PHASE REPORT

Numira Ref. Number: 4047

MICROCT ANALYSES OF APOE -/- MOUSE AORTAS

CONFIDENTIAL

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For Numira, Inc.:

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16 September 2014
Date

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1.0 Personnel

Principal Investigator	Heather Ramsay, Ph.D. Scientific Director Numira, Inc.
MicroCT Analysis	Neha Shah Director of Operations Numira, Inc.
Management	Hal Widlansky CEO Numira, Inc.
Sponsor's Monitoring Scientist	Emilija Veljkovic Manager, Study Management Team Philip Morris International

2.0 Summary

Thirty (30) thoracic regions from ApoE mice were submitted for analysis. Thirty aortas were scanned using micro-computed tomography (microCT) to visualize atherosclerotic plaques and to quantify various parameters of the aorta and amount of plaque. The specimens were processed to obtain 3D images in addition to quantitative measurements of the plaques. Following microCT, all specimens were histologically processed and the slides and blocks returned to the Sponsor.

Results showed that mean plaque volume, plaque surface area, and percent occlusion were highest in the 3R4F group and lowest in the Sham group for the aorta, left common carotid, and left subclavian artery. For the brachiocephalic trunk, the highest mean measures were still found in the 3R4F group, but the lowest mean measures were in the Cessation group. The 3R4F group was significantly higher than all groups for mean aortic plaque volume and mean aortic percent occlusion and significantly higher than the Sham group for mean plaque surface area in the aorta, left common carotid, and the left subclavian artery. This group was also significantly higher than the Sham group and the Cessation group for mean plaque volume in the left subclavian artery.

The pMRTP, Cessation, and Switch treatment groups are only marginally different than Sham for most aortic variables. For the brachiocephalic trunk, left common carotid, and left subclavian artery these three groups typically fell between the Sham group and the 3R4F group for most plaque measures. There were no significant differences between these three groups and the Sham group for any measures.

Most plaque deposition patterns in the aorta and the three branching vessel can be attributed to hemodynamics and vessel architecture, with deposition occurring in lower wall shear stress locations, such as the inner curvature of the aortic arch and the ostias of the brachiocephalic trunk and left subclavian artery.

3.0 Study Conduct

Unless otherwise specified, all procedures described herein were conducted in accordance with Numira, Inc. Standard Operating Procedures.

4.0 Regulatory Compliance

Although this study was conducted according to Numira, Inc. SOPs, it was not subject to monitoring by the Numira Quality Assurance Unit.

5.0 Test System

5.1 Animals

Species:	Mouse

6.0 Experimental Procedures

6.1 Study Design Table

Thirty mouse thoracic regions were received from Philip Morris, Singapore, fixed in 10% neutral buffered formalin (NBF). Samples were stored at room temperature in

10% NBF until processing. Samples were trimmed to remove any extraneous tissue and expose the heart and vessels; the spinal cord was left intact to preserve the integrity of the aorta. The samples were stained using Numira's proprietary staining agent and then were scanned using MicroCT. All samples were scanned and analyzed blind to treatment assignment. Following analysis completion, the sample code was broken and the data grouped for statistical analysis. The samples were assigned to one of five groups by Philip Morris and are detailed in Table 1.

Table 1: Treatment Groups

Group	Treatment	Number of Samples
1	Sham	6
2	3R4F	6
3	pMRTP	6
4	Cessation	6
5	Switch	6

7.0 Experimental Procedures: *Ex Vivo*

7.1 Computed Tomography

All samples were scanned on a high-resolution, volumetric microCT scanner (Varian BIR 130/150, Varian Medical Systems, Lincolnshire, IL). The image data was acquired at 15-µm isotropic voxel resolution.

7.1.1 Data Processing and Image Generation

The microCT-generated image slices were combined into an image volume using the Teem (http://teem.sourceforge.net/) software package. All datasets were then cropped and reoriented to capture with the same anatomic region with similar orientation using Microview (GE Healthcare, Milwaukee, WI).

7.1.2 Segmentation

Numira's segmentation software, VHLab (www.Numira.com) was used to create a label map associated with the regions of interest (aorta, arteries, and plaque) from the imaging data for each specimen.

7.1.3 Measurements

After the segmentation process, the following measurements were reported for the aorta:

- Plaque Volume – Total volume of plaque in aorta, brachiocephalic trunk, left common carotid, and left subclavian artery
- Plaque Surface Area – Total surface area of plaque in aorta, brachiocephalic trunk, left common carotid, and left subclavian artery

- Mean Occlusion – Percent of the vessel cross-section that is occluded with plaque in aorta, brachiocephalic trunk, left common carotid, and left subclavian artery
- Thickness Map – Thickness of plaque translated into color-mapped visualizations of plaque along entire aorta length
- 5-Panel movies – Generated for each sample, the movie integrates the 3D aorta data with various measurements calculated throughout the specimen. The movie animates a cutting plane that moves along the aorta and brachiocephalic trunk, showing a perpendicular cross-section of the microCT volume in each frame. The movie is split into five panels, described below:
    - 3D rendering frame (left) – a 3D rendering of the aorta is displayed with a perpendicular slice. The vessel is shown in transparent gray, the plaque in dark yellow. A centerline embedded in the aorta is pseudo-colored to indicate the cross-sectional area of plaque at each point along the aorta. At the bottom of this frame, the slice distance and plaque cross-sectional area are reported, as well as total measurements (average occlusion, total plaque volume, total plaque surface area) for each of the regions (sinus, aortic arch, thoracic aorta, brachiocephalic trunk).
    - Linear Distance Measurements (top-right) – for each slice along the curved centerline, the average aorta radius, maximum plaque thickness, and average plaque thickness are plotted. As the animation proceeds, a black time-bar indicates the current slice distance along the graph.
    - Percent Measurements (middle-right) – For each slice along the curved centerline, the percent coverage (percent of the vessel wall that has plaque attached) and percent occlusion (percent of the vessel cross-section that is occluded with plaque) are plotted. As the animation proceeds, a black time-bar indicates the current slice distance along the graph.
    - Planar slice (bottom-right) – the grayscale slices cut through the aorta in an orientation centered around and perpendicular to the centerline. The right side is displayed with segmented aortic plaque overlaid in red, and segmented brachiocephalic trunk plaque overlaid in blue.

All metrics and 3D movies were created for the aortas using SCIRun (Scientific Computing and Imaging Institute, University of Utah). Individual sample results are displayed in the Appendices.

8.0 Statistical Analysis

All statistical analyses was performed using R, version 3.0.1 (R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org/).

Mean and standard deviation were calculated for all quantitative data.

For each variable, an ANOVA was performed followed by a *post hoc* Tukey's HSD test to compare each treatment group to each other, if the overall treatment effect was significant by ANOVA.

All tests will be considered statistically significant at the 5% probability level.

9.0 Records to be maintained

The following will be placed in the Numira, Inc. archives upon completion of the study:

All raw data including but not limited to:

- Raw and processed microCT data
- Data pertaining to the receipt of samples
- Correspondence related to the study
- The final phase report

10.0 Archiving

Records created by Numira, Inc. including but not limited to, all raw data and reports will be retained in the archives at Numira, Inc. for a period of 1 year from the date the final report is signed by the Principal Investigator. At the end of this period the Sponsor will be contacted in writing and disposition of these materials will be at the discretion of the Sponsor. At the end of the archival period additional archiving will be subject to additional charges.

A signed copy of the final report with any subsequent amendments or supplementary reports will be retained by the Testing Facility as their property and will not be returned to the Study Sponsor or destroyed at the end of the archive period.

11.0 References

[1] Feintuch, A. *et al.* 2007 Hemodynamics in the mouse aortic arch as assessed by MRI, ultrasound, and numerical modeling. *Am. J. Physiol. Heart C* **292**, H884-H892 (doi:10.1152/ajpheart.00796.2006)

[2] Suo, J. *et al.* 2007 Hemodynamic shear stresses in mouse aortas: Implications for atherogenesis. *Arterioscler. Thromb. Vasc. Biol.* **27**, 346-351 (doi: 10.1161/01.atv.0000253492.45717.46)

[3] Vincent, P.E. *et al.* 2011 Blood flow in the rabbit aortic arch and descending thoracic aorta. *J. R. Soc. Interface* **8**, 1708-1719 (doi:10.1098/rsif.2011.0116)

12.0 Results

Summary microCT data is in Table 2. Summary columnar graphs are in Figures 1-6. Individual data and figures are in the Appendices.

