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Clinica Chimica Acta 554 (2024) 117786

Contents lists available at ScienceDirect

Clinica Chimica Acta

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

Targeted proteomics and metabolomics for biomarker discovery in abdominal aortic aneurysm and post-EVAR sac volume

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ARTICLE INFO

Keywords:
Abdominal aortic aneurysm
EVAR
Volume
Biomarkers
Multi-omics

ABSTRACT

Background and aims: Abdominal aortic aneurysm (AAA) patients undergo uniform surveillance programs both leading up to, and following surgery. Circulating biomarkers could play a pivotal role in individualizing surveillance. We applied a multi-omics approach to identify relevant biomarkers and gain pathophysiological insights.
Materials and methods: In this cross-sectional study, 108 AAA patients and 200 post-endovascular aneurysm repair (post-EVAR) patients were separately investigated. We performed partial least squares regression and ingenuity pathway analysis on circulating concentrations of 96 proteins (92 Olink Cardiovascular-III panel, 4 ELISA-assays) and 199 metabolites (measured by LC-TQMS), and their associations with CT-based AAA/sac volume.
Results: The median (25th-75th percentile) maximal diameter was 50.0 mm (46.0, 53.0) in the AAA group, and 55.4 mm (45.0, 64.2) in the post-EVAR group. Correcting for clinical characteristics in AAA patients, the aneurysm volume Z-score differed 0.068 (95 %CI: (0.042, 0.093)), 0.066 (0.047, 0.085) and −0.051 (-0.064, -0.038) per Z-score valine, leucine and uPA, respectively. After correcting for clinical characteristics and orthogonalization in the post-EVAR group, the sac volume Z-score differed 0.049 (0.034, 0.063) per Z-score TIMP-4, −0.050 (-0.064, -0.037) per Z-score LDL-receptor, −0.051 (-0.062, -0.040) per Z-score 1-OG/2-OG and −0.056 (-0.066, -0.045) per Z-score 1-LG/2-LG.
Conclusions: The branched-chain amino acids and uPA were related to AAA volume. For post-EVAR patients, LDL-receptor, monoacylglycerols and TIMP-4 are potential biomarkers for sac volume. Additionally, distinct markers for sac change were identified.

Abbreviations: AAA, abdominal aortic aneurysm; BCAAs, branched-chain amino acids; CI, confidence interval; CT, computed tomography; DNA, deoxyribonucleic acid; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; EVAR, endovascular aneurysm repair; IPA, ingenuity pathway analysis; LF, latent factor; NO, nitric oxide; ROS, reactive oxygen species; sPLS, sparse partial least squares.

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https://doi.org/10.1016/j.cca.2024.117786
Received 10 May 2023; Received in revised form 27 December 2023; Accepted 14 January 2024
Available online 20 January 2024

1. Introduction

An abdominal aortic aneurysm (AAA) is a degenerative progressive dilatation of the abdominal aorta, with rupture as most worrisome complication. Despite optimal treatment, patients with an AAA rupture have a perioperative mortality of 26.8 %.[1] Therefore, timely detection and timely intervention are key in the management of AAA patients. Uniform imaging protocols remain the only, and stagnant, option for surveillance in AAA patients.[1,2] Measurement of circulating biomarkers may contribute to more appropriate personalized surveillance programs.[1,3,4].

When the AAA reaches a certain size or growth speed, an endovascular or open intervention is indicated. After endovascular aneurysm repair (EVAR), follow-up remains warranted due to a persisting, albeit notably lower, risk of rupture.[5] Aneurysm sac growth after EVAR is associated with rupture risks as it implicates continued stress on the AAA wall.[5–8] Similar to the preoperative surveillance, biomarkers corresponding to postoperative sac size and behaviour could contribute to risk estimation.[9].

At both stages of the disease, previous studies have attempted to identify biomarkers of progression through genetic profiling or limited sets of pre-selected proteins.[3,4,10,11] The main limitation of genetic variants is their inability to reflect adaptive processes of the body to the environment, and knowledge-based pre-selected markers are unlikely to lead to novel pathophysiological insights. The advantage of metabolomics lies in its more downstream position from DNA to phenotype. It can provide insight into disease mechanisms, while capturing most gene-environment interactions. Combining metabolite information with more upstream protein data could allow the discovery of mechanistically correlated biomarkers, which offers the twofold benefit of multiple biomarker discovery and substantiated evidence for their pathophysiological role. This motivated the multi-omics interest across the biomedical field. However, no studies have been published on multi-omics concerning AAA or post-EVAR surveillance.

Therefore, we implemented a multi-omics approach to identify potential circulating biomarkers in two separate AAA populations, namely AAA patients under surveillance and patients post-EVAR. A total of 96 proteins and 199 metabolites, as measured in blood, were related to CT-based AAA or sac volume.

2. Materials and methods

2.1. Study design

In the current cross-sectional analysis, two study groups (AAA & post-EVAR) were assembled from the ongoing prospective observational BIOMArCS-AAA study, carried out since March 2017 in two hospitals in the Netherlands. The AAA group consisted of patients with an AAA $>$ 40 mm, who are under periodic surveillance. The post-EVAR group includes patients with a history of EVAR. A full description of the in- and exclusion criteria and sample size calculation are provided in the supplements (supplementary text and Figure S1). Blood samples were collected without constraints regarding fasting or time of sampling, and processed and stored at −80 °C until analysis. A CT scan was performed for study purposes, unless available through standard care. The study was approved by the medical ethics committee Erasmus MC (MEC-2017–019) and conducted in accordance with the Declaration of Helsinki. All patients provided written informed consent. The study has been registered in ClinicalTrials.gov (NCT03703947).

2.2. CT measurements

CT measurements additional to the standard medical care report were obtained through 3Mensio Vascular software (Medical Imaging B. V., Bilthoven, The Netherlands). These measurements are based on the semi-automatically generated centre lumen line reconstruction.[12] The volume was measured between 10 mm distal to the lowermost renal artery and 10 mm above the aortic bifurcation and included lumen, intraluminal thrombus, calcifications and aortic wall; a previously validated method that established a low intra- and interobserver variability.[12,13].

2.3. Biomarker measurements

All biomarkers were measured in EDTA plasma, with a detailed description in the supplementary text. The protein measurements were performed via the Olink Cardiovascular III proximity extension assay (Olink Proteomics AB, Uppsala, Sweden), consisting of 92 proteins with established or expected associations to cardiovascular disease. Biomarker values are expressed as $\log_2$-transformed relative protein abundance across all samples. Four internal controls and two external controls were added to each of the six assays.[14] The intra- and interassay coefficient of variation were 6 % and 12 %, respectively. Four additional circulating biomarkers were measured with commercially available ELISA assays (MyBiosource Inc., San Diego, California, USA). Fibrillin-1, homocysteine, microfibrillar associated protein 4 and type 3 procollagen N-terminal propeptide were chosen based on their importance in hereditary aortic disease.[15–19] All ELISA kits had an interassay coefficient of variation of $<$ 12 %. The combination of the Olink Cardiovascular III panel and ELISA assays are further referred to as the proteomics panel.

