

**Integrated Plasma and Tissue Proteomics Reveals
Attractin Release by Intraluminal Thrombus of Abdominal Aortic Aneurysms
and Improves Aneurysm Growth Prediction in Humans**

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Abstract

Objective

Discovery of novel biomarkers for abdominal aortic aneurysm growth (AAA) prediction.

Background

Novel biomarker of AAA growth is a recognised priority in research. Our prior work implicated intraluminal thrombus (ILT) in AAAs to be a potential source of systemic mediators during AAA progression. Here we applied a mass spectrometry proteomics pipeline to discover novel biomarkers for AAA growth prediction.

Methods

Patients were prospectively recruited. Plasma samples were collected at baseline (n=62). AAA growth was recorded at 12 months. In Experiment 1, plasma samples from the fastest and slowest growth patients (n=10 each) were compared. In Experiment 2, plasma samples were collected before and at 10-12 weeks after surgery (n=29). In Experiment 3, paired ILT and omental biopsies were collected intra-operatively during open surgical repair (n=3). In Experiment 4, tissue secretome was obtained from *ex-vivo* culture of these paired tissue samples. Samples were subjected to a liquid chromatography tandem mass spectrometry (LC-MS/MS) workflow to discover novel biomarkers.

Results

We discovered 3 proteins that are: (i) present in ILT; (ii) released by ILT; (iii) reduced in circulation after AAA surgery; (iv) differs between fast and slow growth AAAs. One of these is Attractin. Plasma Attractin correlates significantly with future AAA growth (Spearman $r=0.35$, $P<0.005$). Using Attractin and AAA diameter as input variables, the AUROC for predicting no growth and fast growth of AAA at 12 months is 85% and 76%, respectively.

Conclusion

We show that ILT of AAAs releases mediators during the natural history of AAA growth. These are novel biomarkers for AAA growth prediction in humans.

Introduction

Abdominal aortic aneurysms (AAAs) are pathological dilatations of the abdominal aorta to larger than 30mm in diameter. Left untreated, it eventually results in AAA rupture and high mortality. International guidelines all recommend a standard diameter threshold (≥ 5.5 cm) for consideration of elective AAA repair in men^{1, 2}. However, real world data shows drastic discrepancies in the adherence to these established guidelines^{3,4}. Methods for the prediction of AAA growth is considered as a priority for research in the opinions of our peers⁵. It can guide different aspects of clinical management in terms of the frequency of monitoring of AAAs and the optimal timing for surgery.

The Oxford Abdominal Aortic Aneurysm (OxAAA) Study has previously reported a method of AAA growth prediction by incorporating 9 circulating proteins (derived using a commercially available protein array), AAA diameter, and flow mediated dilatation of brachial artery (FMD, a physiological marker of systemic endothelial function)⁶. We had also observed that brachial artery FMD deteriorates during the natural history of AAA growth and is reversed by AAA repair. FMD also inversely correlates with future AAA growth⁷.

Most AAAs contain intra-luminal thrombus (ILT)⁸. Since ILT is either removed or excluded from circulation after successful repair of AAAs, we hypothesise that ILT is a source of mediators that contribute to AAA growth. We had previously developed a liquid chromatography tandem mass spectrometry (LC-MS/MS) proteomics workflow to discover novel proteins released by ruptured coronary artery atherosclerotic plaques in humans – a pathological event that can result in acute coronary syndrome⁹. In this study, we apply a similar approach and combine analyses on blood, thrombus tissue, and tissue supernatant collected from

patients during the natural history of AAA progression to discover novel predictors of AAA growth in humans.

Methods

Participants, blood sampling, and AAA growth rate measurement

Details regarding the OxAAA study cohort and recruitment process have been published⁷. In brief, this single centre prospective study (Ethics Ref: 13/SC/0250) recruited patients in the National Health Service setting. Each participant gave written informed consent. Baseline assessments were performed. In addition to the measurement of AAA antero-posterior diameter by ultrasound imaging, a fasting venous blood sample was collected in EDTA tubes. Platelet-poor plasma (PPP) was prepared immediately after blood collection at room temperature using two-staged centrifugation (1st stage: 1300gx12min; 2nd stage: 2500gx15min) as previously described¹⁰. These were stored at -80°C for subsequent analysis.

