Aortic Growth

The maximum arch/ DTA diameters in my cohort of patients ranges from 3.2cm to 7.9cm. While 3.2cm would not normally be considered aneurysmal, in this case it reflects the disparity that can arise between measurements of the aortic arch taken from axial CT slices and those taken from Corelab-type software. The mean change in arch/ DTA diameter was observed to be 3.14mm/yr, which is in line with other reports in the literature. I observed the mean change in arch/ DTA volume to be 38ml/yr – there is sparse published data against which to compare this. The baseline aortic diameters, dAoD/dt and dAoV/dt were noted to be normally distributed, as determined by the Shapiro-Wilk test (p 0.32, 0.36 and 0.13 respectively).

Of interest, the first 2 patients in Table 3.6 were noted to have regression of their aneurysms in terms of rate of change of diameter. One of these patients had a slight increase in arch/ DTA volume over the period of follow-up and chose to undergo surgery electively - despite his arch measuring less than the guideline 5.5cm - having suffered a previous Type A dissection. The other patient with aneurysmal regression was a young man whose aneurysm was thought to have been induced by a blunt chest trauma in the past – review of his scans prior to enrolment in this study indicated a consistent regression in his aortic diameters over a total of 4 years.

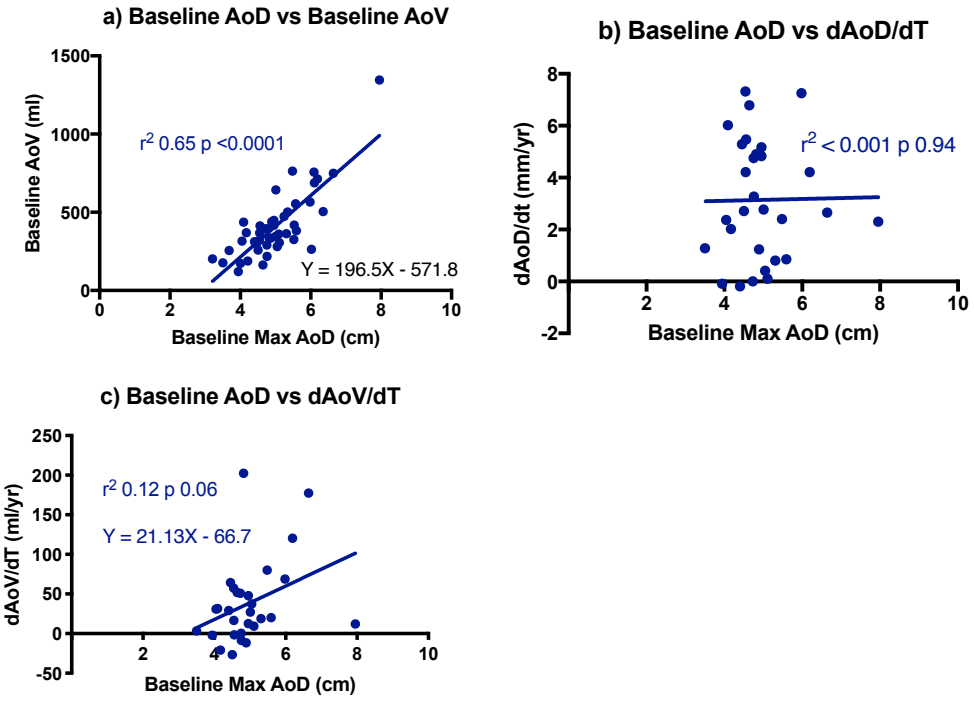
As expected, there is a tight correlation between aortic diameter and volume (Figure 3.9a). Consequently, I will henceforth limit the results to display correlations of biomarkers against AoD only, leaving out AoV.

In this dataset, maximum aortic diameter does not correlate well with rate of change of aortic diameter over time (Figure 3.9b). There is a better correlation between baseline aortic diameter and rate of change of volume over time (Figure

3.9c), but even this fails to reach statistical significance (p=0.06) and the coefficient of determination is low (r2 = 0.12).

Figure 3.9 – correlations between baseline aortic diameter and baseline aortic volume and aortic growth rates

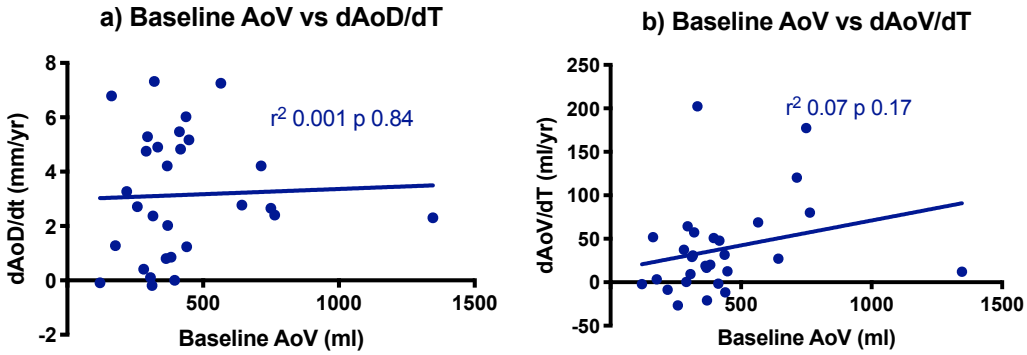
The following scatter plots demonstrate the relationship between a) aneurysm diameter and aortic segmental volume; b) aneurysm diameter and aneurysm growth (in terms of rate of change of maximal diameter); and c) aneurysm diameter and aneurysm growth (in terms of rate of change of aortic volume). The equations of the regression lines are provided within the graphs where the analyses indicated statistically significant correlations. The coefficient of determination (r^2) and the p value are also provided within each graph.



The baseline aortic volume similarly fails to correlate with observed aortic growth, as shown in the linear regression graphs in Figure 3.10.

Figure 3.10 – correlations between baseline aortic volume and aortic growth rates

The following scatter plots demonstrate the relationship between aneurysm volume and a) aneurysm growth in terms of rate of change of maximal diameter; and b) aneurysm growth in terms of rate of change of aortic volume. The equations of the regression lines are not given here since none of the analyses indicated statistically significant correlations. However the coefficient of determination (r^2) and the p value are provided within each graph.



Plasma Proteins and Aortic Outcomes

The summarised results of the plasma protein assays in the longitudinal cohort is described below in Table 3.7. The n number reflects that in some samples, the analyte of interest fell outside the detectable range of the assay. For the univariate analyses, the missing datapoints were simply excluded from the regression, with no attempt made at imputing the missing values given the relatively small size of the dataset.

Table 3.7 – statistical description of the measured candidate biomarkers.

This table provides information on the plasma proteins measured in the 46 AD patients of the longitudinal study. The second column displays the detectable range of the assay and then goes on (in the 3rd column) to show the number of samples in which the analyte fell outside the range detectable by the assay. Each sample was tested twice, so where there was a large difference in the results between the two

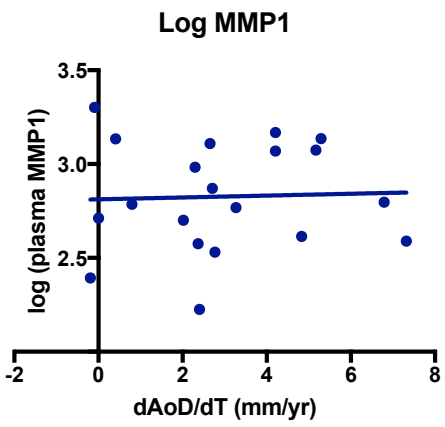
technical duplicates, these were assigned as unreliable results – this information is shown in the 4th column 'no reliable result'. The final three columns therefore show how many samples contribute data for each plasma protein, the mean concentration of that protein and the standard error of the mean. It should be noted that samples were diluted up to a factor of 1:50. The analyte is measured in the diluted sample, but multiplied back up to quantify the concentration in the sample. Hence some of the mean values 'appear' to lie well above the maximum detectable threshold.

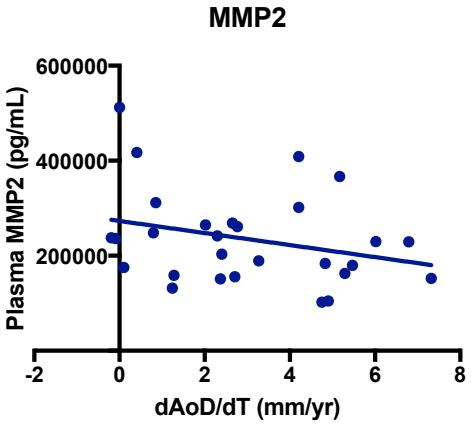
	Assay detectable range	**Samples falling outside detectable range**	**No reliable result**	**N**	**Mean**	**SEM**
MMP1 (pg/mL)	1.1 – 25700	8 below minimum	4	33	850	91
MMP2 (pg/mL)	12.6 – 63100			43	224675	13687
MMP3 (pg/mL)	7.3 – 25700			40	18364	3388
MMP7 (pg/mL)	6.6 – 55850			42	2707	260
MMP9 (pg/mL)	13.7 – 73100			43	44239	8483
MMP10 (pg/mL)	3.2 – 31000			39	2545	301
TIMP-1 (pg/mL)	14 – 10000			41	76017	4396
TIMP-2 (pg/mL)	44 – 32000			41	85243	3128
TIMP-4 (pg/mL)	7 - 5200			43	1758	122
TGFβ1 (pg/mL)	31.2 – 2,000	8 above maximum		38	2953	319
TGFβ2	31.2 – 2,000	8 below minimum		38	110	8

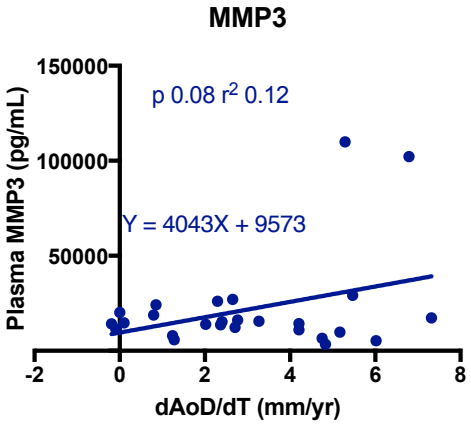
(pg/mL)						
IFNγ (pg/mL)	0.20 – 938	1 below minimum	3	42	14.1	2.3
TNFα (pg/mL)	0.04 – 248			43	2.9	0.3
CRP (mg/L)	0.1 – 50			43	6.2	1.2
OP (ng/mL)	0.04 – 200			43	24.4	1.9
IL2 (pg/mL)	0.09 - 938	31 below minimum	3	12	1.2	0.3
IL6 (pg/mL)	0.06 – 488	1 below minimum	3	42	1.9	0.2
IL8	0.04 – 375			43	7.5	0.7
IL10	0.03 – 233	5 below minimum	3	38	0.4	0.04
IL13	0.24 – 353	17 below minimum	4	25	1.8	0.3

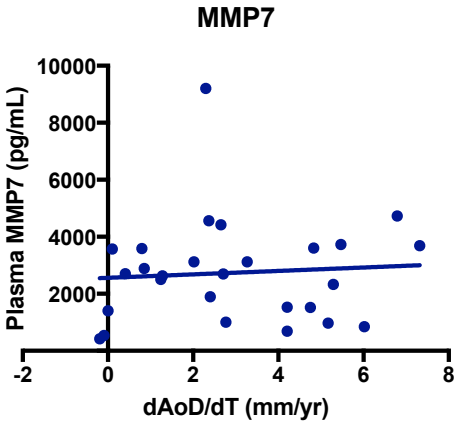
Figure 3.12 – graphs of correlations between plasma proteins and dAoD/dT.

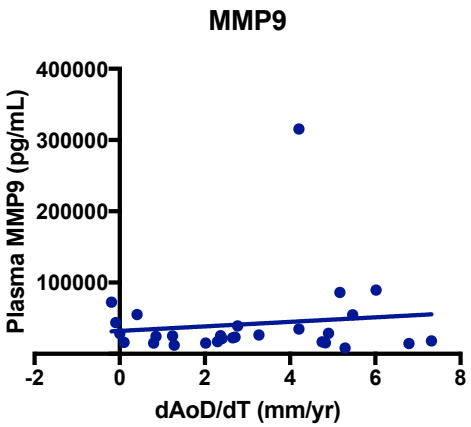
The following scatter plots demonstrate the relationship between aneurysm growth (in terms of rate of change of maximal diameter) and the various plasma proteins measured in the AD group. The equations of the regression lines, coefficients of determination (r^2) and the p value are provided on those graphs where the analysis yielded $p<0.1$.

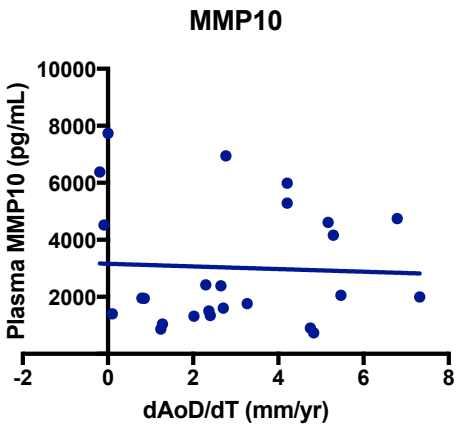

Log MMP1
log (plasma MMP1)
3.5
3.0
2.5
-2
0
2
4
6
8
dAoD/dT (mm/yr)

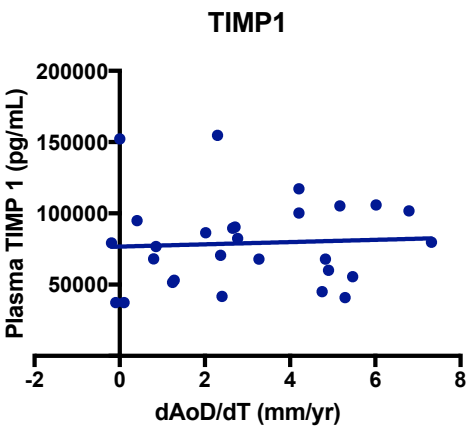

MMP2
Plasma MMP2 (pg/mL)
600000
400000
200000
-2
0
2
4
6
8
dAoD/dT (mm/yr)

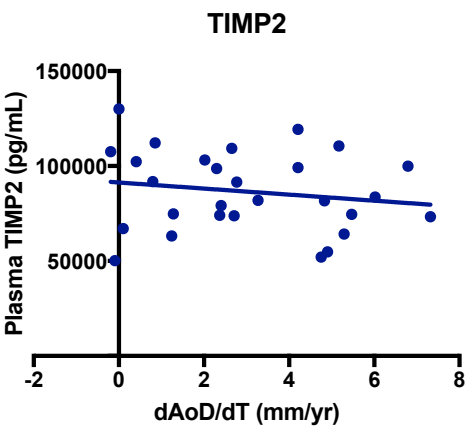

MMP3
Plasma MMP3 (pg/mL)
150000
100000
50000
p 0.08 r2 0.12
Y = 4043X + 9573
-2
0
2
4
6
8
dAoD/dT (mm/yr)

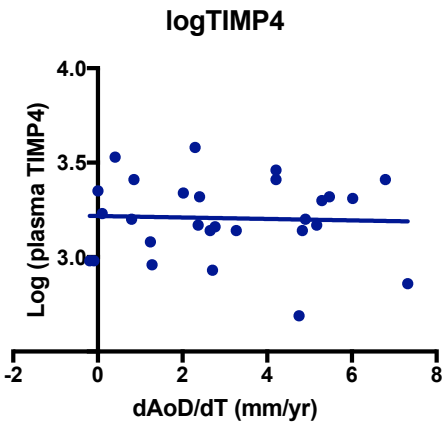

MMP7
Plasma MMP7 (pg/mL)
10000
8000
6000
4000
2000
-2
0
2
4
6
8
dAoD/dT (mm/yr)

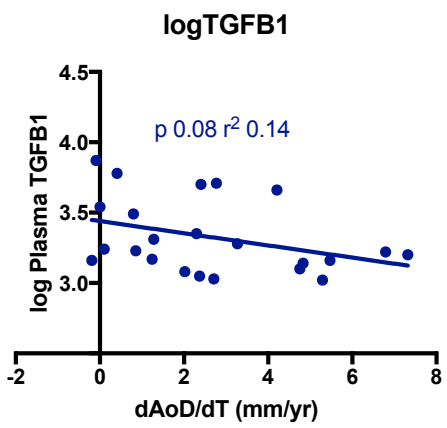

MMP9
Plasma MMP9 (pg/mL)
400000
300000
200000
100000
-2
0
2
4
6
8
dAoD/dT (mm/yr)

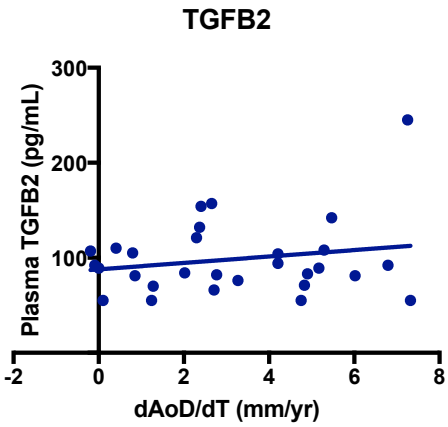

MMP10
Plasma MMP10 (pg/mL)
10000
8000
6000
4000
2000
-2
0
2
4
6
8
dAoD/dT (mm/yr)

