

## Supporting Information

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### **Cold atmospheric plasma treatment of melanoma and glioblastoma cancer cells**

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#### **Supplementary Materials & Methods**

##### a. pH measurement

The pH was measured with a pH 1100H meter (VWR, Leuven, Belgium) before and directly, 24 h and 72 h after treatment.

##### b. Peroxynitrite and hydroxyl radical detection

Malme-3M (18750 cells) and LN229 (15000 cells) were cultured in 96-well plates for 72 hours. Next, cells were loaded before treatment with hydroxyphenyl fluorescein (HPF from Reactive Oxygen Species Detection kit, Cell Technology Inc) to a final concentration of 5  $\mu$ M according to the manufacturer's guidelines. 125  $\mu$ l plasma-activated medium (7 min treated for Malme-3M, 11 min treated for LN229) was added to the cells for a total volume of 200  $\mu$ l. Following treatment, cells were incubated for 24 h and 72 h before flow cytometric measurement on a BD FACScan. Analysis was performed using FlowJo. Statistical analysis using SPSS was performed by parametric testing due to small sample sizes. Treatment conditions (untreated and gas mixtures) were analyzed using one-way ANOVA followed by a post-hoc Tukey. Comparison of treatment times per condition were analyzed using the independent samples T-test with Levene's test for equal variances.

