

Supplementary Data to: Mitochondrial Inhibitor Atovaquone Increases Tumor Oxygenation and Inhibits Hypoxic Gene Expression in Patients with Non-Small Cell Lung Cancer

SUPPLEMENTARY FIGURES

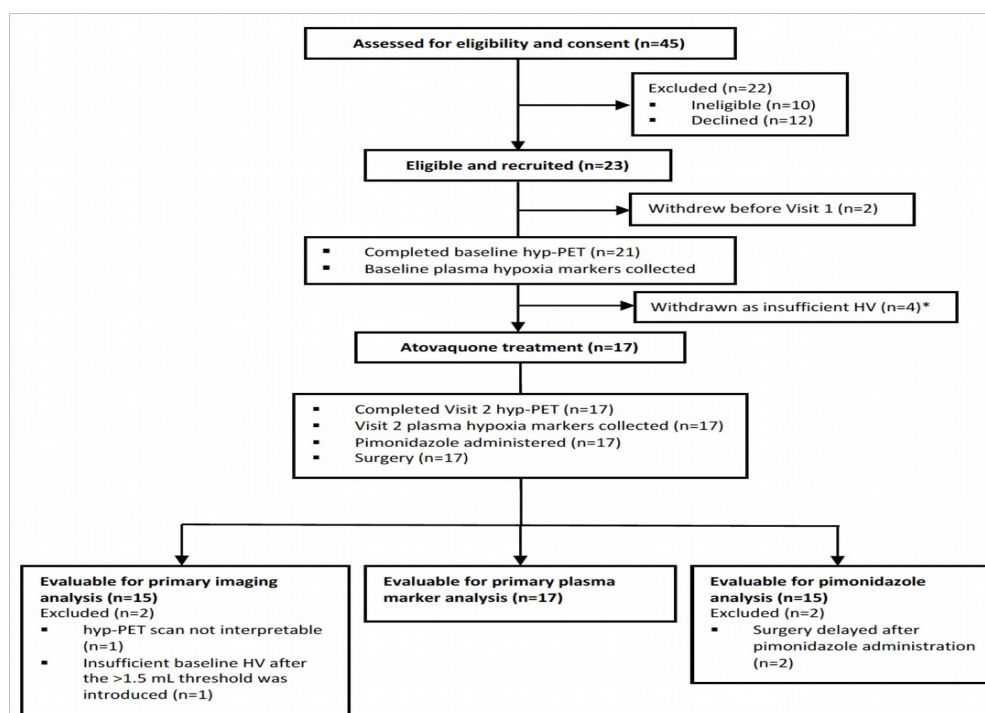


Figure S1. CONSORT diagram for Cohort 1. Recruitment and evaluability of patients for primary endpoints in Cohort 1. *One of the four patients with insufficient hypoxic volume at baseline received atovaquone. This patient is not included in the number of patients treated with atovaquone. All four excluded patients went on to have surgery but are not included in the number who had surgery in this diagram. Abbreviations: hyp-PET, hypoxia positron emission tomography; HV, hypoxic volume.

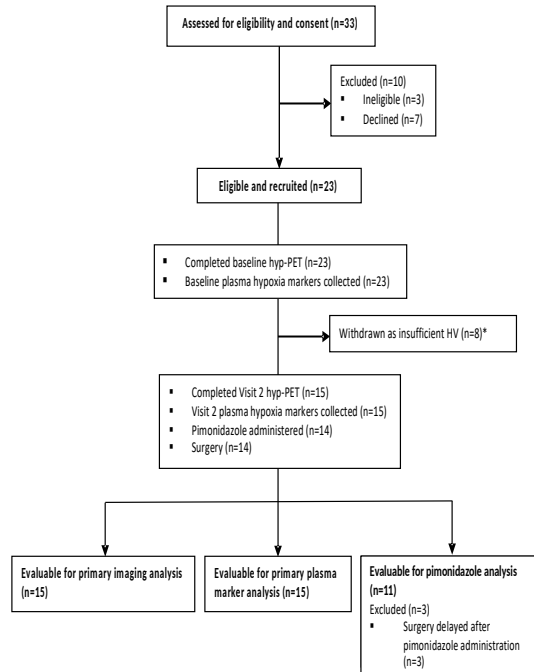


Figure S2. CONSORT diagram for Cohort 2. Recruitment and evaluability of patients for primary endpoints in Cohort 2. *All 8 patients with insufficient hypoxia at baseline went on to have surgery but are not included in the number who had surgery in this diagram. Abbreviations: hyp-PET, hypoxia positron emission tomography; HV, hypoxic volume.