Aorta

For mean aortic plaque volume (Figure 1), the highest value was seen in Group 2 (3R4F), with a volume of 1.270 mm^3, and the lowest in Group 1 (Sham), with a volume of 0.599 mm^3. The mean plaque volume in Group 2 was significantly higher than all other groups. Group 3 (pMRTP), Group 4 (Cessation), and Group 5 (Switch) did not differ significantly from the Sham group (Group 1).

Mean plaque surface area (Figure 2) and mean percent occlusion (Figure 3) were also highest in Group 2 (21.342 mm^2 and 6.550%, respectively). As with mean plaque volume, the lowest measures for mean plaque surface area and mean percent occlusion were found in the Sham group (Group 1) (11.923 mm^2 and 3.682%, respectively). The 3R4F group (Group 2) was significantly higher than the Sham group for mean plaque surface area, but did not differ significantly from Groups 3-5, despite having a mean surface area nearly one third larger than the next largest group (Group 4; Cessation). Group 2 had a significantly larger mean percent occlusion than all other groups. Groups 3-5 did not differ significantly from the Sham group (Group 1).

The anatomical location of the majority of the aortic plaques (Appendix A) lay along the inner curvature of the aortic arch. This coincides with low wall shear stress (WSS)[1-3] and vortices that form in blood flow[1,3]. Low WSS also correlates with monocyte adhesion[2], which is associated with early endothelial lesion development, and lipid deposition[1,3]; both measures contributing to plaque formation.

Table 2: Summary MicroCT Results

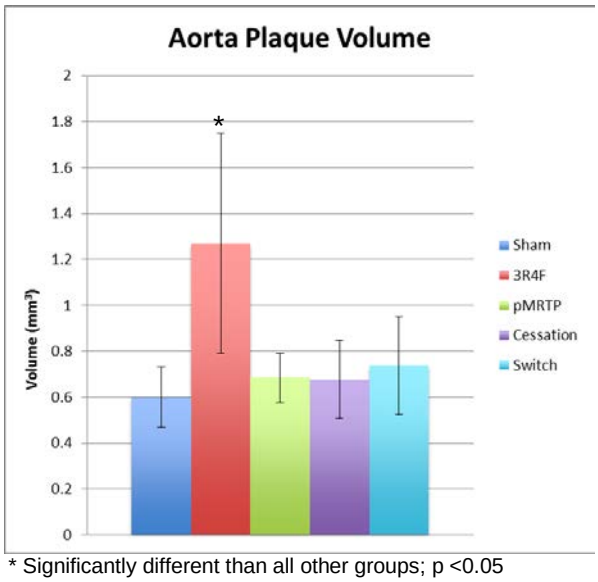
Means and standard deviations (Mean ± SD) by treatment group and region of interest (ROI).

		Aorta			Brachiocephalic Trunk			Left Common Carotid			Left Subclavian Artery		
Group	Sample	Plaque Volume (mm^3)	Plaque Surface Area (mm^2)	Mean Occlusion (%)	Plaque Volume (mm^3)	Plaque Surface Area (mm^2)	Mean Occlusion (%)	Plaque Volume (mm^3)	Plaque Surface Area (mm^2)	Mean Occlusion (%)	Plaque Volume (mm^3)	Plaque Surface Area (mm^2)	Mean Occlusion (%)
Sham	Mean	0.599	11.923	3.682	0.223	4.158	42.536	0.002	0.071	0.768	0.021	0.514	12.746
	SD	0.132	2.271	1.122	0.050	0.536	10.046	0.005	0.173	1.881	0.021	0.469	13.752
3R4F	Mean	1.270[a]	21.342[b]	6.550[a]	0.284	4.788	49.655	0.045	0.911[b]	20.314	0.107[c]	1.964[b]	51.751
	SD	0.478	7.363	1.603	0.053	0.805	6.325	0.046	0.806	19.475	0.064	1.096	27.061
pMRTP	Mean	0.685	14.042	3.858	0.269	4.420	46.601	0.014	0.326	10.512	0.077	1.497	42.836
	SD	0.108	1.782	0.568	0.071	0.893	7.179	0.017	0.375	13.843	0.049	0.728	23.876
Cessation	Mean	0.676	15.165	3.892	0.201	3.822	39.878	0.024	0.639	11.939	0.029	0.722	17.127
	SD	0.170	4.000	0.634	0.043	0.550	5.624	0.018	0.283	10.205	0.041	0.963	24.270
Switch	Mean	0.738	14.779	4.318	0.231	3.896	46.976	0.015	0.395	8.735	0.046	0.854	36.474
	SD	0.213	4.494	1.304	0.074	1.179	10.153	0.014	0.347	9.772	0.040	0.708	34.357

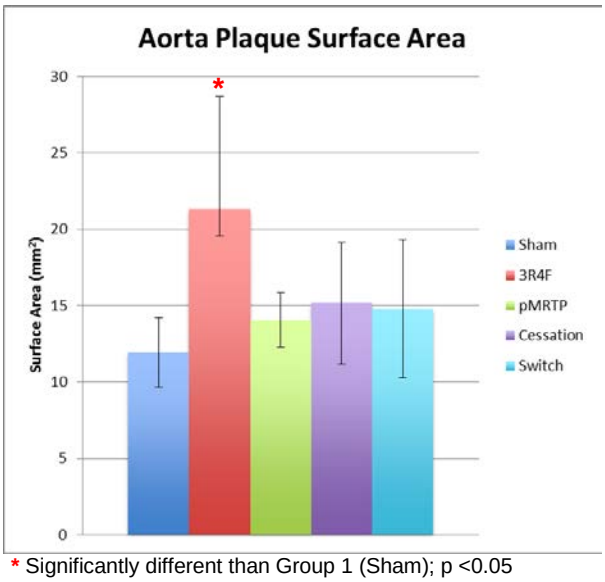
[a] Significantly different than all other groups; $p < 0.05$

[b] Significantly different than Group 1 (Sham); $p < 0.05$

[c] Significantly different than Group 1 (Sham) and Group 4 (Cessation); $p < 0.05$

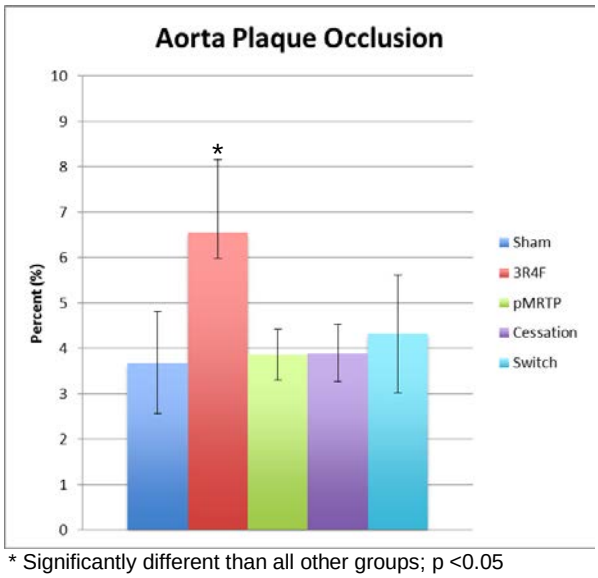


* Significantly different than all other groups; p <0.05



* Significantly different than Group 1 (Sham); p <0.05

Figures 1 & 2. Plaque Volume and Plaque Surface Area in the aorta by treatment group.



* Significantly different than all other groups; p <0.05

Figure 3. Mean Plaque Occlusion in the aorta by treatment group.

Brachiocephalic Trunk

Mean plaque volume (Figure 5) was highest in Group 2 (3R4F), with a volume of 0.284 mm^3, and lowest in Group 4 (Cessation), with a volume of 0.201mm^3. Mean plaque surface area (Figure 6) and mean percent occlusion (Figure 7) were also highest in Group 2 (4.788 mm^2 and 49.655%, respectively) and lowest in the Cessation group (Group 4) (3.822 mm^2 and 39.878%, respectively). However, none of the measures were significantly different in any of the groups.