Supplementary Figures & Tables

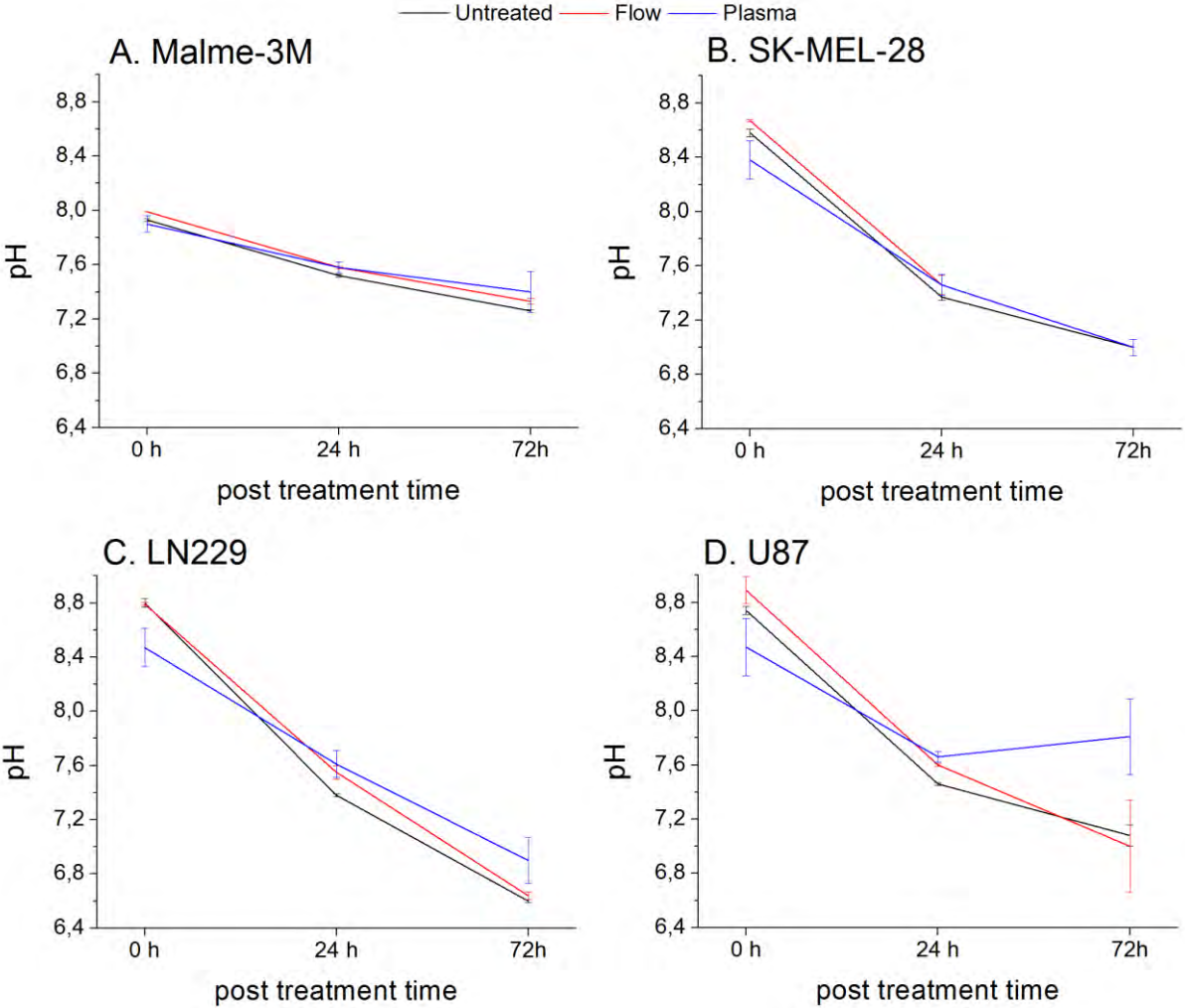


Figure S1. The pH was monitored before, during and after treatment for the untreated, flow-treated and plasma-treated cells. The effect on pH is similar for the three different cases.