The metabolomics panel consisted of 199 metabolites with known or expected associations with aortic disease and were analysed by liquid chromatography triple quadrupole mass spectrometry (LC-TQMS).[20–23] The panel consisted of two platforms including 146 signalling lipids and 53 amines. The analysis was performed in five batches for each platform, quality control and blank samples were included in each batch. The data was presented as relative metabolite abundance. The monoacylglycerols subspecies for the attachment of the lipid on the glycerol residue could not be fully baseline separated, furthermore, acyl migration might occur during the metabolomics analysis.[24] Therefore, they are described according to the following example: oleoylglycerol (1-OG/2-OG).

One protein and 17 metabolites presented missingness in more than 20 % of the samples and were excluded from the analysis. Z-scores were used for all biomarkers.

2.4. Outcome

The primary outcome in both groups was CT-based AAA/sac volume (mL). As a sensitivity analysis in the post-EVAR group, change in diameter (mm) between the preoperative scan and the cross-sectional measurement was examined, taking their time interval into account.

2.5. Statistical analysis

Categorical variables are presented as counts (percentages) and continuous variables either by median (25th-75th percentile) or by mean (standard deviation), according to variable distribution. Normality was confirmed by Shapiro-Wilk test. AAA/sac volume, and ELISA and metabolite biomarker levels, before further corrections for confounders, were $\log_2$-transformed to approach a normal distribution. Samples were defined as outliers if more than 25 % of the biomarkers in one omics panel had an absolute Z-score $>$ 3.

Our final models (multi-block sparse partial least squares regression (sPLS)) do not allow for inclusion of categorical variables. Therefore, correction for all clinical characteristics within this model was not possible. To address this issue, an outcome value (i.e. aneurysm (sac) volume or sac growth) independent of confounding factors was first calculated. A weighted least squares regression was performed, wherein the outcome was predicted by age, sex, body surface area, arterial hypertension, diabetes mellitus, smoking habit, coronary artery disease,

congestive heart failure, peripheral arterial occlusive disease, family history of AAA, antiplatelet medication use and lipid lowering medication use. Additionally, the time interval between surgery and the current measurements was included in the post-EVAR group. Residuals from this regression represent outcome variation independent of these variables; these residuals are hereafter referred to as corrected outcome.[25].

A first exploration of the data was done by principle component analysis (PCA) of both the proteomics and metabolomics panels in both study groups to investigate whether the most important biomarker variation also reflected differences in outcome (AAA or sac volume), before continuing with a supervised method. In both groups (AAA and post-EVAR), a randomly chosen 20 % of the patients were selected as a test set (i.e. holdout set), and only used for evaluation of the final model's performance. The training-test partition was performed per outcome quartile to assure a representative test set. All analyses described below, as well as the results presented, were performed in the remaining 80 % of patients, wherein repeated (n = 10) 10-fold cross-validation was used to optimize model parameters (90 % training, 10 % validation). The parameters optimized were the number of latent factors and the degree of sparsity on each block. Model performance was evaluated using the cumulated Q^2 (Q^2_{cum}) in the training set and the out-of-sample R^2(R^2_{oos}) test set, where an R^2_{oos} of zero indicates identical performance of the model in the test and training set.

Relevant biomarkers were preselected by a p-value < 0.2 in a regression of biomarker levels with the uncorrected outcome. Using this preselected set of biomarkers, a multi-block sPLS model was constructed in each study group. By projecting the biomarkers to latent factors (LF) and optimizing this projection by maximizing the covariance with the outcome, this method can adequately summarize highly correlated biomarker information while capturing the relationship with the outcome variable(s).[26] Model optimization with Q^2 statistic (cut-off > 0)[26,27], and confidence interval and p-value calculation were performed through repeated (n = 10) 10-fold cross-validation.[28,29] Additionally, a second multi-block sPLS model with the same parameters was constructed, now using the corrected outcome. Lastly, for each model, an orthogonal PLS (OPLS) variant was constructed by including all biomarkers with a significant loading on the multi-block sPLS model as predictive component(s) and one or more orthogonal factors. The selection of latent factors was again guided by Q^2 statistic and by permutation testing.

Exploring similar and opposite loadings of biomarkers on each latent factor might implicate pathophysiological connections. Thus, subsequently, the biomarkers selected by the corrected multi-block sPLS and OPLS models were used as input for a pathway analysis using Ingenuity Pathway Analysis (IPA) (QIAGEN Inc., https://digitalinsights.qiagen.com/IPA). For IPA, an absolute regression coefficient over 0.025 and a p-value below 0.05 were used as the thresholds for variable selection. IPA compares the observed abundant markers against a reference set to determine which pathways are up- or downregulated. To account for our targeted selection of cardiovascular biomarkers, this selection was defined as the reference set, rather than the full IPA knowledge base. All proteins were mapped, while 34 metabolites (mainly lipids) remained unmapped.

The analyses were performed in R (version 4.0.3), using the mixOmics[30] and ropls[31] packages.

3. Results

3.1. Patients and samples

A total of 316 participants were eligible for biomarker analysis. Four samples were excluded due to errors in the aliquoting process of the metabolomics analysis. Four patients with outlier samples were excluded from the analysis (supplementary Figure S2). This resulted in a final study population of 108 AAA patients, with a mean age of 71.7 (SD 7.1) and including 90.7 % men, and 200 post-EVAR patients, who were on average 74.3 (SD 7.6) years old and included 92.5 % men. In this post-EVAR group, the median time between EVAR-procedure and blood sampling was 38.36 months, 25th-75th percentile (9.15, 68.52). Other patient characteristics are displayed in Table 1.

A PCA was performed for each omics panel in both groups. As portrayed in Fig. 1, most of the biomarker variation did not relate to differences in aneurysm/sac volumes, thus warranting further exploration with a supervised PLS model in order to investigate biomarker variation specifically associated with AAA/sac volume.

3.2. AAA group

The optimal multi-block sPLS model in the AAA group was

Table 1
Clinical characteristics of the included patients in both study arms.