Prospective AAA annual growth rates in the AAA surveillance arm (n=62) were calculated based on the antero-posterior diameter (APD) measurements in the subsequent yearly AAA monitoring ultrasound scan: $(\Delta\text{APD}/\text{APD at baseline})/(\text{number-of-days-lapsed}/365\text{days})$. Based on the prospectively recorded aneurysm growth rates, we selected a subset of patients [fastest (n=10) vs slowest (n=10), **Experiment 1**] for a discovery analysis. PPP samples were also collected in a separate group of participants undergoing AAA surgery (n=29). In each patient, samples were collected before surgery and at 10-12 weeks after surgery (before vs after, **Experiment 2**). Samples were pooled in their respective groups for the discovery proteomics analyses detailed below.

Intra-operative tissue biopsy

Intraoperative biopsies of paired tissue samples were performed in 3 patients (**Experiment 3**). In addition to ILT, a wedge of omentum containing omental vessels was biopsied to serve as control biological tissue and to compare against the proteomics signature of ILT lysate/secretome. Tissue samples were processed immediately in the operating theatre. Tissue were rinsed thoroughly with normal saline to remove contaminating blood. A fraction of the ILT and omental tissue were snap frozen and stored in -80°C for subsequent analysis (Experiment 3). The rest of the ILT and omental tissue were transferred in ice-cold RPMI 1640 + FCS 5% for the tissue secretome experiments (**Experiment 4**).

Preparation of tissue homogenate

Frozen tissues (ILT, omentum) were placed on a dry ice chilled Steel BioPulverizer (BioSpec, USA) and crushed with mechanical force. 20mg of pulverised tissue were homogenised in beads-beater tubes (Stretton, UK) containing RIPA lysis buffer (25mM Tris HCl, pH 7.6, 150mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) for 4 times at 6,500 Hz for 40s. The samples were centrifuged at 10,000g for 5min at 4°C to remove insoluble tissue debris to obtain tissue homogenate, and stored at -80°C for analysis.

Preparation of tissue secretome

Under sterile aseptic conditions, each tissue sample was dissected out in 3mm x 3mm blocks and placed in a 6-well plate (Corning, USA) and resuspended in 2mL of RPMI 1640 + 5% FCS. This was incubated at 37°C with 5% CO₂ in an incubator. After an hour, samples were removed and washed with pre-warmed PBS (Lonza) and then serum free RPMI media (Lonza) was added. This was incubated for a further 24 hours. The conditioned media (containing the tissue secretome) was collected and centrifuged at 3000g for 10 mins to pellet the remaining cells and cellular debris and stored at -80°C.

In-Solution trypsin digest of proteins

Protein concentration in the tissue homogenates and tissue supernatant were determined by BCA assay (Thermo Fisher). Forty micrograms of total proteins were retrieved from each sample. Ten microlitres from individual plasma samples were used. To enable detection of low abundance proteins in plasma samples, the 12 most abundant proteins were depleted using an immunity affinity spin column (Thermo Pierce Top 12 Spin Column). Disulfide bonds were reduced by adding 200mM of DTT (Sigma) to a final concentration of 5mM for 30 mins at room temperature. Free cysteine residues were alkylated by adding 200mM of iodoacetamide (Sigma) to a final concentration of 20mM and incubated for 30mins at dark. The samples were topped up to 200µl with 6M urea, 100mM TrisHCL pH 8.5.

Methanol/chloroform protein precipitation was used to remove detergents before tryptic digestion. In brief, 600 µl of Methanol and 150µl of Chloroform were added and mixed. Then 450µl of MilluQ-H₂O was added to the bottom layer and centrifuged for 1min at 12,000g. The upper aqueous layer was carefully removed without disturbing protein pellets between layers and 450µl of Methanol were added to the bottom layer and centrifuged at 12,000g for 5 min. The supernatant was removed and the protein pellets resuspended in 50µl of 6M Urea, 100mM TrisHCl, pH 8.5. Urea concentration was reduced to 1M by adding 250µl of MillQ-H₂O. Samples were digested at 37°C overnight with Trypsin added in 1:30 ratio (trypsin:protein). The peptides were acidified and purified by a SepPak C18 cartridge (Waters), dried by Speed Vac centrifugation and resuspended in 80µl of resuspension buffer A (2% acetonitrile 0.1% formic acid) for LC-MS/MS analysis.

