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

Regent Lee^{1,2}, Ismail Cassimee², Honglei Huang^{1,2}, Pierfrancesco Lapolla¹,
Anirudh Chandrashekar¹, Philip Charles², Benedikt Kessler², Roman Fischer², Ashok Handa¹

⁺On behalf of the Oxford Abdominal Aortic Aneurysm Study

¹Nuffield Department of Surgical Sciences, University of Oxford, UK

²Target Discovery Institute, Nuffield Department of Medicine, University of Oxford, UK

Corresponding Author

Regent Lee
regent.lee@nds.ox.ac.uk

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1 Abdominal aortic aneurysms (AAAs) are pathological dilatation of the abdominal aorta to
2 larger than 30mm in diameter. Left untreated, it eventually results in AAA rupture and high
3 mortality. Methods for the prediction of AAA growth is considered as a priority for research
4 in the opinions of our peers¹. It can guide different aspects of clinical management in terms
5 of the frequency of monitoring of AAAs and the optimal timing for surgery.

6 The Oxford Abdominal Aortic Aneurysm (OxAAA) Study has previously reported a method of
7 AAA growth prediction by incorporating 9 circulating proteins (derived using a commercially
8 available protein array), flow mediated dilatation of brachial artery (FMD, a physiological
9 marker of systemic endothelial function), and AAA diameter². We had also observed that
10 systemic endothelial function (measured by brachial artery FMD) deteriorates during the
11 natural history of AAA growth and is reversed by AAA repair³.

12 Most AAAs contain intra-luminal thrombus (ILT)⁴. Since ILT is either removed or excluded
13 from circulation after successful repair of AAAs, we hypothesise that ILT is the source of
14 mediators that contribute to AAA growth. In this report, we utilised mass spectrometry
15 analyses on blood, thrombus tissue, and tissue supernatant collected from patients during
16 the natural history of AAA progression to discover novel predictors of AAA growth in
17 humans.

18 Details regarding the OxAAA study cohort and recruitment process have been published³. In
19 brief, this prospective study (Ethics Ref: 13/SC/0250) recruited patients in the National
20 Health Service setting. Baseline assessments were performed. In addition to the
21 measurement of AAA antero-posterior diameter by ultrasound imaging, fasting blood
22 sample was collected and Platelet-poor plasma was prepared using two-staged
23 centrifugation as previously described⁵ and stored at -80°C for subsequent analysis.

24 Prospective AAA annual growth rates were calculated based on the diameter measurements
25 in the subsequent AAA monitoring ultrasound scans.

26 Plasma samples were collected at baseline and at 1 year from each patient (n=59). Based on
27 the prospectively recorded aneurysm growth rates, we selected a subset of patients [fastest
28 (n=10) vs slowest (n=10)]. These were pooled for the initial discovery analysis. Plasma
29 samples were also collected before and at 10-12 weeks after surgery from each patient
30 (n=29). *Paired* aneurysm wall, ILT, omental biopsies were collected intra-operatively during
31 open surgical repair (n=3). In addition to analyses of the tissue, supernatant was obtained
32 from *ex vivo* culture of these paired tissue samples. We utilised a similar approach for
33 plasma biomarker discovery as recently described⁶. Samples were subjected to Liquid
34 Chromatography Tandem Mass Spectrometry (LC-MS/MS) proteomic analysis to discover
35 protein level differences⁶. LC-MS/MS data were analysed using the Progenesis Q1 software
36 (NonLinear Dynamics), and included only proteins with at least two matched peptide
37 sequences.

38 The median AAA size at baseline was 48 mm. The median growth rate of AAA was 3.8%/year
39 (IQR 1.9% to 6.8%). Comparison between patients with the fastest vs the slowest AAA
40 growth showed 116 proteins (listed by the UniProt Protein IDs in Figure Panel 1) to be
41 differentially expressed in their plasma (Figure panel 2-A). Among these proteins, 35 also
42 changed significantly before and after AAA repair (Figure panel 2-B), suggesting their origin
43 from the AAA complex. Comparison of the proteomics profile of aneurysm tissue, ILT, and
44 omental artery show 128 proteins to be uniquely present in ILT (Figure panel 2-C).