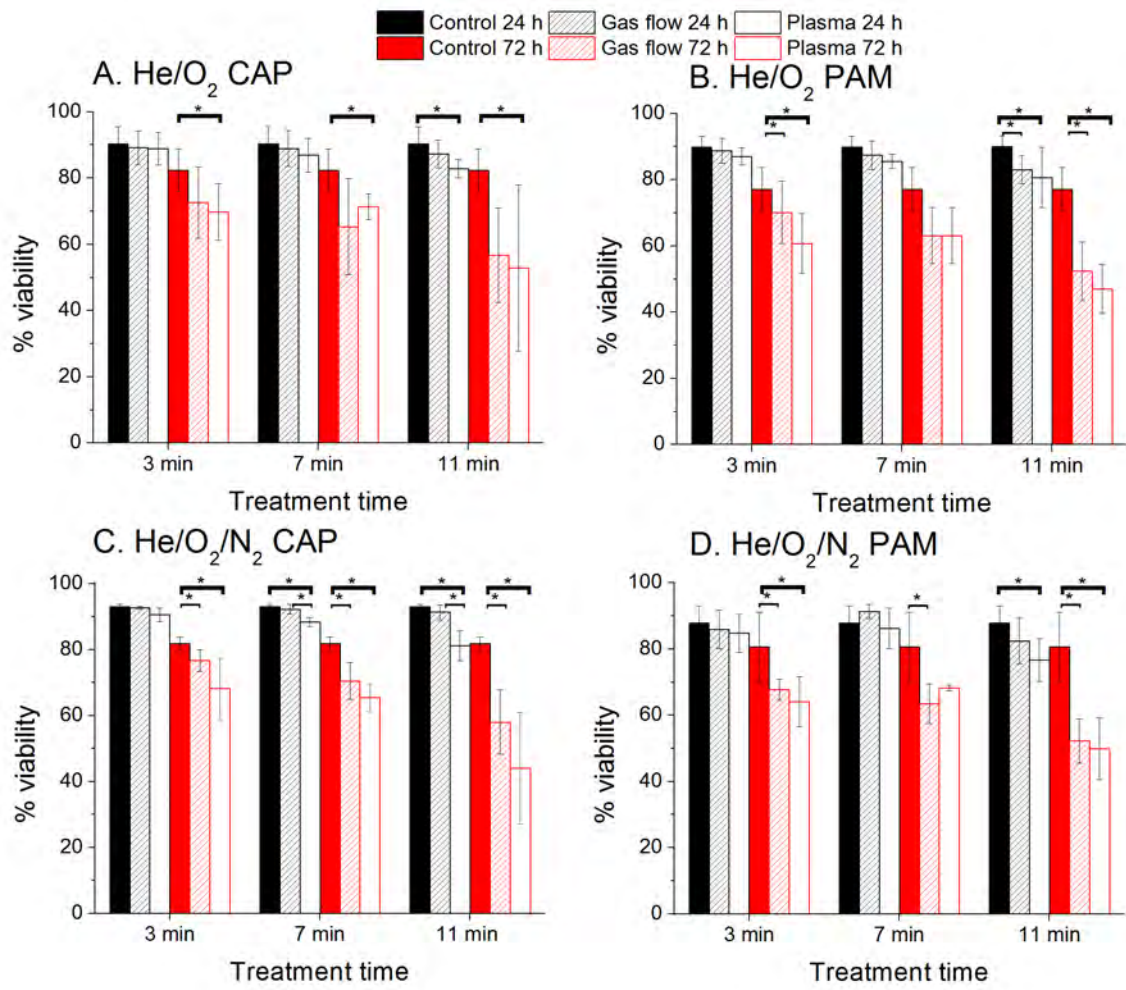


Figure S2. Comparison of the effect of gas flow and plasma on the cell viability for SK-MEL-28. \*  $p < 0.05$ ; CAP: Cold Atmospheric Plasma; PAM: Plasma Activated Medium.