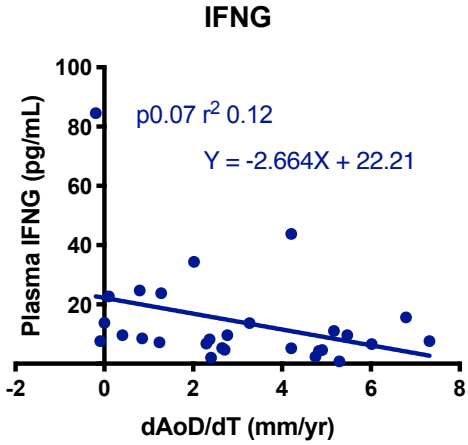

TIMP1
Plasma TIMP 1 (pg/mL)
200000
150000
100000
50000
-2
0
2
4
6
8
dAoD/dT (mm/yr)

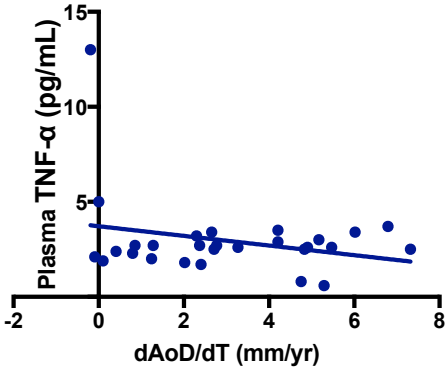

TIMP2
Plasma TIMP2 (pg/mL)
150000
100000
50000
-2
0
2
4
6
8
dAoD/dT (mm/yr)

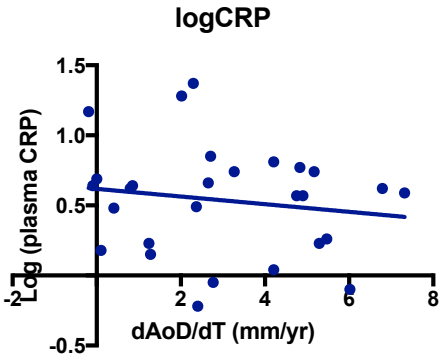

logTIMP4
Log (plasma TIMP4)
4.0
3.5
3.0
-2
0
2
4
6
8
dAoD/dT (mm/yr)

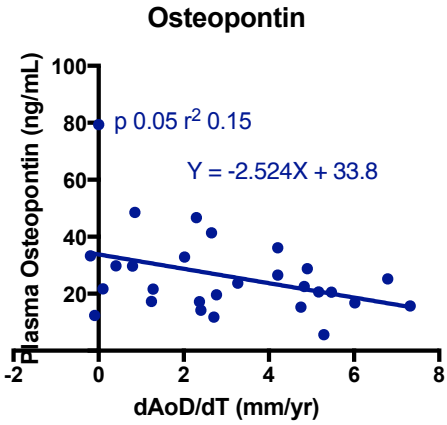

logTGFB1
log Plasma TGFB1
p 0.08 r2 0.14
4.5
4.0
3.5
3.0
-2
0
2
4
6
8
dAoD/dT (mm/yr)

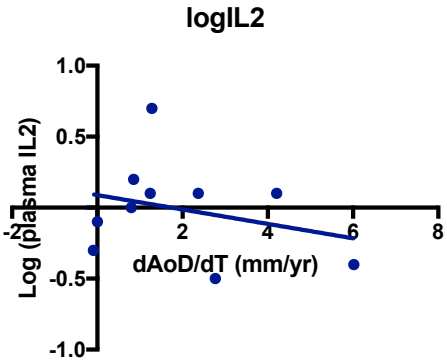

TGFB2
Plasma TGFB2 (pg/mL)
300
200
100
-2
0
2
4
6
8
dAoD/dT (mm/yr)

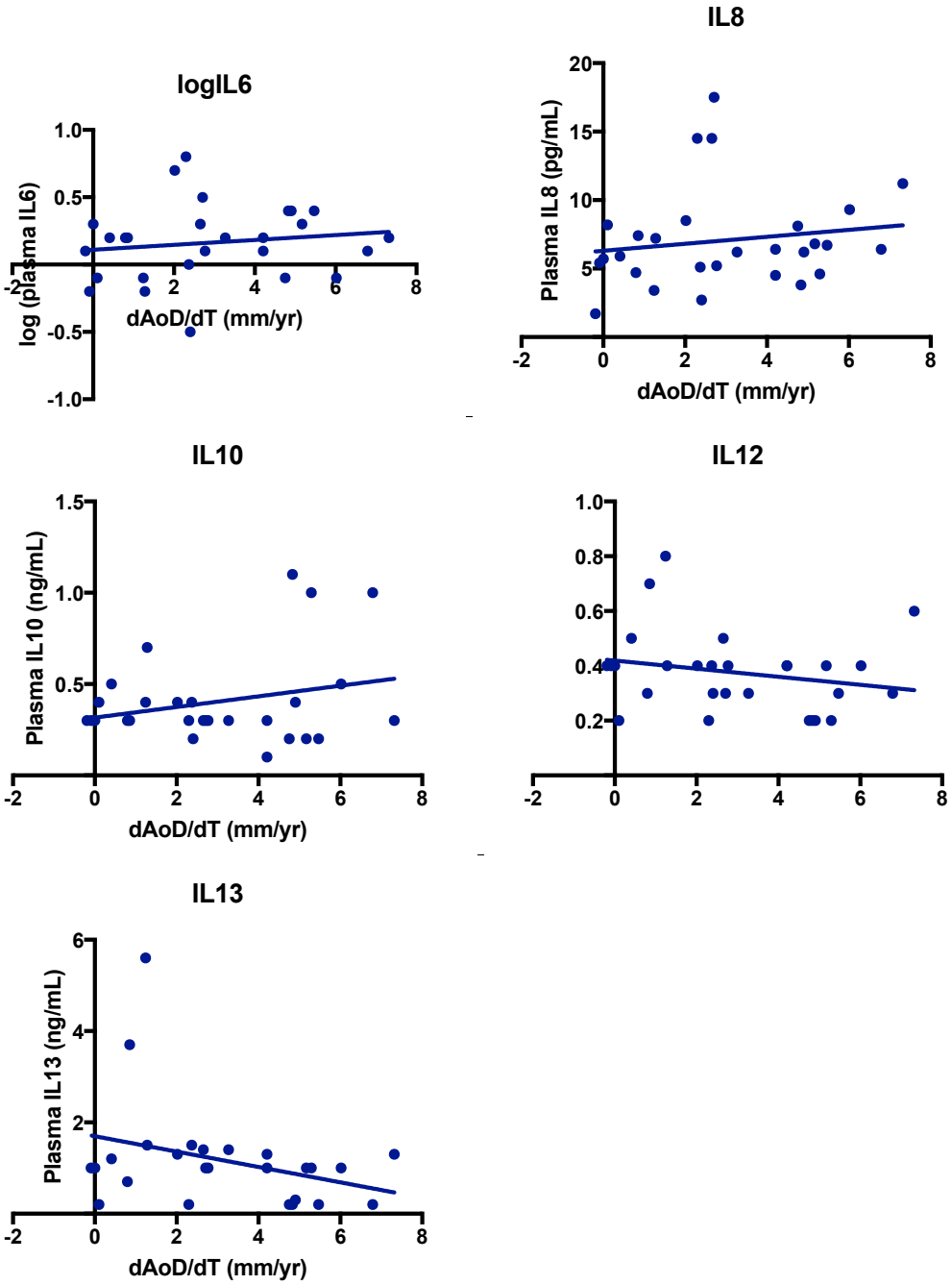

IFNG
Plasma IFNG (pg/mL)
p0.07 r2 0.12
Y = -2.664X + 22.21
100
80
60
40
20
-2
0
2
4
6
8
dAoD/dT (mm/yr)


TNFa
Plasma TNF-α (pg/mL)
15
10
5
-2
0
2
4
6
8
dAoD/dT (mm/yr)


logCRP
Log (plasma CRP)
1.5
1.0
0.5
-0.5
-2
2
4
6
8
dAoD/dT (mm/yr)


Osteopontin
Plasma Osteopontin (ng/mL)
p 0.05 r2 0.15
Y = -2.524X + 33.8
100
80
60
40
20
-2
0
2
4
6
8
dAoD/dT (mm/yr)

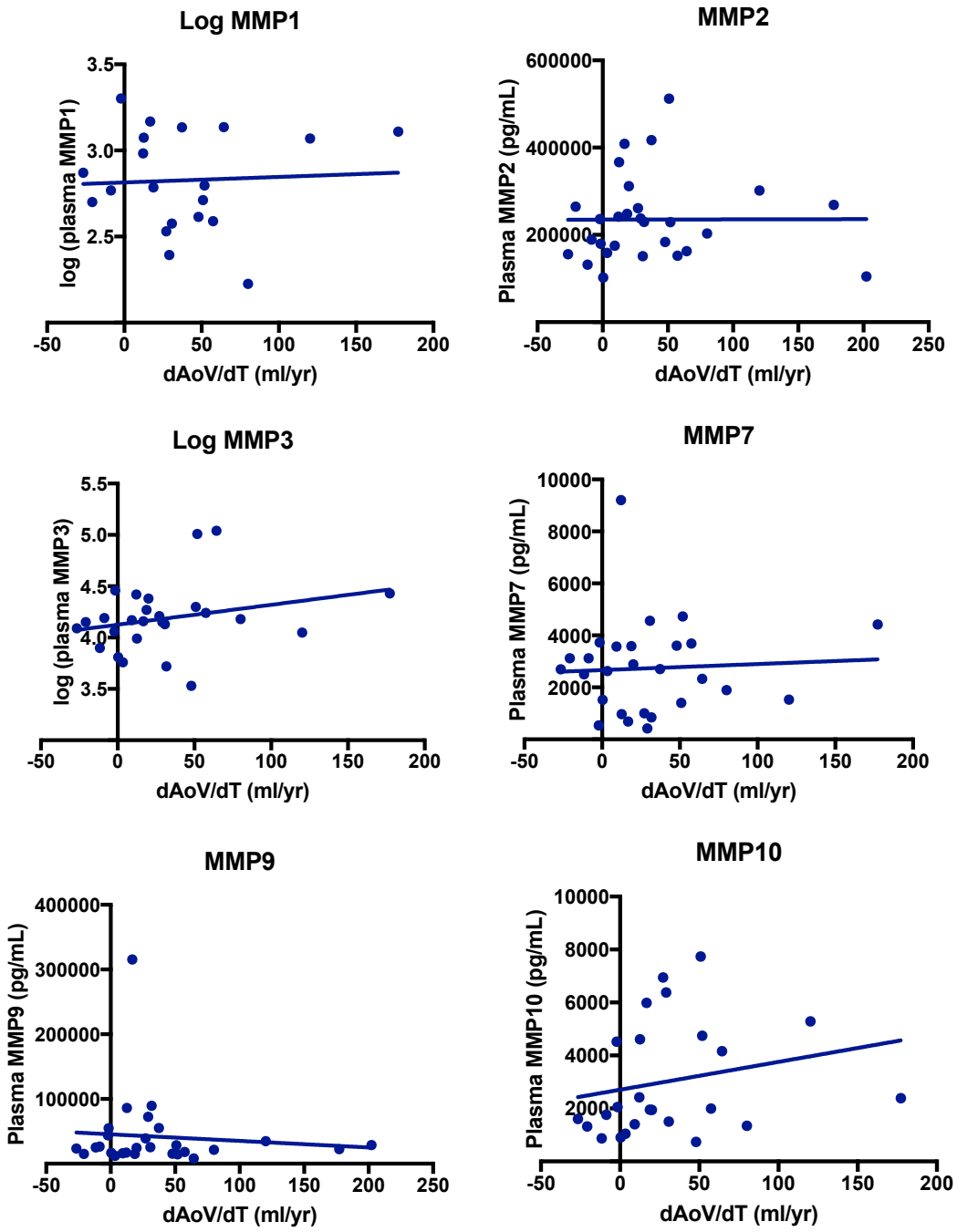

logIL2
Log (plasma IL2)
1.0
0.5
-0.5
-1.0
-2
2
4
6
8
dAoD/dT (mm/yr)

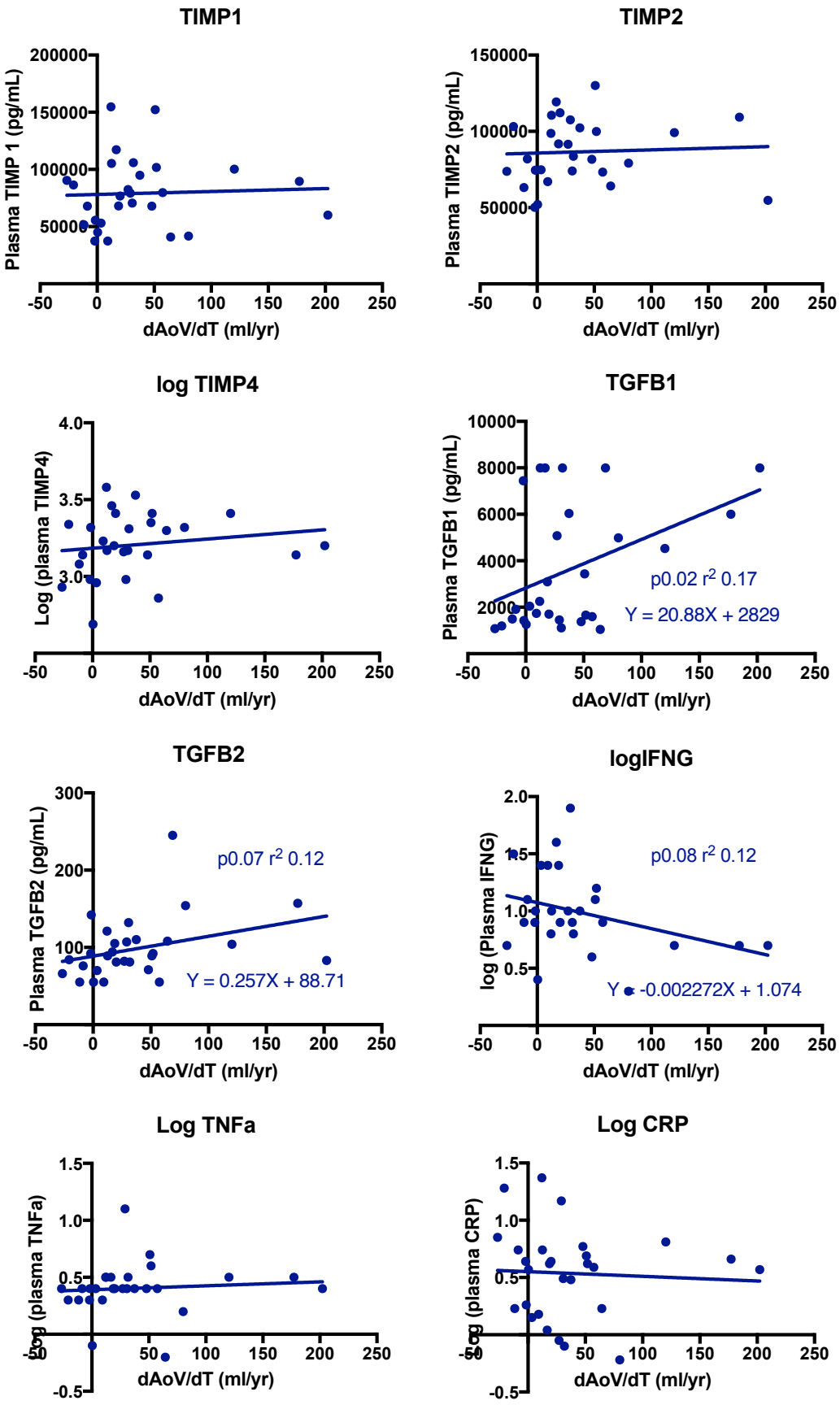


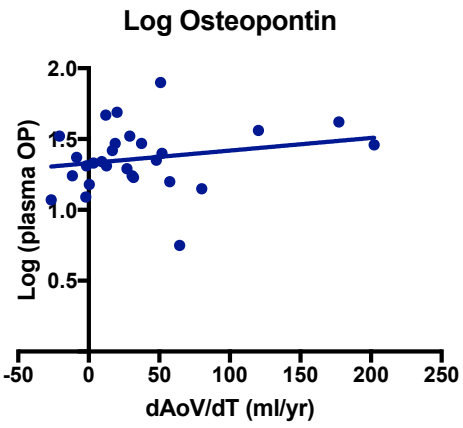
Studying correlations with rate of change of volume, again TGFβ1 (p 0.02), TGFβ2 (p 0.07) and log IL13 (p 0.08) show a statistically significant positive relationship with dAoV/dT, with r^2 values from 0.12-0.21 confirming that each of these analytes individually has only a weak correlation with rate of aneurysm growth.

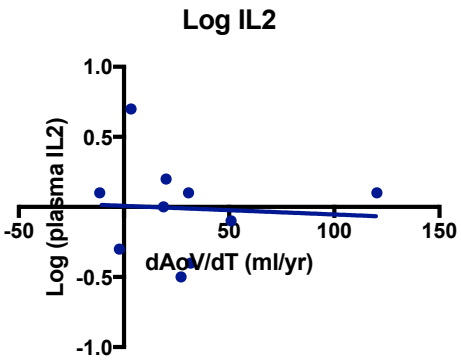
Figure 3.13 – graphs of correlations between plasma proteins and dAoV/dT.

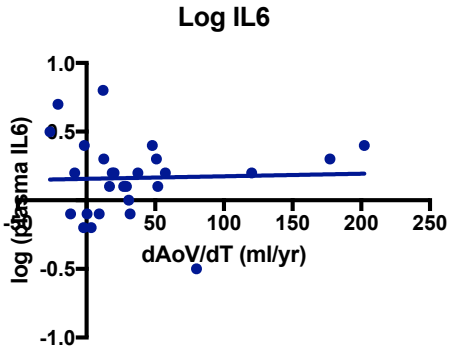
The following scatter plots demonstrate the relationship between aneurysm growth (in terms of rate of change of aortic arch/ DTA volume) and the various plasma proteins measured in the AD group. The equations of the regression lines, coefficients of determination (r^2) and the p value are provided on those graphs where the analysis yielded $p<0.1$.