LC-MS/MS workflow

For peptide analysis, an Ultimate300 uHPLC was coupled to an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific). Each of the pooled plasma samples was run as technical replicates. Tissue lysate and tissue secretome were run individually as biological replicates. All samples were processed in the same batch. One microliter of 0.5 µg/µL tryptic digested peptides were injected into the mass spectrometer for analysis. Peptides were separated on an EASY-Spray C18 column (75 µm ID x 50 cm, Thermo Fisher) at a flow rate of 250 nL/min. The mobile phases consisted of 0.1% formic acid and 5% DMSO in water (buffer A) and 0.1% formic acid and 5% DMSO in Acetonitrile (buffer B). Peptides were eluted with a 60 minutes linear gradient from 3% buffer A to 35% buffer A 60 minutes linear gradient from 3% to 35% of buffer A.

Protein data analysis

Analyses of the dataset was performed using the commercially available Progenesis QI™ software pipeline (Waters). A Mascot search engine (Matrix Science) against the UniProt Human database was used for peptide identification in this pipeline. At least two unique peptides were used for protein quantitation using match between runs. The false discovery rate (FDR) was set to 1% for protein and peptide identification. Label free quantitation (LFQ) intensity data were used for further statistical analysis to compare across the different groups of samples. Differentially expressed proteins in the Progenesis QI™ analyses pipeline were defined as proteins presenting a statistical difference (ANOVA $P < 0.05$) across the comparing groups (ie fast vs slow, before vs after). For tissue lysate and secretome analyses, a protein was considered to be uniquely present in the ILT/ILT secretome if it was significantly higher in ILT/ILT secretome (ANOVA $P < 0.05$) compared to omentum/omentum secretome. Technical

validation of protein measurement was performed using commercially available enzyme linked immunosorbent assay (ELISA).

Statistical analyses

GraphPad Prism (V8.4) and SPSS (IBM V25) were used for other statistical analyses. Spearman correlation was used to examine the association between variables. Mann-Whitney test was used for comparison between groups. A generalised linear model was used to assess the potential variables as predictors of 12-month AAA growth rate. Receiver operating curve analyses were used to assess the predictive model for discriminating 12-months AAA growth in two categories: No/Slow growth was defined as no increase in AAA AP diameter over 12 months; Fast growth was defined as the upper tertile of AAA diameter growth over 12 months observed in the surveillance cohort. Statistical comparisons between the receiver operating characteristics (ROC) curves were performed using the NCSS data analysis software (V2020).

Results

Characteristics of participants undergoing AAA surveillance

Prospectively measured 12-month AAA growth was recorded in 62 participants (male n=54). Baseline characteristics of this group is summarised in Table 1. The median AAA size at baseline was 48mm (range=40-54mm). Median growth rate of AAA was 3.8%/year (Interquartile range=1.9% to 6.8%). The upper tertile of growth rate observed was 5.7%/year – this is set as the threshold of ‘fast growth’ in this cohort. Those with a past history of coronary artery disease (HxCAD, either managed medically or with coronary intervention) experienced faster 12-month AAA growth compared to those without HxCAD (median growth rate of 4.6%/year vs 2.3%/year, Mann-Witney P=0.044). Other baseline characteristics did not affect future AAA growth rate.

LC-MS/MS proteomics analyses

In **Experiment 1**, comparison between patients with the fastest vs the slowest AAA growth showed 116 proteins to be differentially expressed in their plasma (Figure 1-‘A’). These are listed by their UniProt IDs in Table 2. In **Experiment 2**, we observed 258 proteins that were significantly changed *within* the patient after AAA repair. Among these proteins, 35 were also shown to be differentially expressed between those with fastest vs slowest growth in Experiment 1 (Figure 1-‘B’). In **Experiment 3**, comparison of the proteomics profile of tissue lysate showed 128 proteins to be uniquely present in ILT (Figure 1-‘C’). Analyses of the tissue culture supernatant (**Experiment 4**) further revealed 3 proteins that were: (i) uniquely present in ILT; (ii) released by ILT secretome; (iii) reduced in systemic circulation after AAA surgery; (iv) different between fast and slow growth AAAs (Figure 1-‘D’). These were: Attractin (UniProt ID O75882), complement C8 (UniProt ID P07360), and heat shock protein AA5P (UniProt ID Q58FG0).

