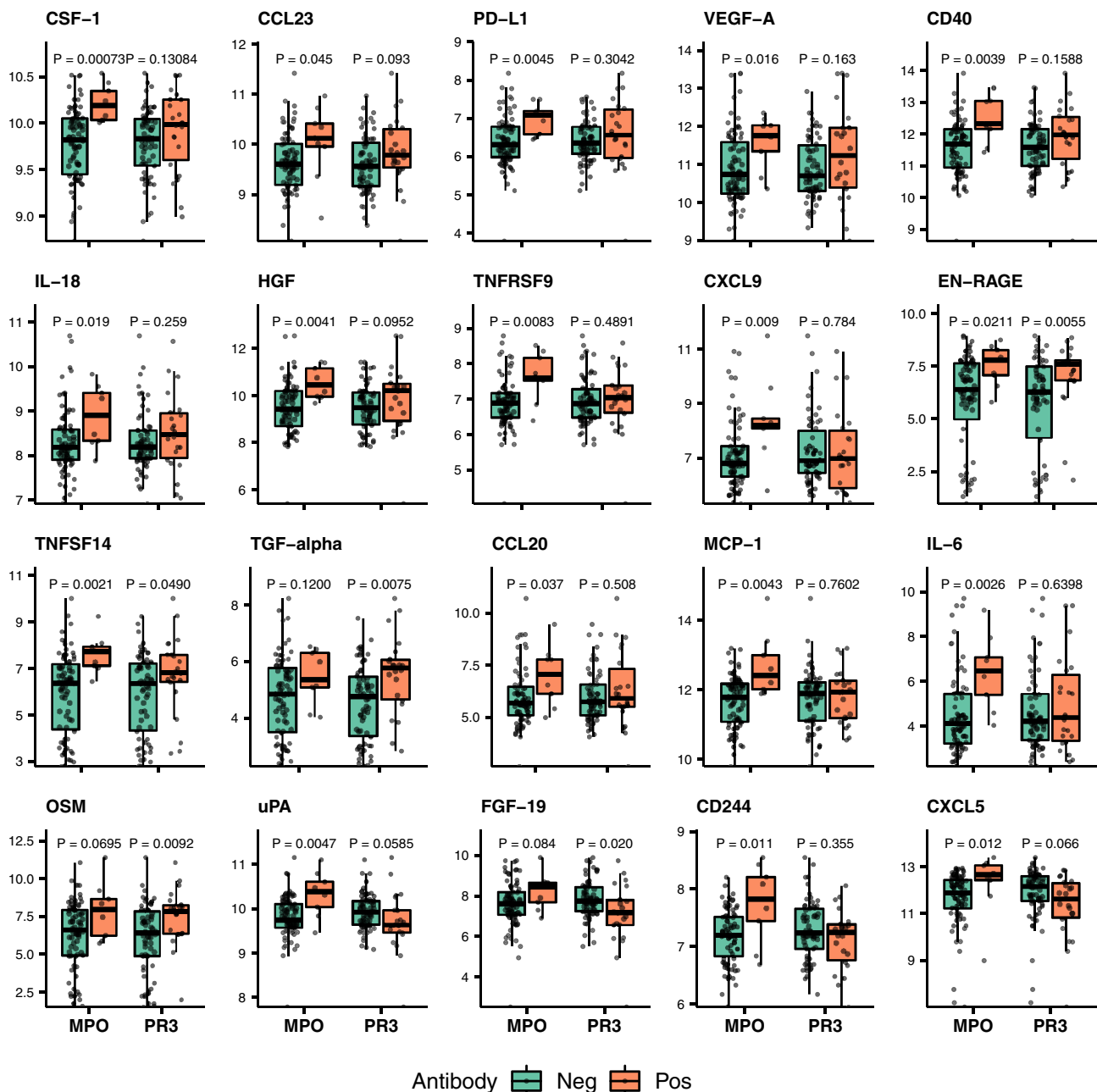


Figure 3. Levels of the 20 protein markers significantly associated with positivity for either myeloperoxidase–antineutrophil cytoplasmic antibodies (MPO–ANCA) or proteinase 3 (PR3)–ANCA in presymptomatic individuals with ANCA-associated vasculitis (AAV) in the adjusted model. Nonparametric tests were used to compare protein levels between presymptomatic AAV individuals who were positive and those who were negative for MPO–ANCA and PR3–ANCA. *P* values were not corrected for the number of comparisons. CSF1 = colony-stimulating factor 1; PD-L1 = programmed death ligand 1; IL-18 = interleukin-18; TNFSF14 = tumor necrosis factor ligand superfamily 14; TGF α = transforming growth factor α ; OSM = oncostatin M; uPA = urokinase (see Figure 1 for other definitions).