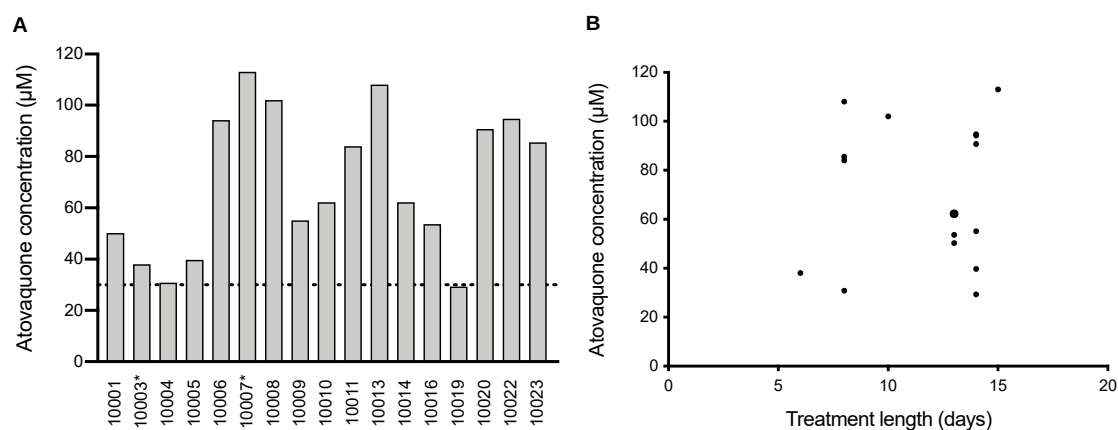


Figure S3. Atovaquone plasma levels. A) Plasma concentration of atovaquone for all patients who received treatment. The dashed line represents the concentration of atovaquone known to reduce tumor oxygen consumption in preclinical experiments. B) Lack of association between length of atovaquone treatment and plasma atovaquone concentration. The larger data point represents two overlapping data points. Patients marked with asterisks were not evaluable for primary imaging analysis.

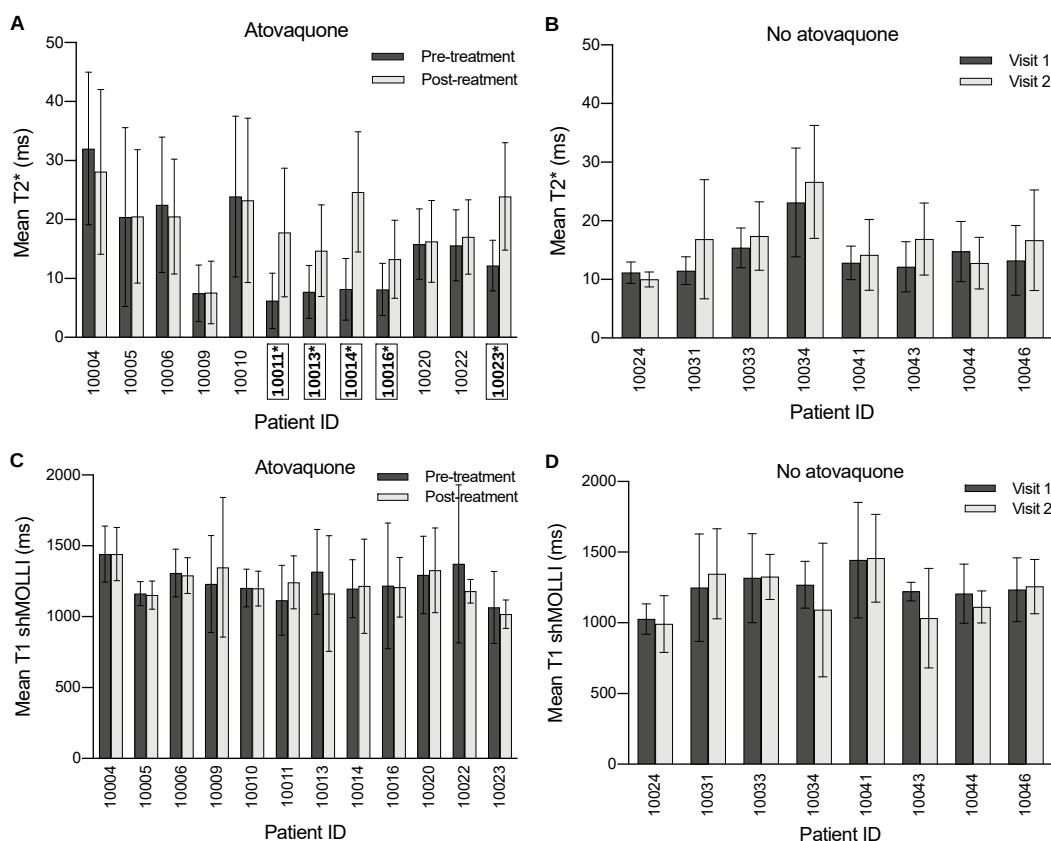


Figure S4. Change in MRI-derived parameters relating to tumor venous oxygenation and cellularity. Data obtained from both imaging visits for all patients with complete MRI data sets. T2* for A) atovaquone-treated and B) untreated patients. T1 shMOLLI for C) atovaquone-treated and D) untreated patients. All values are means with standard deviations. Highlighted patients had a meaningful change in parameter between imaging visits.

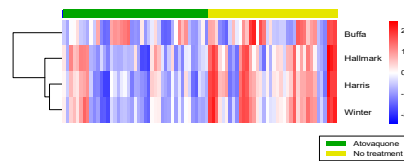


Figure S5. Tumor hypoxia gene expression signatures measured by RNAseq. Expression of Buffa, Hallmark, Harris and Winter hypoxia signatures from multiple regions of resected tumor per patient using the Gene Set Variation Analysis summarization score.

Figure SXX. Evaluation of the main parametric and non-parametric methodologies to compute signature scores, for all hypoxia signatures used in this study (Buffa, Hallmarks, Harris and Winter, reference in main manuscript). Very high correlation was observed among all scores. GSVA was used throughout the study, but results are also provided for Median and ssGSEA scores. For more information and discussion on these methodology see our protocol for systematic evaluation of gene signatures (Dhawan A, Barberis A, Cheng W, Domingo E, West C, Maughan T, Scott JG, Harris AL, Buffa FM. Nat Protoc 14, 1377–1400, 2019, <https://doi.org/10.1038/s41596-019-0136-8>). The R package sigQC v0.1.21 was used.