	AAA n = 108	Post-EVAR n = 200
Age	71.7 (7.1)	74.3 (7.6)
Male	98 (90.7)	185 (92.5)
BSA (m^2)	2.0 (0.2)	2.0 (0.2)
Medical history		
Smoking		
Current	33 (30.6)	50 (25.0)
Former	72 (66.7)	140 (70.0)
Never	3 (2.8)	10 (5.0)
Hypertension	76 (70.4)	161 (80.5)
Diabetes mellitus	27 (25.0)	35 (17.5)
Coronary heart disease[a]	41 (38.0)	68 (34.0)
Heart Failure	9 (8.3)	13 (6.5)
Cerebrovascular disease	16 (14.8)	38 (19.0)
Peripheral arterial occlusive disease	25 (23.1)	30 (15.0)
Medication use		
Antiplatelet	75 (69.4)	156 (78.0)
NOAC	6 (5.6)	9 (4.5)
Coumarin	12 (11.1)	25 (12.5)
Beta-blocker	50 (46.3)	114 (57.0)
ACE inhibitor	31 (28.7)	60 (30.0)
Angiotensin II receptor antagonist	32 (29.6)	58 (29.0)
Thiazide diuretic	20 (18.5)	41 (20.5)
Lipid-lowering drug	84 (77.8)	166 (83.0)
Aneurysm characteristics		
Anatomical classification AAA - juxtarenal	6 (5.6)	21 (10.5)
Maximal diameter AAA/sac (mm)	50.0 [46.0, 53.0]	55.4 [45.0, 64.2]
AAA/sac volume (mL)	105.4 [93.0, 129.0]	146.8 [104.2, 209.9]
Iliac aneurysm	18 (16.7)	56 (28.0)
Familial AAA[b]	24 (22.2)	50 (25.0)
Surgical characteristics		
Type of surgery		
Primary EVAR		168 (84.0)
Reintervention		32 (16.0)
AAA rupture at admission		13 (6.5)
ASA classification		
ASA I or II		80 (40.0)
ASA III, IV or IV		98 (49.0)
Unknown		22 (11.0)
Interval EVAR - blood sample collection (months)		38.36 [9.15, 68.52]
Interval EVAR – CT (months)		38.08 [8.80, 67.59]

Continuous variables are presented as mean (standard deviation) when normally distributed, otherwise median (25th – 75th percentile) was used. Categorical variables are expressed as count (percentage).
AAA: abdominal aortic aneurysm, ACE: angiotensin converting enzyme, ASA classification: Physical Status Classification System by the American Society of Anesthesiologists, BSA: body surface area, CT: computed tomography, EVAR: Endovascular Aneurysm Repair, NOAC: non-vitamin K oral anticoagulant.
[a] Coronary heart disease: history of myocardial infarction and/or percutaneous coronary intervention or coronary artery bypass grafting.
[b] Familial AAA: defined as at least one first-degree relative affected with aortic aneurysm, based on anamnestic information

constructed with one LF (Q^2_{cum} = 0.094, R^2_{oos} = -0.141). Of the 89 preselected markers, 26 proteins and 4 metabolites were significantly related to AAA volume within the PLS model. Secondly, a model with the same parameters was constructed, now correcting aneurysm volume for possible confounders, with a similar performance to that of the uncorrected model (Q^2_{cum} = 0.081, R^2_{oos} = -0.156). Within this PLS model corrected for clinical characteristics, 27 proteins and 4 metabolites were now significantly associated with AAA volume. To assist with further interpretation, both the uncorrected and corrected model were orthogonalized. However, both orthogonolized models were overfit (uncorrected Q^2_{cum} = -0.001, R^2_{oos} = -0.244 & corrected Q^2_{cum} = -0.011, R^2_{oos} = -0.311). Therefore, the results of these OPLS models are only presented in the supplements and should be interpreted with care, as they are not generalizable to AAA patients outside our study population.

In the uncorrected multi-block sPLS model, the strongest positive association with aneurysm volume was observed for the branched-chain amino acids (BCAAs). Per unit difference in biomarker Z-score the AAA volume Z-score differed 0.093, 95 % CI (0.070, 0.117) for valine and 0.085, 95 % CI (0.070, 0.117) for leucine. The largest negative differences in AAA volume Z-score per unit increase in biomarker Z-score were found for urokinase-type plasminogen activator (uPA) (-0.060, 95 % CI (-0.070, −0.050)) and bleomycin hydrolase (-0.051, 95 % CI (-0.057, −0.044)). Other strong associations were found for lysine, isoleucine and JAMA (Fig. 2 & supplementary Table S1).

Similar results were found when correcting aneurysm volume for clinical characteristics (Fig. 2, supplementary Table S1), although all biomarker associations with the outcome became notably weaker. The strongest associations were still observed for valine and leucine; for each unit difference in biomarker Z-score, the AAA volume Z-score respectively differed with 0.068, 95 % CI (0.042, 0.093) and 0.066 (0.047, 0.085).

Using these biomarkers, IPA was able to identify multiple possible canonical pathways involved, which did not remain significant after adjusting for multiple testing (Table 2).

3.3. Post-EVAR group

In the post-EVAR group, the optimal multi-block sparse PLS model was constructed using two LF (Q^2_{cum} = 0.093, R^2_{oos} = 0.048). Of the 95 preselected biomarkers, 31 proteins and 41 metabolites were significantly related to sac volume within the PLS model. Secondly, a model with the same parameters was constructed, now using the corrected outcome, resulted in a model with a slightly lower performance (Q^2_{cum} = 0.059, R^2_{oos} = 0.019). Within the corrected model, 31 proteins and 43 metabolites were identified as significantly associated with sac volume. Both models were orthonogalized to assist with the interpretation (uncorrected Q^2_{cum} = 0.124, R^2_{oos} = 0.091 & corrected Q^2_{cum} = 0.086, R^2_{oos} = 0.036).