Regarding PR3-ANCA, another 7 proteins were found to be significant (CCL25, CASP-8, TRANCE, CCL23, HGF, CSF-1, CD40; $P < 0.01$ – 0.05 , $P_{\text{corr}} > 0.05$).

After adjustments, ANCA-screen was associated with 17 of the proteins covering both MPO-ANCA and PR3-ANCA associations (Table 3). However, in a few of the analyses in the adjusted models, the associations were not significant for ANCA-screen

but were significantly related to either MPO-ANCA or PR3-ANCA. This divergence between the antibodies was particularly evident for associations in which protein levels were disparate for the MPO-ANCA and PR3-ANCA serotypes (uPA, FGF-19, CD244, CXCL5, and DNER) (Table 3).

In the replication cohort, the frequency of positivity for PR3-ANCA and MPO-ANCA was too low to warrant any further

analysis; 1 individual was PR3-ANCA+, 1 individual was MPO-ANCA+, and 4 individuals had ANCA-screen above 1. The low frequency can be explained by the low number of samples collected close to symptom onset due to the longer predating time in the replication cohort (median 7.6 years [IQR 4.0–11.1]) compared to the original cohort (median predating time for PR3-ANCA+ individuals 2.7 years [IQR 0.3–7.7] and median predating time for MPO-ANCA+ individuals 2.0 years [IQR 0.9–3.5]) (6).

DISCUSSION

AAV is a severe autoimmune disease with a low prevalence. Here, we present the results of the first analysis, to our knowledge, of cytokines and growth factors in blood samples from presymptomatic individuals who were later diagnosed as having AAV. In addition, we have identified a replication cohort with blood samples predating the onset of AAV. Based on complementary analyses including between-group comparisons, pathway dissections, and kinetics of cytokines and growth factors, we propose a plausible model for presymptomatic AAV-related events.

NEA of the 20 proteins showing the most significant differences between presymptomatic AAV individuals and controls in the original cohort suggested involvement of metabolism-related pathways in 7 of the 11 examined pathways (KEGG 00100, KEGG 00902, Reactome HSA-163359, HSA-375281, HSA-163685, and HSA-422356, and cholesterol biosynthesis), of inflammation in 2 of the 11 pathways (Reactome HSA-392451 and HSA-397795), and of thromboxane A2 in 1 pathway (Reactome HSA-428930) (15,16) (Figure 1). A close link between metabolism and inflammation has been described extensively in the setting of several autoimmune diseases, including both rheumatoid arthritis (RA) and AAV (17–19), making it plausible that metabolic alterations could contribute to AAV susceptibility. Whether this association would reflect immune cell-related adaptations to an ongoing immune response or a more basic metabolic alteration by other tissues remains to be determined. The thromboxane A2 pathway could reflect an ongoing inflammatory process, as thromboxane A2 is closely associated with inflammation (20). Interestingly, NEA of the replication cohort identified the same pathways, and their combined *P* values were highly significant. Besides the 8 proteins identified in both the original and the replication cohort, the other 10 proteins found in the replication cohort could be found within the previously identified pathways.

As described above, there was overlap in pathway enrichment between the original and replication cohorts, even though comparisons of individual proteins did not always yield the same results. It was noted that in the original cohort many of the proteins actually had a lower expression level in presymptomatic AAV patients compared to controls. One possibility based on this

observation is that this represents a down-regulation in the immune system resulting from intrinsic (i.e., genetic) and/or extrinsic factors such as infections, which have been suggested to be etiologic factors in vasculitis (21). Taking these findings together, it is plausible that mild immune alterations in general could contribute to the initiation of AAV pathogenesis.