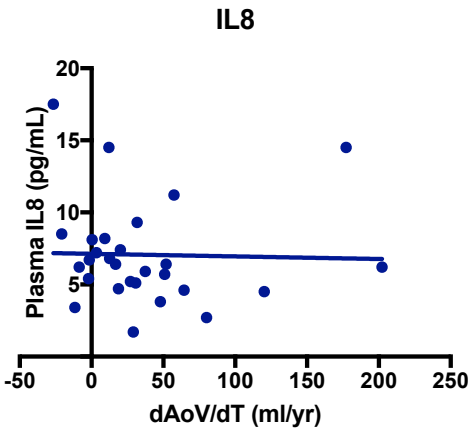


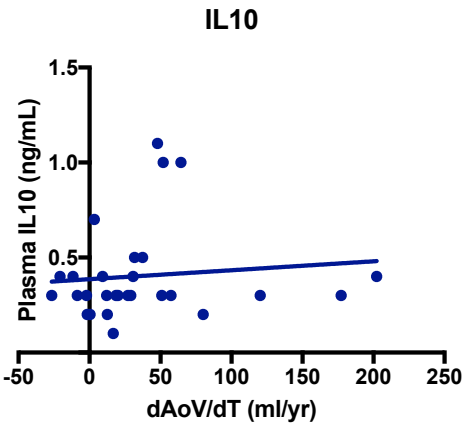

TIMP1
Plasma TIMP 1 (pg/mL)
200000
150000
100000
50000
-50
0
50
100
150
200
250
dAoV/dT (ml/yr)
TIMP2
Plasma TIMP2 (pg/mL)
150000
100000
50000
dAoV/dT (ml/yr)
log TIMP4
Log (plasma TIMP4)
4.0
3.5
3.0
dAoV/dT (ml/yr)
TGFB1
Plasma TGFB1 (pg/mL)
10000
8000
6000
4000
2000
p0.02 r2 0.17
Y = 20.88X + 2829
dAoV/dT (ml/yr)
TGFB2
Plasma TGFB2 (pg/mL)
300
200
100
p0.07 r2 0.12
Y = 0.257X + 88.71
dAoV/dT (ml/yr)
logIFNG
log (Plasma IFNG)
2.0
1.5
1.0
0.5
p0.08 r2 0.12
Y = -0.002272X + 1.074
dAoV/dT (ml/yr)
Log TNFa
Log (plasma TNFa)
1.5
1.0
0.5
-0.5
dAoV/dT (ml/yr)
Log CRP
Log (plasma CRP)
1.5
1.0
0.5
-0.5
dAoV/dT (ml/yr)

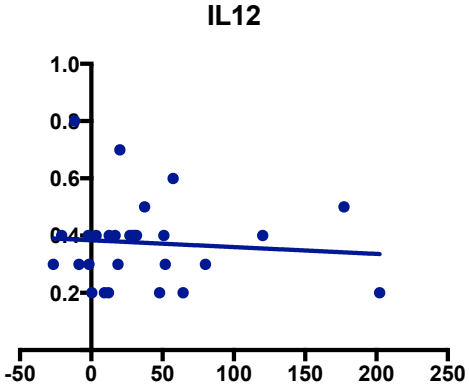

Log Osteopontin
Log (plasma OP)
2.0
1.5
1.0
0.5
-50
0
50
100
150
200
250
dAoV/dT (ml/yr)

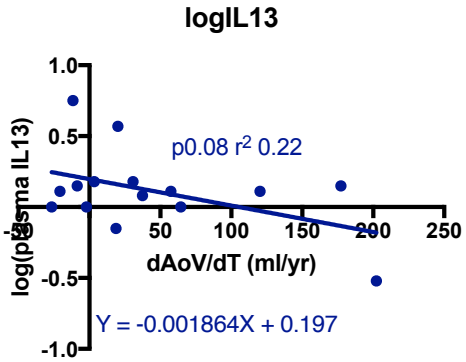

Log IL2
Log (plasma IL2)
1.0
0.5
-0.5
-1.0
-50
50
100
150
dAoV/dT (ml/yr)


Log IL6
1.0
0.5
-0.5
-1.0
50
100
150
200
250
dAoV/dT (ml/yr)


IL8
Plasma IL8 (pg/mL)
20
15
10
5
-50
0
50
100
150
200
250
dAoV/dT (ml/yr)


IL10
Plasma IL10 (ng/mL)
1.5
1.0
0.5
-50
0
50
100
150
200
250
dAoV/dT (ml/yr)


IL12
1.0
0.8
0.6
0.4
0.2
-50
0
50
100
150
200
250


logIL13
1.0
0.5
-0.5
-1.0
50
100
150
200
250
dAoV/dT (ml/yr)
p0.08 r2 0.22
Y = -0.001864X + 0.197

Differential White Cell Count and Aortic Outcomes

The differential white cell counts were compared in AD versus CTRL patients. The results are shown in Table 3.8 and indicate that there is no statistically significant difference in the two groups. However, although differential WBC is therefore not diagnostic, there is a weak correlation between neutrophil and lymphocyte counts and aneurysm diameter (Figure 3.14). These two variables also correlate weakly with rate of change of aortic diameter, although the p values (0.08 and 0.12 respectively) indicate that this is a less robust finding that would require a larger sample of patients to verify. Figures 3.15 and 3.16 demonstrate the linear regression of differential WBC against aortic growth.

Table 3.8 – differential WBC results of the AD group patients.

This table shows the average white cell count and its constituent parts in patients in the AD and CTRL groups of patients. The last column provides the p value derived by subjecting the AD and CTRL datasets to a two-tailed Student T test.

	AD group (n=46)		CTRL group (n=12)		P (AD vs CTRL)
	Mean	SEM	Mean	SEM	
Total white cell count	7.0	0.2	7.1	0.2	0.71
Neutrophil count (%)	63.8	1.2	64.4	2.2	0.82
Lymphocytes (%)	23.7	1.1	23.9	1.8	0.90
Monocytes (%)	8.4	0.4	8.0	0.6	0.55
Eosinophils (%)	2.8	0.3	2.8	0.3	0.96
Basophils (%)	0.6	0.1	0.5	0.1	0.68

Figure 3.14 Correlations between WBC and baseline aortic diameter

The following scatter plots demonstrate the relationship between the maximum aortic diameter at baseline and the white cell counts (total and constituent parts) of the 46 AD patients. The equations of the regression lines are given where the analysis indicated statistically significant correlations. The coefficient of determination (r^2) and the p value are provided within each graph.

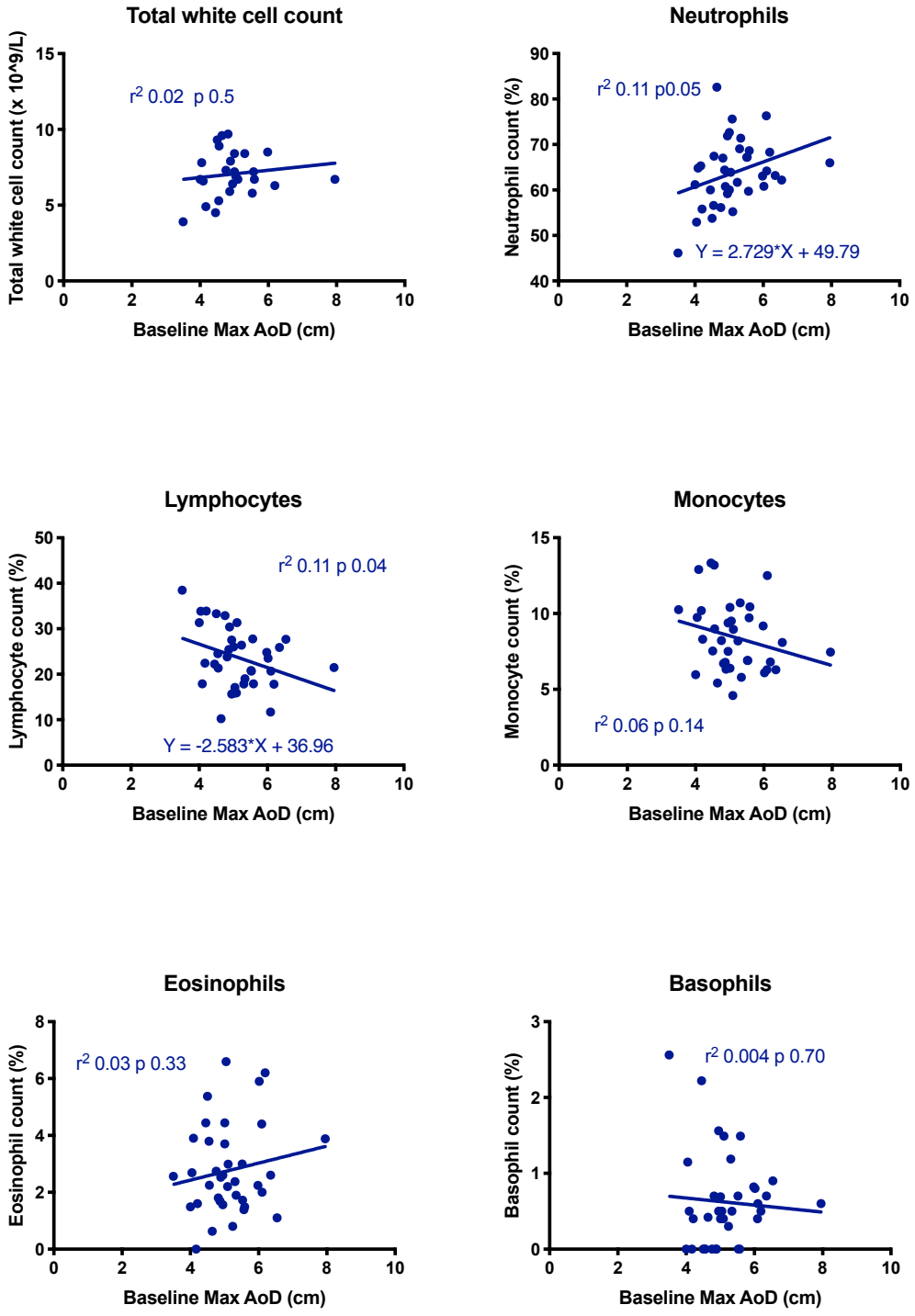
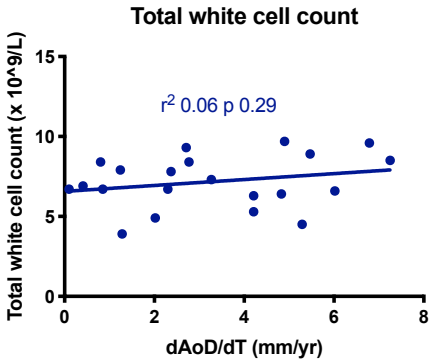
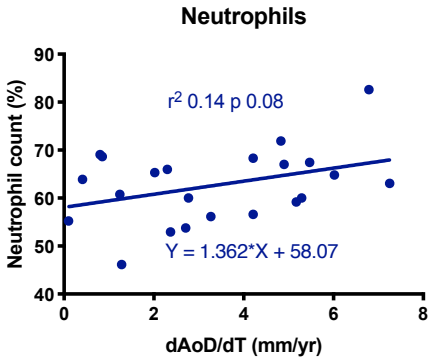
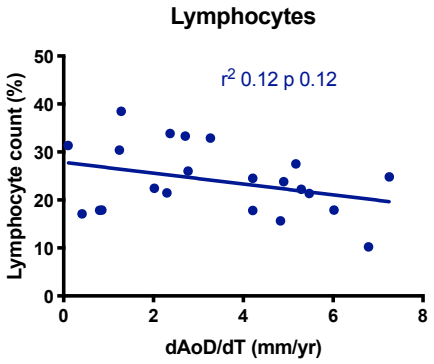


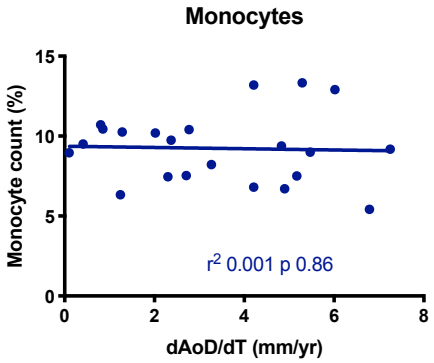
Figure 3.15 - correlations between WBC and aortic growth (dAoD/dT)

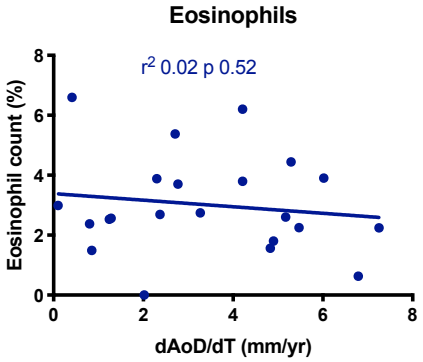
The following scatter plots demonstrate the relationship between aneurysm growth (in terms of rate of change of maximal diameter) and the white cell counts (total and constituent parts) of the 46 AD patients. The equation of the regression line is given in the graph of neutrophil count versus dAoD/dT, where the analysis indicated a weak correlation approaching statistical significance (p 0.08). The coefficient of determination (r^2) and the p value are also provided within each graph.











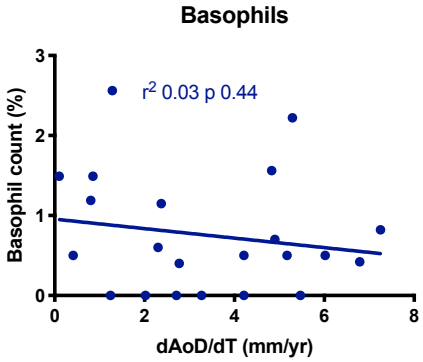
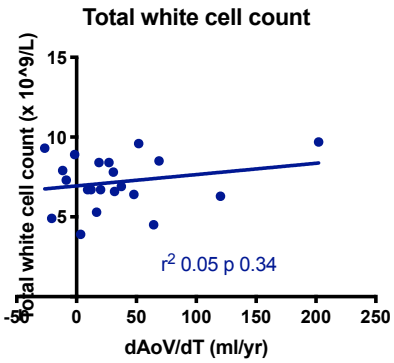
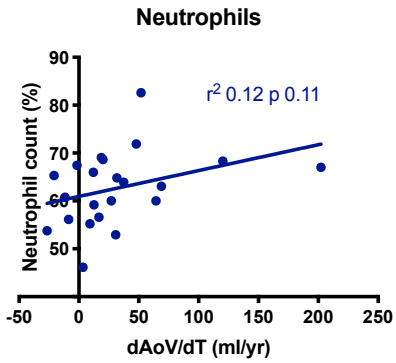
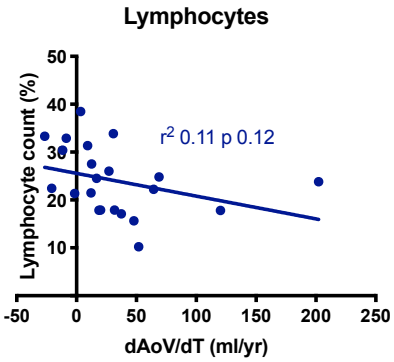


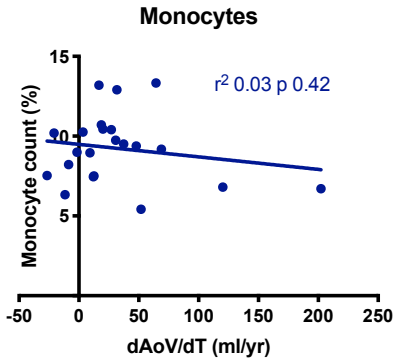
Figure 3.16 - correlations between WBC and aortic growth (dAoV/dT)

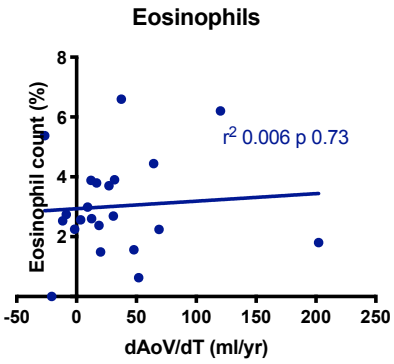
The following scatter plots demonstrate the relationship between aneurysm growth (in terms of rate of change of aortic volume) and the white cell counts (total and constituent parts) of the 46 AD patients. The equations of the regression lines are not given here since none of the analyses indicated statistically significant correlations. However the coefficient of determination (r^2) and the p value are provided within each graph.