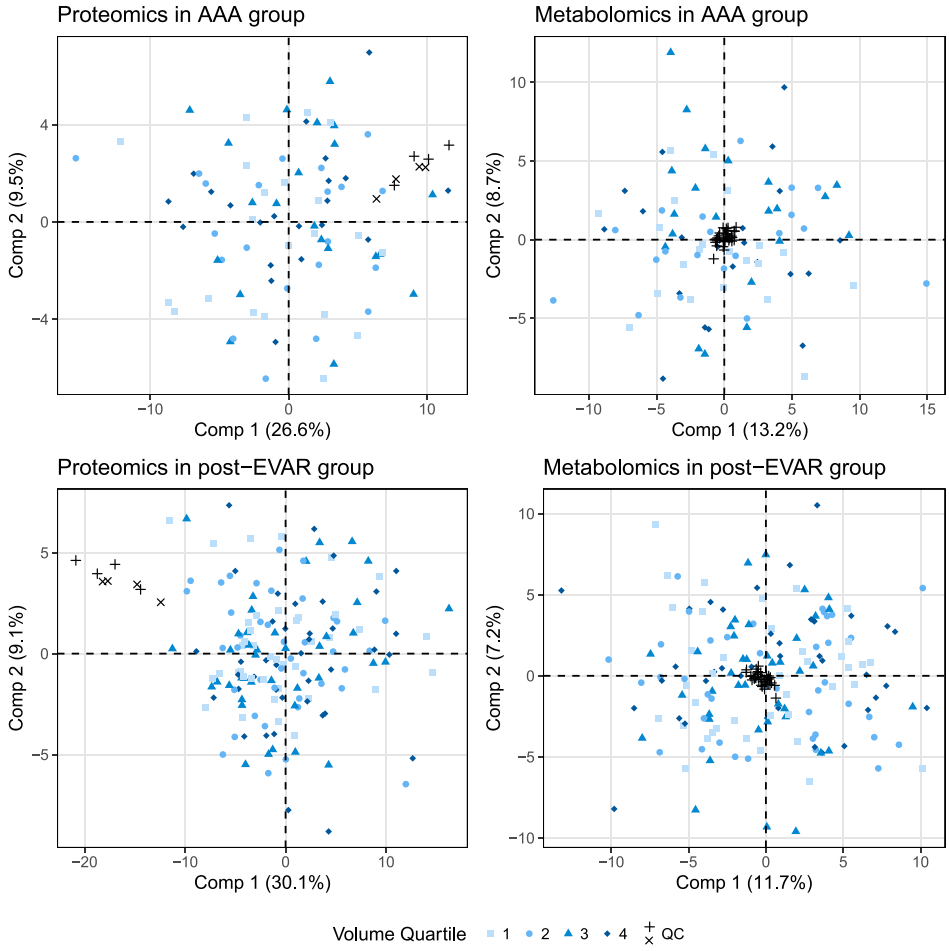

Fig. 1. Principal component analysis score plots. A principle component analysis was performed for each –omics panel in both groups. The scores of each patient on the first two components is summarized in this figure. The percentage of explained biomarker variance by each component is given in brackets. The shape and colour of each dot correspond to the aneurysm (sac) volume quartile of that patient. The quality control samples are represented as black plus sings. In the proteomics analysis, the second quality control samples were denoted by the black 'x' signs. The proteomics analyses for our study were performed in parallel with another study, which also resulted in combined quality control samples. This was not the case for the metabolomics analyses, where the quality control samples represent pooled samples only from our study.

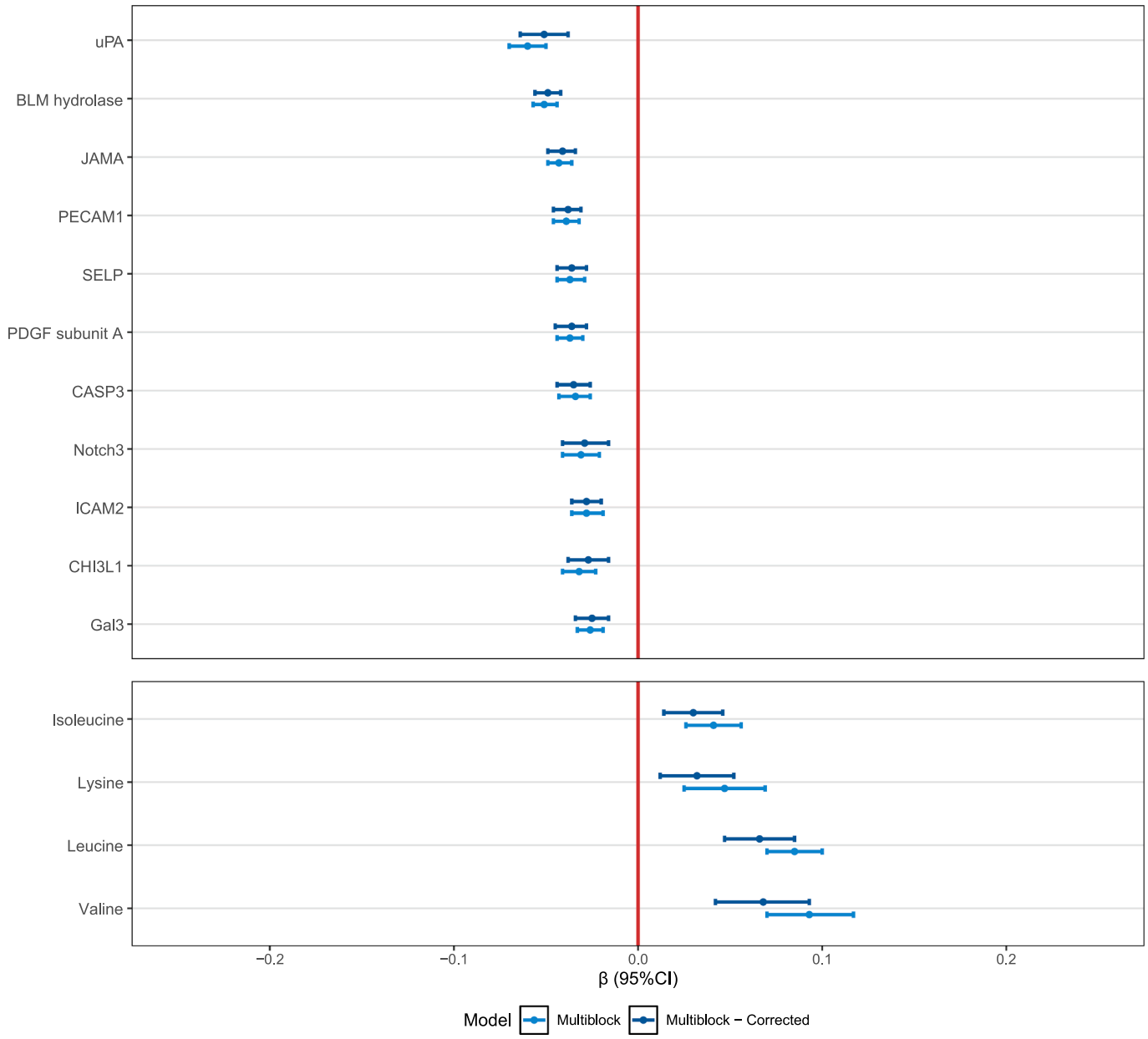

Fig. 2. Regression coefficients on aneurysm volume in the AAA group. In this figure, a summary of the results of the uncorrected (light) and corrected (dark) PLS regression in AAA patients are portrayed. The multi-block models are blue, the orthogonal models are not presented. The depicted biomarkers are the most influential proteins and metabolites that were selected by the corrected multi-block PLS regression model. The results are presented as mean effect (β) with 95 % confidence interval (CI) of a unit increase of the biomarker Z-score on AAA volume. The volume is expressed as standard deviation of $\log_2$ mL (Z-score). Marker abbreviations can be found in supplementary Table S4.