Using the RF model and noting that the lower boundary of the 95% CI for the AUC was above 0.5, we established that the protein markers taken together had predictive ability for AAV in the replication cohort. Therefore, it will be interesting to further clarify their role in processes leading up to AAV.

In the metaanalysis based on the results from the 2 independent cohorts, 3 of the proteins with the lowest *P* values in the subgroups of individuals whose samples were taken ≤ 5 years before symptom onset remained significant in both cohorts ($P < 0.05$). Additionally, 2 other proteins from the original cohort and 3 other proteins from the replication cohort also remained significant in the analysis of the 20 proteins with the lowest *P* values, corroborating our initial findings.

Indeed, analysis of samples collected up to 5 years before disease onset offered a unique chance to study the initiation and progression of AAV and its related underlying molecular causes. For this purpose, the original cohort was stratified into 2 subgroups: those whose samples were collected ≤ 1 year before disease onset and those whose samples were collected ≤ 5 years before disease onset. Comparisons of samples from presymptomatic AAV individuals collected ≤ 5 years before symptom onset and matched controls showed that CCL23 was positively associated with presymptomatic AAV. CCL23, a trypsin-like serine protease, has been linked to chronic rhinosinusitis and nasal polyps (22), which are a problem particularly in PR3-AAV. Moreover, CCL23 is known to promote chemotaxis of T cells, monocytes, and neutrophils (23). Up-regulation of HGF and VEGF-A were also noted in samples collected ≤ 5 years before symptom onset, suggesting ongoing angiogenesis, possibly due to initial vascular damage (24,25). In addition, we observed down-regulation of Flt-3L, which is involved in both hematopoietic stem cell precursor development and dendritic cell maturation (26). VEGF can negatively regulate Flt-3L, thus supporting the finding of the reciprocal level of these 2 cytokines (27). Furthermore, increased levels of VEGF-A, HGF, and CCL23 were found in the replication cohort in samples collected ≤ 5 years before symptom onset, although the significance values were only at borderline. In samples collected ≤ 5 years before symptom onset in the original cohort, the chemokine CCL20 showed a significant up-regulation in presymptomatic AAV individuals compared to matched controls. CCL20 is involved in the recruitment of T cells in inflamed joints in the setting of RA and is up-regulated in active vasculitis (28). In samples from presymptomatic AAV patients collected ≤ 1 year prior to diagnosis, we observed a cytokine pattern that indicated a progression in pathogenesis. CCL20, HGF, and VEGF-A were

still significantly increased in presymptomatic AAV patients compared to controls. In addition, we found up-regulation of CXCL1, which promotes neutrophil chemotaxis, MMP10, which is involved in extracellular matrix degradation, and IL-6, a crucial inflammation-related cytokine (29,30). Thus, in this time window, an ongoing process of vasculitis pathogenesis was noted, although the individuals were without symptoms.

We analyzed the associations between cytokines and ANCA-screen and found that 17 of the 73 originally analyzed proteins were significantly associated with ANCA positivity. Among those 17 proteins, only 1 (CCL23) was also present in the initial analysis, distinguishing the whole group of pre-patients compared to controls. Thus, the development of ANCA positivity occurs, at least in part, secondary to initially different cytokine profiles and potential susceptibility to developing AAV. We acknowledge, however, that the relatively low proportion of ANCA+ individuals could be a limitation in this comparison. An alternative explanation is that the development of ANCAs is a separate, parallel process that together with an altered cytokine environment drives the pathogenesis of AAV.

Furthermore, additional analysis of PR3-ANCA and MPO-ANCA subgroups revealed almost mutually exclusive subphenotypes based on the associated cytokines. Thus, MPO-ANCA positivity was strongly associated with CSF-1, TNFSF14, MCP-1, uPA, FGF-19, and CD244. CSF-1 is a pleiotropic cytokine with a well-documented effect on macrophage development (31). Interestingly, it has been suggested that CSF-1 has a role in kidney disease (32), which is consistent with the renal involvement found in MPO-ANCA+ patients in particular (33). Furthermore, CD244, TNFSF14, and MCP-1 are important for the regulation of T cells during infection and tissue injury and imply T cell involvement in MPO-ANCA+ vasculitis, a notion that is supported by animal experiments (34).