45 Analyses of the tissue culture supernatant further revealed 3 proteins that were: (i) uniquely
46 present in ILT; (ii) released by ILT; (iii) reduced in systemic circulation after AAA surgery; (iv)

different between fast and slow growth AAAs (Figure panel 2-D). These are: attractin (UniProt ID O75882), complement C8 (UniProt ID P07360), heat shock protein AA5P (UniProt ID Q58FG0). To technically validate the LC-MS/MS data, attractin level in individual patient was measured by ELISA (R&D Quantikine DATRN0). Consistent with the LC-MS/MS data, plasma attractin level is significantly higher in patients with fast AAA growth (Figure panel 3, median 28.5 vs 21.9 ng/ml, $P < 0.001$). Plasma attractin level correlates significantly with future AAA growth rate (Figure panel 4, Spearman $r = 0.35$, $P < 0.005$).

We tested the utility of a generalised linear model to predict aneurysm growth in these patients. 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 growth (>0% growth) for outcomes at 12 months. Using attractin and AAA diameter as input variables, the area under receiver operating characteristics (AUROC) for predicting no growth of AAA at 12 months is 85% (Figure panel 5, asymptotic $P < 0.001$) as compared to 76% with AAA diameter alone.

This report is a significant breakthrough from our previous work². By focusing on the role of thrombus as a source of systemic mediator release, we discover novel proteins that are released from thrombus and drive AAA growth in humans, tested in a prospectively recruited cohort. This is the first report in which novel proteins that correlate to future AAA growth have been discovered through a mass spectrometry workflow. The validity of the mass spectrometry discovery workflow is demonstrated by the precise replication of the LC-MS/MS data by ELISA measurements on individual patient samples.

Since the first description of attractin in 1998⁷, there has been little in the reported literature regarding its biological role in disease. Evidence points toward its release by

70 activated T-cell. It is involved in the initial immune cell clustering during inflammatory
 71 response and it regulates chemotactic activity of chemokines⁷. There is mounting evidence
 72 of T-cells being active in AAA ILT and that it plays a role in AAA pathophysiology⁸. This report
 73 is the first to implicate attractin in human AAA progression and warrants further
 74 mechanistic investigations.

75 It is important for external cohorts to replicate the efficacy of our biomarker panel. We
 76 hope this work serves as a primer to generate interest in the vascular surgical community
 77 and stimulates future efforts to validate the prediction algorithm.

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*Contributors to the OxAAA Study include: Amy Jones, Felicity Woodgate, Nicholas Killough, Kirthi Bellamkonda, Elisha Ngetish, Joel Ward, Members of the Oxford Regional Vascular Services, and the Jackie Walton Vascular Studies Unit.

Figure

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

AAA growth rates were prospectively recorded in 59 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. Plasma samples were also collected from patients before and after AAA repair (n=29). Paired aneurysm wall, ILT, omental biopsies were collected intra-operatively during open surgical repair (n=3). In addition to analyses of the tissue, supernatant 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 (listed by the UniProt Protein IDs in Panel 1) to be differentially expressed in their plasma (2-A). Among these proteins, 35 also changed significantly before and after AAA repair (2-B), suggesting their origin from the AAA complex. Comparison of the proteomics profile of aneurysm tissue, ILT, and omental artery showed 128 proteins to be uniquely present in ILT (2-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 (2-D). These are: attractin (UniProt ID O75882), complement C8 (UniProt ID P07360), heat shock protein AA5P (HSPAA5P, UniProt ID Q58FG0). Attractin level in individual patient was further measured by ELISA (R&D Quantikine DATRN0). Plasma attractin level is significantly higher in patients with fast AAA growth (panel 3, median 28.5 vs 21.9 ng/ml, $P<0.001$). Plasma attractin level correlates significantly with future AAA growth rate (Figure panel 4, Spearman $r=0.35$, $P<0.005$). We regressed the measured values of attractin in combination with AAA diameter against a categorical response with levels of 'Slow/no' growth (0%) or growth (>0% growth) for outcomes at 12 months. Using attractin and AAA diameter as input variables, the AUROC for predicting no growth of AAA at 12 months is 85% (panel 5, asymptotic $P<0.001$).