Technical validation of LC-MS/MS data

To technically validate the LC-MS/MS data, Attractin level in individual patient was measured by ELISA (R&D Systems Quantikine DATRN0). This choice was based on the availability of a reliable commercial assay. The median level of plasma Attractin was 24.1ng/ml (IQR 20.4 to 27.9 ng/ml). Consistent with the LC-MS/MS data, plasma Attractin level was significantly higher in patients with fast AAA growth compared to slow AAA growth (Figure 2, median Attractin level of 28.5 vs 21.9 ng/ml, Mann Whitney $P < 0.001$).

Correlation between Attractin with baseline AAA size and 12-month AAA growth

There was no correlation between plasma Attractin and baseline AAA diameter (Spearman $\rho = -0.006$, $P = 0.96$). There was no difference in plasma Attractin between those with and

without HxCAD (Mann Whitney $P=0.46$). Plasma Attractin level correlated significantly with future AAA growth rate (Figure 3, Spearman $\rho=0.35$, $P<0.005$). We utilised a generalised linear model to assess whether Attractin associated with 12-month AAA growth rate as a continuous variable. In univariate analysis, there was a significant association between Attractin and future 12-month AAA growth rate ($P=0.02$). In multivariate analysis, the association between Attractin and 12-month AAA growth rate remained statistically significant ($P<0.05$) after adjusting for the effect of HxCAD and AAA diameter. The association of HxCAD and 12-month AAA growth was no longer significant after adjusting for the effect of Attractin. The β co-efficient and 95% confidence intervals for the univariate and multivariate models are presented in Supplemental Table 1 and 2.

Prediction of AAA growth at 12 months

We used the measured values of Attractin in combination with the measured AAA diameter as the independent variables of a logistic regression model against a categorical outcome of 'Slow/no' growth (0%) or more ($>0\%$ growth) at 12 months. We separately tested these against the categorical outcome of 'Fast' growth ($\geq 5.7\%/year$) or less ($<5.7\%/year$) as outcomes at 12 months. Using Attractin and AAA diameter as the input variables, the area under receiver operating characteristics (AUROC) curve for predicting slow/no growth at 12 months is 0.85 (95% CI=0.69 to 0.93, asymptotic $P<0.001$). AUROC for predicting fast growth of AAA at 12 months is 0.76 (95% CI=0.59 to 0.87, asymptotic $P=0.001$). These represented improvements as compared to using AAA diameter as the lone input variable: AUROC is 0.76 (95% CI=0.59 to 0.87) for slow/no growth, AUROC is 0.52 (95% CI=0.35 to 0.66) for fast growth. These AUROC comparisons were statistically significant: $P<0.05$ for the slow/no growth predictions, $P<0.01$ for the fast growth predictions (Figure 4).

For the prediction of slow/no growth, the above logistic regression analysis generated a probability of an individual's AAA being slow/no growth in the subsequent 12 months. Using a cut off value of 0.39 for the probability ('Aneurysm Slow Growth Index', ASGI), the prediction algorithm gave the following statistics: sensitivity 54% (7/13), specificity 94% (46/49), accuracy 85% (53/62). For the prediction of fast growth, the above logistic regression analysis generated a probability of an individual's AAA being fast growth in the subsequent 12 months. Using a cut off value of 0.42 for the probability ('Aneurysm Fast Growth Index', AFGI), the prediction algorithm gave the following statistics: sensitivity 66% (14/21), specificity 85% (35/41), accuracy 79% (49/62).