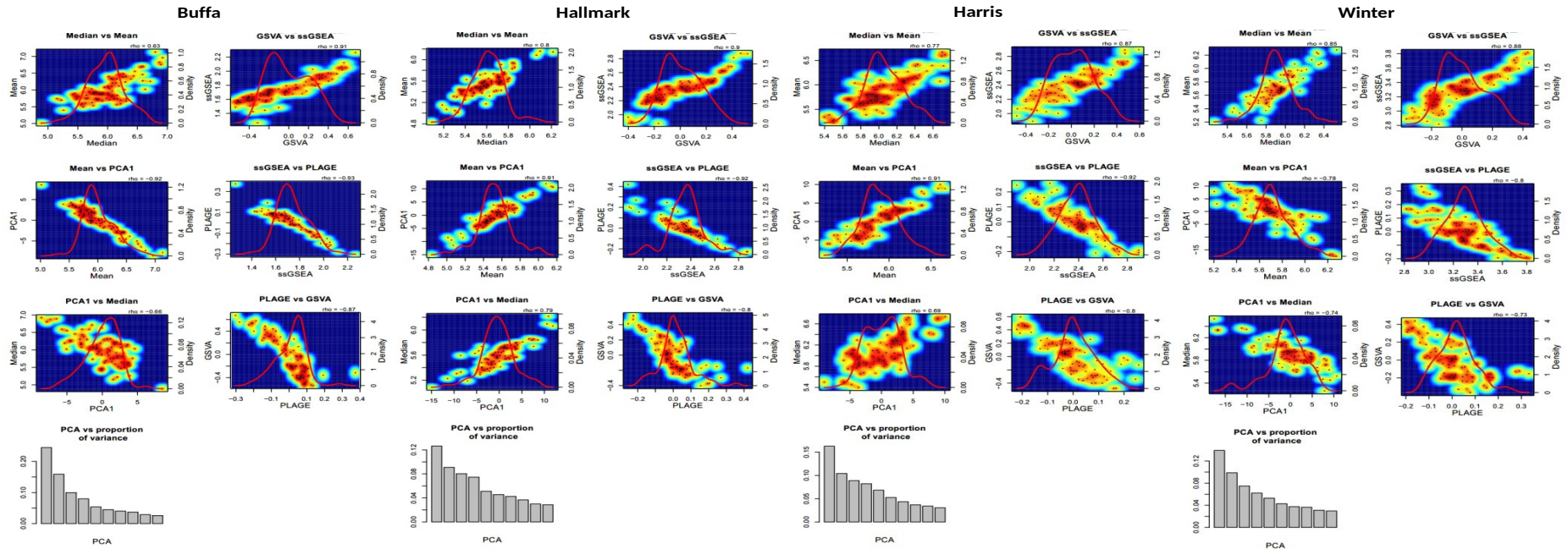


Figure S6. Hypoxia signature expression metrics. Evaluation of the main parametric and non-parametric methodologies to compute signature scores for Buffa, Hallmark, Harris and Winter hypoxia signatures. High correlation was observed amongst all scores. GSVA was used throughout the study but results were also provided for median and ssGSEA scores. For more information and discussion on methodology used, see our protocol for systematic evaluation of gene signatures (1). The R package sigQC v0.1.21 was used (<https://cran.r-project.org/>).

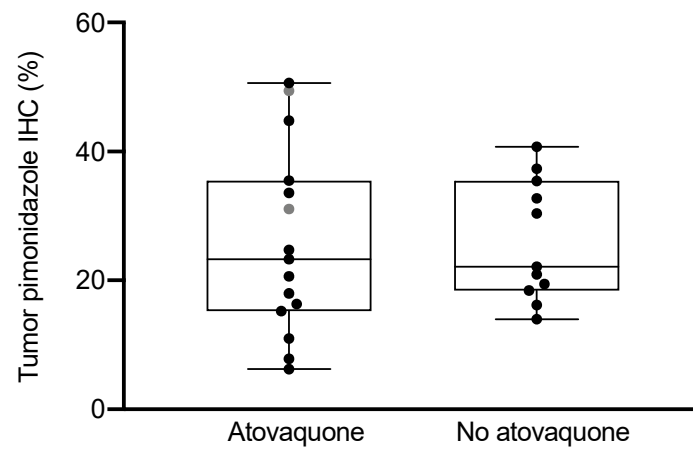


Figure S7. Inter-cohort comparison of tumor pimonidazole immunohistochemistry. Box and whisker plots comparing tumor pimonidazole IHC percentage scores between all atovaquone-treated and untreated patients. Median levels with interquartile and absolute ranges are shown. Grey data points represent patients who were unevaluable for primary imaging analysis. Abbreviation: IHC, immunohistochemistry.

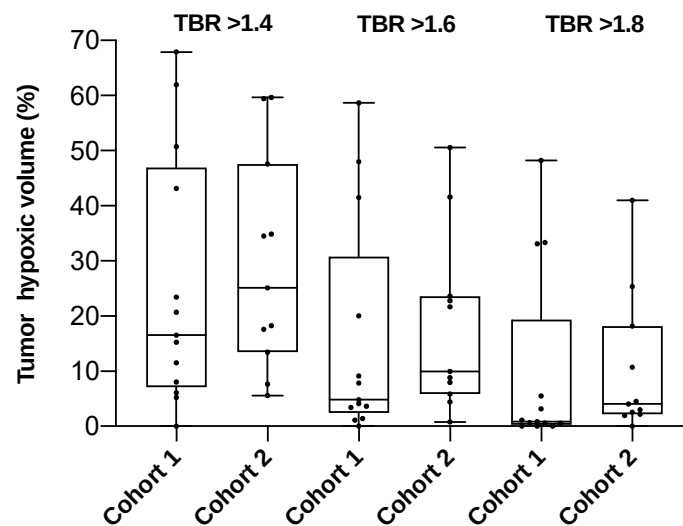


Figure S8. Inter-cohort comparison of tumor hypoxic volume prior to surgery. Box and whisker plots comparing hypoxia PET-CT-derived tumor hypoxic volume percentage at Visit 2 for atovaquone-treated (Cohort 1) and untreated (Cohort 2) patients using different PET tracer uptake thresholds to define hypoxic volume. Median, interquartile range and absolute range are shown. Only data for patients evaluable for both hypoxia PET-CT and pimonidazole endpoint analysis is shown. Abbreviation: TBR, tumor-to-blood uptake ratio.