In the uncorrected multi-block sPLS model, the largest positive differences in sac volume Z-score per unit increase in biomarker Z-score were found for insulin-like growth factor-binding protein 2 (IGFBP-2) (0.064, 95 % CI (0.052, 0.075), LF 1) and metalloproteinase inhibitor 4 (TIMP-4) (0.055, 95 % CI (0.043, 0.067), LF 1). The strongest negative association was observed for LDL-receptor, for each unit increase in LDL-receptor Z-score, the sac volume Z-score differed −0.196, 95 % CI (-0.237, −0.155), LF 2. Other strong negative associations were found for the monoacylglycerols (MAGs) 1-OG/2-OG, 1-LG/2-LG and 1-AG/2-AG, as well as the valine and LPE 18:0 (Fig. 3, Supplementary Table S2). Additionally, asymmetric dimethylarginine (ADMA), homo-arginine, leucine, 15S-HETrE, NT-proBNP, tryptophan, OPG, homocysteine and cyclic LPA 18:1 were positively or negatively associated with the post-EVAR sac volume (Fig. 3, Supplementary Table S2). After orthogonalization, the strongest negative associations with sac volume, although notably weaker, were still observed for LDL-receptor (-0.050, 95 % CI (-0.062, −0.039)), 1-OG/2-OG (-0.051, 95 % CI (-0.062, −0.041)) and 1-LG/2-LG (-0.051, 95 % CI (-0.063, −0.040)), alongside 1-AG/2-AG and the BCAAs. The strongest positive association, again weaker, was observed for TIMP-4 (0.049, 95 % CI (0.037, 0.061)), alongside OPG. While all other associations were considerably weaker, LPE 14:0 and TFPI were now more prominently associated with the outcome (Fig. 3, Supplementary Table S2).

When correcting sac volume for clinical characteristics, the biomarker profile associated with sac volume was similar to the uncorrected analysis, although all associations were somewhat weaker (Fig. 3, Supplementary Table S2). The most prominent association between biomarker and sac volume was still observed for LDL-receptor (-0.157, 95 % CI (-0.197, −0.117), LF 2). Correspondingly, correcting sac volume for clinical variables barely changed the associations found

Table 2
Canonical pathways related to aneurysm volume in AAA patients.

Pathway	Multi-block		Molecules
	z-score	Adj. p-value	
Branched-chain amino acid catabolism	–	0.158	L-isoleucine, L-leucine, L-valine
Response to elevated platelet cytosolic Ca2+	–	0.279	PDGFA, PECAM1, SELP
IL-13 Signaling Pathway	–	0.279	CHI3L1, PDGFA
DHCR24 Signaling Pathway	–	0.279	CASP3, PDGFA
Oncostatin M Signaling	–	0.279	CHI3L1, PLAU
Sphingosine-1-phosphate Signaling	–	0.279	CASP3, PDGFA
PAK Signaling	–	0.279	CASP3, PDGFA
Cell surface interactions at the vascular wall	–	0.282	F11R, PECAM1, SELP
Integrin cell surface interactions	–	0.282	F11R, ICAM2, PECAM1
Role of PKR in Interferon Induction and Antiviral Response	–	0.282	CASP3, PDGFA
Tryptophan catabolism	–	0.282	L-isoleucine, L-leucine, L-valine
tRNA Charging	−2,000	0.282	L-isoleucine, L-leucine, L-lysine, L-valine

Biomarkers with a significant relationship to AAA volume according to the corrected multi-block sPLS and orthogonal PLS model were used in an ingenuity pathway analysis (IPA). Biomarkers with strong associations with the outcome ($|\beta| > 0.025$ and p-value < 0.05) were included in the IPA. All significant pathways before multiple testing correction are included in the table. As the orthogonal model was overfit in the AAA group, these results are not presented. Results of the IPA are presented as the activation z-score, a measure of up- or downregulation of the canonical pathway, and Benjamini-Hochberg adjusted p-value.
Abbreviations used: AAA: abdominal aortic aneurysm, Adj. p-value: Benjamini-Hochberg adjusted p-value, CASP3: caspase-3, CHI3LI: chitinase-3-like protein 1, DHCR24: 24-dehydrocholesterol reductase, F11R: junctional adhesion molecule A (JAMA), ICAM2: intercellular adhesion molecule 2, IL: interleukin, PAK: p21-activated kinase, PDGFA: platelet-derived growth factor subunit A, PECAM1: platelet endothelial cell adhesion molecule, PKR: protein kinase RNA, PLAU: urokinase-type plasminogen activator (uPA), RNA: ribonucleic acid, SELP: p-selectin.

by the uncorrected OPLS model. However, cyclic LPA 18:0, 11-HDoHE, bleomycin hydrolase, platelet glycoprotein VI (GP6) and JAMA now showed more prominently positive association with sac volume (Fig. 3, Supplementary Table S2).

As a sensitivity analysis of our results in the post-EVAR group, the analysis was repeated, now using the biomarker levels to predict sac growth instead of sac volume, using one LF ($Q^2_{cum} = 0.058$, $R^2_{oos} = 0.044$). Of the 72 preselected biomarkers, 4 proteins and 21 metabolites were significantly loaded by the model. Next, a model with the same parameters was constructed, now correcting sac growth for clinical variables, resulting in a model with a similar performance ($Q^2_{cum} = 0.046$, $R^2_{oos} = 0.002$). From the same set of preselected biomarkers, 2 proteins and 22 metabolites were significantly associated with sac growth after correction for clinical characteristics. Both models were orthogonalized (uncorrected $Q^2_{cum} = 0.093$, $R^2_{oos} = -0.057$ and corrected $Q^2_{cum} = 0.077$, $R^2_{oos} = -0.163$).

Similar to sac volume, the uncorrected multi-block sPLS model for sac growth identified IGFBP-2, TIMP-4, the BCAAs and LDL-receptor as important biomarkers. Additionally, 2-aminoadipic acid (2-AAA) showed a negative relationship to sac growth (Fig. 3 & supplementary Table s3). After orthogonalizing the model, we observed more prominent negative association between the previously identified BCAAs, LDL-receptor and 2-AAA, while the associations between IGFBP-2 and TIMP-4, and sac growth were shrank. Similar to the sac volume model, the MAGs, homo-arginine and tryptophan showed important negative associations. More interestingly, contrary to the sac volume model, lithocholic acid 3-sulfate (LCA-3S), 11-HDoHE and oleoylethanolamide demonstrated strong positive associations, and LPE 14:0 a strong negative association with sac growth. Per unit change in the biomarker Z-score, the diameter change Z-score differed 0.068, 95 % CI (0.051, 0.086) for LCA-3S, 0.059, 95 % CI (0.045, 0.073) for 11-HDoHE, 0.053, 95 % CI (0.039, 0.066) for oleoylethanolamide, and −0.052, 95 % CI (-0.072, −0.031) for LPE 14:0.