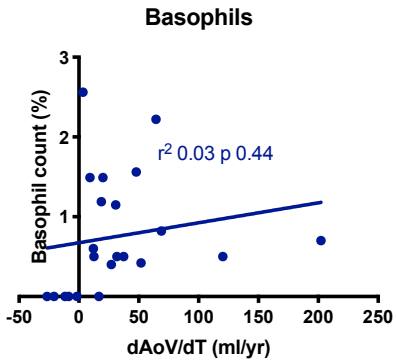

Total white cell count
Total white cell count (x 10^9/L)
15
10
5
r2 0.05 p 0.34
-50
0
50
100
150
200
250
dAoV/dT (ml/yr)


Neutrophils
Neutrophil count (%)
90
80
70
60
50
r2 0.12 p 0.11
-50
0
50
100
150
200
250
dAoV/dT (ml/yr)


Lymphocytes
Lymphocyte count (%)
50
40
30
20
10
r2 0.11 p 0.12
-50
0
50
100
150
200
250
dAoV/dT (ml/yr)


Monocytes
Monocyte count (%)
15
10
5
r2 0.03 p 0.42
-50
0
50
100
150
200
250
dAoV/dT (ml/yr)


Eosinophils
Eosinophil count (%)
8
6
4
2
r2 0.006 p 0.73
-50
0
50
100
150
200
250
dAoV/dT (ml/yr)


Basophils
Basophil count (%)
3
2
1
r2 0.03 p 0.44
-50
0
50
100
150
200
250
dAoV/dT (ml/yr)

PBMC-RNAs and Aortic Outcomes

The pilot study concluded with the validation of 7 targets as being differentially expressed in AD versus CTRL patients: ADAMTS2, BPI, CAMP and LYZ at the $p<0.05$ level of significance; and MSR1, PRTN3 and SYCE1 at the $p<0.1$ level of significance. The relationship between the expression of these 7 RNA targets and AD aneurysm size and growth rate was explored using linear regression (Figures 3.17-3.19), but no significant associations were identified.

Figure 3.17 correlations between RNAs and baseline aortic diameter

The following scatter plots demonstrate the relationship between the maximum aortic diameter at baseline (the time of recruitment) and the 7 genes shown to be differentially expressed after the qPCR validation of the 2nd RNA sequencing results. The equations of the regression lines are not given here since none of the analyses indicated statistically significant correlations. However the coefficient of determination (r^2) and the p value are provided within each graph.

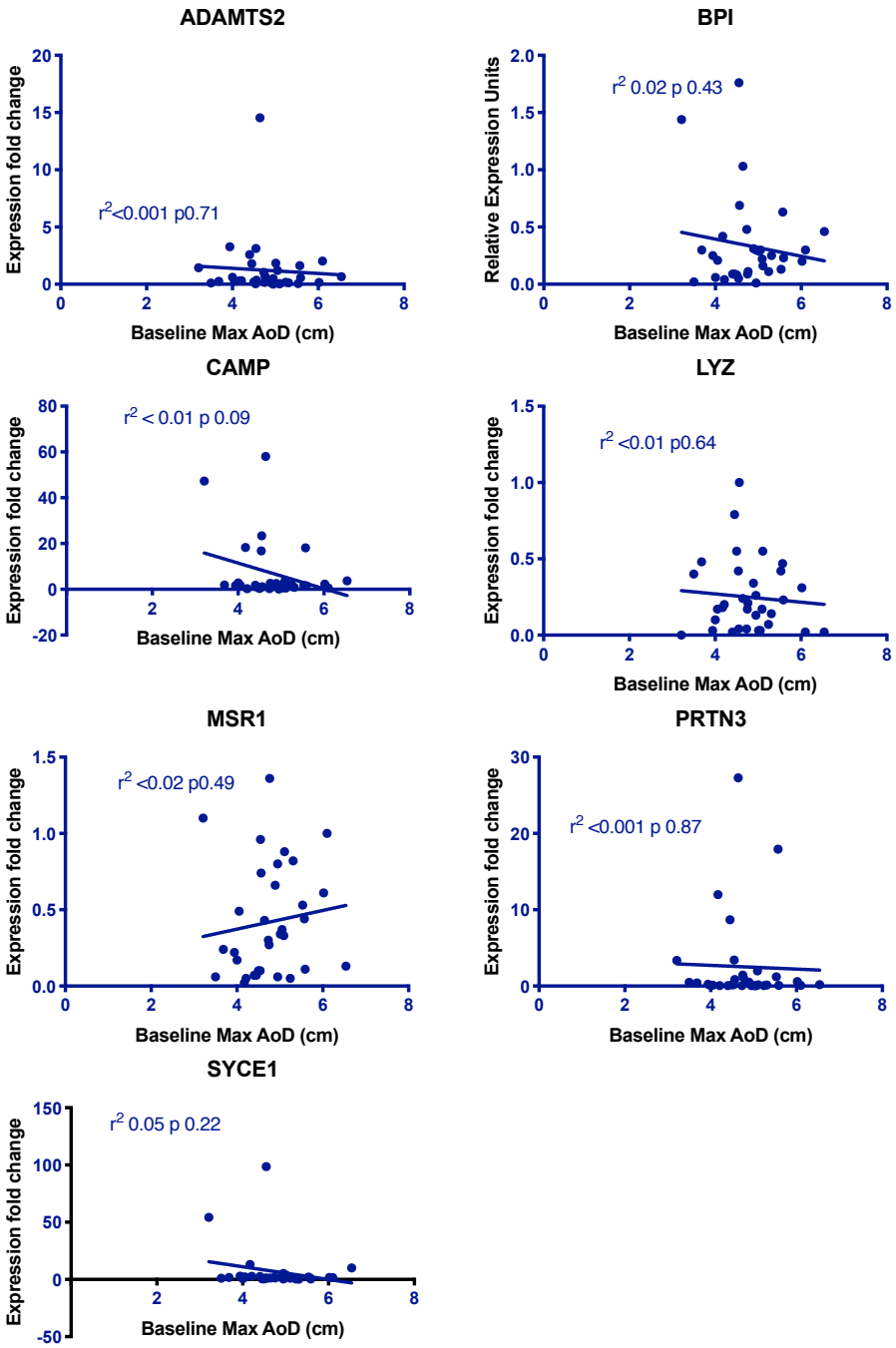


Figure 3.18 - correlations between RNAs and aortic growth (dAoD/dT)

The following scatter plots demonstrate the relationship between aneurysm growth (in terms of rate of change of maximal diameter) and the 7 genes shown to be differentially expressed after the qPCR validation of the 2nd RNA sequencing results. The equations of the regression lines are not given here since none of the analyses indicated statistically significant correlations. However the coefficient of determination (r^2) and the p value are provided within each graph.

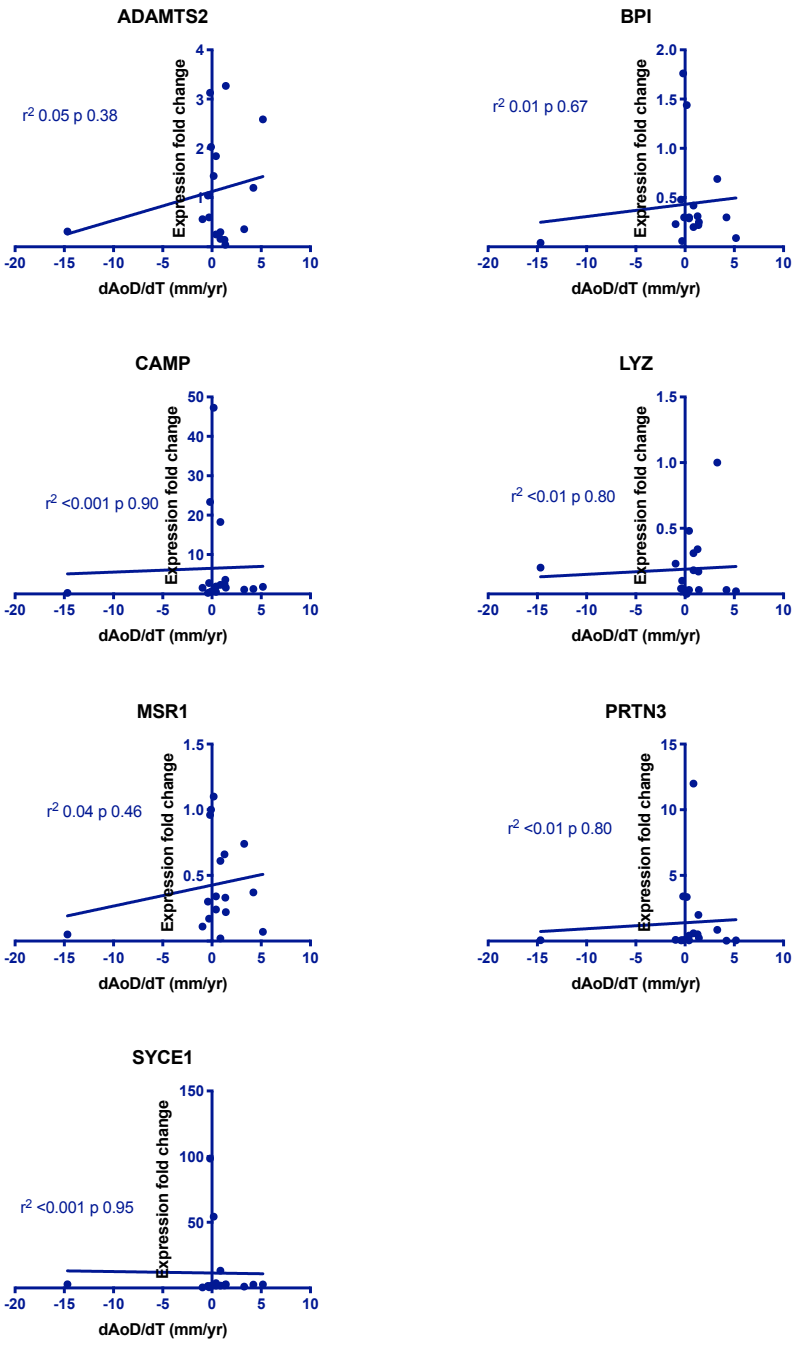
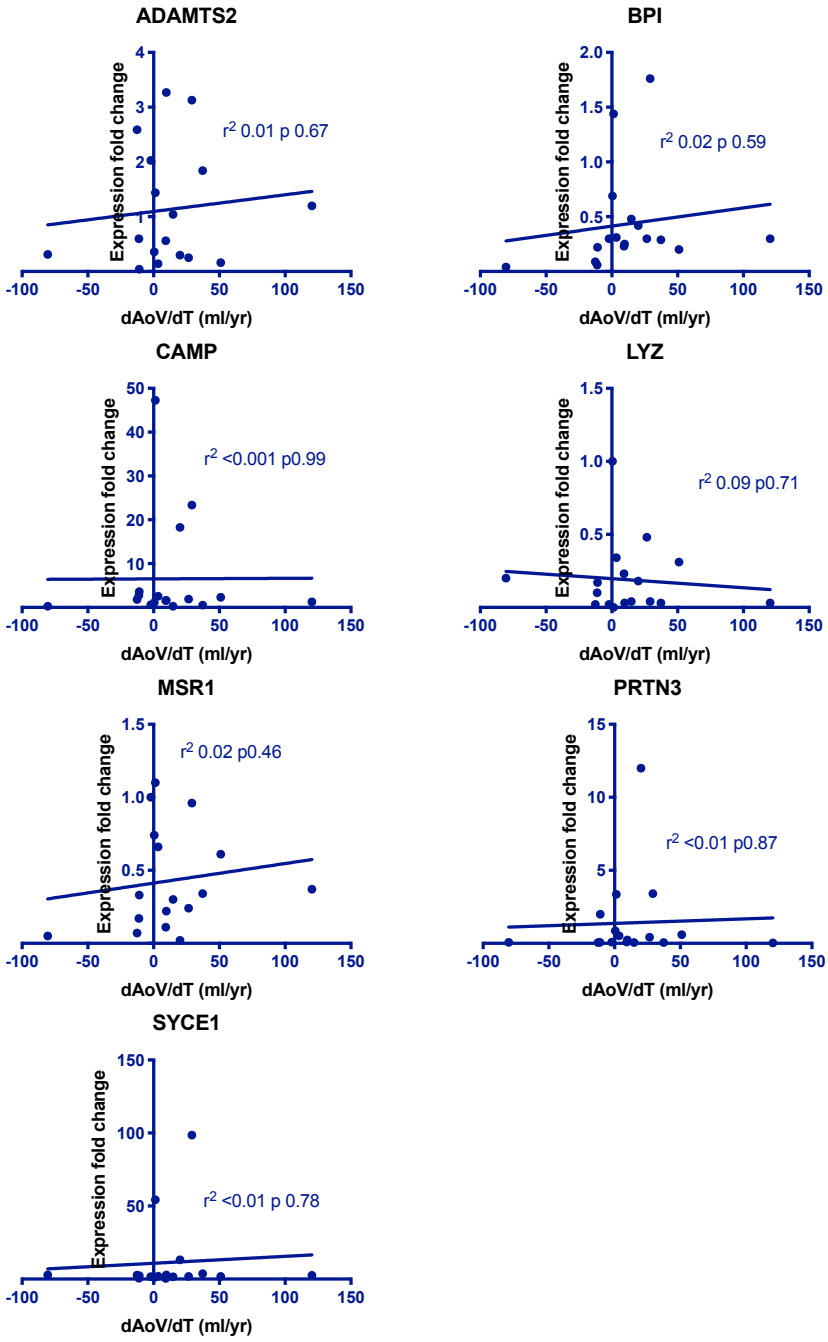


Figure 3.19 - correlations between RNAs and aortic growth (dAoV/dT)

The following scatter plots demonstrate the relationship between aneurysm growth (in terms of rate of change of aortic volume) and the 7 genes shown to be differentially expressed after the qPCR validation of the 2nd RNA sequencing results. The equations of the regression lines are not given here since none of the analyses indicated statistically significant correlations. However the coefficient of determination (r^2) and the p value are provided within each graph.



Wall Stress and Aortic Outcomes

Numerical models were constructed as described in the methods section and simulated in 6 cases of true DTA aneurysms (Figure 3.20). The mean wall stress was found to be strongly associated with baseline AoD (r^2 0.83, p 0.01, Figure 3.21). This is to be expected due to the Law of Laplace, which states that stress within the wall should be proportional to the diameter of the vessel. However in

practice a perfect correlation is never achieved in nature, as the vascular bed is a very complicated system. Other factors such as the 3-dimensional curvature of aorta, presence of ILT and calcium and variation in blood pressure will all contribute to the wall stress. In particular, the ILT acts as a mechanical barrier separating the blood flow and aortic wall. This is known as the cushion effect and lowers the wall tension. This principle is well demonstrated in the case with the largest baseline AoD (Figure 8.6c). In this case, there was a considerable volume of ILT, but blood pressure was also reasonably controlled (138/83 mmHg). As a result, the mean wall stress of the subject was lower compared with subjects with similar AoD.

Figure 3.20 – Wall stress plots of the 6 cases modelled.

This figure depicts band plots of the von Mises stress (in kPa) in the 6 cases (a-f) with true aneurysm of the DTA. Red represents high wall stress regions and blue represents low stress in each case. High stress concentrations were observed in regions with inflection, sharp changes of geometry or material mismatch (for example in cases a, e and f).

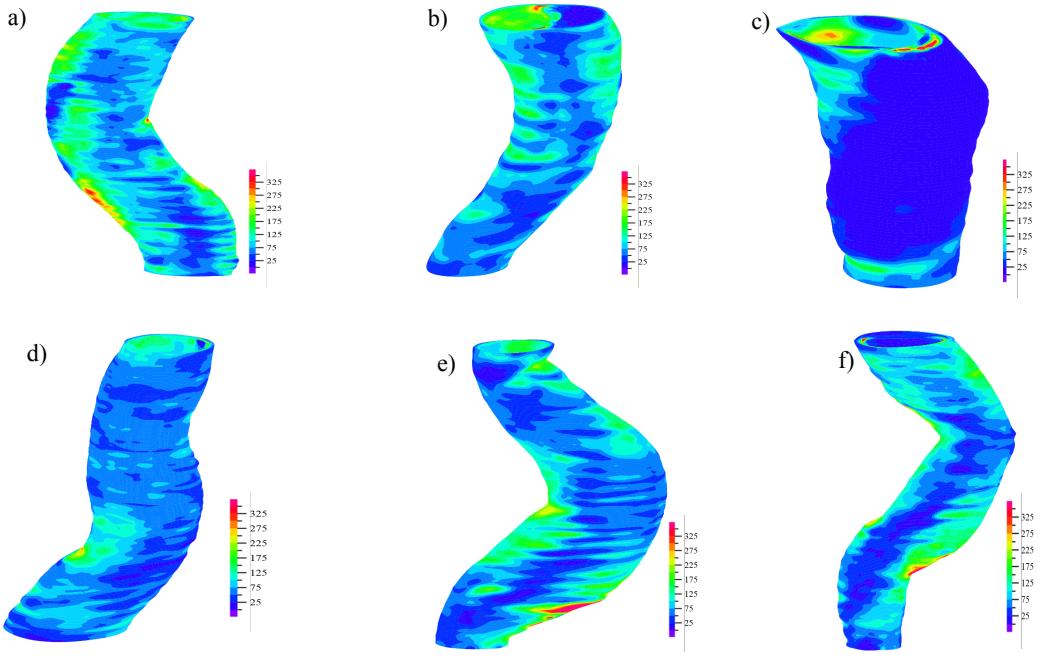
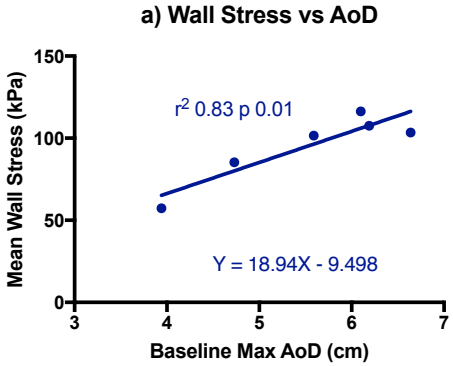


Figure 3.21 – Correlation between mean wall stress and baseline AoD

This figure demonstrates the results of univariate regression analysis comparing the mean wall stress in 6 cases of DTA true aneurysm against maximum aortic diameter at baseline. As previously, the equations of the regression lines, the coefficient of determination (r^2) and the p value are also provided and indicate a strong relationship between the estimated wall stress (derived from the CT scan) and aneurysm size, with $r^2 > 0.7$ and $p < 0.05$.



