

## FULL PAPER

# Cold atmospheric plasma treatment of melanoma and glioblastoma cancer cells

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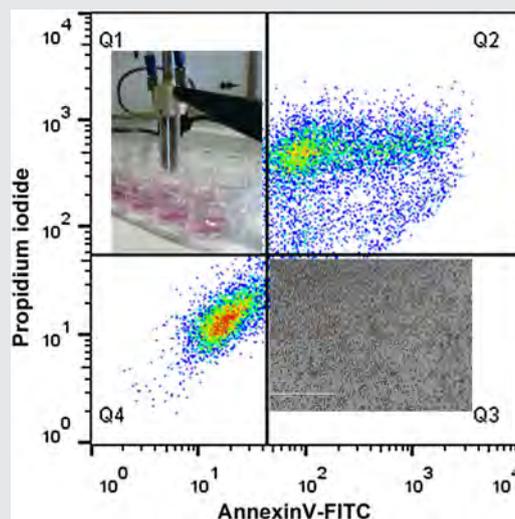
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In this paper, two types of melanoma and glioblastoma cancer cell lines are treated with cold atmospheric plasma to assess the effect of several parameters on the cell viability. The cell viability decreases with treatment duration and time until analysis in all cell lines with varying sensitivity. The majority of dead cells stains both AnnexinV (AnnV) and propidium iodide, indicating that the plasma-treated non-viable cells are mostly late apoptotic or necrotic. Genetic mutations might be involved in the response to plasma. Comparing the effects of two gas mixtures, as well as indirect plasma-activated medium versus direct treatment, gives different results per cell line. In conclusion, this study confirms the potential of plasma for cancer therapy and emphasizes the influence of experimental parameters on therapeutic outcome.

**KEYWORDS**

cancer cell, viability, experimental parameters, glioblastoma, melanoma, non-thermal plasma

## 1 | INTRODUCTION

Plasmas are ionized gasses which can be seen as a reactive chemical cocktail consisting of electrons, ions, neutral

species (radicals, molecules, or excited species), and photons. In particular, the reactive oxygen species (ROS) and reactive nitrogen species (RNS) are the most important for the biomedical applications of plasmas.<sup>[1]</sup> Over the last few decades, mainly cold atmospheric pressure plasmas (CAPs), which are characterized by a non-equilibrium between the high-temperature electrons and the low-temperature heavy molecules, have attracted a lot of attention in these

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applications.<sup>[2,3]</sup> Due to the non-equilibrium in CAPs, it is possible to treat living or heat-sensitive surfaces, which enables the use of these plasmas for the treatment of cancer cells.

Cancer is still the second leading cause of death worldwide (www.cancer.gov), leading to more than eight million deaths a year. Treatment of this disease is very difficult due to, e.g., drug resistance, therefore, new modalities for cancer therapy are an urgent need.<sup>[4]</sup> In recent years, the anticancer capacity of CAPs has been illustrated in cancer cell lines of breast, skin, lung, pancreas, cervix, and brain.<sup>[5–20]</sup> In the literature, both the floating-electrode dielectric barrier discharge (FE-DBD), developed by Fridman et al., as well as the atmospheric pressure plasma jet (APPJ) setup are used as CAP sources for the treatment of cancer cells.<sup>[3,13]</sup> These CAP sources can be directly applied to cancer cells, or they can be used to irradiate media, thereby generating plasma-activated medium (PAM), which is applied to the cancer cells afterwards.<sup>[21]</sup>

The mechanism of the interaction between plasma and cell is not yet fully understood, but it seems that ROS and RNS are key players in this process.<sup>[22]</sup> When these species interact with the eukaryotic cell membrane, they might either penetrate and/or form reactive oxygen and nitrogen species (RONS) or lipid peroxidation products.<sup>[17]</sup> This causes a disturbance of the oxidative balance. Excessive production of oxidative stress, exceeding the cellular antioxidative defense, can lead to cell death in both normal and aberrant cells by activating intracellular signaling pathways.<sup>[23]</sup> Cellular antioxidant systems will respond to plasma-generated RONS by activating antioxidative enzymes to protect the cells. It is hypothesized that healthy cells can deal better with this disturbance and restore their oxidative balance, because they a) take up less exogenous RONS, and b) neutralize RONS by more efficient antioxidant systems. In contrast, tumor cells contain higher steady state RONS concentrations and bear malfunctioning antioxidant mechanisms.<sup>[10,24,25]</sup> Therefore, the increasing RONS concentration can be used as an effective and selective treatment against cancer. Indeed, CAPs have been demonstrated to selectively induce cell death in cancer cells compared to normal cells.<sup>[6,8,19,26]</sup>

In this paper, we investigate the plasma treatment of melanoma and glioblastoma (GBM) cells with two different gas mixtures and different treatment times, and we compare direct CAP treatment with indirect PAM treatment, to reveal which treatment conditions are effective for different types of cancer cell lines. Melanoma is the most aggressive form of skin cancer with a high prevalence, a median overall survival of less than 2 years, and resistance to current modalities of cancer therapy.<sup>[27]</sup> GBM is the most common malignant primary brain tumor, with an median survival time of 15 months after diagnosis and for which standard therapy is inadequate.<sup>[28]</sup> By investigating two different cancer cell types and by choosing two different cell lines for each cancer

type, we aim to obtain more information on cell sensitivity to plasma treatment comparing different treatment parameters per cell type.