After correcting for clinical characteristics, a similar biomarker pattern was associated with sac growth as before the correction, although all associations were, again, weaker. This applied to the orthogonalized model as well, with two exceptions. LDL-receptor was no longer included in the corrected OPLS model, while palmitoylethanolamide was included in the corrected, but not the uncorrected, model. Both are attributable to variable selection, rather than for biological reasons (Fig. 3 & supplementary Table s3).

Multiple IPA-pathways were associated with sac volume, but none remained significant after correcting for multiple testing. Although several pathways could be related to post-EVAR sac growth, only branched-chain amino acid catabolism remained significant after adjusting for multiple testing (Table 3A & 3B).

4. Discussion

In AAA patients under periodic surveillance, uPA and the BCAAs strongly correlated with aneurysm volume, regardless of clinical characteristics. Analogously, in the post-EVAR group, LDL-receptor and the MAGs were identified as promising biomarkers for sac volume, alongside a number of other potential markers, regardless of clinical factors. Lastly, alongside LDL-receptor and the MAGs, the BCAAs, oleoyl- and palmitoylethanolamide, lithocholic acid 3-sulfate, 11-HDoHE, LPE 14:0 and 2-aminoadipic acid might hold promise as markers for sac growth following EVAR. Branched-chain amino acid catabolism might play an important role in post-EVAR sac changes.

The current study offers several advantages over previous literature. The use of volume instead of maximal diameter allowed for a more comprehensive approach to the disease and was more sensitive to differences in size.[13] Secondly, to the best of our knowledge this is the first study combining proteomic and metabolomics data in patients with an AAA, and omics approaches have not yet been used to investigate sac behaviour after EVAR. Importantly, identifying possible shared pathophysiological processes through a multi-omics approach substantiated our findings for individual markers. Lastly, the current methodology further allowed us to form hypotheses concerning the underlying mechanisms of disease.

4.1. AAA group

In the AAA group, OPLS models could not be constructed. A possible explanation might be that the biomarker covariance unrelated to the outcome is fairly limited due to the targeted –omics approach, and forced orthogonalization thus removes predictive information. Therefore, we interpret only the results of the multi-block models, but have to keep in mind that some of the identified biomarkers might be more related to other predictive markers rather than to the outcome itself.

The essential BCAAs (leucine, isoleucine and valine) have been linked diverse physiological functions, including cell growth and autophagy regulation. They are mentioned in relation to several cardiovascular diseases, among which their association with insulin resistance is the most substantiated.[32] Interestingly, one recent publication links increased catabolism of this group to platelet activation and arterial thrombosis.[33].

The relationship between AAA size and uPA, one of the plasminogen activators, is ambiguous.[34–38] Interestingly, the murine study by Uchida et al. related uPA deficiency to AAA ruptures, which in turn might reflect accelerated or intensified disease progression.[38] We correspondingly found a negative association between circulating uPA

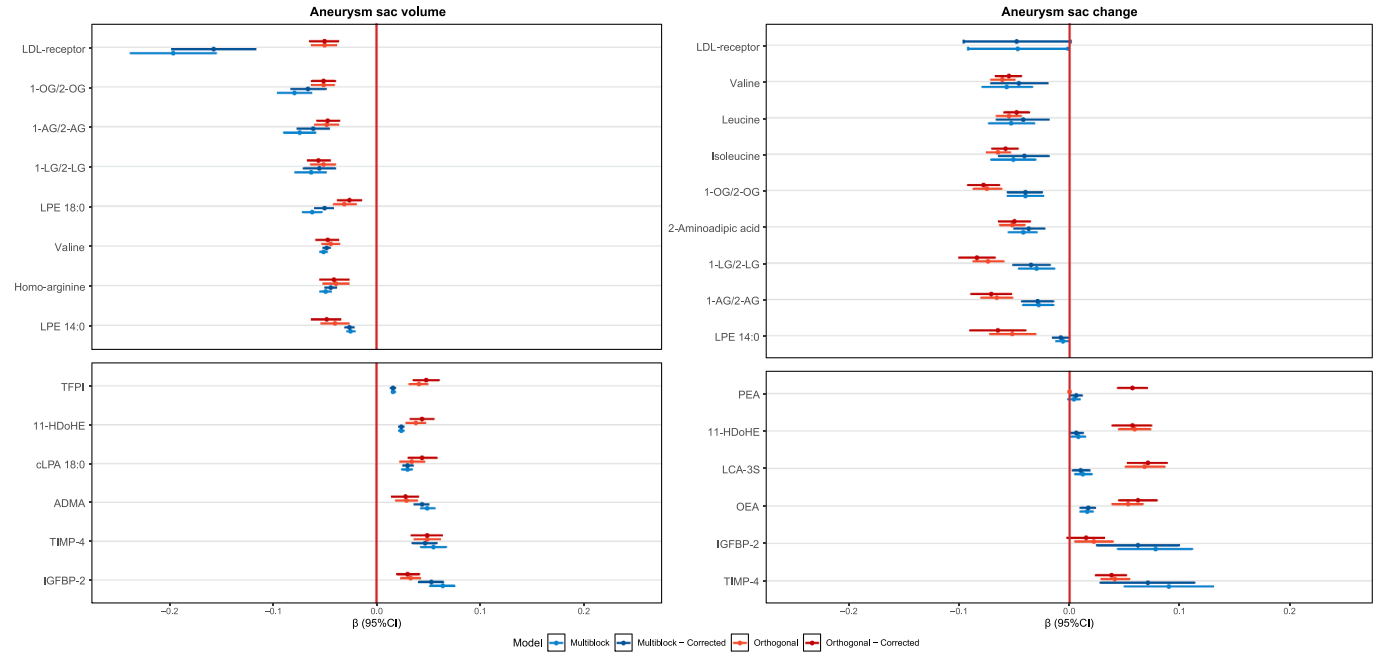

Fig. 3. Regression coefficients on sac volume and sac change in the post-EVAR group. In this figure, a summary of the results of the uncorrected (light) and corrected (dark) PLS regression in patients following EVAR are portrayed. The multi-block models are blue, the orthogonal models are red. The depicted biomarkers are the most influential proteins and metabolites that were selected by the corrected multi-block or orthogonal PLS regression model. The results are presented as mean effect (β) with 95 % confidence interval (CI) of a unit increase of the biomarker Z-score on sac volume. The volume is expressed as standard deviation of $\log_2$ mL (Z-score). Marker abbreviations can be found in supplementary Table S4.

and AAA volume, warranting further investigation into this biomarker.