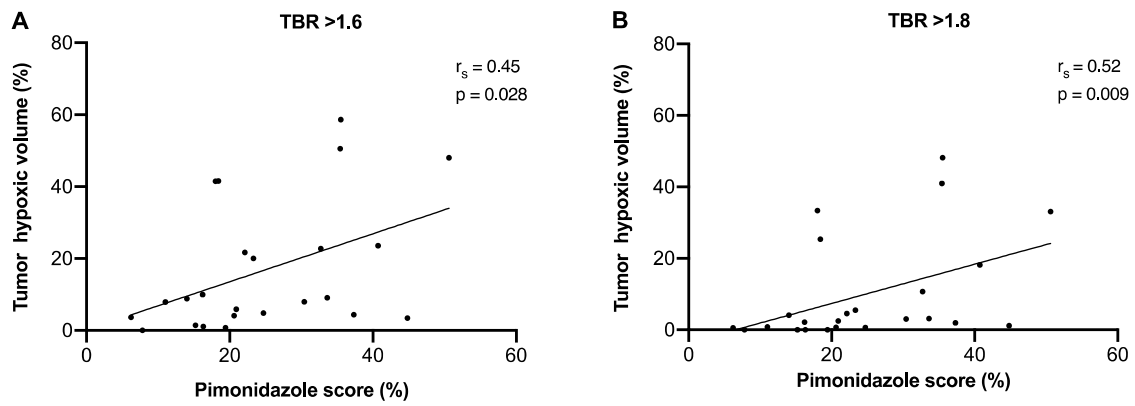


Figure S9. Correlation of Tumor Pimonidazole Score and Hypoxic Volume. Positive correlation between tumor pimonidazole immunohistochemistry percentage score and hypoxia PET-CT-derived tumor hypoxic volume percentage using the higher PET tracer uptake thresholds A) TBR >1.6 and B) TBR >1.8. Spearman's correlation coefficients are shown. Data is for patients evaluable for both hypoxia PET-CT and pimonidazole endpoints. Abbreviation: TBR, tumor-to-blood uptake ratio.

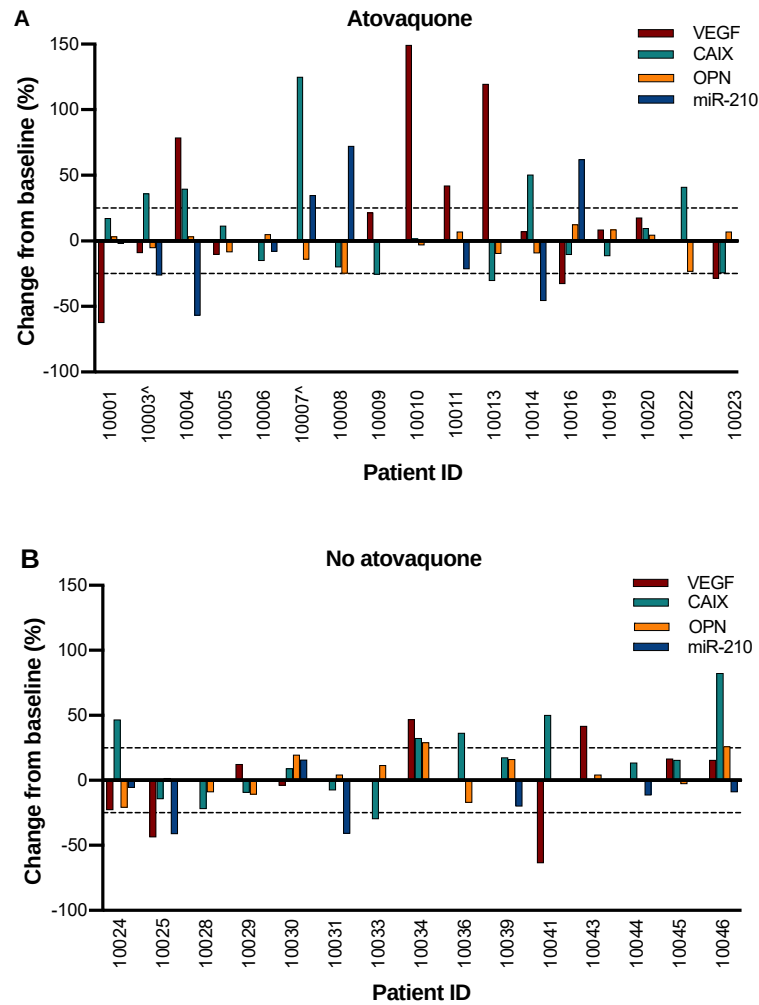


Figure S10. Change in putative plasma tumor hypoxia markers. Percentage change from baseline in plasma levels of VEGF, CAIX, OPN and miR-210 per individual A) atovaquone-treated and B) untreated patients. Dashed lines indicate the margin of error when measuring percentage change in levels of VEGF, CAIX and OPN using ELISA. Only results from non-hemolyzed miR-210 samples are shown. Highlighted patients were not evaluable for primary imaging analysis. Abbreviations: VEGF, vascular endothelial growth factor; CAIX, carbonic anhydrase IX; OPN, osteopontin.