Similar to the inner curvature of the aortic arch, the proximal ostia of the brachiocephalic trunk has a lower WSS[2], allowing for lipid deposition and plaque formation. Although the 3R4F group exhibited a trend towards a higher plaque volume compared to all other groups, differences were not statistical significant. The lack of significant differences between groups in plaque formation along this aortic branch may be the result of a plaque formation process that is already maximal at the time point examined (8 months). A time-course analysis of these lesions upon exposure to the conventional cigarette 3R4F might help to discern whether the rate of plaque progression in the brachiocephalic trunk is different than the rate of progression in the aortic arch or the thoracic aorta.

Left Common Carotid

The highest value for mean plaque volume (Figure 5) was seen in the 3R4F group (Group 2; 0.045 mm^3) and the lowest in the Sham group (Group 1; 0.002 mm^3). None of the groups were significantly different from each other and the mean volume for Group 3 (pMRTP), Group 4 (Cessation), and Group 5 (Switch) fell approximately half way between the highest and lowest groups.

Mean plaque surface area (Figure 6) was also highest in Group 2 (0.911 mm^2) and lowest in Group 1 (0.071 mm^2). The 3R4F group (Group 2) was significantly higher than the Sham group for mean plaque surface area, but did not differ significantly from Groups 3-5, despite having a mean surface area one third larger than the next largest group (Group 4; Cessation).

Mean percent occlusion (Figure 7) was highest in Group 2 (20.314 %) and lowest in the Sham group (Group 1) (0.768%). There were no significant differences between groups for this variable.

Lower measure results in the left common carotid (LCC) than for either the brachiocephalic trunk or the left subclavian artery may be attributed to hemodynamics and vessel architecture. Though the blood flow rates in the LCC are roughly equivalent to the left subclavian artery[1], the diameter of the LCC is the smallest of the three branches[2]. As shear is calculated as force per unit area, the same blood flow rate through the smaller vessel diameter yields a higher WSS in the LCC[2], which would impede plaque formation. And, as previously indicated, the brachiocephalic trunk has higher plaque measures, due to a lower WSS and inclination toward plaque formation.

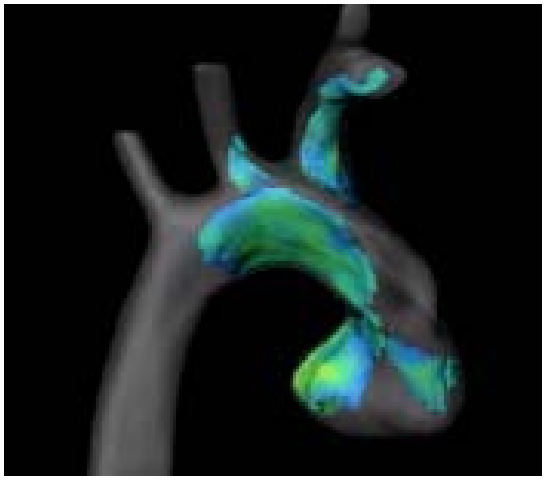
Left Subclavian Artery

For mean plaque volume (Figure 5), the highest value was seen in Group 2 (3R4F), with a volume of 0.107 mm^3, and the lowest in Group 1 (Sham), with a volume of 0.021 mm^3. The mean plaque volume in Group 2 was significantly higher than Group 1 (Sham) and Group 4 (Cessation). Mean plaque volumes for Group 3 (pMRTP) and Group 5 (Switch) fell between the highest and lowest groups but were not significantly different compared to Sham or 3R4F.

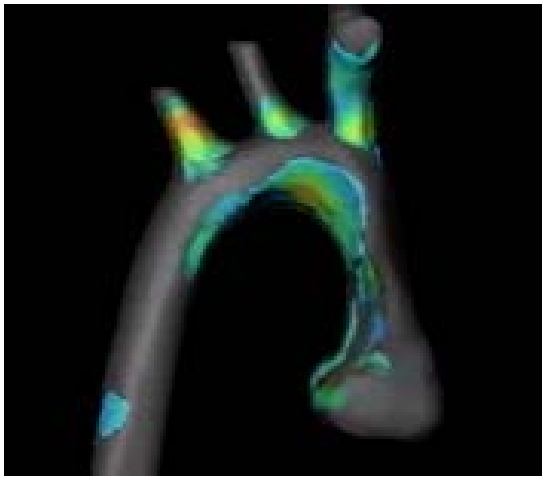
Mean plaque surface area (Figure 6) was also highest in Group 2 (1.964 mm^2) and lowest in Group 1 (0.514 mm^2). The 3R4F group (Group 2) was significantly higher than the Sham group for mean plaque surface area, but did not differ significantly from Groups 3-5. Plaque surface area:volume ratios in the LSA were more similar to the aorta. The SA:V of the plaques in the brachiocephalic trunk had a higher profile (more volume per unit surface area) than the LSA, while the LCC was flatter.

Mean percent occlusion (Figure 7) was highest in Group 2 (51.751 %) and lowest in the Sham group (Group 1) (12.746%). There were no significant differences between groups for this variable. Mean percent occlusion results for Group 3 (pMRTP), Group 4 (Cessation), and Group 5 (Switch) ranged between the highest and lowest groups, with Cessation at 17.127%, Switch at 36.474%, and pMRTP at 42.836%.

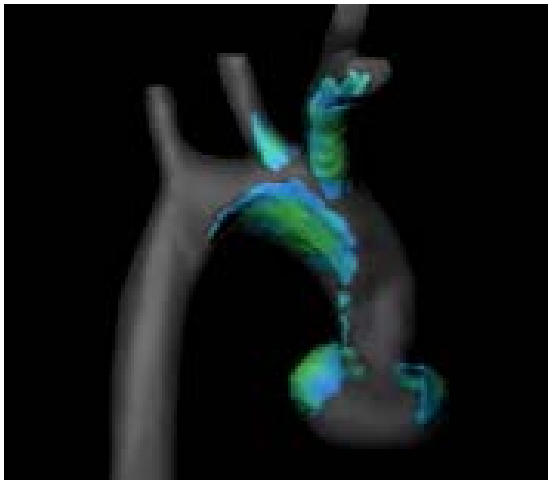
The variability in percent occlusion for the LSA, relative to the other aortic branches may be due, in part, to anatomical variation. The width of the span to the next closest arterial branch (LCC), the degree of bend in the aortic arch, and the angle of vessel branching would strongly impact the velocity of blood flow and the incidence of vortices formed at the LSA ostia, which, in turn, would impact plaque formation. Representative images below in Figure 4 indicate that LSA's with the highest percent occlusion are on aortas with steeper arches and narrower spans at the ostias between the LSA and the LCC, relative to the LSA's with 0% occlusion.



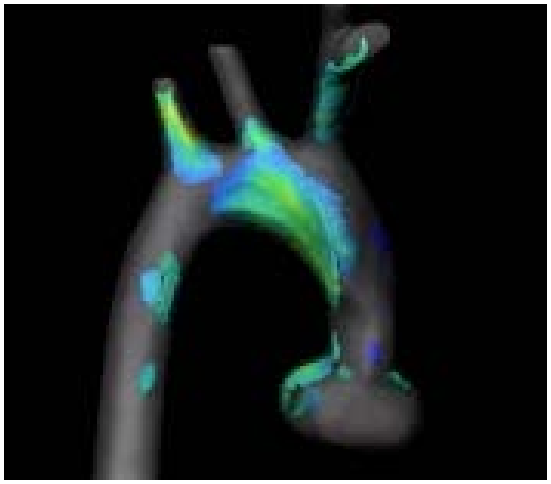
Sample 921063; Group 1
0.000% LSA occlusion