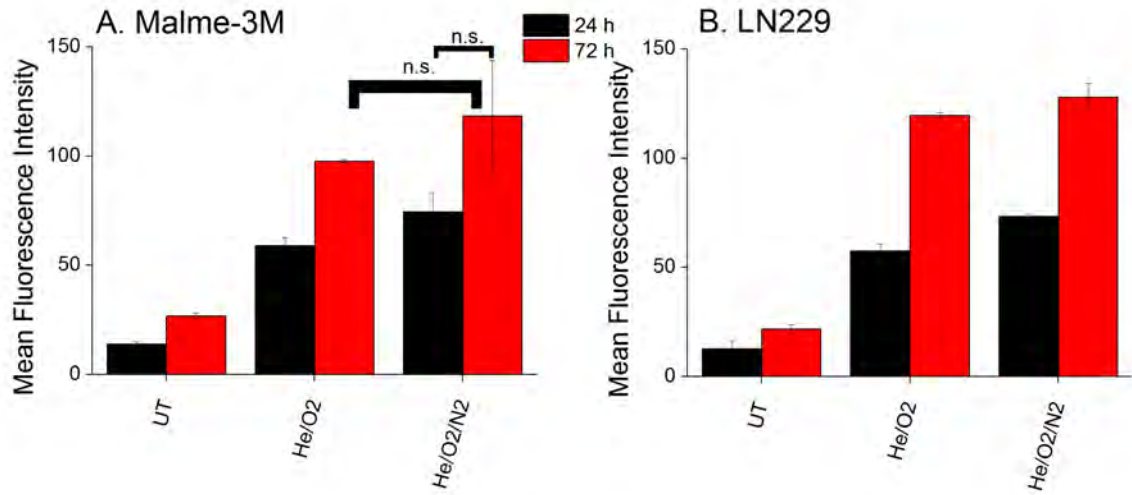


Figure S3. The presence of reactive oxygen and nitrogen species (hydroxyl radicals and peroxyntirite) using Hydroxyphenyl fluorescein was analyzed after 24 and 72 hours of treatment. Malme-3M and LN229 cells were incubated with 7 min and 11 min treated PAM respectively. All comparisons are significant, except when denoted with n.s. (not significant).

Table S1. Overview of the flow cytometry data, analysed with FlowJo. Using an AnnV-FITC and PI staining, a distinction can be made between AnnV-FITC and PI negative cells (AnnV- PI-, 'viable cells'); AnnV-FITC positive and PI negative cells (AnnV+ PI-, 'early apoptosis'); AnnV-FITC and PI positive cells (AnnV+PI+, 'late apoptosis and necrosis'); AnnV-FITC negative and PI positive cells (AnnV- PI+, 'necrosis'). Statistics were performed on the cell viability between the untreated and the treated groups, like mentioned in the experimental section. \*p < 0.05. A. Direct treatment with He+0.6%O<sub>2</sub> B. Indirect treatment with He+0.6%O<sub>2</sub> C. Direct treatment with He+0.35%O<sub>2</sub>+0.06%N<sub>2</sub> D. Indirect treatment with He+0.35%O<sub>2</sub>+0.06%N<sub>2</sub>