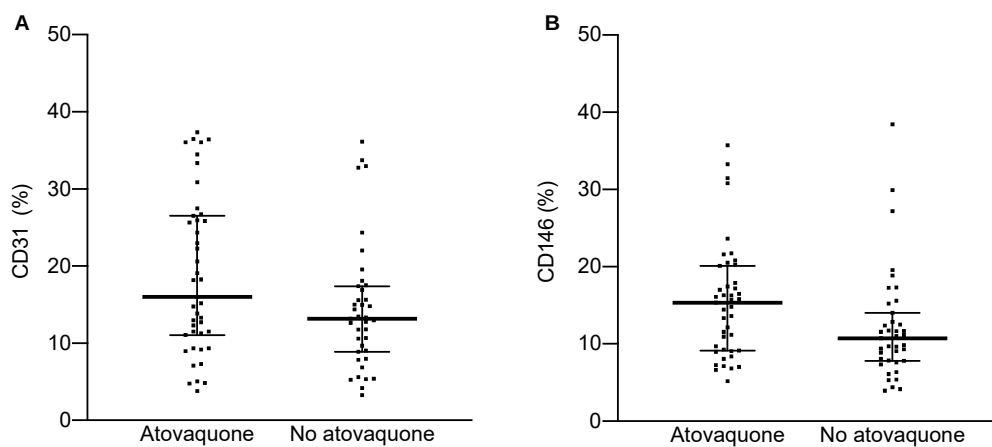


Figure S11. Inter-cohort comparison of A) CD31 and B) CD146 immunohistochemistry. Scores are percentage of tumor tissue positive for each marker with data for patients evaluable for primary imaging analysis shown. Median levels with interquartile ranges are presented.

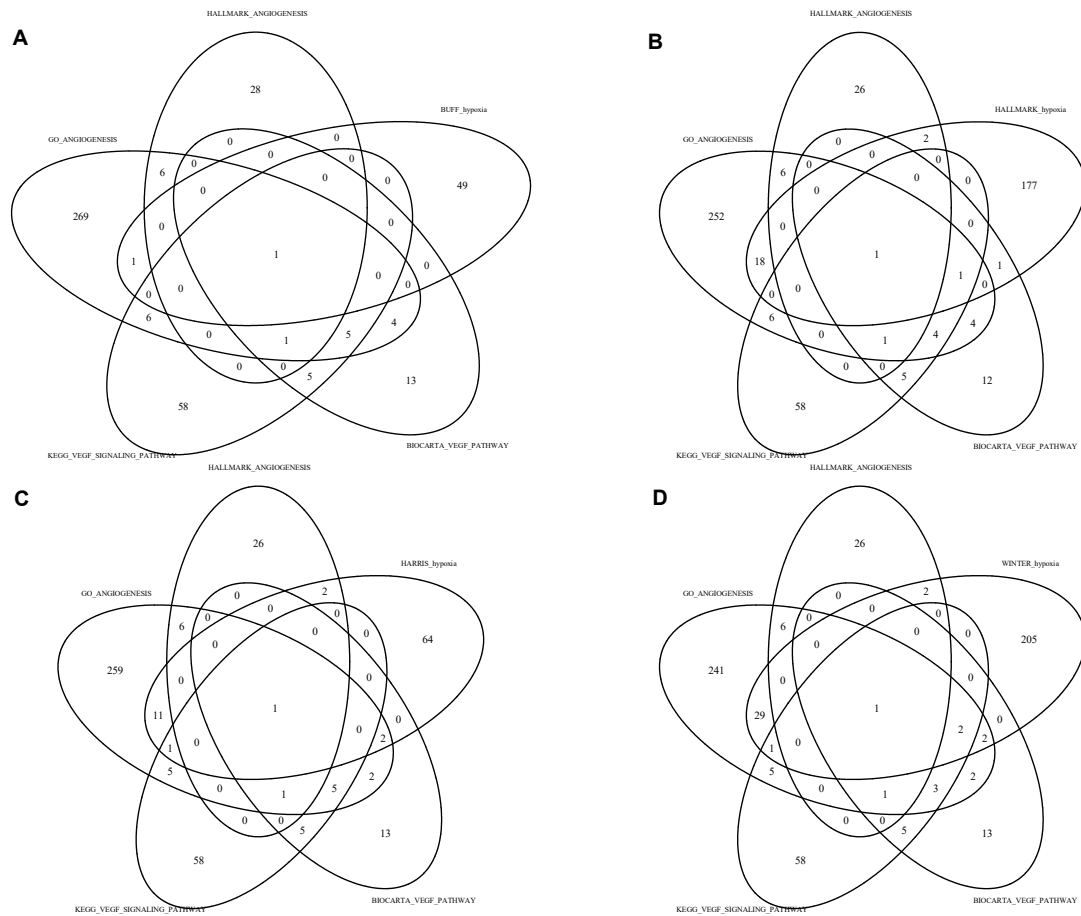


Figure S12. Venn diagrams illustrating overlap of A) Buffa, B) Hallmark, C) Harris and D) Winter hypoxia signature genes with angiogenesis and VEGF pathway genes.

SUPPLEMENTARY TABLES

Table S1. Complete hypoxia PET-CT data obtained for each patients.