Sample 920929; Group 3
77.294% LSA occlusion

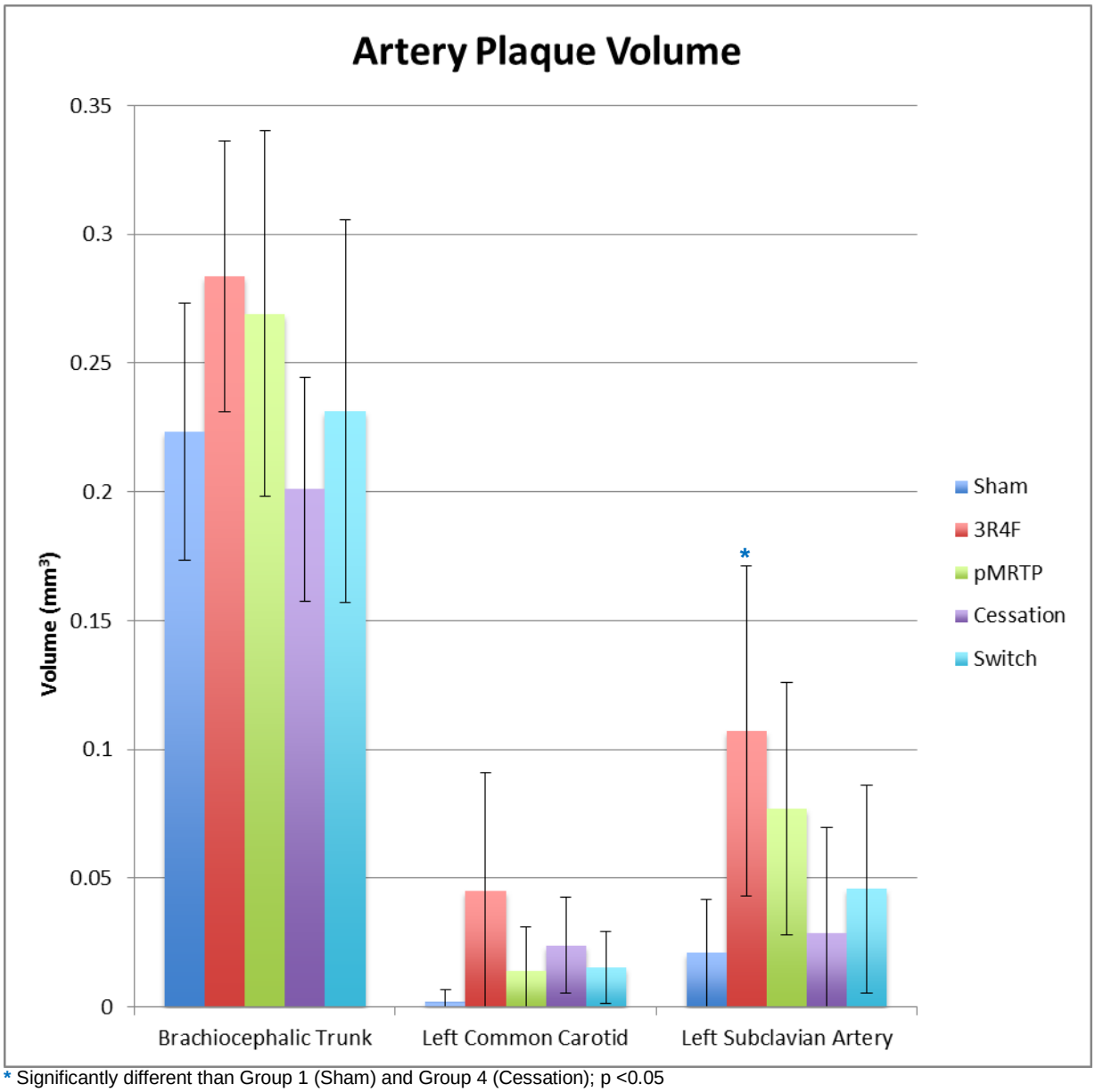


Sample 920567; Group 4
0.000% LSA occlusion



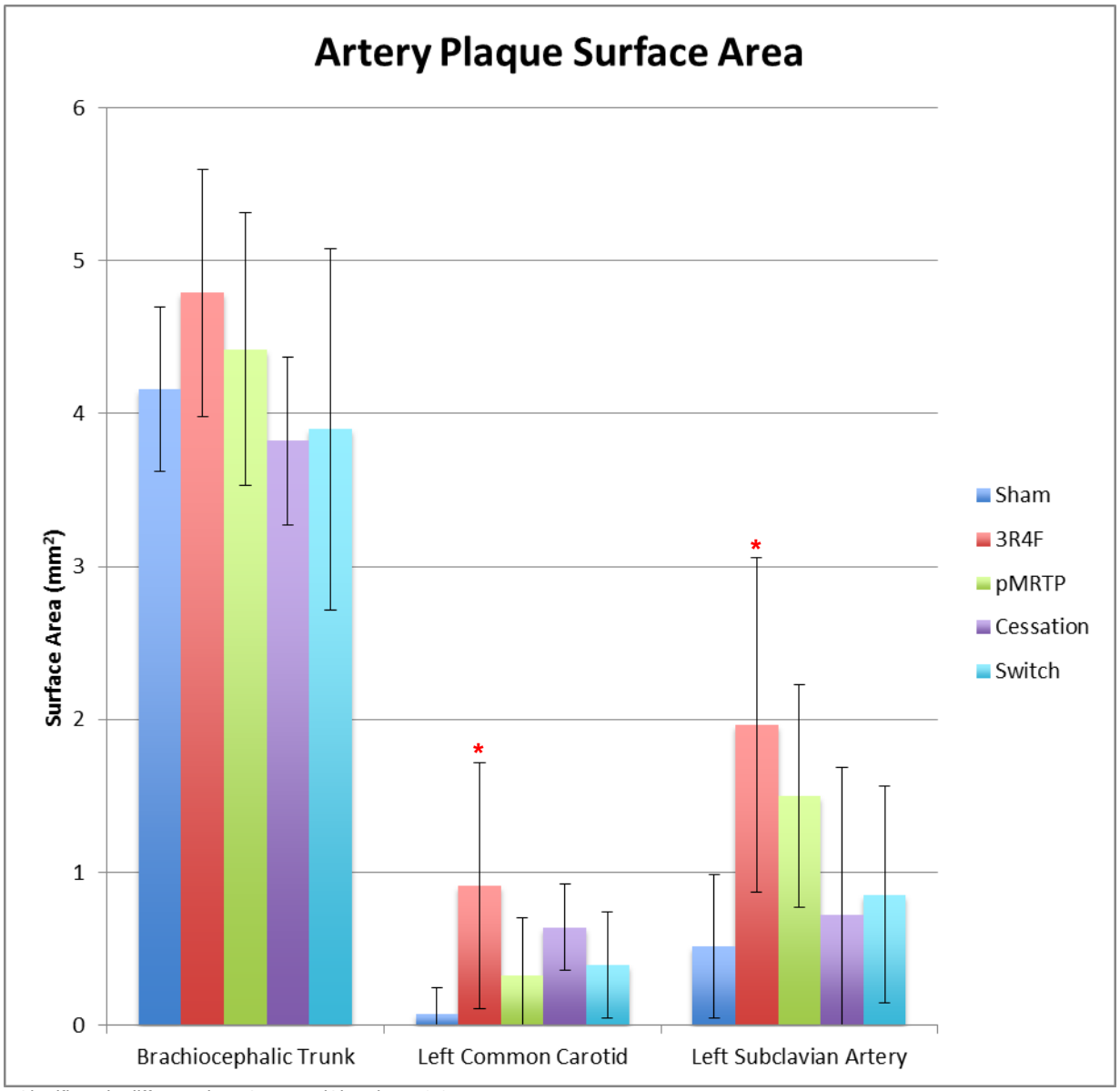
Sample 920691; Group 5
81.052% LSA occlusion

Figure 4. MicroCT 3D model images of the aortic branches from representative samples.



* Significantly different than Group 1 (Sham) and Group 4 (Cessation); p <0.05

Figure 5. Mean Plaque Volume in arteries by treatment group.



* Significantly different than Group 1 (Sham); p <0.05

Figure 6. Plaque Surface Area in arteries by treatment group.