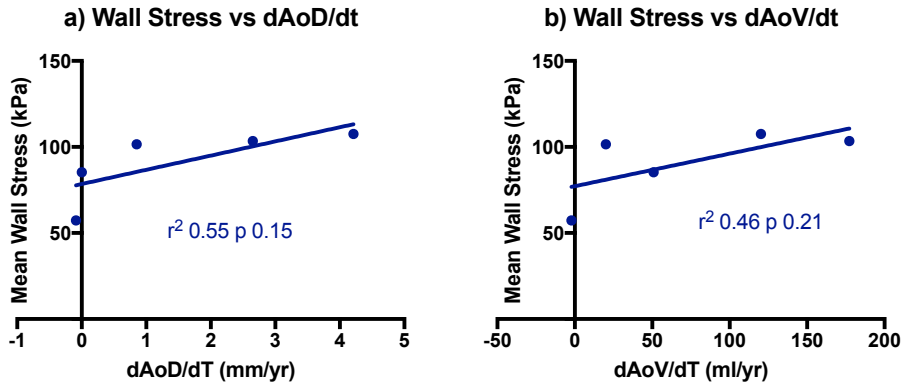
Of the 6 cases modelled, one patient underwent surgery and therefore follow-up data was not available. For the remaining 5 cases, wall stress was compared against prospectively observed growth rate. The linear regressions are shown in Figure 3.22.

There appears to be a reasonable correlation between mean wall stress and dAoD/dT (r^2 0.55 p0.15, Figure 8.8a) and also dAoV/dT (r^2 0.46, p0.21, Figure 8.8b), but this failed to reach statistical significance – possibly due to the small number of cases.

Figure 3.22 – Correlation between wall stress and aneurysm growth rates.

This figure demonstrates the results of univariate regression analysis comparing the mean wall stress (estimated from baseline CT scans) in 6 cases of DTA true aneurysm against a)rate of change of aneurysm diameter over time and b) rate of change of AD aneurysm volume over time. Visually there appears to be a reasonable correlation, but the coefficients of determination (r^2 values) indicate the relationship is not as strong as that with aneurysm size (Figure 8.7). The p

values fail to achieve statistical significance, but this may simply be due to the small number of datapoints.



Multivariate regression

The results above describe the efforts made to identify correlates of aneurysm progression in this study. Univariate regression analysis has shown that any statistically significant correlations are weak. It is of particular interest that aneurysm size at baseline (which is currently used as the key determinant of whether intervention is required) does not demonstrate a statistically significant correlation with growth rate.

At the outset, the goal was to use multivariate regression to identify a panel of biomarkers that could be more predictive of aneurysm progression than a single biomarker alone. There are several challenges in this project that make multivariate regression difficult. Firstly, many (40) baseline variables were collected in a few (46) patients. Such a high number of variables overwhelms standard packages for multivariate regression analysis (most of which cater for 5-10 covariates). Secondly, when the number of covariates is similar to the number of samples, advanced statistical techniques are required. Thirdly, some of the analytes were not consistently quantifiable in all the samples, leading to a degree of missingness across analytes and across samples. Despite these challenges, having invested the effort to obtain the dataset it was decided to

pursue multivariate modelling further with the help of a specialist statistician (Dr Sarah Marley of Select Statistics) as a consultant. Her report is paraphrased below.

Missing data

There are various unquantified values in the dataset – either because the analyte was truly unquantifiable or because its level fell outside the thresholds of the assay (Table 9.1). It is necessary to complete the dataset as far as possible to facilitate multivariate regression and there are different strategies that can be used to tackle each of types of missingness:

1. Missing values:

   These need to either be imputed or discarded on a case-by-case basis. Both of these strategies could potentially introduce bias into the results. Given that the dataset from which to impute values is relatively small, it was decided that omitting the missing values was a better approach. Therefore, where a value was completely missing, that patient was omitted from any elements of the analysis where the corresponding variable is included. For other analyses not requiring the corresponding missing variable, that patient is reintroduced to maintain the maximum sample size possible.

2. Where values are reported as "less than" or "more than" the assay limits, there are slightly different options.
   a. These values can be dropped from the analysis. However as these are the low and high extremes, removing them would likely bias the results.
   b. Alternatively, the values could be imputed with either the minimum detectable limit or a suitably low value for the "less

thans"; and equivalently the maximum detectable limit or a suitably high value for the "more thans". As there was no clear clinical or biological basis on which to infer what the most likely "true" values were in these cases, it was agreed to run both of the these options (a and b) in the analysis.

Table 9.1 –missingness and sub- and supra-threshold values in the biomarker dataset

This table shows the number of samples which did not provide exact values of each biomarker. The first column lists the biomarkers assayed, the next column details how many values were missing out of the 46 samples assayed. The column entitled "less thans" shows the number of samples in which the analyte was not abundant enough to be measured by the assay. Similarly the column entitled "more thans" shows the number of samples in which the abundance of the analyte exceeded the assay's capacity to quantify it. The final column provides the number of samples that therefore provided exact measurements each analyte.

Biomarker	**Missing (blanks)**	**"Less thans"**	**"More thans"**	**Non-missing and not less than/more than**
MMP1	5	8	0	33
TGFB1	0	0	8	38
TGFB2	0	8	0	38
IFNγ	3	1	0	42
IL2	3	31	0	12

IL6	3	1	0	42
IL10	3	5	0	38
IL12	3	20	0	23
IL13	4	17	0	25

Analysis Workflow

The distribution of the data was checked, confirming that aortic growth outcomes were normally distributed. However, there is some evidence of the size outcomes and biomarkers being skewed, indicating a possible lack of normality and providing motivation to log these values in the analysis. Having said that, it is important to note that the assumption of normality for a linear regression model is that the model residuals (difference between the observed and predicted values) are normally distributed, which does not necessarily rely upon the outcomes or predictor variables themselves being normally distributed. Therefore, we explored both the logged (taking the natural log, i.e., inverse of the exponential function) and raw/unlogged values of the biomarkers and size outcomes in the subsequent analysis. For the two key outcomes (dAoD/dT and dAoV/dT), the following steps were then followed:

1. Univariate analysis
2. Principal component analysis
3. Multivariate modelling

Univariate analyses

In an echo of the simple univariate analysis done in earlier chapters, but this time with imputed values where relevant, individual linear regression models were fitted to the dAoD/dT and dAoV/dT outcomes. This time, the univariate regression included one potential predictor variable at a time, i.e. not controlling for any other potential confounders or risk factors. Logged and unlogged biomarkers were modelled, and the approaches of a) not imputing the "less than"/"more than" values; b) imputing these with the quoted values and c)

imputing these with the minimum/maximum values as described above were all tested. Thus there was weak evidence ($p<0.1$) of an association between dAoD/dT and the variables reported in Table 3.9. Imputing values using the strategies described did not dramatically change any of the results identified in my own univariate analysis.

Table 3.9 – associations between biomarkers and dAoD/dT

This table summarises the results of univariate regression analysis performed by SelectStatistics on the dataset of potential biomarkers in the 46 AD patients. This univariate regression exercise used imputation to recover any missing values. Imputations either assumed less thans and more thans to equate to the thresholds of the assay, or assumed that they equated to the minimum and maximum values quantified in any of the samples. The regressions were repeated in each case to look for the best regression model. Any 'biomarkers' that subsequently yielded a statistically significant correlation against aneurysm growth (dAoD/dT) at the p<0.1 level are listed in this table.

Outcome = dAoD/dT	**Predictor**	**Coefficient**	**p-value**	**Adjusted r^2**	**(Number of patients included in model)**
Blood components	Neutrophils	0.111	0.069	0.12	21
	Lymphocytes	-0.111	0.098	0.092	21
	log(neutrophils)	6.75	0.075	0.113	21
	log(lymphocytes)	-2.56	0.092	0.097	21
Plasma	MMP2	-	0.066	0.098	26

proteins		0.0000081			
	MMP3	0.0000305	0.071	0.101	24
	TGFβ1	-0.000505	0.052	0.135	22
	Osteopontin	-0.0525	0.066	0.098	26
	IL10	3.43	0.059	0.115	24
	log(MMP2)	-1.94	0.074	0.091	26
	log(TGFβ1)	-1.57	0.045	0.146	22
	log(IL13)[1]	-0.818	0.0798	0.086	26
	log(IL2) [2]	-1.04	0.0275	0.153	26

[1]'Less than' values imputed as the minimum detectable threshold of the assay;

[2] 'Less than' values imputed as the minimum value measured in the cohort.

For the blood components above, examining the model fit (via residual plots), we find that the unlogged models appear to better meet the model assumptions, therefore we would recommend focussing on the models with the unlogged blood components above. Conversely, for the biomarkers MMP2 and TGFB1, the logged models appear to fit better. The model fit for the other models listed above appears reasonable, though it should be noted that these are based on relatively low numbers of observations. Also, the p-values for the coefficients in the above models are generally greater than 0.05 (but less than 0.1) indicating only weak evidence of an association with the outcome (at the 10% significance level). Furthermore, we have run lots of models and therefore statistical tests here, and so we should be cautious not to over-interpret the results given the level of multiple testing (each test has a chance of returning a false positive result and by running lots of tests we increase our chances of finding a false positive).

Regarding dAoV/dT, there is evidence (p-value<0.1) for an association with the variables shown in Table 3.10.

Table 3.10 - associations between biomarkers and dAoV/dT

This table summarises the results of univariate regression analysis performed by SelectStatistics on the dataset of potential biomarkers in the 46 AD patients. This univariate regression exercise used imputation to recover any missing values. Imputations either assumed less thans and more thans to equate to the thresholds of the assay, or assumed that they equated to the minimum and maximum values quantified in any of the samples. The regressions were repeated in each case to look for the best regression model. Any 'biomarkers' that subsequently yielded a statistically significant correlation against aneurysm growth (dAoV/dT) at the p<0.1 level are listed in this table.

Outcome = dAoV/dT	**Predictor**	**Coefficient**	**p-value**	**Adjusted r^2**	**n (Number of patients)**
Biomarkers	TGFβ1[1]	0.00995	0.013	0.191	27
	TGFβ 2[2]	0.455	0.082	0.081	27
	IFNγ [2]	-1.83	0.088	0.08	26
	TGFB2[3]	0.455	0.082	0.081	27
	IFNγ [3]	-1.83	0.088	0.08	26
	log(IFNγ)	-27.2	0.069	0.099	25
	log(IL13)	-49.6	0.083	0.153	15
	log(TGFB1)[1]	36	0.010	0.205	27

	log(TGFB2)[2]	55.4	0.061	0.099	27
	log(IFNγ)[2]	-22.7	0.069	0.095	26
	log(TGFB2)[3]	55.4	0.061	0.099	27
	log(IFNγ)[3]	-22.7	0.069	0.095	26
	TGFβ1[4]	0.0107	0.0049	0.247	27
	log(TGFβ1)[4]	37.3	0.0062	0.234	27
Risk factors	BSA	-115	0.033	0.137	27
	log(BSA)	-245	0.027	0.148	27
Aneurysm sizes	Baseline MaxAoD	21.2	0.071	0.09	27
	Baseline Indexed AoD	36.4	0.072	0.089	27
	log(BaselineMax AoD	121	0.053	0.108	27
	log(Baseline Indexed AoD)	105	0.042	0.121	27
	log (BaselineAoV)	35.2	0.093	0.073	27
	Baseline Max AoD(<5.5cm versus ≥5.5cm)	-49.8	0.066	0.094	27

[1] 'More than' values imputed as the maximum detectable threshold of the assay;
[2] 'Less than' values imputed as the minimum detectable threshold of the assay;
[3] 'Less than' values imputed as the minimum value measured in the cohort;
[4] 'More than' values imputed as the maximum value measured in the cohort;

Principal Component Analysis (PCA)

After running some initial PCAs on the imputed plasma protein values, it was apparent that in order to capture a reasonable proportion of the variation in the

values, we need a relatively high number of the principal components. For example, to capture 50% of the variation we would need at least 3 components, to capture 75% of the variation we would need at least 7 and to capture 90% of the variation we would need at least 10 components. (This is further exacerbated if we also include the numeric risk factors and/or blood components in the PCA.) Given the sample size available, this would be too many components to include in a multiple variable regression. It would have been desirable to use just one or two composite measures to include in the models, but in this case this would likely capture too little of the information contained in the dataset to make this effective (especially given the added difficulty in interpreting the principal components). Consequently, rather than a traditional multivariate regression model, we aimed instead to create a forward stepwise model, whereby the first 5 principal components were evaluated for their ability to improved growth prediction on top of baseline aortic diameter. This is a pragmatic approach when data is limited and also reflects the clinical practice of first assessing risk according to baseline aortic size.

Multiple variable modelling

Adding in the most informative biomarkers and/or blood components (based on the adjusted r^2 values in the univariate modelling), on top of the log Baseline Indexed AoD as a predictor variable, we obtain the following "final" model (Table 3.11). As noted above, we've only allowed a couple of additional predictors into the final model here as we have a limited sample size.

Table 3.11 – the final multivariate model of best fit for predicting dAoD/dT

This table displays the results of the multivariate regression modelling exercise undertaken by Select Statistics, looking for predictors of aneurysm growth in terms of rate of change of aortic diameter. Here the relationship between baseline aneurysm diameter is taken as the starting point for the model, given that this is current clinical practice, even though the correlation with future growth is poor.

The biomarkers that provided the best 'improvement' to this basic model are listed in the table, beneath log(Baseline Indexed AoD). The p-value (from Spearman rank correlation) is shown next to each biomarker, followed by an overall F-test p value for the multivariate model. The coefficient of determination appears in the penultimate column – 0.24 indicating the regression model does not model the data particularly well. Finally the last column shows how many complete patient datasets contributed to the model.

Predictor variable	**Coefficient**	**p-value**	**Overall model F-test p-value**	**Adjusted r^2**	**Number of patients included in**
log(Baseline IndexedAoD)	1.33	0.5041	0.0476	0.240	22
log(TGFβ1)	-1.47	0.0513			
log(IL2) (Less than imputation = Min)	-0.85	0.0745			

The table above translates into a 'predictive' formula as follows:

$$dAoD/dT = 0.43(\text{baseline indexed AoD}) - 1.5(\log TGF\beta 1) - 0.86(\log IL2)$$

The overall F-test reported above tests whether the combination of variables included in the model significantly improves the fit compared to an intercept-only model (i.e., a model simply based on the mean outcome). An F-test p value of 0.05 suggests that it does. However, the r^2 value is relatively low (0.24) suggesting that this equation or combination of variables does not describe the variation in dAoD/dt particularly well.

As with the univariate modelling results, it is important to note that we only find weak evidence of the above effects and that the results are based on a sample of

the patients available (due to the missing data in both the outcome and predictor variables) and a relatively small overall sample size. Furthermore, for the IL2 biomarker, a reasonably large proportion of the values were only available as "less than" a certain figure and have been imputed with the minimum observed/quoted value for this variable.

Similarly, adding in the most informative biomarkers and/or blood components (based on the adjusted r^2 values), on top of the log Baseline Indexed AoD as a predictor variable, we obtain the following "final" model for dAoV/dT (Table 3.12).

Table 3.12 - the final multivariate model of best fit for predicting dAoV/dT

This table displays the results of the multivariate regression modelling exercise undertaken by Select Statistics, looking for predictors of aneurysm growth in terms of rate of change of aortic volume. Here the relationship between baseline aneurysm diameter is taken as the starting point for the model, given that this is current clinical practice, even though the correlation with future growth is poor. The biomarkers that provided the best 'improvement' to this basic model are listed in the table, beneath log(Baseline Indexed AoD). The p-value (from Spearman rank correlation) is shown next to each biomarker, followed by an overall F-test p value for the multivariate model. The coefficient of determination appears in the penultimate column – 0.24 indicating the regression model does not model the data particularly well. Finally the last column shows how many complete patient datasets contributed to the model.

Predictor variable	**Coefficient**	**p-value**	**Overall model F-test p-value**	**Adjusted r^2**	**Number of patients included in model**
log(Baseline Indexed	157	0.0221	0.0045	0.594	15

AoD)					
TGFB1[1]	0.0125	0.0321			
log(IL13)	-22.5	0.2987			

[1] 'More than' values imputed as the maximum detectable threshold of the assay;

As above, this translates to a 'predictive' formula as follows:

$$dAoV/dT = 1.57(\text{baseline indexed AoD}) + 0.01(\log TGFB1) - 22.5(\log IL13)$$

In this multivariate model, the F-test value is good (<0.001) and the r^2 value of 0.59 indicates this equation more robustly models the variation in dAoV/dT. However, all the caveats regarding sample size and the limitations incurred by imputing values hold true for this model as well as the one for dAoD/dT.

SUMMARY

Multivariate regression modelling of these results is challenging because the dataset is relatively small and also several measurements need imputation. Consequently it was necessary to take aneurysm diameter as the starting point and test which potential biomarkers added greatest predictive value to it. With the caveat that the model is based on a small number of complete biomarker panels, multivariate analysis did establish a model for predicting aneurysm growth rate. In each case, the proposed model predicts growth better than baseline aneurysm size alone. For the prediction of dAod/dT, TGFβ1 and IL2 provide additional predictive capacity, but the adjusted r^2 of the model is only 0.24. For the prediction of dAoV/dT, TGFβ1 and IL13 add predictive value to baseline aortic diameter. The final model gives an r^2 of 0.59 and p value less than 0.05. These models obviously require validation in a larger, virgin, dataset and this prospect will be discussed further in the final chapter.