Patient ID	Cohort	Tumor volume (mL)	PET Tracer	4-hour hypoxia PET						Dynamic PET					
				Baseline Visit			Pre-surgery Visit			Baseline Visit			Pre-surgery Visit		
				TBR ≥ 1.4 HV (mL)	TBR ≥ 1.6 HV (mL)	TBR ≥ 1.8 HV (mL)	TBR ≥ 1.4 HV (mL)	TBR ≥ 1.6 HV (mL)	TBR ≥ 1.8 HV (mL)	v_B (%)	K_1 (mL/min/g)	k_5 (1/min)	v_B (%)	K_1 (mL/min/g)	k_5 (1/min)
10001	1	89.5	FMISO	31.8	24.0	16.5	22.9	7.7	0.8	5.3	0.63	0.0050	4.9	0.73	0.0036
10004	1	184.9	FAZA	97.7	63.1	30.9	83.6	38.8	10.7	0.2	0.54	0.0035	0.1	0.62	0.0038
10005	1	61.0	FMISO	8.2	3.5	1.7	2.8	0.6	0.0	5.3	0.42	0.0028	-	-	-
10006	1	179.5	FMISO	50.8	22.2	7.5	28.5	7.7	1.3	5.7	0.37	0.0038	4.9	0.40	0.0029
10007	1	19.4	FMISO	0.6	0.1	0.0	0.7	0.1	0.0	4.7	0.40	0.0018	5.0	0.50	0.0018
10008	1	17.8	FMISO	1.7	0.7	0.2	1.4	0.6	0.2	2.1	0.36	0.0022	4.4	0.34	0.0058
10009	1	59.3	FMISO	33.6	26.8	19.5	37.4	29.0	20.0	2.6	0.26	0.0049	2.7	0.16	0.0214
10010	1	371.1	FMISO	188.3	135.3	90.2	127.2	61.4	20.6	0.2	0.27	0.0088	2.2	0.19	0.0069
10011	1	24.6	FMISO	1.8	0.3	0.1	1.7	0.4	0.0	-	-	-	2.2	0.19	0.0069
10012	1	15.2	FMISO	0.0	0.0	0.0	-	-	-	11	0.68	0.0016	-	-	-
10013	1	15.5	FMISO	5.4	2.0	0.6	0.8	0.1	0.0	11	0.53	0.0027	9.1	0.53	0.0020
10014	1	42.2	FMISO	2.7	0.1	0.0	0.0	0.0	0.0	16	0.73	0.0026	11	0.47	0.0010
10015	1	37.1	FMISO	0.0	0.0	0.0	-	-	-	6.2	0.28	0.0001	-	-	-
10016	1	30.6	FMISO	6.7	2.8	1.1	5.9	2.6	0.9	-	-	-	4.4	0.26	0.2344
10017	1	24.4	FMISO	0.0	0.0	0.0	-	-	-	13	0.41	0.0005	-	-	-
10018	1	2.4	FMISO	0.0	0.0	0.0	-	-	-	19	0.24	0.0287	-	-	-
10019	1	366.9	FMISO	201.7	172.9	146.2	202.6	165.7	133.1	2.0	0.36	0.0082	5.1	0.43	0.0086
10020	1	85.3	FMISO	35.3	22.1	10.8	13.7	4.0	0.5	3.7	0.55	0.0035	5.1	0.48	0.0031
10022	1	45.9	FMISO	28.9	23.7	17.2	32.4	28.0	23.0	0.1	0.42	0.0119	1.3	0.36	0.0105
10023	1	33.4	FMISO	6.9	3.8	1.4	4.1	1.3	0.2	6.8	0.43	0.0043	8.7	0.37	0.0037
10024	2	45.8	FMISO	26.2	18.7	11.7	26.0	18.2	11.1	0.5	0.27	0.0062	0.5	0.16	0.0017

10025	2	40.8	FMISO	4.9	0.9	0.1	6.2	0.8	0.0	6.5	0.15	0.0041	5.7	0.42	0.0042
10026	2	43.4	FMISO	0.0	0.0	0.0	-	-	-	2.0	0.70	0.0032	-	-	-
10027	2	27.9	FMISO	0.0	0.0	0.0	-	-	-	0.0	0.24	0.0049	-	-	-
10028	2	20.6	FMISO	11.8	9.8	8.0	13.1	11.1	9.0	4.7	0.84	0.0065	4.9	0.58	0.0072
10029	2	24.1	FMISO	1.6	0.3	0.0	3.2	1.4	0.6	1.4	0.52	0.0016	2.5	0.37	0.0035
10030	2	243.0	FMISO	44.2	27.9	18.4	51.0	24.7	11.4	0.1	0.25	0.0038	0.1	0.24	0.0033
10031	2	5.4	FMISO	1.9	1.5	1.1	1.9	1.3	1.0	0.1	0.28	0.0044	0.1	0.37	0.0066
10032	2	11.1	FMISO	0.6	0.0	0.0	-	-	-	-	-	-	-	-	-
10033	2	6.3	FMISO	3.5	2.6	1.9	2.6	1.7	0.8	3.7	0.29	0.0096	3.1	0.32	0.0046
10034	2	43.7	FMISO	21.7	16.2	11.7	19.6	12.9	8.6	5.3	0.18	0.0109	4.6	0.17	0.0077
10035	2	2.5	FMISO	0.8	0.6	0.4	-	-	-	0.1	0.12	0.0172	-	-	-
10036	2	35.9	FMISO	1.7	0.2	0.0	2.1	0.3	0.0	2.1	0.38	0.0042	2.8	0.26	0.0051
10037	2	19.1	FMISO	0.1	0.0	0.0	-	-	-	6.3	0.63	0.0022	-	-	-
10038	2	32.7	FMISO	0.2	0.0	0.0	-	-	-	4.3	0.58	0.0026	-	-	-
10039	2	144.8	FMISO	34.0	18.3	8.8	49.8	21.8	8.2	2.3	0.27	0.0057	3.7	0.22	0.0046
10040	2	2.9	FMISO	0.1	0.0	0.0	-	-	-	8.2	0.31	0.0027	-	-	-
10041	2	20.0	FMISO	4.2	1.5	0.3	5.8	2.3	0.5	6.1	0.47	0.0023	8.2	0.42	0.0028
10042	2	7.6	FMISO	0.0	0.0	0.0	-	-	-	2.5	0.18	0.0049	-	-	-
10043	2	80.7	FMISO	40.5	21.5	4.8	37.7	17.2	3.6	0.1	0.90	0.0047	0.1	0.80	0.0058
10044	2	45.3	FMISO	2.6	0.7	0.2	3.5	2.0	0.9	0.1	0.43	0.0028	0.1	0.44	0.0033
10045	2	185.4	FMISO	44.8	23.9	11.9	32.7	14.8	5.6	-	-	-	-	-	-
10046	2	57.5	FMISO	11.4	3.6	0.7	13.9	5.9	2.6	-	-	-	6.5	0.35	0.0058