In the PR3-ANCA subgroup, there was an inverse association between PR3-ANCA positivity and levels of CXCL5 and DNER. Both proteins are involved in macrophage response, suggesting its potential impairment in this group (35). That inference is consistent with the proposition that PR3-ANCA is particularly associated with prior infections, such as *Staphylococcus aureus* infections. Moreover, TGF α is produced by macrophages and is a stimulator of epithelial cells, OSM is a potential proinflammatory agent in the endothelium (36), and S100A12 (ENRAGE) has been linked to systemic inflammation, indicating chronic ongoing systemic inflammation in PR3-ANCA+ individuals.

For one cytokine, FGF-19, we observed a reciprocal association between increased expression levels in MPO-ANCA+ individuals and decreased expression levels in PR3-ANCA+ individuals. Circulating FGF-19 is increased in patients with end-stage renal disease (37). Presymptomatic MPO-ANCA+ individuals more frequently present with renal manifestations of disease and subsequently develop MPA vasculitis, which is more commonly related to renal disease (6). Although these presymptomatic

individuals did not have end-stage renal disease, the increased levels of FGF-19 could imply an ongoing process in the kidneys, which would not be the case to the same extent in PR3-ANCA+ vasculitis. It was not possible to analyze the relationship between autoantibodies and protein levels in the replication cohort due to longer predating times, resulting in few ANCA+ individuals in that cohort.

The strengths of this study include the large-scale protein marker from a well-defined, unique cohort of presymptomatic AAV individuals who were sampled years before symptom onset. We had the opportunity to use matched controls from the same biobanks, decreasing the risk for collection bias. One limitation of our study was that it comprised only 1 sample per individual, precluding analysis of progression of protein markers on the individual level. Although, to our knowledge, this study is the only analysis to date of presymptomatic AAV individuals, the number of included samples in biobanks are small by default. Because this is an exploratory analysis of presymptomatic markers, further studies that target the most interesting proteins and pathway analyses identified in this study are encouraged. Furthermore, since the Olink platform is designed to detect inflammatory proteins in general, it is plausible that there may be additional AAV-related proteins not included in the platform that are yet to be uncovered (12). The proteins and corresponding pathways involved in AAV were different from those described in our previously presented findings in individuals with presymptomatic RA (12). However, it should be emphasized that the selection of proteins included in the panels as well as the analysis methods were different (12).

There were significant differences between the original cohort and the replication cohort that can explain some of the disparate results in the analyses. Although a few proteins were found to be early biomarkers for AAV in both cohorts, more importantly, we found that identical pathways were involved in disease progression in both of the cohorts. Thus, we conclude that a panel of biomarkers that are involved in pathways, rather than a few separate biomarkers, more preferentially identify the onset of AAV and characterize disease progression. The cytokine profiles related to PR3-ANCA positivity and MPO-ANCA positivity were almost mutually exclusive, which suggests that there are different disease-related mechanisms for these 2 groups. Taking these results together, we believe that we have, at least to some extent, identified molecular fingerprints for PR3-ANCA+ and MPO-ANCA+ vasculitis. AAV is a complex disease with individual variations in symptom onset and disease progression, which poses a challenge for identifying predisposing molecular mechanisms. Based on this study using samples from presymptomatic individuals who were later diagnosed as having AAV, we suggest that a combination of early mild immunodeficiency and overlapping, albeit distinct, molecular pathways in PR3-ANCA+ vasculitis and MPO-ANCA+ vasculitis could clarify the basis of AAV, and explain the substantial differences in the pathogenesis of these two AAV diseases.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Brink had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Brink, Berglin, Rantapää-Dahlqvist.

Acquisition of data. Brink, Berglin, Mohammad, Gjertsson, Rantapää-Dahlqvist.

Analysis and interpretation of data. Brink, Lundquist, Alexeyenko, Lejon, Rantapää-Dahlqvist.

ADDITIONAL DISCLOSURES

Author Alexeyenko is an employee of Evi-networks.

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