DISCUSSION

BRIEF SUMMARY OF RESEARCH QUESTIONS AND ANSWERS

The key aim of this thesis was to establish whether there is scope for a panel of biomarkers that would predict the future clinical course for an aneurysm of the arch or descending thoracic aorta. Accordingly a variety of plasma proteins, blood cell counts and PBMC derived RNAs were quantified, a range of demographic features were recorded and aortic wall stress was estimated in a cohort of patients with 3 years of follow-up clinical and aortic growth data.

Q1 – are there measurable biomarkers in the circulation that uniquely identify patients with AD aneurysms?

Yes. In comparison with 'CTRL' patients with coronary artery disease:

- The expression of PBMC-RNAs ADAMTS2, BPI, LYZ and MSR1 is reduced while the expression of CAMP, PRTN3 and SYCE1 is raised in AD patients;
- Plasma levels of IL8, MMP9, MMP9:TIMP1 and MMP9:TIMP2 are significantly lower and MMP2:MMP9 is significantly higher in aneurysm patients;
- The MMP2:MMP1 ratio is substantially higher in AD patients (425) compared to both controls (233) and ASC patients (117).

Q2 – are there measurable biomarkers in the circulation that correlate with growth or progression of AD aneurysms?

Yes, univariate regression suggests that:

- log (Plasma TGFβ1) and log (plasma IL2) correlate with dAoD/dT at the $p<0.05$ level; and
- TGFβ1 and BSA correlate with dAoV/dT at the $p<0.05$ level.

Multivariate regression modelling suggest that the following equations provide a better prediction of aneurysm growth than using baseline diameter alone, as is the current clinical practice.

- dAoD/dT = 0.43(baseline indexed AoD) -1.5(logTGFB1) -0.86(logIL2)
- dAoV/dT = 1.57(baseline indexed AoD) +0.01(logTGFB1) -22.5(logIL2)

Wall stress, estimated mathematically from baseline CT scans suggested that there is a robust relationship between baseline wall stress and future aneurysm growth. Unfortunately the sample size used for this analysis did not allow for wall stress to be included in the multivariate modeling exercise.

In the sections that follow, I will describe how these findings fit with existing knowledge, discuss the limitations (including sample size and selection of controls) of the current work and lastly explore some options for extending this work further.

PUTTING MY RESULTS IN CONTEXT

Before attempting to set my results in the wider context of what is already known, it is worth emphasising the paucity of literature that is directly relevant to this type of aneurysm. As mentioned in the introductory chapter, the majority of work published on aneurysm biomarkers focuses either on other species or different types of aneurysm (ascending, abdominal or genetically mediated). This limitation notwithstanding I will proceed to describe how my results sit in the context of existing knowledge.

Biomarkers that uniquely identify patients with AD aneurysms

PBMC-RNAs

The results chapter described the differences in the PBMC transcriptome between AD and CTRL patients. To clarify, the PBMC extract predominantly comprises monocytes (circa 85%), with a lesser contribution from lymphocytes (circa 10-15%) and some inevitable granulocyte (neutrophil) contamination. Comparing the pooled RNA from these cell types in AD versus CTRL patients, it was noted that the expression of ADAMTS2 (A Disintegrin and Metalloproteinase with Thrombospondin Motifs 2), BPI (Bactericidal Permeability Increasing Protein), LYZ (Lysozyme) and MSR1 (Macrophage Scavenger Receptor 1) were all significantly reduced, while CAMP (Cathelicedin Antimicrobial Protein), PRTN3 (Proteinase 3) and SYCE1 (Synaptonemal Complex Central Element Protein 1) were all increased. The small sample size has resulted in a small number of genes that are verified as being differentially expressed, rather than a larger scale 'picture' of the transcriptome. While it is therefore challenging to draw any conclusions about the behaviour of PBMCs in AD aneurysms, it is possible to discuss the potential roles of these RNAs – or rather their corresponding proteins.

ADAMTS2 is a proteinase whose function is to trim procollagen molecules so that they can be assembled into fibrils in the extracellular matrix. Mutations in ADAMTS2 have been identified in cases of Ehlers-Danlos syndrome [84], a connective tissue disorder which predisposes to aneurysm formation and dissection in the ascending aorta. ADAMTS2 mutations have also been associated with 'idiopathic' cerebral aneurysms [85]. However, it has also been demonstrated that ADAMTS2 is upregulated in the context of myocardial ischaemia [89, 90]. The observation therefore that ADAMTS2 expression is higher in my control group is most likely to reflect its up-regulation in patients with coronary disease rather than down-regulation in patients with arch/ DTA aneurysm. Although given its function, it is clear that under-expression of ADAMTS2 could lead to faulty construction of the ECM and subsequent aneurysm formation. In the absence of a healthy control group, it is impossible therefore to interpret this finding.

BPI is usually found in neutrophil granules and is involved with the innate immune defence against Gram-negative bacteria. No association with aneurysms (or coronary disease) has previously been reported.

Lysozyme is an antimicrobial agent produced by macrophages and neutrophils. It contributes to innate immunity against bacteria by destroying peptidoglycan in the bacterial cell wall. One study of human AAA tissue samples identified a 14-fold increase in LYZ RNA compared to controls [88]. The authors proposed that this could be due to bacterial infiltration and a localised infection in aneurysm-related thrombus. No other associations with aneurysms have been reported, and biologically it is difficult to make a mechanistic link between down-regulation of LYZ and AD aneurysm development. It is probably therefore more logical that the differential expression reflects up-regulation of LYZ in the CTRL group, where perhaps the bacterial infiltration hypothesis could be involved with those patients' coronary vessel disease.

MSR1, as the name suggests, is a receptor found on the surface of macrophages. Its purpose is to scavenge low density lipoproteins by endocytosis and accordingly it has been reported to be upregulated in samples of atherosclerotic

plaque and abdominal aneurysm [90]. It is particularly interesting to note one study that showed MSR1 expression correlated well (r 0.55) with AAA diameter in a cohort of 31 patients [90]. Sadly no reports of MSR1 in thoracic aneurysm were found. However studies have shown that PBMC-MSR expression is raised in patients after an acute coronary event [91]. Therefore as with ADAMTS2, it may be that I have corroborated a biomarker of coronary artery disease rather than TAA.

Turning to the genes that were up-regulated: SYCE1 is involved with meiosis while CAMP is an antimicrobial protein produced by macrophages and neutrophils. Neither has previously been associated with aneurysms or cardiovascular disease. PRTN3 has some biological credibility as a biomarker of TAA since it is a serine protease which breaks down elastin, type IV collagen and many other ECM components. Again though, there is no published literature drawing a connection between PRTN3 and aneurysms.

The piece of literature most directly relevant to this discussion of the PBMC transcriptome in TAA is the work done by Wang et al in 2007 [56]. This group from Yale compared the PBMC transcriptome of TAA patients (mostly ASC, with a mixture of genders and aetiologies, but excluding Marfan syndrome) against spousal controls. The team identified a panel of 41 genes which, when measured in a 'virgin' group of patients, predicted the existence and location of TAA with 70% sensitivity and 90% specificity. Looking at the genes that segregated TAA cases from controls, the up-regulated genes featured in three main pathways – interleukin signalling (mainly IL10), fibroblast growth factor signalling and endothelin signalling. The down-regulated genes were found to contribute to T-cell activation, apoptosis pathways and Wnt signalling. In their discussion, the group proposed that this pattern of increased IL10 and a shift towards a Th1 T cell- response, signified an anti-inflammatory, protective response in the chronic phase of the aneurysms. None of the 7 genes from my study feature in the Yale group's panel of 41. Conversely, searching for the 41 'Yale genes' in my RNAseq results revealed agreement in only 3 genes. That is to say that JAK3, SYNGAP1

and TBL1X were found in my RNAseq results also, with the same pattern of expression (up/ down-regulated) as the Yale group's results. However, the latter two genes had a false discovery rate reported at 98%. JAK3 – a mediator of interleukin signalling was found to be up-regulated in both studies, but was associated with a FDR of 29% in my results and was therefore not carried forwards for validation by qPCR. Of the other 38 genes in the Yale panel, 11 genes were identified in my RNAseq, but with the opposite pattern of expression and high FDRs, and the remainder were not found in my results. Given that my results do not corroborate many in the Yale study, it is important to consider the validity of my findings, especially in light of the technical challenges I had in securing good quality RNA for sequencing.

The first point I would highlight is that my study compares AD aneurysm patients against coronary disease patients, while the Yale study compared TAA patients against healthy controls. There were also differences in the patient selection process and differences in RNA methods. In this project, I endeavoured to make my patient cohort as homogeneous as possible, focussing on adult males only, excluding those with any history of diabetes, cancers or family history of aneurysms, and separating aneurysms by location. In contrast, the Yale study had a relatively heterogeneous group – both genders, all aetiologies apart from Marfan syndrome, and the aneurysms could be located anywhere in the thoracic aorta. The influence of this heterogeneity is evidenced in the Yale study itself, where they go on to show how the PBMC transcriptome differs between ascending and descending aneurysms and between sporadic and familial cases. Unfortunately each disease group was not compared against the controls in their paper. Nonetheless, it is far to say that the two studies consider very different patient groups.

It is also relevant that although the Yale PBMC study is larger than mine (58 TAAs vs. 36 controls), both studies are relatively small if one considers the size of the PBMC transcriptome and the degree of variation within it. For definitive

genomic, transcriptomic, or indeed any –omic studies, sample sizes into the hundreds or thousands would be desirable.

With these factors in mind, it is perhaps not surprising that the two studies have not shown convergent results. Taking a step back, the original premise was that there is a characteristic and quantifiable change in the PBMC transcriptome in response to TAA, and that these changes reflect the activity within the aneurysm. However, a recent review of monocyte and macrophage function (the dominant component of PBMCs) in AAA suggests that this premise over-simplifies the situation [92]. The traditional thinking is that inflammatory mediators at the aneurysm site recruit circulating monocytes. They then transform into macrophages which proceed to participate in ECM remodelling. However there are two major challenges to this paradigm – (i) the discovery that vascular-resident macrophages are not all derived from circulating (bone marrow derived) monocytes; and (ii) the demonstration of multiple subtypes of monocytes and macrophages with different functions.

Concerning the origin of macrophages present in the aorta, a large proportion derive from progenitor cells that migrated during embryonic life and this population of macrophages is maintained independently from bone-marrow progenitors [93, 94]. On the other hand, the macrophages that accumulate during AAA development originate from bone marrow-derived circulating monocytes. In murine models of AAA, depleting circulating monocytes reduces (but does not eliminate) macrophage accumulation in the aorta, and does not prevent aneurysm formation [95], suggesting that both populations of macrophages have complementary, but distinct roles in aneurysm formation.

Regarding the subtypes of monocytes, in healthy individuals the majority (~ 90%) are so-called classical monocytes responsible for innate immune responses, while the remaining 'non-classical' monocytes are involved with immune surveillance and tissue repair. However in AAA patients, it has been shown that the proportion of classical monocytes is reduced in favour of an

intermediate phenotype of monocytes with a different pattern of cell surface markers and transcriptomic profile [92]. Oddly enough, in mouse models of AAA, it appears to be classical-type monocytes that are more associated with aneurysm progression [95-98], so the exact role of each subtype merits further investigation.

Similarly there are several sub-types of macrophage. M1 macrophages produce pro-inflammatory cytokine and proteolytic enzymes. In contrast M2 macrophages generally produce anti-inflammatory cytokines and participate in tissue repair. The different subtypes arise in response to different triggers in vitro, and there are even suggestions that specific monocyte subsets give rise to specific macrophage subsets. Studies of macrophage polarisation in human AAA tissue have indicated that the different macrophage subtypes congregate in different layers of the aneurysm [99, 100]. It has been difficult to explore this further since the criteria by which M1 and M2 macrophages are identified in humans is not agreed. In animal models of AAA, however, studies have suggested that there is dominance of the M1 phenotyope during aneurysm initiation, and that this then switches to M2 dominance as the aneurysm expands [101, 102]. Thus, it seems probable that there are different roles for the different macrophage subtypes, which are pathologically and temporally distinct.

These last three paragraphs illustrate that there is a potentially a vast chasm between the behaviour (i.e. the transcriptome) of the circulating monocyte and that of the macrophage responding to the aneurysm. Pooling the RNA from all PBMCs as I did in this study may have clouded the issue. It may be prove more fruitful in future work to separate and quantify the different monocyte subtypes, characterise the transcriptome of each and seek to correlate this to the tissue macrophage subtypes and the state of the aneurysm. This could lend a degree of mechanistic credibility to any biomarker thus identified.

Plasma proteins

In the initial pilot study of ascending aneurysm (ASC) patients, arch/ descending (AD) patients and controls, there appeared to be differences in the plasma levels of IL8, MMP1, MMP2 and MMP9 in the three groups:

1. **Plasma IL8 levels were also lower in both aneurysm groups** (AD mean 6.02 pg/mL, ASC mean 6.69 pg/mL, CTRL mean 9.40 pg/mL, p0.06).
2. **Plasma MMP9 levels were lower in both aneurysm groups** (AD mean 87248 pg/mL, ASC mean 85118 pg/mL) compared to controls (CTRL mean 135927 pg/mL, p 0.04).
3. **In AD patients, MMP2 levels were substantially higher** (mean 320115 pg/mL) than in controls (mean 258803 pg/mL) and ASC patients (mean 229844 pg/mL); **and MMP 1 levels were significantly lower** (CTRL mean 2249 pg/mL, ASC mean 5424 pg/mL, AD mean 1156). Consequently the MMP2:MMP1 ratio was particularly high in AD patients.

Low levels of IL8 and MMP9 in TAA patients

Interleukin 8 (also known as CXCL8) is a pro-inflammatory cytokine. Therefore it would be logical to expect IL8 levels to be raised in chronic inflammatory conditions like TAA. IL8 is produced predominantly by macrophages and endothelial cells when they detect inflammatory signals (for example IL-1b and TNFα). It is secreted locally and induces nearby endothelial cells to increase their expression of cell surface receptors that will bind neutrophils and (to a lesser extent) other granulocytes. This facilitates migration of these leukocytes from the circulation into the injured region to effect repair -hence IL8s alternative name of neutrophil chemotactic factor. Thus IL8 resides largely within tissue rather than in the circulation - a phenomenon that allows radiolabelled IL8 to be diagnostically useful in inflammatory conditions such as pneumonia [103], osteomyelitis [104] and colitis [105]. In the specific context of aneurysms, various studies have demonstrated increased expression of IL8 in aneurysmal tissue taken from the abdominal aorta [106, 107].

High tissue levels of IL8 could perhaps explain the low serum levels. It may be that macrophages producing IL8 (pro-inflammatory M2 macrophages) are held in the tissue at the aneurysm site and are consequently diminished in the circulation. However an important part of IL8's role is to mobilise granulocytes from the bone marrow. Given this requirement for IL8 to function via the circulation following an inflammatory stimulus, serum levels would still be expected to increase rather than decrease in aneurysm patients. Certainly a rise in serum IL8 is observed in inflammatory conditions such as sepsis [108], pyelonephritis [109] and prostatitis [110] and the correlation is sufficiently robust that serum IL8 has been proposed as a biomarker of these conditions. With this in mind, my observation of reduced serum IL8 levels in TAA patients still remains to be explained.

The default supposition would be that there is genuinely less inflammation in my cohort of thoracic aneurysm patients compared to my control group. Given that the control group was not healthy individuals but patients with severe coronary atherosclerosis, the accurate conclusion of my data is that serum IL8 levels were lower in TAA patients compared to patients with coronary artery disease. This could just signify that thoracic aneurysm is a less potent inflammatory stimulus than severe coronary artery disease. The only guiding literature I could find comes from Juvonen et al, who published data on cytokine levels in patients with a) no apparent disease, b) coronary artery disease and c) abdominal aortic aneurysms [111]. They showed that the pro-inflammatory markers IL1b, IL6, TNFα and IFNγ were highest in the aneurysm group ($p<0.05$) and moreover that there was no significant difference between the healthy controls and the coronary disease group. This paper did not report IL8 levels in the three groups. Although their data suggests that the aneurysm patients were in a more pro-inflammatory state than CAD and control patients, it is again worth noting that they assayed AAA patients and we have already detailed the various disparities between TAA and AAA. Therefore my discussion of low IL8 levels in AD patients

must conclude with a hypothesis that perhaps TAAs provoke less inflammation than CAD or indeed AAAs.

One plausible explanation though, could be the anti-inflammatory effects of various cardiovascular medications. Pharmaceutical inhibition of inflammatory cascades would also explain why the serum levels of IL8 are relatively low in all 3 of my study groups. As described in the results chapter, mean serum IL8 was 9.4pg/mL in the control group, 6.7pg/mL in the ASC group and 6.0 pg/mL in the AD group.

The 'normal' range quoted by assay manufacturers is 1.48 to 1720 pg/mL, while clinical studies have published levels in healthy cohorts in the 10-30 pg/mL range [112-114].

Statins are known to have an anti-inflammatory effect aside from their main function to lower cholesterol levels. Simvastatin in particular has been demonstrated to inhibit IL8 production, along with various other pro-inflammatory cytokines such as IL1a and IL6 [115]. In my cohorts, however, statin prescription is near ubiquitous in the control group (95%) that had the higher levels of IL8, and much lower (50%) in the aneurysm groups that had the lower levels of IL8. Hence statin use alone does not contribute a good explanation for the observed differences in IL8.

ACE-inhibitors and angiotensin receptor blockers (ARBs) also have a potent anti-inflammatory effect and both are known to specifically reduce IL8 levels [116]. This is thought to be mediated by suppressing angiotensin II signalling through its type 1 receptors, which in turn suppresses NF-kB mediated pro-inflammatory signals. A study in 2014 used data from a clinical study of abdominal aneurysm patients to show that serum IL8 levels were indeed lower in patients taking the ACE inhibitor ramipril. In the aneurysm groups in my study (ASC+AD), 42% were prescribed ACEI's and 19% were prescribed ARBs, compared to 30% and

10% respectively in the control group. The greater use of ACEIs and ARBs in the aneurysm groups did not reach statistical significance, but could potentially explain why serum IL8 levels were lower than in the control group. Returning to the original data, unfortunately there is no significant difference in the IL8 levels of those aneurysm patients on ACEIs and/or ARBs compared to those who weren't (7.6 pg/mL vs. 6.9 pg/mL, p0.55), but this hypothesis may warrant further exploration.