Discussion

A growing body of evidence supports the active biological role of ILT throughout the natural history of AAA disease, rather than as just a bystander. For example, we recently reported that AAA ILT contains enzymatically oxidized phospholipids (exoPL), and that exoPL modulates AAA growth in a murine model¹¹. We also reported that the spatial morphology of ILT influences the likelihood of type 2 endoleak after endovascular stenting repair of AAA⁸. Successful surgical repair of AAA either removes ILT completely (open repair) or excludes ILT from systemic circulation (EVAR). This results in improved systemic vascular endothelial function after surgery to levels similar to healthy volunteers without an AAA⁷. Such evidence supports our hypothesis that ILT thrombus as a source of systemic mediators during AAA progression.

This report is a significant breakthrough from our previous methods of AAA growth prediction^{6,12}. Our prior predictive models required the inclusion of a physiological measurement (FMD of brachial artery). This requires a dedicated ultrasound measurement

and cannot be derived by plasma sample measurement alone. By focusing on the role of thrombus as a source of systemic mediator release, we discover novel proteins that have a specific utility for AAA growth prediction. We achieved this by applying an established mass spectrometry discovery pipeline to a robustly curated AAA clinical bioresource.

Our previous model also included 9 proteins (Thrombospondin, CXCL10, IL6, IL8, , RAGE, MIP1a, MIP1b, leptin, ICAM1) selected by the analysis of plasma samples of fast vs slow/no growth patients using an antibody array (R&D Proteome Profiler). Attractin has the same utility for AAA growth prediction as compared to the other 9 proteins combined (see Supplemental Table 3). With only 2 input variables (AAA diameter and Attractin), this iteration of the prediction algorithm could be readily measured in an outpatient setting. With the development of a point of care testing device (to measure Attractin and others), it will be feasible to apply this lean algorithm at the time of AAA screening and surveillance scans. This will facilitate validation of this algorithm on a large scale, and its eventual application in clinical settings.

Multiple testing is an issue with datasets that compare numerous targets. We overcame this by a combination of approaches. Firstly, we designed separate experiments to address the same hypothesis from different angles. We combined the results from these experiments and accepted only a handful (n=3) of proteins that fulfilled the stringent test within each experiment (i.e. at the intersection of the Venn diagram in Figure 1). In addition, we applied a low FDR threshold in conjunction with the statistical testing within the Progenesis QI™ analysis pipeline. The validity of our mass spectrometry discovery workflow was demonstrated by the precise replication of the LC-MS/MS data by ELISA measurements of Attractin on individual patient samples.

We are gradually gaining insight to the biological role of Attractin in disease since its first description in 1998¹³. Attractin is a dipeptidyl peptidase IV/CD26-like enzyme typically expressed on human peripheral blood monocytes and also released by activated T-cells. It is involved in the initial immune cell clustering during an inflammatory response and regulates chemotactic activity of chemokines¹³. Attractin further plays a role in the regulation of differentiation of T cells¹⁴. There is mounting evidence of T-cells being active in AAA ILT and play a role in AAA pathophysiology¹⁵. Here we describe a novel role of Attractin in human AAA progression which warrants further mechanistic investigations.

Although the study utilised a relatively small cohort, we show that it is feasible to discover novel biomarkers in this context through a robust experimental pipeline. We did not attempt to technically measure the other two potential biomarkers (Complement C8, HSP AA5P) due to the lack of reliable commercial ELISAs. Given the demonstrated utility of Attractin in prediction of AAA growth, one would be optimistic that the predictive accuracy would be further enhanced should all three novel proteins be measured. It will be critical to perform external validations of our findings, either through established biobank samples or through prospectively recruited cases. It is important to note that measurement of plasma biomarkers can be prone to pre-analytic factors, especially due to incomplete removal of blood cells in plasma¹⁰. We have applied a stringent protocol to procure platelet poor plasma in this study. Future validation efforts will rely on samples collected with rigor to avoid the confounding pre-analytic effects.

Conclusion

Intraluminal thrombus is a source of systemic Attractin release during the natural history of AAA. Plasma level of Attractin can be utilised to predict slow or fast growth of AAA in humans.

276 We hope this work serves as a primer to generate interest in the vascular surgical community
277 and stimulates future efforts to validate the prediction algorithm.

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Data Sharing and Availability

Access to the samples curated by the Oxford Abdominal Aortic Aneurysm Study requires relevant regulatory approval and material transfer agreement. Data underpinning this manuscript (raw or synthesised) can be available upon reasonable request.