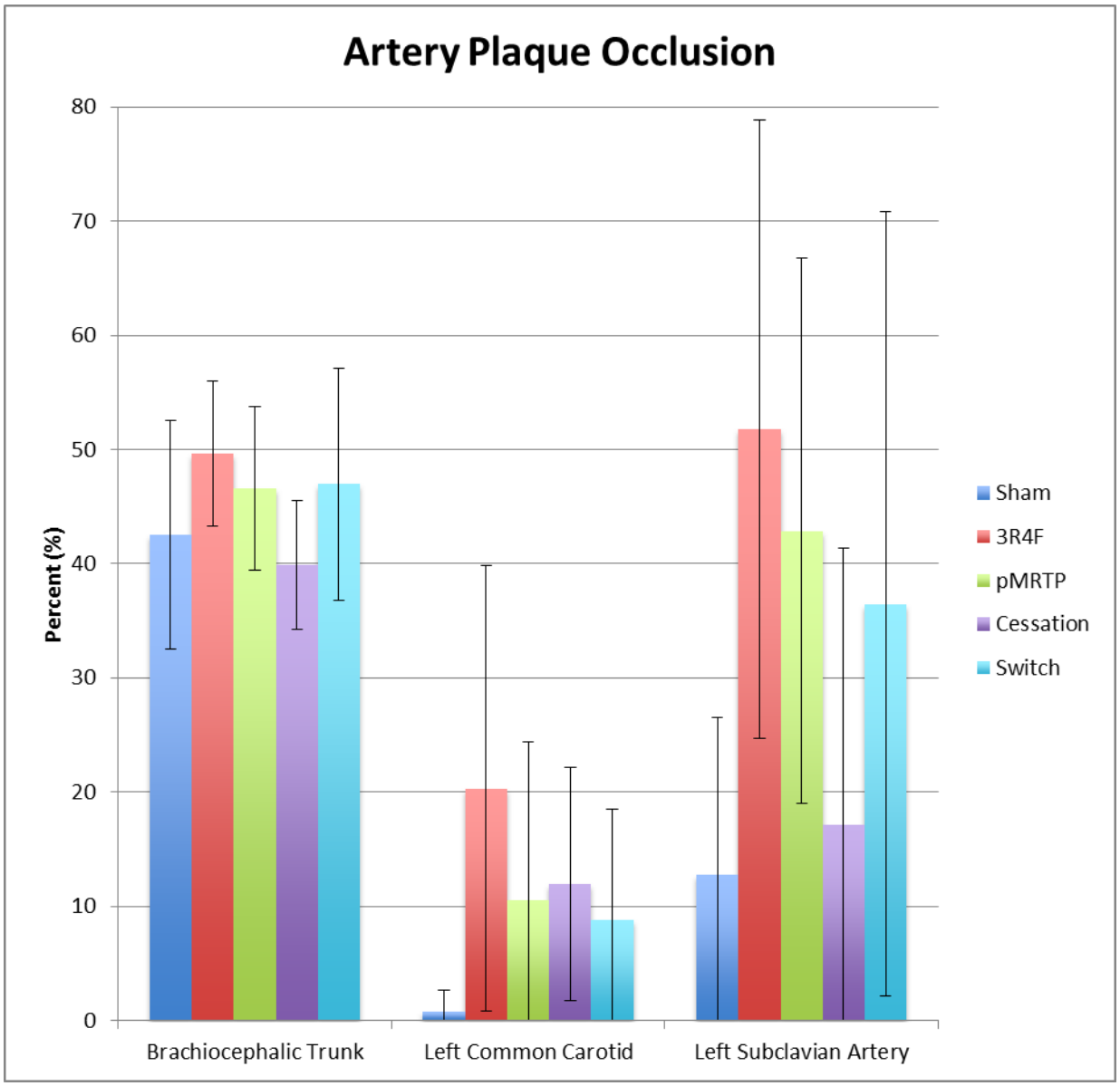


Figure 7. Mean Plaque Occlusion in arteries by treatment group.

13.0 Conclusions

Mean plaque volume, plaque surface area, and percent occlusion were highest in the 3R4F group and lowest in the Sham group for the aorta, left common carotid, and left subclavian artery. For the brachiocephalic trunk, the highest mean measures were still found in the 3R4F group, but the lowest mean measures were in the Cessation group. The 3R4F group was significantly higher than all groups for mean aortic plaque volume and mean aortic percent occlusion and significantly higher than the Sham group for mean plaque surface area in the aorta, left common carotid, and the left subclavian artery. This group was also significantly higher than the Sham group and the Cessation group for mean plaque volume in the left subclavian artery.

The pMRTP, Cessation, and Switch treatment groups are only marginally different than Sham for most aortic variables. For the brachiocephalic trunk, left common carotid, and left subclavian artery these three groups typically fell between the Sham group and the 3R4F group for most plaque measures. There were no significant differences between these three groups and the Sham group for any measures.

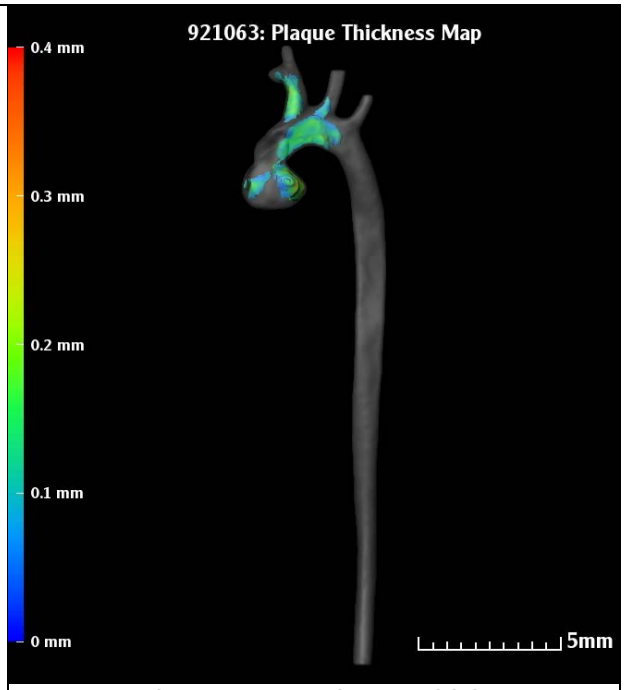
Most plaque deposition patterns in the aorta and the three branching vessel can be attributed to hemodynamics, anatomical variation, and vessel architecture, with deposition occurring in lower wall shear stress locations, such as the inner curvature of the aortic arch and the ostias of the brachiocephalic trunk and left subclavian artery. Additionally, variability in atherosclerotic measures will be higher in the branches than the aorta, as minute architecture differences in the smaller vessels translate more immediately into changes in the plaque deposition environment.