A. He + 0.6% O <sub>2</sub> Direct treatment			24h				72h			
			Ann V – PI -	Ann V + PI -	Ann V + PI +	Ann V – PI +	Ann V – PI -	Ann V + PI -	Ann V + PI +	Ann V – PI +
<b>Malme-3M</b>	<i>Untreated</i>		96.8 ± 1.1	0.5 ± 0.3	1.3 ± 0.4	1.4 ± 0.7	93.7 ± 5.0	0.3 ± 0.2	2.4 ± 1.6	3.8 ± 3.5
	<i>Plasma</i>	3min	94.5 ± 2.6	0.5 ± 0.7	2.3 ± 1.4	2.9 ± 1.9	91.0 ± 8.2	0.4 ± 0.3	3.7 ± 2.8	4.9 ± 5.5
		7min	83.9 ± 7.5 *	1.0 ± 1.0	9.7 ± 5.0	5.4 ± 3.3	62.4 ± 13.6 *	1.9 ± 2.1	18.5 ± 9.2	17.3 ± 15.9
		11min	32.2 ± 15.6 *	1.7 ± 3.0	33.2 ± 10.2	32.9 ± 18.9	3.0 ± 3.8 *	0.6 ± 0.4	75.5 ± 22.2	20.9 ± 18.9
<b>SK-MEL-28</b>	<i>Untreated</i>		90.3 ± 5.1	1.2 ± 0.7	4.0 ± 2.7	4.6 ± 1.9	82.3 ± 6.5	0.4 ± 0.2	3.1 ± 1.0	14.6 ± 8.0
	<i>Gas flow</i>	3min	89.1 ± 5.1	1.6 ± 1.1	4.5 ± 2.5	4.9 ± 2.6	72.6 ± 10.8	0.4 ± 0.4	3.6 ± 1.1	22.4 ± 11.5
		7min	88.9 ± 5.4	1.3 ± 0.8	4.2 ± 1.9	5.6 ± 3.4	65.3 ± 14.5	0.3 ± 0.3	2.6 ± 1.3	31.9 ± 15.0
		11min	87.3 ± 4.2	1.5 ± 0.7	4.8 ± 1.5	6.4 ± 2.9	56.7 ± 14.3	0.5 ± 0.2	4.8 ± 1.8	38.0 ± 14.8
	<i>Plasma</i>	3min	88.8 ± 4.9	1.3 ± 0.8	4.7 ± 2.5	5.2 ± 2.3	69.7 ± 8.5 *	0.4 ± 0.3	4.1 ± 1.3	25.8 ± 9.1
		7min	86.9 ± 5.1	2.0 ± 1.0	5.8 ± 2.4	5.3 ± 2.5	71.3 ± 3.9 *	1.3 ± 1.4	6.8 ± 3.4	21.3 ± 8.5
		11min	82.8 ± 2.8 *	2.2 ± 1.5	6.5 ± 2.8	8.5 ± 3.1	52.9 ± 25.1 *	2.6 ± 3.1	18.2 ± 12.4	26.2 ± 17.4
<b>LN229</b>	<i>Untreated</i>		85.9 ± 3.2	4.7 ± 1.3	3.1 ± 1.1	6.3 ± 2.4	88.1 ± 1.2	4.0 ± 0.4	3.3 ± 0.5	4.6 ± 1.4
	<i>Plasma</i>	3min	86.4 ± 3.2	4.5 ± 0.9	3.3 ± 0.7	5.8 ± 3.4	81.9 ± 1.5	7.8 ± 1.2	5.2 ± 0.9	5.1 ± 1.1
		7min	91.3 ± 2.3 *	3.2 ± 1.1	2.8 ± 0.8	2.7 ± 1.9	63.7 ± 21.8 *	7.6 ± 2.3	25.5 ± 24.2	3.2 ± 1.5
		11min	55.2 ± 11.7 *	6.7 ± 2.2	26.1 ± 12.6	12.1 ± 4.7	14.4 ± 11.1 *	3.9 ± 2.5	79.9 ± 14.6	1.8 ± 1.5
<b>U87</b>	<i>Untreated</i>		94.3 ± 1.7	2.0 ± 0.9	1.7 ± 0.8	2.0 ± 0.7	95.8 ± 1.1	1.2 ± 1.0	1.4 ± 0.4	1.6 ± 0.8
	<i>Plasma</i>	1min	93.5 ± 1.2	2.5 ± 0.7	2.1 ± 0.5	1.9 ± 0.6	96.4 ± 1.2	5.9 ± 1.4	1.3 ± 0.5	1.0 ± 0.3
		3min	72.4 ± 13.3 *	7.1 ± 7.5	16.6 ± 14.3	3.9 ± 2.2	76.2 ± 6.4 *	5.9 ± 1.4	13.5 ± 4.9	4.5 ± 1.9
		5min	44.5 ± 25.2 *	2.7 ± 1.0	48.3 ± 25.5	4.6 ± 1.4	30.6 ± 26.1 *	5.1 ± 2.5	56.8 ± 26.8	7.5 ± 6.4
		7min	6.6 ± 6.2 *	2.3 ± 1.5	86.1 ± 5.8	5.0 ± 1.9	4.3 ± 3.7 *	1.7 ± 0.9	88.6 ± 5.5	5.3 ± 2.9
		11min	1.0 ± 0.9 *	2.7 ± 1.0	93.0 ± 1.0	3.3 ± 1.4	0.7 ± 0.7 *	1.1 ± 0.5	93.9 ± 2.5	4.3 ± 2.1