Table and Figure Legends

Table 1: Demographic Data of Study Participants Under AAA Surveillance

Table 2: UniProt IDs of Proteins that are differentially expressed between patients with fast and slow growing abdominal aortic aneurysm in the subsequent 12 months. Prospectively measured 12-month AAA growth was recorded in 62 participants. Prospective AAA annual growth rates were calculated based on the antero-posterior diameter measurements in the subsequent yearly AAA monitoring ultrasound scan. Based on the prospectively recorded aneurysm growth rates, we selected a subset of patients [fastest (n=10) vs slowest (n=10)] for a discovery analysis by a LC-MS/MS workflow. Plasma samples were pooled in their respective groups for analysis. Other samples were analysed individually. Data were analysed using the Progenesis QI software (NonLinear Dynamics) and included only proteins with at least two matched peptide sequences. False discovery rate of protein identification was set at 1%. Comparison between patients with the fastest vs the slowest aneurysm growth showed 116 proteins to be differentially expressed in their plasma sample. Proteins here are listed as their UniProt IDs. O75882: Attractin, P07360: Complement C8, Q58FG0: Heat Shock Protein AA5P.

Figure 1: Integrated Plasma and Tissue Proteomics Reveals Attractin Release by Intraluminal Thrombus of Abdominal Aortic Aneurysms. In Experiment 1, AAA growth rates were prospectively recorded in 62 patients. Based on the growth rate in the subsequent 12 months, we selected a subset of patients (fastest vs slowest, n=10 each) for the initial discovery analysis. In experiment 2, plasma samples were also collected from patients before and after AAA repair (n=29). In experiment 3, *Paired* intraluminal thrombus (ILT) and omental biopsies were collected intra-operatively during open surgical repair (n=3). In addition to analyses of the tissue lysate, tissue secretome was obtained from *ex vivo* culture of these paired tissue samples. Samples were subjected to Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) proteomic analysis to discover protein level differences. Plasma samples were pooled in their respective groups for analysis. Other samples were analysed individually. LC-MS/MS data were analysed using the Progenesis QI software (NonLinear Dynamics) and included only proteins with at least two matched peptide sequences. Comparison between patients with the fastest vs the slowest aneurysm growth showed 116 proteins to be differentially expressed in their plasma ('A'). Among these proteins, 35 also changed significantly before and after AAA repair ('B'), suggesting their origin from the AAA complex. Comparison of the proteomics profile of ILT, and omental artery tissue showed 128 proteins to be uniquely present in ILT ('C'). Analyses of the tissue culture supernatant further revealed 3 proteins that were: (i) uniquely present in ILT; (ii) released by ILT; (iii) systemic levels reduced after AAA surgery; (iv) different between fast and slow growth AAAs ('D').

Figure 2: Technical validation of mass spectrometry data on Attractin. Attractin level in individual patient was further measured by ELISA (R&D Quantikine DATTRN0). Plasma attractin level is significantly higher in patients with fast AAA growth (panel 3, median 28.5 vs 21.9 ng/ml, $P<0.001$).

Figure 3. Plasma attractin level correlates significantly with future AAA growth rate.

Figure 4: Receiver Operation Characteristics (ROC) Curves of AAA growth prediction using Attractin and AAA diameter as input variables. We regressed the measured values of Attractin in combination with the measurements of AAA diameter against a categorical response with levels of 'Slow/no' growth (0%) or more ($>0\%$ growth), and with levels of 'Fast' growth ($\geq 5.7\%/year$) or less ($<5.7\%/year$) as outcomes at 12 months. The threshold of fast growth is determined by the upper tertile of growth observed in this cohort. Using Attractin and AAA diameter as input variables, the area under receiver operating characteristics (AUROC) for predicting slow/no growth at 12 months is 85% (solid line, asymptotic $P<0.001$). AUROC for predicting fast growth of AAA at 12 months is 76% (solid line asymptotic $P=0.001$). These represent improvements as compared to using AAA diameter as the lone input variable (dotted lines, AUROC is 76% for slow growth and 52% for fast growth).