14.0 Appendix A – Representative MicroCT Images

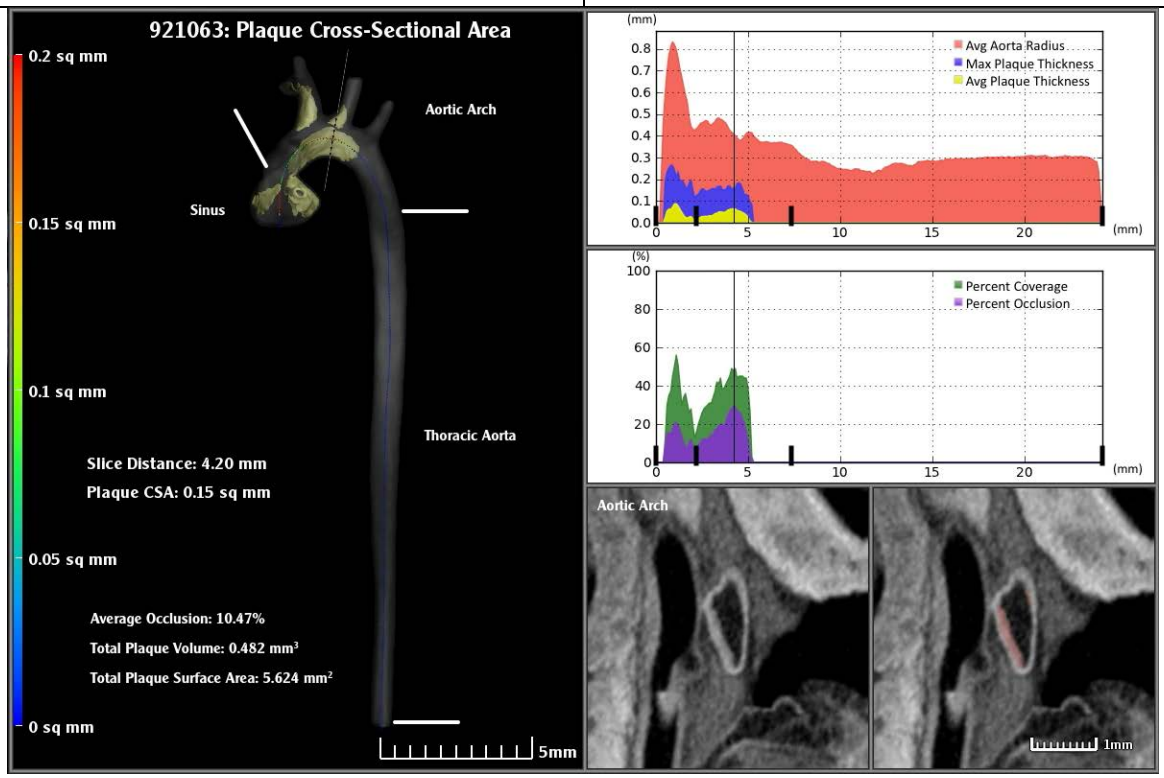
14.1 Sham Group



Sample 921063 – Segmentation Slice



Sample 921063 – Plaque Thickness

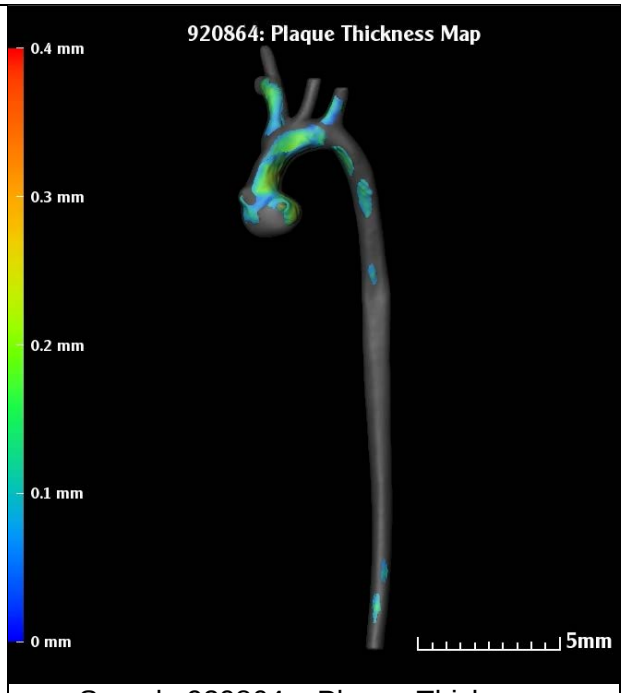


Sample 921063 – Multipanel

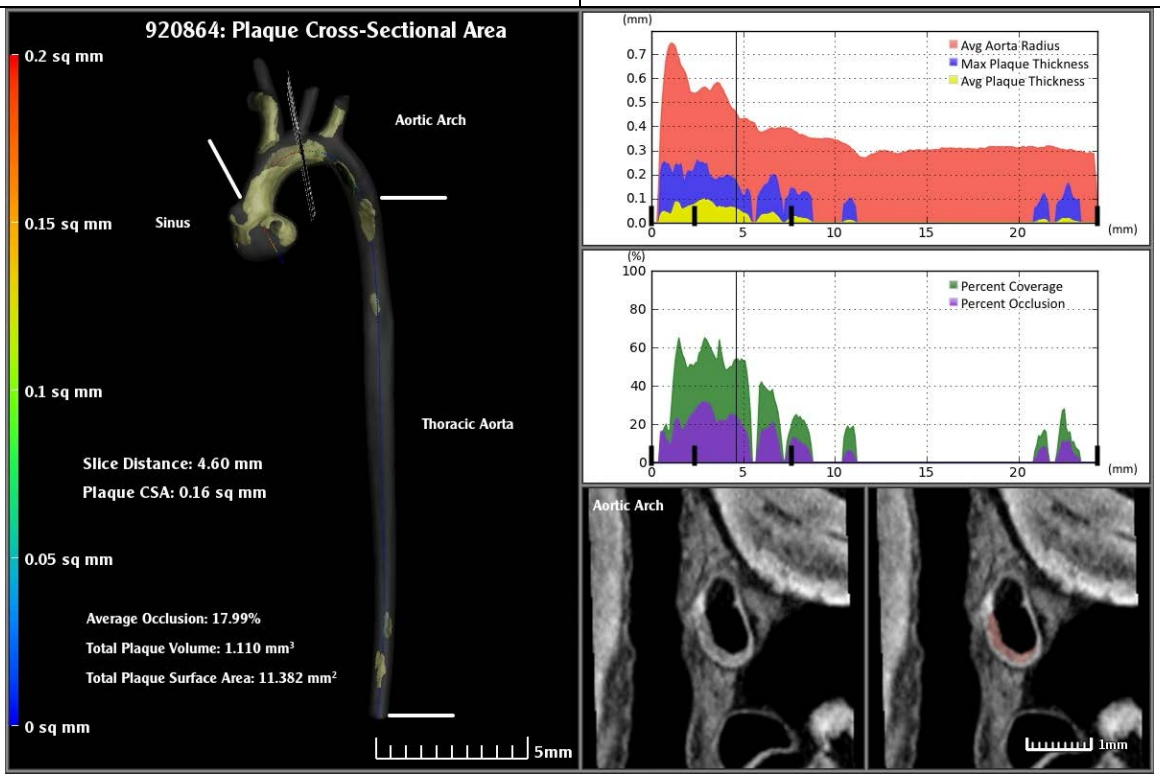
14.2 3R4F Group



Sample 920864 – Segmentation Slice

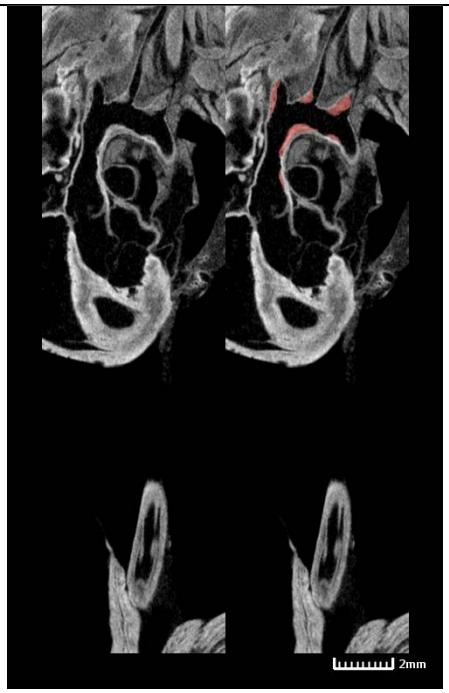


Sample 920864 – Plaque Thickness

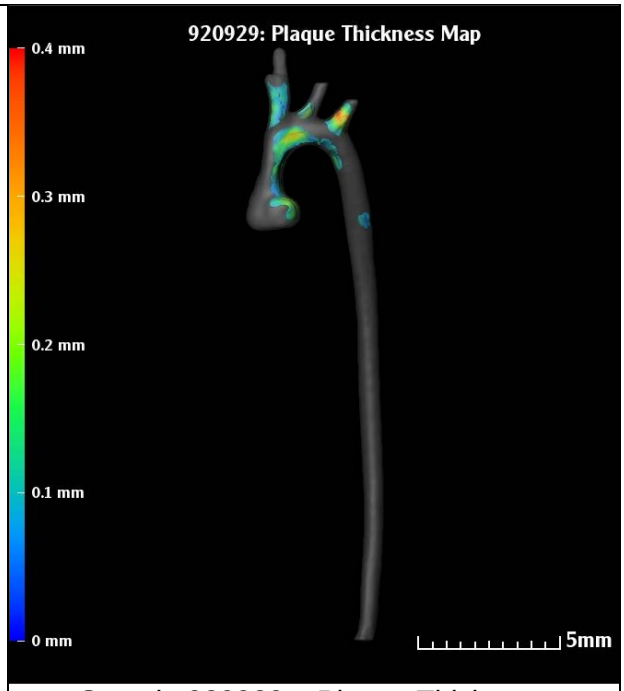


Sample 920864 – Multipanel

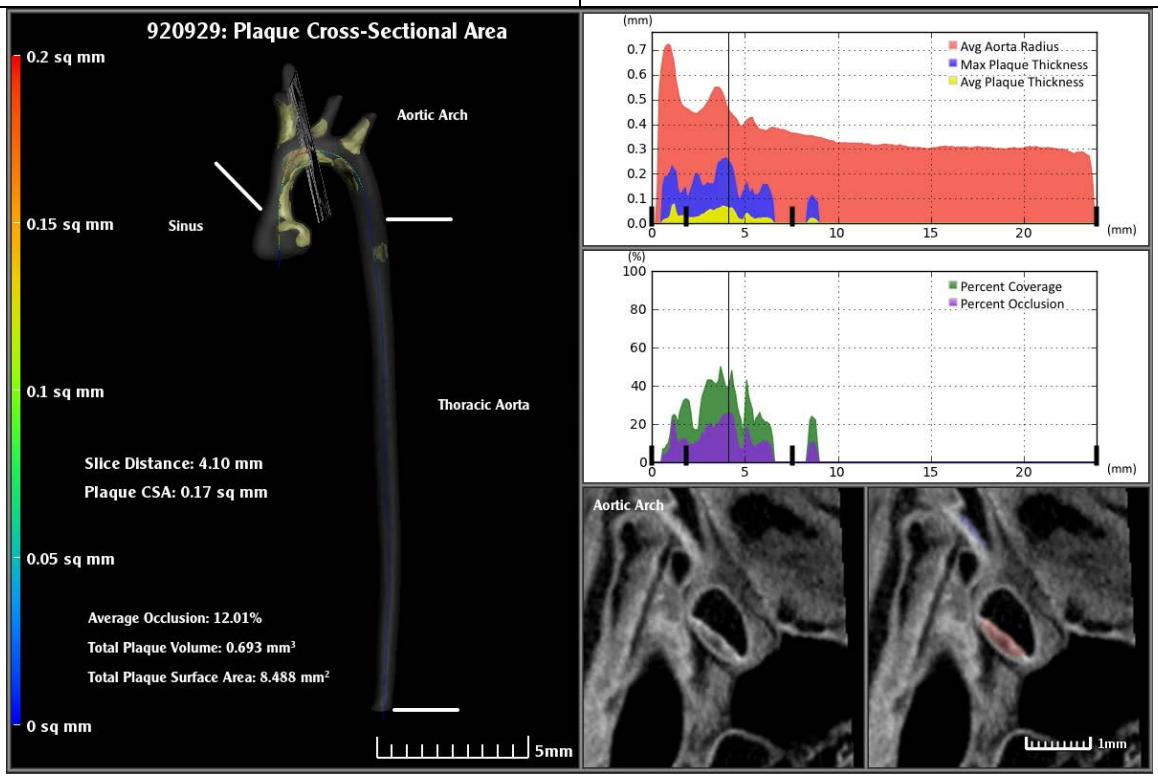
14.3 pMRTP Group



Sample 920929 – Segmentation Slice



Sample 920929 – Plaque Thickness

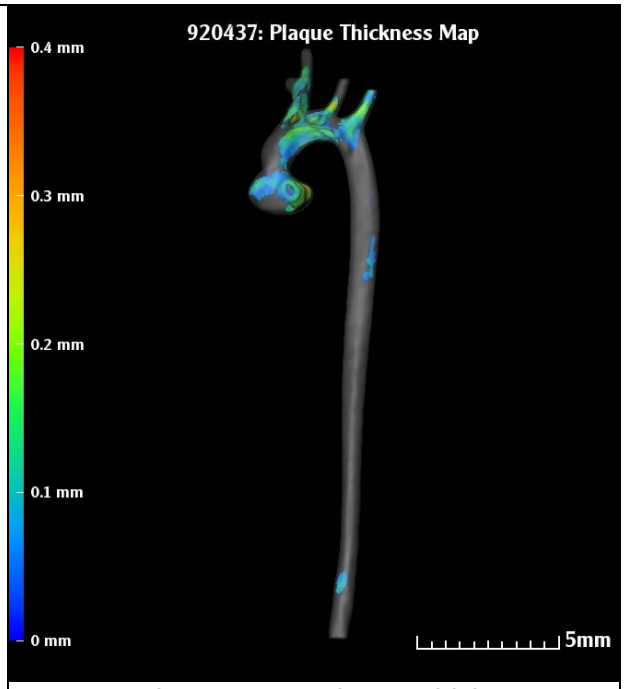


Sample 920929 – Multipanel

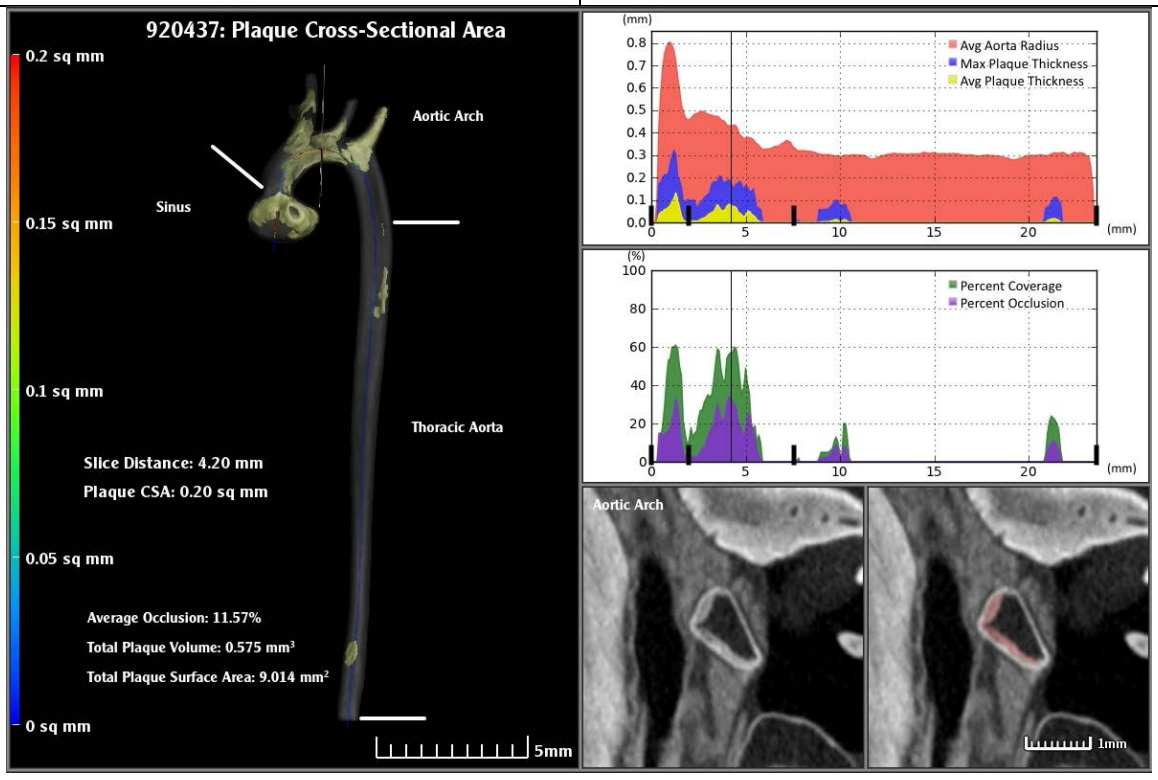
14.4 Cessation Group



Sample 920437 – Segmentation Slice



Sample 920437 – Plaque Thickness

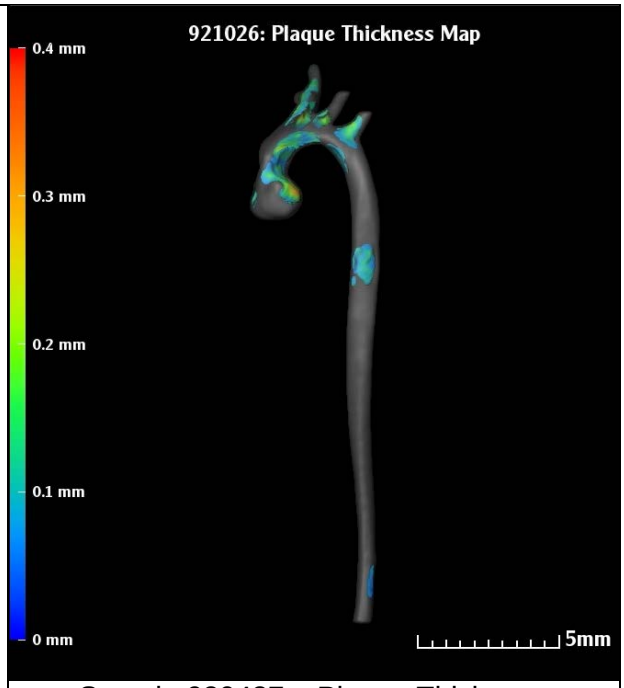


Sample 920437 – Multipanel

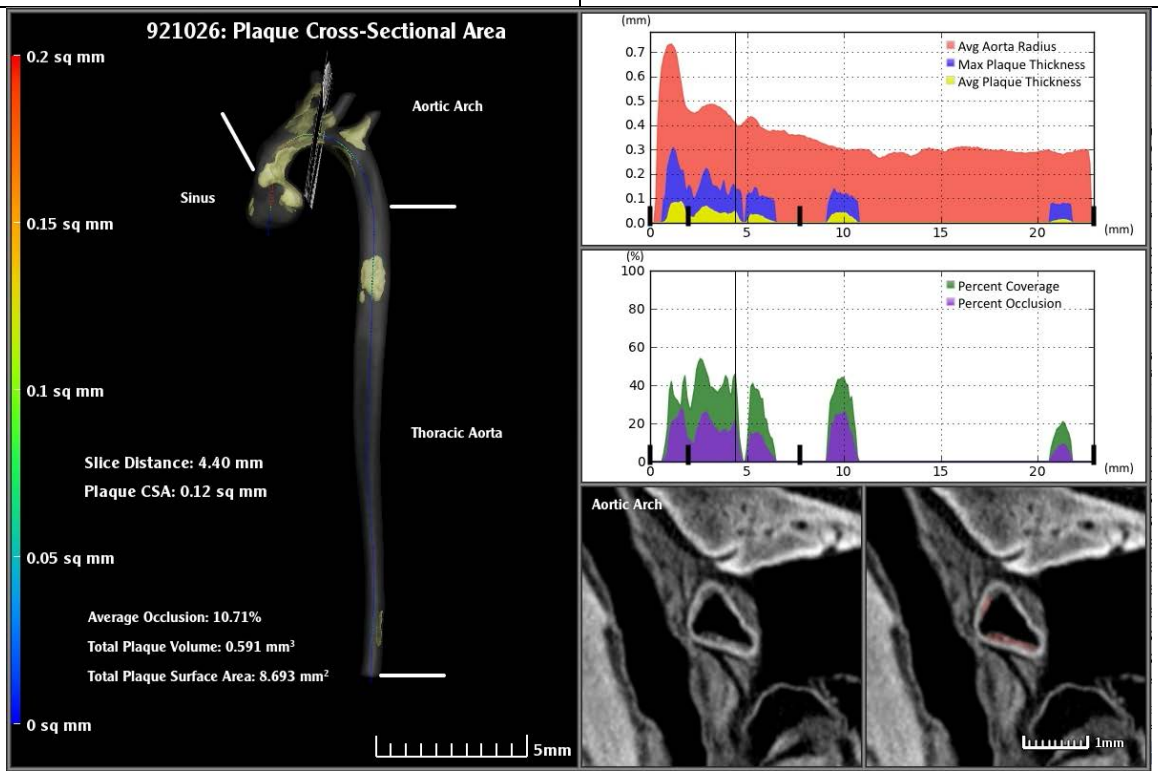
14.5 Switch Group



Sample 920437 – Segmentation Slice



Sample 920437 – Plaque Thickness



Sample 920437 – Multipanel

15.0 Appendix B – Individual Data

			Aorta			Brachiocephalic Trunk			Left Common Carotid			Left Subclavian Artery		
Group	Coded ID	Uncoded ID	Plaque Volume (mm^3)	Plaque Surface Area	Mean Occlusion (%)	Plaque Volume (mm^3)	Plaque Surface Area	Mean Occlusion (%)	Plaque Volume (mm^3)	Plaque Surface Area	Mean Occlusion (%)	Plaque Volume (mm^3)	Plaque Surface Area	Mean Occlusion (%)