<b>B. He + 0.6% O<sub>2</sub></b> <i>Indirect treatment</i>			<b>24h</b>				<b>72h</b>			
			<b>Ann V – PI -</b>	<b>Ann V + PI -</b>	<b>Ann V + PI +</b>	<b>Ann V – PI +</b>	<b>Ann V – PI -</b>	<b>Ann V + PI -</b>	<b>Ann V + PI +</b>	<b>Ann V – PI +</b>
<b>Malme-3M</b>	<i>Untreated</i>		91.2 ± 3.3	0.6 ± 0.3	4.3 ± 1.1	3.9 ± 2.6	91.4 ± 3.6	1.1 ± 0.5	4.7 ± 2.5	2.7 ± 1.2
	<i>Plasma</i>	3min	89.7 ± 1.9	0.9 ± 0.4	6.1 ± 1.5	3.4 ± 1.7	90.8 ± 4.1	1.7 ± 0.8	5.3 ± 2.5	2.2 ± 0.9
		7min	87.8 ± 3.2	1.2 ± 0.6	6.7 ± 2.1	4.4 ± 1.3	84.9 ± 4.4 *	2.3 ± 1.1	9.6 ± 3.3	3.1 ± 0.6
		11min	76.2 ± 7.1 *	1.7 ± 0.5	13.8 ± 3.9	8.3 ± 3.9	43.1 ± 13.7 *	2.8 ± 0.9	33.7 ± 6.3	20.5 ± 10.0
<b>SK-MEL-28</b>	<i>Untreated</i>		89.8 ± 3.3	0.7 ± 0.6	3.8 ± 1.7	5.7 ± 1.7	77.1 ± 6.6	0.6 ± 0.3	4.9 ± 4.9	17.3 ± 6.5
	<i>Gas flow</i>	3min	88.7 ± 3.8	0.7 ± 0.5	4.0 ± 1.0	6.5 ± 3.4	70.1 ± 9.4	0.7 ± 0.5	5.4 ± 2.8	23.9 ± 7.7
		7min	87.4 ± 4.3	0.7 ± 0.5	4.4 ± 2.0	7.5 ± 2.5	63.1 ± 8.4 *	1.0 ± 0.5	6.6 ± 1.3	29.4 ± 9.0
		11min	83.0 ± 4.2 *	1.1 ± 0.8	5.4 ± 1.1	10.6 ± 4.4	52.3 ± 8.9 *	1.8 ± 1.1	10.4 ± 5.5	35.5 ± 8.0
		<i>Plasma</i>	3min	87.0 ± 2.6	1.1 ± 1.0	5.2 ± 1.8	7.1 ± 2.6	60.7 ± 9.1 *	0.7 ± 0.5	6.2 ± 2.3
	7min		85.5 ± 2.2	1.2 ± 1.0	5.5 ± 1.1	7.8 ± 3.0	63.1 ± 8.4	1.1 ± 0.4	6.6 ± 1.3	29.4 ± 9.0
	11min		80.6 ± 9.1 *	1.7 ± 1.5	1.2 ± 1.7	4.6 ± 5.5	47.0 ± 7.4 *	2.0 ± 1.2	20.7 ± 5.5	30.3 ± 10.6
<b>LN229</b>	<i>Untreated</i>		86.0 ± 3.5	5.1 ± 1.9	2.9 ± 1.2	6.0 ± 2.6	88.8 ± 1.2	3.7 ± 0.6	3.2 ± 0.5	4.3 ± 1.3
	<i>Plasma</i>	3min	89.2 ± 4.5	5.8 ± 3.0	2.4 ± 1.6	2.6 ± 0.5	82.6 ± 2.9 *	7.3 ± 2.0	5.3 ± 0.9	4.8 ± 1.1
		7min	89.0 ± 6.0	4.7 ± 3.1	3.7 ± 2.6	2.6 ± 1.6	79.0 ± 5.1 *	7.5 ± 1.3	10.1 ± 5.1	3.4 ± 0.9
		11min	46.5 ± 18.3 *	5.8 ± 2.4	32.6 ± 15.5	15.2 ± 6.8	19.4 ± 13.1 *	5.0 ± 1.9	73.3 ± 15.1	2.3 ± 0.5
<b>U87</b>	<i>Untreated</i>		94.5 ± 1.5	2.0 ± 0.7	1.7 ± 0.7	1.9 ± 0.7	95.7 ± 0.9	1.2 ± 0.6	1.5 ± 0.5	1.6 ± 0.7
	<i>Plasma</i>	1min	94.1 ± 1.7	2.5 ± 1.4	1.8 ± 0.6	1.7 ± 0.8	96.6 ± 0.8	1.3 ± 0.8	1.4 ± 0.3	0.8 ± 0.7
		3min	81.3 ± 9.2 *	4.9 ± 2.5	10.3 ± 8.2	3.5 ± 1.5	86.4 ± 5.1 *	4.2 ± 1.9	6.2 ± 2.7	3.2 ± 2.1
		5min	62.3 ± 18.2 *	4.2 ± 2.5	28.8 ± 17.9	4.7 ± 1.8	50.8 ± 24.5 *	5.5 ± 2.6	37.1 ± 23.6	6.6 ± 2.3
		7min	14.0 ± 7.6 *	2.4 ± 1.2	76.4 ± 7.8	7.2 ± 3.1	13.3 ± 5.5 *	3.4 ± 1.3	77.8 ± 6.8	5.6 ± 2.4
		11min	3.9 ± 4.1 *	1.8 ± 0.9	89.8 ± 3.9	4.5 ± 1.4	1.3 ± 1.4 *	1.1 ± 0.7	93.9 ± 3.1	3.8 ± 1.2