Each latent factor represents a distinct pattern of association with the outcome, and markers are loaded on these factors according to their importance in such a pattern. The latent factor in the AAA group was mainly explained by the inverse associations of uPA and bleomycin hydrolase, alongside JAMA, PECAM-1, PDGF-A, P-selectin, and caspase-3, while the branched-chain amino acids and lysine were positively associated. As such, this factor might represent an inverse relationship between AAA volume and the intertwined activation of thrombocytes and the provoked monocyte response (supplementary text). Pathway analysis, however, could not corroborate these pathways, which might be related to the targeted –omics approach and/or the lack of extensive background knowledge on metabolomics, as reflected by the number of unmapped molecules.

Aneurysm size remains the best predictor for growth, risk of rupture and survival.[39] Patients with an AAA diameter < 40 mm will unlikely be considered for surgery during their lifetime, while most patients with AAA's > 45 mm will at some point become eligible for intervention. [2,40,41] Thus, patients with a larger AAA might originate from a subgroup particularly susceptible to aneurysm growth. Interpreting our findings of increased BCAAs and decreased uPA levels, and their related markers, with increasing aneurysm volume accordingly, might indicate that patients with a reduced platelet activation and immune response are more susceptible to growth. A different assumption could be that patients with a larger AAA volume represent advanced disease in a homogenous AAA population. In this case, our findings might indicate that inflammation, possibly guided by the interactions between intraluminal thrombus and vessel wall, initiates the disease. As the aneurysmal destruction progresses, the aortic wall becomes progressively acellular and less reactive, while fibrosis takes over. Both concepts align with the shifting paradigm of AAA pathology, which followed from the unsuccessful exploration of pharmaceutical strategies to limit abdominal aneurysms progression based on preclinical research.[42,43].

4.2. Post-EVAR group

LDL-receptor was one of biomarkers that strongly and inversely related to sac volume, even after correction for clinical factors, including

Table 3A
Canonical pathways related to sac volume in post-EVAR patients.

Pathway	Multi-block		Orthogonal		Molecules
	z-score	Adj. p-value	z-score	Adj. p-value	
Branched-chain amino acid catabolism	–	0.538	–	0.687	L-isoleucine, L-leucine, L-valine
Transport of bile salts and organic acids, metal ions and amine compounds	−1,633	0.681	–	–	L-isoleucine, L-leucine, L-lysine, L-tryptophan, L-valine, Taurolithocholate-3-sulfate
Tryptophan catabolism	−2,000	0.681	–	–	L-isoleucine, L-leucine, L-tryptophan, L-valine
Transport of inorganic cations/anions and amino acids/ oligopeptides	−2,449	0.681	–	–	Homo-arginine, L-isoleucine, L-leucine, L-lysine, L-tryptophan, L-valine
tRNA Charging	2,236	0.681	–	–	L-isoleucine, L-leucine, L-lysine, L-tryptophan, L-valine
Cell surface interactions at the vascular wall	–	–	1,342	0.687	EPCAM, F11R, GP6, PECAM1, SELP
Insulin processing	–	–	–	0.687	L-isoleucine, L-leucine, L-valine
Amino acids regulate mTORC1	–	–	–	0.687	L-arginine, L-lysine
Endothelin-1 Signaling	–	–	–	0.687	L-arginine, L-leucine

Table 3B
Canonical pathways related to sac growth in post-EVAR patients.

Pathway	Multi-block		Orthogonal		Molecules
	z-score	Adj. p-value	z-score	Adj. p-value	
Branched-chain amino acid catabolism	**–**	**0.012**	**–**	**0.085**	**L-isoleucine, L-leucine, L-valine**
Transport of inorganic cations/anions and amino acids/ oligopeptides	−2,236	0.058	−2.449	0.087	Homo-arginine, L-alanine*, L-isoleucine, L-leucine, L-lysine, L-valine
tRNA Charging	2,000	0.098	2.236	0.087	L-alanine*, L-isoleucine, L-leucine, L-lysine, L-valine
Tryptophan catabolism	–	0.098	−2.000	0.087	L-alanine*, L-isoleucine, L-leucine, L-valine
Transport of bile salts and organic acids, metal ions and amine compounds	−2,000	0.107	−1.633	0.136	L-alanine*, L-isoleucine, L-leucine, L-lysine, L-valine, Taurolithocholate-3-sulfate*
Lysine Degradation V	–		–	0.149	L-lysine, S-2-amino-hexanedioic acid
Lysine Degradation II	–		–	0.244	L-lysine, S-2-amino-hexanedioic acid

Biomarkers with a significant relationship to AAA volume according to the corrected multi-block sPLS and orthogonal PLS model were used in an ingenuity pathway analysis (IPA). Biomarkers with strong associations with the outcome ($|\beta| > 0.025$ and p-value < 0.05) were included in the IPA. All significant pathways before multiple testing correction are included in the table. If pathways were identified by both models, but the molecules of each model contributing to that pathway differed, the molecules originating only from the multi-block sPLS model are indicated by † and when originating only from the OPLS model by *.
Results of the IPA are presented as the activation z-score, a measure of up- or downregulation of the canonical pathway, and Benjamini-Hochberg adjusted p-value.
Abbreviations used: EVAR: endovascular aneurysm repair, Adj. p-value: Benjamini-Hochberg adjusted p-value, EPCAM: epithelial cell adhesion molecule, F11R: junctional adhesion molecule A (JAMA), GP6: platelet glycoprotein VI, mTORC1: mechanistic target of rapamycin complex 1, PECAM1: platelet endothelial cell adhesion molecule, RNA: ribonucleic acid, SELP: p-selectin.

lipid lowering therapy. This transmembrane protein is essential in the endocytosis of LDL-cholesterol.[44] Genome-wide association studies have associated variants of the LDL-receptor gene as an important contributor to the overall cardiovascular risk, including AAA incidence.[45] Despite its clear pathophysiological implication, the relationship between the circulating, shedded variant and the transmembrane LDLR protein have not been fully established.[46].