Sham	921063	1062006	0.605	11.897	3.145	0.192	3.831	37.346	0.012	0.424	4.608	0	0	0
	920176	1062027	0.665	13.300	3.319	0.188	3.827	35.747	0	0	0	0.008	0.298	5.326
	920389	1062033	0.782	14.620	4.964	0.317	5.130	52.415	0	0	0	0	0	0
	920063	1062039	0.623	13.188	5.002	0.186	4.367	30.080	0	0	0	0.037	0.901	16.455
	920472	1062040	0.534	9.935	3.555	0.225	4.117	55.853	0	0	0	0.034	0.871	19.424
	921003	1062045	0.387	8.598	2.109	0.232	3.676	43.775	0	0	0	0.047	1.013	35.268
3R4F	920881	2032045	1.208	19.260	6.413	0.327	5.170	54.252	0.100	1.725	45.550	0.107	2.053	61.884
	920817	2032046	0.880	16.707	5.072	0.229	3.291	59.422	0	0	0	0	0	0
	920779	2062040	0.915	17.328	4.980	0.329	5.604	49.883	0.055	1.473	30.332	0.175	3.273	66.854
	920829	2062041	1.295	18.107	7.216	0.220	4.567	45.206	0.020	0.630	10.364	0.132	2.390	67.235
	920864	2062044	1.136	20.540	6.300	0.264	4.994	42.111	0	0	0	0.072	1.690	43.999
	921086	2062046	2.187	36.107	9.319	0.333	5.103	47.054	0.096	1.638	35.640	0.158	2.376	70.534
pMRTP	920866	3032045	0.676	15.149	4.431	0.251	3.723	47.586	0.042	0.861	34.850	0.035	1.034	25.319
	920183	3062039	0.771	14.971	4.578	0.328	5.416	49.136	0	0	0	0.007	0.240	8.198