C. He + 0.35% O<sub>2</sub> + 0.06%N<sub>2</sub>  
Direct treatment

			24h				72h			
			Ann V – PI -	Ann V + PI -	Ann V + PI +	Ann V – PI +	Ann V – PI -	Ann V + PI -	Ann V + PI +	Ann V – PI +
<b>Malme-3M</b>	<i>Untreated</i>		94.3 ± 1.2	0.6 ± 0.3	2.9 ± 1.2	2.3 ± 0.7	86.3 ± 4.4	0.8 ± 0.8	5.4 ± 1.2	7.4 ± 4.4
	<i>Plasma</i>	3min	93.2 ± 1.4	0.4 ± 0.3	3.0 ± 1.2	3.3 ± 0.7	86.7 ± 4.2	1.0 ± 1.0	7.3 ± 2.6	5.0 ± 2.5
		7min	88.3 ± 3.5 *	1.0 ± 0.6	6.5 ± 2.4	4.2 ± 1.6	64.8 ± 29.5	2.3 ± 1.3	22.3 ± 21.5	10.6 ± 9.7
		11min	65.2 ± 19.5 *	1.4 ± 0.2	21.2 ± 14.9	12.2 ± 5.1	22.8 ± 18.3 *	2.1 ± 1.8	54.3 ± 22.7	20.8 ± 14.4
<b>SK-MEL-28</b>	<i>Untreated</i>		93.0 ± 0.8	0.4 ± 0.1	2.2 ± 0.4	4.4 ± 0.5	81.9 ± 2.0	0.6 ± 0.3	3.6 ± 0.8	13.9 ± 2.4
	<i>Gas flow</i>	3min	92.7 ± 0.4	0.7 ± 0.5	2.3 ± 1.1	4.2 ± 1.1	76.7 ± 3.3 *	0.9 ± 0.5	4.6 ± 1.2	17.9 ± 4.6
		7min	92.3 ± 1.4	1.2 ± 1.0	3.1 ± 0.9	3.8 ± 1.1	70.5 ± 5.6 *	0.8 ± 0.3	5.4 ± 0.9	23.3 ± 6.7
		11min	91.4 ± 2.2	1.0 ± 1.2	3.1 ± 2.1	4.4 ± 1.3	58.0 ± 9.8 *	1.1 ± 0.8	12.6 ± 4.8	36.2 ± 12.6
		<i>Plasma</i>	3min	90.6 ± 2.1	1.2 ± 1.5	4.1 ± 1.1	4.1 ± 1.0	68.2 ± 9.3 *	0.7 ± 0.5	4.5 ± 1.0
	7min		88.4 ± 1.4 *	2.0 ± 1.2	4.8 ± 1.2	4.6 ± 1.9	65.5 ± 4.3 *	3.0 ± 1.2	11.6 ± 3.4	19.9 ± 4.8
	11min		81.2 ± 4.6 *	1.9 ± 0.7	6.0 ± 2.8	10.9 ± 4.4	44.0 ± 16.9 *	1.9 ± 0.7	27.4 ± 16.7	24.5 ± 6.8
<b>LN229</b>	<i>Untreated</i>		90.9 ± 1.9	4.1 ± 1.8	1.9 ± 0.6	3.4 ± 1.0	91.3 ± 3.1	4.0 ± 1.8	2.3 ± 1.5	3.5 ± 1.2
	<i>Plasma</i>	3min	91.1 ± 0.5	4.1 ± 0.8	2.1 ± 0.3	2.8 ± 1.1	79.9 ± 5.9 *	8.0 ± 1.7	6.5 ± 6.1	5.6 ± 2.5
		7min	34.7 ± 17.2 *	10.7 ± 4.0	51.2 ± 20.1	3.3 ± 1.3	39.5 ± 13.0 *	5.8 ± 2.1	48.1 ± 12.6	6.6 ± 2.6
		11min	2.7 ± 4.1 *	2.3 ± 1.8	93.3 ± 7.2	1.7 ± 1.4	0.1 ± 0.1 *	0.3 ± 0.2	96.5 ± 4.1	3.2 ± 3.7
<b>U87</b>	<i>Untreated</i>		91.6 ± 3.3	3.8 ± 1.6	2.2 ± 0.8	2.5 ± 1.5	93.8 ± 2.8	1.9 ± 1.5	2.3 ± 1.1	2.0 ± 0.9
	<i>Plasma</i>	1min	92.0 ± 2.0	4.0 ± 0.5	2.1 ± 0.7	2.0 ± 1.1	95.2 ± 1.1	1.8 ± 0.8	1.8 ± 0.7	1.2 ± 0.3
		3min	66.6 ± 27.0 *	6.2 ± 2.8	23.1 ± 26.9	4.1 ± 3.0	52.4 ± 28.6 *	7.0 ± 3.0	36.9 ± 28.8	3.7 ± 2.2
		5min	48.8 ± 21.3 *	4.1 ± 2.8	42.3 ± 22.9	4.8 ± 2.0	25.7 ± 20.4 *	5.5 ± 2.9	63.4 ± 25.1	5.4 ± 2.1
		7min	5.3 ± 8.2 *	1.5 ± 0.2	88.4 ± 10.3	4.8 ± 2.5	1.0 ± 1.3 *	0.6 ± 0.6	97.2 ± 2.7	1.1 ± 0.9
		11min	0.1 ± 0.1 *	1.2 ± 0.8	96.2 ± 1.2	2.6 ± 0.9	0.1 ± 0.1 *	0.4 ± 0.3	98.6 ± 0.9	0.9 ± 0.6