MMP9 is a matrix metalloproteinase synthesised by a wide variety of cell types, including macrophages, neutrophils, vascular smooth muscle cells and fibroblasts. MMP9 is involved in many processes relevant to aneurysm development. Aside from proteolytic degradation of the extracellular matrix, and angiogenesis, MMP9 also promotes the migration of neutrophils into the aneurysm site and their subsequent degranulation. Secretion of MMP9 is triggered by pro-inflammatory signals such as raised levels of IL1b , TNFα and IL8 via the NF-kB pathway. There is also a positive feedback pathway whereby MMP9 acts to increase levels of IL8. Hence it is reassuring that MMP9 serum levels follow the same pattern as IL8, and are low in my aneurysm groups compared to controls. This result corroborates the findings of two other research groups. Karapanagiotidis et al [117] compared serum MMP 9 levels in patients (both male and female) with thoracic aneurysm (ASC + AD), chronic dissection, acute dissection, acute myocardial ischemia and healthy controls. They found that MMP9 levels were lower in aneurysm patients compared to controls and all other aetiologies studied. More recently Ikonomidis et al [57] examined plasma MMP and TIMP levels in a cohort of patients with ascending aneurysms, dividing them into a group that had normal with normal aortic valves and a group with bicuspid aortic valve. In both aneurysm groups, plasma MMP9 levels were lower than in the control patients (2560 pg/mL and 4180 pg/mL versus 8360 pg/mL in the control group). This latter study analysed MMP9 levels in samples of the aneurysm tissue also, and discovered that again MMP9 levels were lower in ASC aneurysm tissue compared to controls. This is directly opposite to what is observed in abdominal aortic aneurysms, where high serum MMP9 levels have

consistently been reported [118]. Given that MMP9 has such a significant role in aneurysm development, this counterintuitive finding deserves some consideration.

The substrates of MMP9 include collagen types I-V, X1, XVI, fibronectin, laminin, osteopontin, thrombospondin 1 and decorin [119], all of which are found abundantly in all regions of the aorta. The suggestion made in the IL8 paragraphs was that low levels were due to the anti-inflammatory effects of ACEI's and ARBs. These drugs are also known to reduce MMP9 secretion and activity [120, 121], so this could explain the observation of low levels compared to controls. However, it does not adequately explain why serum MMP9 levels would be low in thoracic aneurysm patients but high in abdominal aneurysm patients, since the use of these drugs is just as prevalent in both groups [122]. The most logical reason for different MMP9 levels in different aortic segments would be that the pathological process is different in the two segments. The descending aorta to the naked eye looks like one entity, divided into a thoracic and abdominal component merely for convenience. However this is far from the truth. The regional heterogeneity in the aorta was the subject of a review article by Ruddy et al. [123]. In it she describes myriad differences between the thoracic and abdominal aorta, some of which may influence biomarker profiles:

- Embryologically, the ascending aorta and aortic arch originates from neural crest cells while the abdominal aorta derives from mesoderm;
- The thoracic aorta has a far greater elastin content than the abdominal segment;
- The media of the thoracic aorta has far more lamellar units, meaning that it has a vascular and avascular zone while the media of the abdominal aorta is totally avascular. This means that there is greater delivery of oxygen, inflammatory mediators and growth factors to the thoracic media which likely influences the remodelling process;

- The thoracic aorta is more resistant to atherosclerotic plaque formation – this is presumed to be due to the differences in blood flow and shear stresses in the two segments.

With these facts in mind, it appears plausible that serum MMP9 levels are genuinely low in TAA whilst being high in AAA cases. This thesis of course was not designed to delve further into the reasons or mechanisms behind potential biomarkers. Nonetheless, it is interesting to speculate why MMP9 should be low – after all heightened activity of MMP9 has been well demonstrated in tissue-based studies of thoracic aneurysm development and progression. One reason could be that MMP9 is produced by different cell types in the two different regions. In the abdomen, MMP9 comes mainly from macrophages, but in the chest, MMP9 is produced by fibroblasts and smooth muscle cells (SMCs). SMCs in healthy thoracic aorta do not produce MMP9 – the SMCs in aneurysms that produce MMP9 have undergone a phenotypic switch into the synthetic phenotype. It may be therefore that MMP9 production occurs on a larger scale in AAA, and in a way that 'communicates' with the circulation in a different way to TAA. It is also interesting to note that a lack of MMP9 (in a murine model) slows aneurysm progression in the thoracic aorta but prevents it altogether in the abdominal aorta, suggesting that perhaps MMP9 is not the dominant proteolytic agent in TAAs [124].

Lastly, there is the 'elephant in the room' – TGFB. It is an understatement to say that the role of TGFB in aneurysm development is complex. Nonetheless it is generally accepted that there is increased signalling via TGFB pathways in TAA, but when TGFB signalling is artificially increased in AAA models, aneurysm progression is slowed down[14]. TGFB thus appears to be a friend in the abdomen, but foe in the chest. Generally MMP9 is considered an activator of TGFB signalling, by liberating it from the latent complex. Perhaps in TAA, any MMP9 that is produced is more rapidly consumed in the process of liberating TGFB and other agents take the lead in proteolysis, while in the abdomen MMP9

is produced in higher volumes and is more readily able to 'leach' into the circulation? This is of course pure speculation, but given the findings of this thesis, an examination into the pathways linking TGFB and MMP9 may be interesting.

Low levels of MMP1 and high levels of MMP2 in AD patients

Of all the plasma proteins tested, the pattern of low MMP1 and high MMP2 distinguished AD aneurysm cases from both ASC cases and controls. As above, this merits a brief discussion of whether this observation can be biologically justified. Like the disparity between the thoracic and abdominal aortae, there is a difference in the embryological origin of the ascending aorta and arch and descending segments too. The ascending and arch portions derive from neural crest cells in the embryo, while the DTA derives from somitic mesoderm. There is also decreasing elastin content as one proceeds away from the aortic root [123]. This is relevant because of the difference in substrate preference between MMPs 1 and 2. MMP1 is a collagenase that acts upon collagen types I-III in their 'normal' helical form. MMP2 is a gelatinase that can act upon collagens which have already been partially broken down (by MMP1, for example). MMP2 also has elastase activity. It is therefore unsurprising that MMP2 should be elevated in aneurysm cases, where degeneration of the elastin in the media is a pathognomonic finding. An investigation of the literature suggests there are few studies of MMP2 activity in TAA compared to the other gelatinase MMP9. However, there are human and murine studies that corroborate my finding of increased MMP2 activity in aneurysmal tissue [124, 125]. It is curious though that MMP2 should be higher in AD aneurysm cases compared to ASCs. Given the greater proportion (relative to collagen) of elastin in the ascending aorta, it might be expected that MMP2 would be higher in the ASC cases. One possible explanation may be that there is a greater contribution of atherosclerosis in the pathology of my AD cases compared to my ASC cases. It has long been noted that the thoracic aorta in general and the ascending aorta in particular are more resistant to the development of atherosclerotic plaques [123]. Tissue-based

studies comparing DTA cases to normal aorta have shown that MMP2 activity is elevated when the DTA is atherosclerosis related, but not when the DTA is related to other aetiologies (syndromic TAA for example) [126-129]. My study of course focussed on degenerative TAA, excluding any genetically determined cases. It could be argued that since the ASC aneurysms were smaller than the AD ones, the atherosclerosis may have been less well-developed in the ASC cases. However, murine models of TAA development have suggested that MMP2 activity rises in the early phase of aneurysm development and then tails off again, suggesting that its main role is at the instigation of the aneurysm rather than in the subsequent growth [130]. Thus there remains much uncertainty about the role of MMP2 in TAA - some studies have even shown a difference in MMP2 activity between the front and the back of an aneurysm [128]. Suffice to say the finding of uniquely high MMP2 levels in AD cases could be biologically plausible given that other studies have corroborated high MMP2 levels in TAA and yet other studies that have described the regional peculiarities of the arch and DTA.

Turning to MMP1, there are even fewer studies of its role in TAA than MMP2. In the context of AAA, heightened MMP1 activity has been noted in tissue samples [131], and higher plasma MMP1 levels have been demonstrated in ruptured AAAs compared to non-ruptured ones. In 2007, Ikonomidis et al identified lower levels of MMP1 in tissue samples from ASC patients [126], but 6 years later the same group published a study [57] indicating MMP1 levels in aneurysm tissue were higher in ASC patients than in controls. The two studies used different methods to quantify MMP1 (immunoblotting in the 2007 paper and qPCR in 2013), but this alone cannot justify the opposing findings. Given that the literature presents a mixed picture of the role of MMP1 in TAA, we must revisit the biological facts. As described above, MMP1 is a collagenase designed to denature native collagens I-III which predominantly exist in the adventitia. It is perhaps the case that MMP1 levels are low in TAA cases (but high in AAA cases) because there is less collagen relative to elastin in the thoracic aorta compared to the abdominal segment. And perhaps MMP1 is lower in AD cases compared to

ASC cases because the AD aneurysms (being larger) were older than the ASC ones. With increasing chronicity it seems possible that the native collagen has mostly been denatured so that MMP1 levels wane as the gelatinases rise.

Biomarkers that predict progression in patients with AD aneurysms

The over-arching aim at the outset of this project was to find predictors of aneurysm growth rate. TGFB1 correlated significantly with aneurysm growth, both in terms of diameter and volume. In addition, IL2 correlated with dAoD/dT and body surface area correlated with dAoV/dT. The available literature that is relevant to these findings is neatly encapsulated in a systematic review of TAA growth rates published by Oladokun et al in 2016 [53]. The team reviewed not only the growth rates reported but also the factors that were observed to influence growth rates. Much like my own literature review summarised in the introduction, the team identified that aortopathy (Marfan syndrome, bicuspid aortic valve disease) smoking and COPD were the most consistent accelerants of aortic growth. No studies were found that compare cytokine levels and aneurysm progression. Therefore to set my findings in context, it is necessary to step back towards the underlying biology of TGFB1 and IL2.

The pathways of TGFB signalling in TAA have been and continue to be an active area of research since mutations that affect its signalling have been found at the root of aortopathies such as Marfan and Loeys-Dietz syndromes. The research in this field however, is generally based on in vitro or small-animal models of disease, with very little work done on circulating TGFB. A personal literature search did reveal though, one paper examining serum TGFB1 levels in a mouse model of Marfan syndrome [61]. This study hypothesised that serum TGFB1 would be elevated in the Marfan mouse since the mutant fibrillin is unable to properly sequester it. They found this to be true and also found that blocking

TGFB activity in aortic tissue led to a corresponding fall in serum TGFB1 levels. So there is some support for the notion that circulating TGFB1 may indicate the level of TGFB signalling (and therefore 'activity') in the aneurysm itself.

In contrast, there is far less literature to support a mechanistic role for IL2 in TAA progression. IL2 is a cytokine whose main function is to promote the differentiation of immature T cells into regulatory T cells (T_{regs}). There is some accumulating evidence that T_{regs} can slow aneurysm progression in murine models of AAA [132]. Thus it may be that as an aneurysm becomes more chronic, there is an attempt (via IL2) to dampen the immune response. However, it is probably wise to reserve judgement since the statistical significance of the correlation between IL2 and growth rate is dependent on many imputed values, as described in the results section.

The most robust predictor of future growth however, was not plasma proteins, RNAs or patient characteristics but aortic wall stress (r^2 0.46-0.55). This presumable relates to the intrinsic pleiotropy of circulating cytokines and proteins – with so few actors playing so many interconnected roles, it was ambitious to expect a biomarker to standout. Aortic wall stress on the other hand relates far more specifically to TAA progression. Of course it is important to acknowledge that aortic wall stress was not measured, but rather estimated, using the methods described. The process was computationally burdensome, due to the complexity of thoracic aortic morphology and a variety of assumptions were required (for example that mechanical properties measured ex vivo are not too dissimilar to those in vivo). It is also an impractical process in its current form, since it takes a trained person several hours to manually segment each patient's CT scan. Nonetheless, the correlation between baseline wall stress and future growth was good, despite having only 5 cases modelled. This finding is corroborated by other authors [44], but unfortunately the resources and time required have prevented large-scale studies being performed. This is a particular shame because the wall stress dataset was too small to be included in the multivariate regression modelling.

Thus having cast the net wide in my search for a circulating biomarker, the potential candidates identified in this study were only weak correlates of aneurysm progression. It is also worth noting that my study (in line with others [53] found that baseline aortic size was not a robust predictor of either future growth or aortic events. However, aortic diameter is usually the only objective parameter consistently available to surgeons, and therefore this is the parameter used to decide management strategy. Accordingly it seemed reasonable to assess whether the relatively minor influence of the candidate biomarkers could improve the predictive capacity of aortic diameter alone. Multivariate regression modelling indicated that the following equations do indeed provide a better prediction of aneurysm growth than using baseline diameter alone.

- dAoD/dT = 0.43(baseline indexed AoD) -1.5(logTGFB1) -0.86(logIL2)
- dAoV/dT = 1.57(baseline indexed AoD) +0.01(logTGFB1) -22.5(logIL2)

It is immediately obvious that in the two formulae, TGFB1 would appear to have a different direction of correlation to the growth rate: in the first equation, TGFB1 negatively correlates, while it positively correlates in the second. However, the coefficient in the second equation is only +0.01, suggesting that the contribution of 'logTGFB1' to dAoV/dT is minimal. Given the small sample size that ultimately contributed to these formulae, it seems most likely that this 'plus/minus' discrepancy in the role of TGFB1 reflects the lack of statistical power of the sample rather than a biological anomaly.

Two research groups [36, 45] in the past have attempted to derive a formula for projected aortic growth:

Dapunt et al., 1994 Change in diameter = $0.0167(\text{initial aortic diameter})^{2.1}$

	Change in volume = 0.0356 (initial volume)$^{1.322}$
Shimada et al., 1999	Last diameter = initial diameter x $e^{(0.00367 \text{ x time})}$

Neither group had any baseline biochemical values to evaluate, and neither group had validated their formulae in a 'virgin' cohort (at least no such validation has been published). Shimada's group proceeded to publish a more advanced formula a year later, relating final aortic diameter (ADf) to initial size (ADi), time interval (years), and the dichotomous variables thrombus, previous aortic operation (prevoper), and transient ischaemic attack/stroke (TIA/Str): ADf = ADi × e(time × factors), where factors = (0.0433 + (0.0291 [0.007] × thrombus − 0.0243 [0.007] × prevoper + 0.0215 [0.010] × TIA/Str))[10.55]. Like these other groups, a major caveat of my 'formula' is the lack of validation. This issue will be revisited in the section on future work.

LIMITATIONS

First and foremost, this work has been challenged by the small size of the sample. Patients were recruited from the Papworth-Addenbrookes aortic MDT so that samples could be processed within the optimum 2-hour window after collection. This MDT takes patients from across East Anglia, but despite this wide catchment area it was still not possible to recruit more than 50 patients. The ETTAA study, recruiting AD patients across the whole of England, only managed to recruit 244 patients fitting the eligibility criteria in the same time frame. The estimated incidence of the disease suggests that there should be more eligible patients, but unfortunately the majority are discharged from hospital follow-up because treatment is deemed too risky for them. Thus they are 'lost' from any databases. Momentum is building behind a UK registry of aortic disease, but until such a database exists it is likely that studies of this pathology in the UK will be limited in number to the small numbers of 100s. Of course this could be compensated by collaborating with other countries and other registries or

biobanks. To date, the registries that house large volumes of longitudinal data (for example, IRAAD, the Yale Aortic database, GERAADA) do not have a repository of biological samples. Conversely, biobanks rarely have longitudinal follow-up/ radiological data accompanying the samples. Given the burgeoning interest in –omics studies in aortopathies, a bank of aortic data with an accompanying vault of samples would be of immense future value.

Whilst on the topic of biobanks, it is perhaps also appropriate to pass comment on the control group used in this study. I chose male patients presenting with coronary artery disease and presented the justification for this in the Methods section. In short, using male CAD patients (with the same exclusion criteria as my aneurysm patients) allowed me to search for biomarkers that would discern 'aortopaths' from 'arteriopaths'. I felt that if I had chosen 'healthy' controls, then many of the biomarker candidates I was interrogating would likely be different between controls and AD patients simply because the latter have a chronic, inflammatory, vascular endothelial disorder already known to cause changes in circulating cytokines and white cells. Since hypertension and atherosclerosis are such prevalent diseases, any biomarker identified would then have to be tested again to prove that it selected patients with AD aneurysm from those with atherosclerosis and its other manifestations (e.g. peripheral vascular disease). It was also a pragmatic choice, since patients undergoing coronary surgery at Papworth are already invited to give consent for providing blood and tissue for research purposes. Therefore a further ethical consent was not required and the study was able to get underway quickly. Ultimately however, this approach has left me with the converse uncertainty of not knowing whether my down-expressed biomarkers are actually up-expressed markers of CAD or down-expressed markers of AD aneurysm. Going forward, it would obviously be advisable to test this by including a third, healthy control, group. It is also worth questioning how reliable it is to consider my AD group as having non-genetically mediated aneurysms. In the absence of genetically testing these patients, it is difficult to be sure that the AD group do not contain patients who are probands bearing heritable aortic disease. Again this would be worth interrogating and proving in any future work.