	920065	3062041	0.705	15.122	3.669	0.205	3.581	44.138	0.017	0.541	12.062	0.100	1.956	54.203
	920190	3062042	0.546	11.788	3.055	0.297	5.390	53.157	0	0	0	0.097	2.197	47.880
	920355	3062044	0.829	15.504	3.850	0.358	4.826	52.074	0	0	0	0.080	1.796	44.120
	920929	3992006	0.583	11.718	3.565	0.176	3.586	33.514	0.025	0.554	16.158	0.143	1.759	77.294
Cessation	920236	4062040	0.944	20.535	4.840	0.176	3.614	35.844	0.017	0.488	5.056	0.037	1.163	24.938
	920295	4062041	0.674	17.379	3.870	0.218	3.740	40.305	0.010	0.358	3.279	0	0	0
	920437	4062042	0.808	17.980	4.378	0.278	4.914	44.864	0.058	0.992	31.376	0.105	2.418	61.878
	920484	4062043	0.516	10.560	3.601	0.201	3.549	46.472	0.027	0.924	12.821	0.031	0.750	15.946
	920547	4062044	0.575	11.854	3.050	0.179	3.722	40.451	0.007	0.346	7.511	0	0	0
	920567	4062046	0.539	12.684	3.610	0.155	3.390	31.333	0.025	0.724	11.588	0	0	0
Switch	920258	5062039	0.611	12.849	3.576	0.251	3.519	57.116	0.032	0.847	24.702	0.037	0.834	23.089
	921026	5062040	0.743	15.675	5.109	0.258	4.146	44.732	0.029	0.611	13.804	0.064	1.244	47.267
	920641	5062041	0.445	8.846	2.469	0.224	3.957	45.732	0	0	0	0	0	0
	920691	5062042	0.890	17.745	5.377	0.087	1.975	32.407	0.011	0.328	2.599	0.094	1.495	81.052
	920022	5062043	0.686	12.065	3.567	0.290	5.623	41.924	0	0	0	0	0	0
	920582	5062046	1.052	21.495	5.808	0.278	4.154	59.945	0.020	0.585	11.305	0.080	1.548	67.434