Table S2. Pimonidazole immunohistochemistry.

Cohort 1 (n=15)		Cohort 2 (n=11)	
Patient ID	Pimonidazole score (%)	Patient ID	Pimonidazole score (%)
10001	11.0	10024	18.4
10003 ^a	49.4	10028	35.4
10004	23.3	10029	20.9
10005	16.3	10030	14.0
10006	20.6	10031	40.7
10007 ^a	31.1	10033	32.7
10008	44.8	10036	19.4
10009	50.6	10041	16.2
10011	15.2	10043	22.1
10014	7.8	10044	37.3
10016	33.6	10045	30.4
10019	18.0		
10020	24.7		
10022	35.5		
10023	6.2		
Median (IQR)	23.3 [15.2, 35.5]		22.1 [18.4, 35.4]
Mean difference between groups adjusted for tumor volume (95% CI)	1.2 (-8.7, 11.1)		
Unadjusted mean difference between groups (95% CI)	0.3 (-10.1, 10.6)		

^aPatients unevaluable for primary imaging analysis.

Abbreviations: IQR, interquartile range; CI, confidence interval.

Table S3. Summary of plasma osteopontin levels.

	Cohort 1 (n=17)	Cohort 2 (n=15)
Median baseline (IQR)	66.2 [57.2, 77.4]	89.3 [63.2, 107.0]
Median pre-surgery (IQR)	68.0 [52.3, 73.1]	101.3 [63.0, 109.2]
Change from baseline (95% CI)	0.8 (-5.7, 3.3)	2.4 (-4.3, 13.3)
Percentage change from baseline (95% CI)	1.1% (-9.3, 5.1)	2.3% (-7.8, 15.6)
Mean difference between groups adjusted for baseline and tumor volume (95% CI)	10.6 (1.8, 19.3)	
Mean difference between groups adjusted for baseline osteopontin (95% CI)	8.3 (-1.2, 17.7)	

Abbreviations: IQR, interquartile range; CI, confidence interval. Unit of measurement is ng/mL.

Table S4. Summary of plasma CAIX levels.

	Cohort 1 (n=17)	Cohort 2 (n=15)
Median baseline (IQR)	47.3 [21.2, 75.6]	74.9 [40.9, 111.0]
Median pre-surgery (IQR)	60.8 [34.8, 93.0]	60.0 [46.8, 135.7]
Median change from baseline ^a (95% CI)	1.6 (-11.1, 14.3)	12.6 (-6.7, 19.5)
Median percentage change from baseline ^a (95% CI)	5.8% (-17.6, 37.8)	13.7% (-9.1, 35.8)
Mean difference between groups adjusted for baseline CAIX and tumor volume ^a (95% CI)	13.6 (95% CI: -5.1, 32.2)	
Mean difference between groups adjusted for baseline CAIX ^a (95% CI)	10.8 (95% CI: -6.8, 28.4)	

^aAs one patient in Cohort 1 had a baseline level below the lower limit of quantification, change and percentage change from baseline could not be determined and this patient was also not included in the regression analysis. Abbreviations: IQR, interquartile range; CI, confidence interval; CAIX, carbonic anhydrase IX. Unit of measurement is pg/mL.

Table S5. Summary of plasma VEGF levels.

	Cohort 1 (n=17)	Cohort 2 (n=15)
Median baseline (IQR)	63.4 [35.5, 75.9]	52.3 [<LLOQ, 80.6]
Median pre-surgery (IQR)	54.7 [41.3, 88.6]	52.3 [36.1, 81.9]

Four patients in Cohort 1 and five patients in Cohort 2 had levels either at baseline or at both visits below the lower limit of quantification. As a result, changes per cohort for this analyte. Abbreviations: IQR, interquartile range; VEGF, vascular endothelial growth factor; LLOQ, lower limit of quantification. Unit of measurement is pg/mL.

Table S6. Summary of plasma miR-210 levels.

	Cohort 1 (n=10)	Cohort 2 (n=7)
Median baseline $\times 10^{-3}$ (IQR)	1.2 (0.8, 1.5)	1.1 (1.0, 1.7)
Median pre-surgery $\times 10^{-3}$ (IQR)	1.1 (0.8, 1.3)	1.0 (0.9, 1.3)
Median change $\times 10^{-3}$ (95% CI)	-0.0 (-0.8, 0.7)	-0.1 (-0.6, 0.1)
Median percentage change (95% CI)	-5.2% (-39.4, 53.3)	-11.6% (-41.3, 9.2)

Only data for patients with non-hemolyzed samples is shown. Seven patients in Cohort 1 and eight patients in Cohort 2 had evidence of hemolysis of either one or both plasma samples. Values represent miR-210 levels relative to endogenous miR-16 and spike-in miRNA (miR-Cel-39). Abbreviations: IQR, interquartile range; CI, confidence interval.