After quantity, the next most significant challenge was the quality of the samples. Earlier I detailed some of the challenges I experienced with RNA extractions. While this was of course due to my own inexperience, it will have undoubtedly impacted upon the differential gene expression analysis, and cannot be easily offset. For the primary experiment, I was necessarily restricted to using the samples collected at the start of follow-up. Collecting a second set of samples from the recruited patients would have required the follow-up period to be extended beyond the time available. Nor could I test the validity of the seven gene targets as diagnostic markers of AD aneurysms in an animal model, for example, because inter-species variation in RNA species is too great. Similarly in vitro experiments could not be used to validate the targets either, because the whole biological system needs to be replicated in order to recreate the transcriptomic response in the PBMCs. Thus the only way to verify the RNA results requires the experiment to be repeated in a new set of patients.

Finally, and in relation to the issue of sample size, was the necessity of imputing several cytokine values in order to complete the multivariate modelling exercise. In several samples the level of one or more cytokine fell outside the dynamic range of the assay, so that the number of complete datasets was compromised. Similarly the number of aortic size datapoints was small - most patients had only two datapoints and therefore I had little choice but to model their aortic growth as linear, which is probably only true over short time periods. However, this experiment provides the first description of all these plasma proteins AD aneurysm patients. With this in hand, it will be possible to refine the assays used, choosing those with a more appropriate dynamic range where appropriate for future studies.

FUTURE WORK

Unfortunately this dataset has proved too small and the follow-up period too short to provide robust conclusions, although it does give hope that there are differences in these pleiotropic circulating substances that could perhaps be useful biomarkers. The search for a 'crystal ball' for aneurysm patients can bear much further investigation. In particular, I would like to propose three particular avenues of research that could follow on from my study. First a validation study that would aim to verify the findings herein in a new set of patients. Secondly (not necessarily independently), a study that advances the computational modelling of the aorta that we started here. Thirdly, investigations into the mechanisms underlying the observed 'biomarker' changes and linking to the pathological changes in progressing aneurysm.

1. Validation Study

The first and most obvious follow-on is a validation study that could 'test' the ability of the biomarkers proposed herein to predict aneurysm growth. This idea has been discussed with the statistical team that I collaborated with. The automatic question is of course, what sample size would be needed to reasonably validate my biomarker findings. There are challenges in calculating this sample size using the data I have described in this thesis, because the aortic growth model described in my results section was developed using a very small dataset – the number of assumptions needed would render the resulting sample size very unreliable. Consequently, it may be more advisable to use the validation study to explore the consistency of the coefficients if the models were fit to new data, rather than aiming to establish the predictive performance of the models per se. The validation study could also be used to explore more complex models of the outcomes (i.e. whether including more variables improves the model).

2. Computational modelling of the aorta in aneurysm

The wall stress estimates derived from the computational modelling section gave the best correlation with future growth. Consequently it would be interesting to pursue this further, with a larger sample of scans. My sample size was confined as it became apparent after recruitment that only anatomically 'simple' cases could be modelled. Nonetheless the correlation we have demonstrated here could be used as pilot data to seek sufficient funding for the computer- and man-power necessary for a larger sample. It is also encouraging that the basic concept has been tested and the potential difficulties identified. Specifically, we have demonstrated that there is no need for a particular scan protocol, so existing scans could be used, which in turn simplifies the ethics application. Naturally it would be necessary to incorporate a control group of patients in order to establish the specificity and sensitivity of the test. If wall stress estimates were shown to be a reliable indicator of future growth, then this would justify efforts to automate the segmentation process by which constituent parts of the aorta are outlined.

3. Mechanistic studies

Earlier in the discussion, various speculations were made about the roles of plasma MMPs 1, 2 and 9, TGFB1 and IL2 in TAA progression. Of course it would be fascinating to explore the biological roles of these agents in TAA, to establish whether they have any biological credibility as biomarkers. It is difficult to conceive one study that would satisfactorily achieve this aim though, since the phase that was studied (plasma and PBMC transcriptome) is removed from the biological site of the pathology (the aorta).

In the first instance, it would be desirable to quantify my 'biomarkers' in the aneurysm tissue and see whether there is a connection between tissue- and circulating levels. This could be done using banked tissue samples from the few patients in my study who underwent surgery. Of note, this equates to patients who fell within a narrow band of aortic diameters and relative physiological fitness. These patients also did not contribute to the aortic growth data – apart

from one, all patients who were operated had no radiological follow-up after the biomarker samples were collected. Thus the subset for whom parallel plasma and tissue samples are available is a small and highly selected subset.

If plasma and tissue levels are concordant, the next stage would be to assess whether the same biomarker profile was evident in an animal (ideally murine) model of TAA. If so, aneurysm growth could be manipulated to study the impact on the biomarker and also the pathways by which the biomarker relates to aortopathy (whether it is cause or effect). Realistically, this workflow would need to be followed for each biomarker I have proposed – both the diagnostic ones and the prognostic ones.

Exploring whether my 7 genes of interest are differentially expressed in the murine PBMC transcriptome is also possible, but since these were not correlated to growth, this may only be of interest to explore therapeutic options. Validating the gene targets as diagnostic markers of TAA could be better achieved in a second group of patients – perhaps as part of the extension study described above.

FINAL SUMMARY

This body of work has examined a range of easy-to-measure circulating biomarkers in a cohort of 50 patients with aneurysms of the arch and descending thoracic aorta. I have described within this thesis various obstacles (logistical and biological) to identifying biomarkers of aneurysm progression. Despite these challenges, I have created a dataset quantifying plasma proteins and PBMC-RNAs in a cohort of 50 AD patients with measurements of growth or clinical progression over 2 years. Analysis of my dataset suggests that:

e) aortic size alone is not a good predictor of growth
f) prediction of future growth could possibly be improved by combining plasma protein measurements with aortic size:

$$dAoD/dT = 0.43(\text{baseline indexed AoD}) - 1.5(\log TGFB1) - 0.86(\log IL2)$$

$$dAoV/dT = 1.57(\text{baseline indexed AoD}) + 0.01(\log TGFB1) - 22.5(\log IL2)$$

g) wall stress estimations appear to provide even more robust predictions of future growth
h) PBMC-RNA profiling could be useful as a diagnostic tool to detect patients with AD aneurysms.

PARTICIPANT INFORMATION SHEET

Papworth Hospital NHS
NHS Foundation Trust
Papworth Hospital
Papworth Everard
Cambridge
CB23 3RE

Study Title: CAN BLOOD BORNE BIOMARKERS PREDICT LOCATION AND RISK OF CLINICAL EVENTS IN THORACIC AORTIC ANEURYSM?

You are being invited to take part in a research study. Please note that this research is educational and forms part of a PhD project.Before you decide whether to take part, it is important for you to understand why the research is being done and what it will involve. Feel free to discuss the study with others if you wish. Please take time to decide whether or not you wish to take part.

……

1. What is the purpose of this study?

The aim of this study is to discover whether there are particular molecules in the bloodstream that can tell us about the size and growth rate of an aortic aneurysm. We already know that particular genes and proteins are associated with the occurrence of aneurysms, but so far no blood test has been identified that can tell us about the growth rate – which is an important factor in deciding when to plan an operation.

The study is based on patients referred to or being treated at Papworth or Addenbrooke's Hospital with an aneurysm (widening) of their aorta. We are asking for your consent to provide a 20ml blood sample when you attend Papworth or Addenbrooke's hospital for a scan of your aneurysm. Samples will be transferred to Papworth Hospital for processing on the same day. Your sample will be allocated a study ID number as soon as it arrives at Papworth, so that it becomes anonymised. Only the approved research team will have access to your identifying details, so that they can connect your biological results with your scan results and clinical data. All data will be held securely at Papworth.

Your blood samples will be processed in the laboratory to extract RNA and proteins. We will isolate the RNAs and proteins of interest to this study and measure the levels in your blood/ tissue sample. Each time you return for a

scan (up to the end of the study in September 2018), we will ask you for a sample of blood. The results of our study will help to determine whether a blood test for could be used to monitor patients with aneurysms, reducing the number of CT or MR scans required – this would allow more frequent monitoring and perhaps spare many patients the time, discomfort and radiation exposure associated with these scans.

2. Why have I been invited? You have been invited to take part in this study because you have been diagnosed with an aortic aneurysm and have been referred to Papworth or Addenbrooke's for further investigation and/or treatment.

3. Do I have to take part? No. Your participation in this study is entirely voluntary. If you decide to take part you will be asked to sign consent form. You are under no pressure to take part and may withdraw from the study if you wish at any time, without having to explain why. If you decide not to take part, the quality of medical and nursing care you receive will not be affected. With your permission we will keep the information we have already collected about you. You will not be contacted again about the study.

4. What will happen to me if I take part? If you agree to take part, you will be met by the Research Fellow (Priya Sastry) when you attend for your scan or other hospital appointment. You will have the opportunity to ask any questions about the study. The research fellow will then take your blood sample (20mls maximum, about 4 teaspoonfuls) and you will be free to proceed to have your scan/ appointment as normal. There will be no extra needle-sticks at the time of the sample collection as blood will be taken from the cannula inserted for the scan. We will use the measurements of your aorta each time you attend for a scan to allow us to calculate the growth rate of your aorta and seek correlations with the blood tests.

Your treatment or investigations will not be altered in any way by taking part in this study. Your medical team will decide on the best treatment for you, according to the appearances of your aorta and your symptoms or other aspects of your medical history.

5. What are the possible disadvantages and risks of taking part? We don't anticipate any serious disadvantages or risks in this study. The blood sample will be collected when you have your cannula inserted for your scan, so you shouldn't feel any additional discomfort. Very rarely though some patients may find the experience of collecting the blood sample uncomfortable.

6. What are the benefits of taking part? There are no direct benefits to you from taking part in this study but the information we get may help to improve the treatment of other people with aneurysms in the future.

7. What if there is a problem? Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be fully addressed.

If you have a concern about any aspect of this study, you should ask to speak with the research fellow who will do their best to answer your questions. If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from your doctor.

8. Will my taking part in the study be kept confidential? All information that is collected about you during the course of this study will be kept strictly confidential. Paper records regarding your case and your study number will be held securely at Papworth. Electronic information will be kept on computers that are protected by passwords. If you wish, your GP can be notified of your participation. When the study is reported it will not be possible to identify you personally.

9. What will happen to the results of the research study? The results of the study will be published in scientific journals and presented at scientific meetings. You will not be identified in any report/publication.

10. Who is organising and funding the research? The study is being funded, organised and run by Papworth Hospital NHS Foundation Trust.

The doctor and the research team conducting the research are not receiving additional payments for including you in this study.

11. Who has reviewed the study? All research in the NHS is looked at by independent group of people, called a Research Ethics Committee, to protect your interests. The study was reviewed by the Queens Square NRES Ethics Committee, London and the Research and Development Department at Papworth Hospital NHS Foundation Trust.

12. Contact details:

In the first instance please contact the Research Fellow on 01480 364451 or E-mail: priya.sastry@nhs.net.

Thank you for taking time to read this sheet and for considering taking part in this study.

Papworth Hospital NHS
NHS Foundation
Papworth Hospital
Papworth Everard
Cambridge

Consent form

Study Title: CAN BLOOD BORNE BIOMARKERS PREDICT LOCATION AND RISK OF CLINICAL EVENTS IN THORACIC AORTIC ANEURYSM?

	Please initial in each box
1. I confirm that I have read and understood the information sheet (V1.2, dated 26/07/2017 for the above study and have had the opportunity to ask questions.	
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	
3. I understand that sections of my medical notes and information collected during the study may be looked at by responsible individuals from regulatory authorities or from the NHS Trust where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.	
4. I agree to provide a 20ml blood sample whenever I attend Addenbrooke's or Papworth hospital for a scan of my aneurysm	
5. I agree to take part in the above study.	

Name of Patient (PRINT)	Date	Signature

Name of person taking consent (PRINT)	Date	Signature

When completed, 1 for patient; 1 for researcher site file (original); 1 to be kept with hospital notes

Appendix B

Details of the primers and qPCR protocols used to quantify RNAs as described in Chapter 6.

Total RNA was extracted using the RNeasy mini kit (Qiagen). cDNA was synthesised from 250 ng RNA using the Maxima First Strand cDNA Synthesis kit (Fermentas). Quantitative real-time polymerase chain reaction (qRT-PCR) reaction mixtures were prepared with SYBR green PCR master mix (Applied Biosystems) and run on the 7500 Fast Real-time PCR system. C_T values were normalised to the housekeeping gene, GAPDH. Primer sequences are listed in the table below:

GeneName	Forward primer	Reverse primer
ADAMTS2	GTAACCTGTGGCAACGGCA	GGATCTGAGATGTTTCGGGGAC
ALOX15B	TCTCTTCAAGCTGCTGATCCC	CTGCATCCCATCATCACGGT
AZU1	CTGCTTCCAAAGCCAGAACC	TGAAGCAGCATCAGGTCGTT
BPI	CCACCGGCCTTACCTTCTAC	GCAGCAATTCAACCGGGAAG
CAMP	TGATGCCTCTGGCCATCATT	CTGGGTCCCCATCCATCGTG
CLC	GCTACCCGTGCCATACACAG	CGACGACCAAAGCACACTTG
COL19A1	GCCTCTGAGGGGCTCAAATA	AGGGCATGACTCTTCTGTCT
CXCL2	ACCGAAGTCATAGCCACACTC	TCTTAACCATGGGCGATGCG
DEFA4	GCCATGAGGATTATCGCCCT	CCCTTGTTGAGCCTGAAAC
EGR1	TGACCGCAGAGTCTTTTCCTG	GTGGTTTGGCTGGGGTAACT
ELANE	CGTGGCGAATGTAAACGTCC	TTTTCGAAGATGCGCTGCAC
FSTL1	GCGGGGAAGGAGAGGTCTTA	TTCCACATCGTGGTCTGGTC
GAPDH	AACAGCCTCAAGATCATCAGC	GGATGATGTTCTGGAGAGCC
LPL	CCGCCGACCAAAGAAGAGAT	TAGCCACGGACTCTGCTACT
LTF	CACCCTGGGCTTTCTGCTAT	GCTGATCACCCTGAGTTGCT
LYZ	GGAATGGATGGCTACAGGGG	CAGCAAAGCACTGCAGGATA

MSR1	CCGGAAGGCCAGGAAATTCT	AAGAGGGCCCTGCCCTAATA
NRCAM	GTGTGTGAGTCTCAGCAGGA	GGTTGGAGGCTGTACCAAGTC
NRP1	GAAGTGGAAGCCCCTACAGC	CCACCTGTGAGCTGGAAGTC
PCDH9	ACACACCAGACAGTCGCA	CGGAGAGGCCTGGTCATAGA
PRTN3	CGGCATCTGCTTCGGAGAC	AGTCCACGTAGAGGGCTACC
PTGDR2	TGCCTCTTGTCTAGCTGCTG	GACATCGTGGGGCTCTGG
RBM11	GGGGCCACTAACCAAAGTGA	AGAGCGAGAACTCCCAAATCG
SYCE1	CTGGCGAGCTGAGAGGAAAT	TCTAGGCTTCCCACTTTCTGC
TNF	CCCATGTTGTAGCAAACCCTC	TGAGGTACAGGCCCTCTGAT
TRIM58	TCCTGAGCAGAAGTAAGGCTG	GCAGGGCCATGTGTCAAATC
ZNF667	AAGACCGAGCCCTGCCTTAC	AAAGACCAAGCGAGACCAGG
ZNF683	ATCTCAAGGTCCACCTGCGT	GGGTCTTGAGGTTACTGGAGC

The qPCR protocol was as follows:

Step	**Temperature**	**Time**	**Number of cycles**
Initial denature	95	20 sec	1
Denature	95	1 sec	40
Annealing/ extension	60	20 sec	

Appendix C

Details of the formulae and conditions used to create the finite element models of aortic aneurysms

In brief, the deformation of each aneurysmal component (node) was governed by the Cauchy momentum equation,

$$\rho_s \ddot{\boldsymbol{U}} = \nabla \cdot \boldsymbol{\sigma}$$

where $\boldsymbol{U}$ is the displacement vector, $\boldsymbol{\sigma}$ is the stress tensor and ρ_s is the density of each component. Tissues, including wall, ILT and calcium, were assumed to be hyperelastic, homogeneous, isotropic and incompressible with material properties described by the modified Mooney-Rivlin formulation:

$$W = c_1(\bar{I}_1 - 3) + D_1\left[exp\left(D_2(\bar{I}_1 - 3)\right) - 1\right] + \kappa(J - 1),$$

where $\bar{I}_1 = J^{-2/3} I_1$ and $J = det(\boldsymbol{F})$, $\boldsymbol{F}$ is the deformation gradient and I_1 is the first invariant of deformation tensor. κ is Lagrangian multiplier for the incompressibility. c_1, D_1, and D_2 are material parameters derived from previous experimental studies [B1-3]: arterial wall, c_1=0.07 kPa, D_1=6.54 kPa, D_2=5.88; ILT, c_1=0.24 kPa, D_1=8.69 kPa, D_2=0.61; and calcium, c_1=7.24x10^3 kPa, D_1=0.01 kPa, D_2=2.34x10^{-14}.

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16/12/14: Papworth Pump Priming Grant – 'Can m/miRNAs predict the risk of progression in
patients with thoracic aortic aneurysm'. £34381. This grant supported the RNA sequencing costs associated with the pilot project.

15/4/2013: NIHR-HTA 11/147/03 Effective Treatments for Thoracic Aortic Aneurysms (ETTAA study): A prospective cohort study. 6 year multicentre UK project funded by HTA for £1.7million. I am a named co-applicant and composed the grant application and research protocol. This grant has contributed to my salary during this first year of my PhD.

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