**D. He + 0.35% O<sub>2</sub> + 0.06%N<sub>2</sub>**  
**Indirect treatment**

			24h				72h			
			Ann V – PI -	Ann V + PI -	Ann V + PI +	Ann V – PI +	Ann V – PI -	Ann V + PI -	Ann V + PI +	Ann V – PI +
<b>Malme-3M</b>	<i>Untreated</i>		94.3 ± 1.2	0.6 ± 0.3	2.9 ± 1.2	2.3 ± 0.7	86.3 ± 4.4	0.8 ± 0.8	5.4 ± 1.2	7.4 ± 4.4
	<i>Plasma</i>	3min	92.3 ± 1.7	0.8 ± 0.5	4.5 ± 1.5	2.5 ± 0.4	86.5 ± 4.3	2.1 ± 2.3	7.1 ± 1.9	4.4 ± 3.4
		7min	90.5 ± 2.3	1.1 ± 0.6	5.5 ± 1.4	2.9 ± 0.8	77.3 ± 12.2	3.3 ± 2.5	14.9 ± 9.6	4.4 ± 3.6
		11min	76.3 ± 8.2*	2.7 ± 1.4	13.6 ± 5.2	7.5 ± 2.1	23.6 ± 15.6*	2.8 ± 1.9	46.7 ± 18.0	27.1 ± 12.2
<b>SK-MEL-28</b>	<i>Untreated</i>		87.8 ± 5.2	0.5 ± 0.4	3.6 ± 1.5	7.9 ± 5.3	80.7 ± 10.5	0.4 ± 0.3	3.4 ± 2.5	15.5 ± 8.4
	<i>Gas flow</i>	3min	85.9 ± 5.8	1.1 ± 0.4	4.9 ± 1.6	8.1 ± 4.9	67.7 ± 3.2*	1.1 ± 0.6	6.1 ± 1.6	25.1 ± 3.5
		7min	91.4 ± 2.1	1.0 ± 1.2	2.7 ± 1.7	4.9 ± 1.2	63.5 ± 6.0*	1.4 ± 0.9	6.1 ± 2.0	29.0 ± 6.4
		11min	82.5 ± 7.0	1.4 ± 1.4	5.1 ± 1.2	11.0 ± 6.7	52.3 ± 6.7*	2.2 ± 1.2	10.7 ± 6.3	34.8 ± 5.8
	<i>Plasma</i>	3min	84.8 ± 5.7	1.2 ± 0.5	6.2 ± 3.1	7.8 ± 4.0	64.1 ± 7.5*	0.8 ± 0.6	5.9 ± 2.9	29.2 ± 7.0
		7min	86.3 ± 6.1	1.4 ± 1.1	4.6 ± 2.6	7.7 ± 3.8	68.3 ± 0.9	1.1 ± 0.7	8.7 ± 1.2	21.9 ± 0.7
		11min	76.7 ± 6.5*	3.7 ± 2.0	7.3 ± 1.9	12.2 ± 6.6	49.9 ± 9.4*	2.1 ± 1.2	18.4 ± 2.9	29.6 ± 9.5
<b>LN229</b>	<i>Untreated</i>		90.2 ± 3.1	3.5 ± 1.6	1.9 ± 0.7	4.4 ± 1.6	85.5 ± 3.3	3.9 ± 0.9	4.7 ± 1.0	5.9 ± 2.2
	<i>Plasma</i>	3min	90.1 ± 3.2	3.8 ± 1.7	2.2 ± 0.7	3.9 ± 1.7	78.7 ± 7.1	6.9 ± 2.0	6.7 ± 2.6	7.7 ± 3.2
		7min	90.1 ± 2.5	4.7 ± 1.9	2.4 ± 0.5	2.9 ± 2.1	75.6 ± 5.7*	10.8 ± 2.1	9.8 ± 3.8	3.9 ± 2.5
		11min	65.3 ± 18.0*	9.3 ± 4.0	20.7 ± 15.5	4.8 ± 2.3	32.5 ± 14.3*	6.3 ± 1.6	57.4 ± 16.3	3.8 ± 1.2
<b>U87</b>	<i>Untreated</i>		89.6 ± 4.3	4.8 ± 2.7	2.8 ± 1.1	2.9 ± 1.5	94.5 ± 2.3	1.6 ± 0.9	2.1 ± 1.0	1.8 ± 0.9
	<i>Plasma</i>	1min	91.7 ± 1.0	3.7 ± 1.4	2.6 ± 0.6	1.9 ± 0.8	95.8 ± 1.3	1.5 ± 0.7	1.7 ± 0.8	1.0 ± 0.3
		3min	73.3 ± 15.9*	7.0 ± 2.4	15.6 ± 15.0	4.2 ± 3.0	69.6 ± 26.0*	5.1 ± 1.4	21.3 ± 23.0	4.0 ± 3.2
		5min	66.4 ± 3.6*	4.5 ± 0.8	24.0 ± 3.5	5.1 ± 0.8	49.8 ± 20.6*	8.0 ± 2.0	34.7 ± 19.7	7.6 ± 3.1
		7min	4.0 ± 7.0*	0.8 ± 0.6	92.7 ± 8.0	2.6 ± 0.6	1.2 ± 1.8*	1.2 ± 1.2	93.6 ± 4.3	3.9 ± 2.7
		11min	0.1 ± 0.1*	0.6 ± 0.5	98.4 ± 1.1	0.9 ± 0.6	0.9 ± 1.3*	0.5 ± 0.4	93.3 ± 3.5	5.3 ± 3.2