Alongside LDL-receptor, the MAGs also showed a strong negative association with post-EVAR sac volume, even after correction for clinical factors. The MAGs are produced through the hydrolysis of plasma triglycerides (TGs) by lipoprotein lipase (LPL).[47] Among the MAGs, most evidence exists concerning 2-arachidonoylglycerol (2-AG), which has been described to show affinity for the cannabinoid receptor 2 (CB-2). Through the CB-2 receptor, it functions as an atheroprotective substance by limiting macrophage accumulation and foam cell formation, increasing plaque stability and promoting IgM antibody production through B1 cells, which in turn limits LDL oxidation.[48,49] However, as other endocannabinoids were not associated with sac volume, and the other MAGs show a stronger association, the more likely conclusion would be that LPL or TGs explain their loading.[50] Both have shown to be associated with AAA presence in Mendelian randomization studies.[51,52].

The strong negative association observed for LPE 18:0 and the strong positive association observed for IGFBP-2 were drastically reduced after orthogonalization, indicating that these molecules were more associated with the other markers, rather than post-EVAR sac volume.

Although part of its association with post-EVAR sac volume was explained by clinical characteristics, TIMP-4 might be a promising marker in the follow-up after EVAR. The tissue inhibitor of the proteolytic metalloproteinases 2, 3 and 9, has been specifically implicated in AAA, whereas other TIMPs relate to different cardiovascular diseases.[53–55] Thus explaining why previous studies with other TIMPs could not conclude any associations with aneurysm or sac size.[39].

Lastly, the branched-chain amino acids, LPE 14:0 and TFPI might be markers of additional interest for post-EVAR sac volume, as they showed considerable association with this outcome after correction for clinical factors and orthogonalization. TFPI, a protein mainly known for its anticoagulant role, has previously been linked to AAA size in preoperative patients.[56] In post-EVAR patients, our results might indicate a decreased thrombin activation or increased endothelial activation, proportional to sac size. In-depth research into TFPI-isoforms might pinpoint specific pathways related to post-EVAR sac behaviour.[57].

The first latent factor was composed by negatively loading tryptophan, homo-arginine and the branched-chain amino acids (BCAAs), and by the positively loaded IGFBP2, TIMP-4, homocysteine and trimethyllysine. Oxidative stress might be a common attribute among these markers (supplementary text). As the second latent factor was mainly constructed by LDL-receptor, alongside and the monoacylglycerols, a disturbed lipid profile might form the base for this second component (supplementary text).

Contrary to the AAA group, inferences on the biological mechanisms and sac volume after EVAR are not as straightforward. Therefore, we have added a preliminary analysis of sac growth. The BCAAs and the MAGs maintained their importance in this sensitivity analysis after taking clinical factors and orthogonolization into account, further establishing their potential in post-EVAR surveillance. Additionally, LCA-3S, OEA and PEA, which have anti-inflammatory effects in the vasculature[58–61], and 11-HDoHE and LPE 14:0, which might have more pro-inflammatory roles[62], could be of future special interest to reflect sac dynamics after EVAR, and might indicate a stronger inflammatory response to be beneficial to achieve sac shrinkage. Altogether, these results should be considered with caution, as repeated measurements would be more appropriate to evaluate change over time. Future research, including the ongoing BIOMArCS-AAA cohort, will clarify the role of these molecules in sac growth and their potential in personalized follow up after EVAR.

4.3. Limitations

The cross-sectional framework of the current analyses limits causal inference and the ability to distinguish biomarkers of natural progression from those of susceptible subgroups. Additionally, the current associations with relative biomarker abundance might reflect absolute concentrations below the limits of quantitation, which would make such markers irrelevant for clinical practice. Further in-depth research on the currently proposed markers could elucidate their role in the pathophysiology of AAA and importance for stratified surveillance programs. Secondly, the absence of volume measurements pre-intervention in the post-EVAR group prohibited a three-dimensional approach to analyse sac behaviour in relationship to biomarkers. Thirdly, although there was no relationship between the sampling time and aneurysm/sac volume (supplementary Figure S3), maintaining no constraints regarding fasting or time of sampling might have increased the variation in biomarker levels. Therefore it is possible that only strong associations or associations with biomarkers less affected by fasting or circadian rhythm could have been observed. Fourthly, the goal of this study was to provide clues for potential biomarkers in AAA and post-EVAR surveillance. For this purpose, the performance of the PLS model was acceptable in the post-

EVAR group, but we must aware that the models in the AAA group might be slightly overfitted. Still, altogether a large portion of the variation in aneurysm (sac) size remains unexplained by these markers. Future research will need to clarify whether patient subgrouping or a broader –omics coverage might be required. Lastly, although the current model allowed us to make assumptions regarding pathophysiology, these mechanisms could not be corroborated as the integration of metabolomics into pathway analyses is still ongoing at this time.

5. Conclusion

The branched-chain amino acids and uPA in patients with AAA under surveillance, and LDL-receptor, the monoacylglycerols, and to a lesser extent the branched-chain amino acids, LPE 14:0, TFPI and TIMP-4 in patients following EVAR, were correlated with AAA/sac volume. In this post-EVAR group, the branched-chain amino acids and monoacylglycerols, alongside several other distinct markers might specifically reflect sac behaviour over time.

Clinical Trial Registration Information

Registered in ClinicalTrials.gov, number NCT03703947.

CRediT authorship contribution statement

Alexander Vanmaele: Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Elke Bouwens:** Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Sanne E Hoeks:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Alida Kindt:** Formal analysis, Investigation, Methodology, Software, Writing – review & editing. **Lieke Lamont:** Formal analysis, Investigation, Methodology, Resources, Writing – review & editing. **Bram Fioole:** Conceptualization, Investigation, Supervision, Writing – review & editing. **Adriaan Moelker:** Conceptualization, Investigation, Validation, Writing – review & editing. **Sander ten Raa:** Supervision, Writing – review & editing. **Burhan Hussain:** Investigation, Validation, Writing – review & editing. **José Oliveira-Pinto:** Investigation, Methodology, Validation, Writing – review & editing. **Arne S Ijpma:** Investigation, Software, Writing – review & editing. **Felix van Lier:** Investigation, Writing – review & editing. **K. Martijn Akkerhuis:** Conceptualization, Writing – review & editing. **Danielle F Majoor-Krakauer:** Conceptualization, Writing – review & editing. **Thomas Hankemeier:** Formal analysis, Investigation, Methodology, Resources, Writing – review & editing. **Yolanda de Rijke:** Formal analysis, Investigation, Methodology, Resources, Writing – review & editing. **Hence JM Verhagen:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Eric Boersma:** Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing. **Isabella Kardys:** Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing.

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.

Data availability

Anonymized data will be made available by the corresponding author to other researchers for purposes of reproducing the results upon reasonable request and in accordance with a data-sharing agreement.

Acknowledgements

The authors are grateful to Barry Koelewijn (analytical laboratory assistance) and Colinda Koppelaar (data collection) for their contributions to this study.

Research Funding

The current work is supported by Stichting Lijf en Leven.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2024.117786.

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