Table S7. Complete perfusion CT data

Patient ID	Cohort	Unmatched data ^a						Matched data ^b					
		Baseline scan			Pre-surgery scan			Baseline scan			Pre-surgery scan		
		BF	BV	MTT	BF	BV	MTT	BF	BV	MTT	BF	BV	MTT
10001	1	107.9	9.0	6.2	120.2	9.3	7.3	107.9	9.0	6.2	120.2	9.3	7.3
10003 ^c	1	162.0	6.1	2.6	93.8	4.0	2.5	162.0	6.1	2.6	93.8	4.0	2.5
10004	1	63.2	6.7	8.7	61.5	7.5	10.1	63.2	6.7	8.7	61.5	7.5	10.1
10005	1	38.3	2.6	6.0	71.6	4.5	5.8	38.3	2.6	6.0	71.5	4.3	5.7
10006	1	77.6	5.0	6.8	79.8	4.8	6.3	77.6	5.0	6.8	79.8	4.8	6.3
10007 ^c	1	87.8	6.7	6.8	80.4	6.3	8.4	87.8	6.7	6.8	80.4	6.3	8.4
10008	1	115.9	5.6	5.8	133.4	5.6	4.7	115.9	5.6	5.8	133.4	5.6	4.7
10009	1	105.5	5.1	4.8	132.2	5.8	4.3	105.5	5.1	4.8	132.2	5.8	4.3
10010	1	24.5	2.7	9.6	27.9	2.6	8.7	23.3	2.7	9.8	26.5	2.5	8.7
10011	1	111.3	8.4	7.2	104.4	8.1	8.6	111.3	8.4	7.2	104.4	8.1	8.6
10012	1	135.1	8.2	5.5	-	-	-	-	-	-	-	-	-
10014	1	186.8	7.7	3.6	165.2	7.9	5.2	198.1	7.8	3.2	165.2	7.9	5.2
10015	1	109.7	4.8	2.7	-	-	-	-	-	-	-	-	-
10016	1	73.9	4.1	5.6	63.9	3.3	5.3	70.5	3.7	5.4	63.9	3.3	5.3
10017	1	110.3	4.1	3.7	-	-	-	-	-	-	-	-	-
10018	1	52.1	4.8	7.7	-	-	-	-	-	-	-	-	-
10019	1	48.9	7.4	4.9	59.9	7.3	4.9	49.5	7.4	4.9	59.5	7.3	4.9
10020	1	80.9	6.8	7.0	72.0	5.1	6.7	80.9	6.8	7.0	72.0	5.1	6.7
10022	1	64.3	5.6	7.6	45.4	5.6	10.9	64.9	5.7	7.7	45.2	5.4	10.7
10023	1	129.7	8.1	7.6	104.9	6.6	6.6	112.0	7.7	7.9	104.9	6.6	6.6
10024	2	33.3	2.5	11.0	30.1	2.6	10.2	33.5	2.5	11.0	30.1	2.6	10.2
10025	2	59.9	4.0	5.9	41.3	3.0	4.3	48.2	3.5	6.0	44.5	3.3	4.7
10026	2	187.3	8.7	4.4	-	-	-	-	-	-	-	-	-
10027	2	40.4	2.6	4.2	-	-	-	-	-	-	-	-	-
10028	2	62.8	6.2	8.4	70.8	6.4	7.4	62.8	6.2	8.4	70.8	6.4	7.4
10029	2	107.6	6.4	5.9	-	-	-	-	-	-	-	-	-
10030	2	41.1	3.2	6.9	42.2	3.8	6.7	42.1	3.1	6.7	44.8	3.9	6.5
10031	2	-	-	-	121.2	4.9	3.3	-	-	-	-	-	-
10032	2	114.9	4.4	3.2	-	-	-	-	-	-	-	-	-
10033	2	91.7	4.9	7.1	-	-	-	-	-	-	-	-	-
10034	2	43.2	2.3	3.2	-	-	-	-	-	-	-	-	-
10035	2	41.1	2.6	6.4	-	-	-	-	-	-	-	-	-
10036	2	-	-	-	38.4	4.0	8.6	-	-	-	-	-	-
10037	2	80.8	8.3	8.7	-	-	-	-	-	-	-	-	-
10038	2	106.0	5.7	4.6	-	-	-	-	-	-	-	-	-
10039	2	-	-	-	70.7	3.4	4.9	-	-	-	-	-	-
10040	2	161.0	6.4	3.1	-	-	-	-	-	-	-	-	-
10041	2	75.6	4.0	5.1	-	-	-	-	-	-	-	-	-
10042	2	80.0	3.2	4.2	-	-	-	-	-	-	-	-	-
10043	2	92.2	6.9	5.9	65.1	6.6	8.6	98.6	6.8	5.3	65.3	6.5	8.5
10044	2	71.2	7.2	9.0	74.2	7.2	9.1	72.1	7.3	8.9	71.2	7.2	9.3

10045	2	92.0	4.5	6.0	-	-	-	-	-	-	-	-	-
10046	2	-	-	-	68.9	3.1	1.5	-	-	-	-	-	-

^aUnmatched data is all perfusion CT data collected per tumor.

^bMatched data is perfusion CT data only from the same tumor region imaged at both visits.

^cPatients unevaluable for primary imaging analysis.

Abbreviations and units: BF, blood flow (mL/100 g/min); BV, blood volume (mL/100 g); MTT, mean transit time (s).

REFERENCE FOR SUPPLEMENTARY DATA

1. Dhawan A, Barberis A, Cheng W-C, Domingo E, West C, Maughan T, et al. Guidelines for using sigQC for systematic evaluation of gene signatures. Nature Protocols. 2019;14:1377–400.