## 2 | EXPERIMENTAL SECTION

### 2.1 | Cell culture

Human melanoma cell lines, Malme-3M (ATCC<sup>®</sup> HTB-64<sup>™</sup>) and SK-MEL-28 (ATCC<sup>®</sup> HTB-72<sup>™</sup>), as well as human GBM cell lines, LN229 (ATCC<sup>®</sup> CRL-2611<sup>™</sup>) and U87 (Cell Line Service GmbH), were cultured in Roswell Park Memorial Institute 1640 RPMI1640 (LifeTechnologies) supplemented with 10% heat-inactivated fetal bovine serum (FBS, LifeTechnologies) and 0.5% penicillin/streptomycin (LifeTechnologies). The cell lines were maintained at 37 °C in a 5% CO<sub>2</sub> humidified atmosphere.

Before plasma treatment, the cells were cultured in 24-well plates (Malme-3M: 75000; SK-MEL-28: 50000; LN229: 60000; U87; 40000) for 72 h. The cells were harvested by using 0.05% trypsin-EDTA (LifeTechnologies) or Accutase (for U87; Sigma-Aldrich).

### 2.2 | Micro-atmospheric pressure plasma jet ( $\mu$ -APPJ)

The plasma source used in this study is the COST-action reference plasma source (described in detail by Golda et al.).<sup>[29]</sup> It can be divided into three main components: (i) the electrode assembly; (ii) the gas connector; and (iii) the housing (constituting out of the electric connections and the voltage and current probes). The electrode assembly was equipped with two symmetrical stainless steel electrodes with dimensions of  $52.5 \times 12 \times 1 \text{ mm}^3$ , separated by a 1 mm gap through which the feed gas flows (Figure 1). The plasma was generated by applying an AC voltage (with a root mean square voltage of 230 V) between the two electrodes, of which one is grounded. The applied frequency and flow rate used in all treatments were 13.56 MHz and  $1.4 \text{ L} \cdot \text{min}^{-1}$ ,

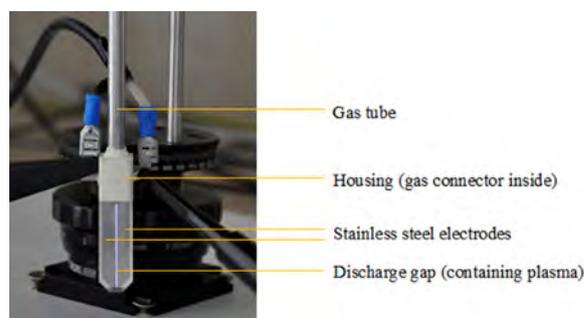


FIGURE 1  $\mu$ -Atmospheric pressure plasma jet used in this study

respectively. The plasma jet was positioned 8 mm above the surface of the sample to be treated.

## 2.3 | Plasma treatment

Two different plasma treatment methods were investigated. In the first method, the cancer cells were placed beneath the plasma jet source, thereby treating them directly in 500  $\mu$ L medium. Afterwards, 300  $\mu$ L fresh medium was added to the cells. Hereafter, this direct method is mentioned as CAP. In the second method, a well containing 500  $\mu$ L medium was treated by the plasma, after which the PAM was transferred to the well containing the cells in 300  $\mu$ L medium. This indirect treatment method will be indicated hereafter as PAM. In both methods, two different flow gasses were examined: (i) He/O<sub>2</sub> (99.4/0.6 mol%) and (ii) He/O<sub>2</sub>/N<sub>2</sub> (99.59/0.35/0.06 mol%). Moreover, the effect of the plasma dose was investigated by applying different treatment times (ranging from 1 up to 11 min). Finally, to be able to differentiate the plasma effect from that of the gas flow or environmental factors, two control conditions were included: (i) no treatment and (ii) gas flow treatment (i.e., without igniting the plasma). Following plasma treatment, the cells were incubated at 37 °C and 5% CO<sub>2</sub> for 24 h or 72 h, after which the viability was analyzed to determine the antitumor activity.

## 2.4 | Cell viability

The cell viability was assessed using flow cytometry based on AnnexinV (AnnV) and propidium iodide (PI) staining. At different time points after treatment, 24 and 72 h, the medium containing dead cells was collected and combined with the subsequently harvested cells. Following centrifugation, the supernatant was discarded and the cells were stained with 2  $\mu$ g AnnV-FITC (BD Biosciences) in 200  $\mu$ L 1 X AnnV Binding Buffer (BD Biosciences). Before measuring on a BD FACScan (Becton Dickinson, Erembodegem, Belgium), 0.5  $\mu$ g PI (LifeTechnologies) was added. Analysis was performed using FlowJo v10 (ThreeStar, Ashland, USA).

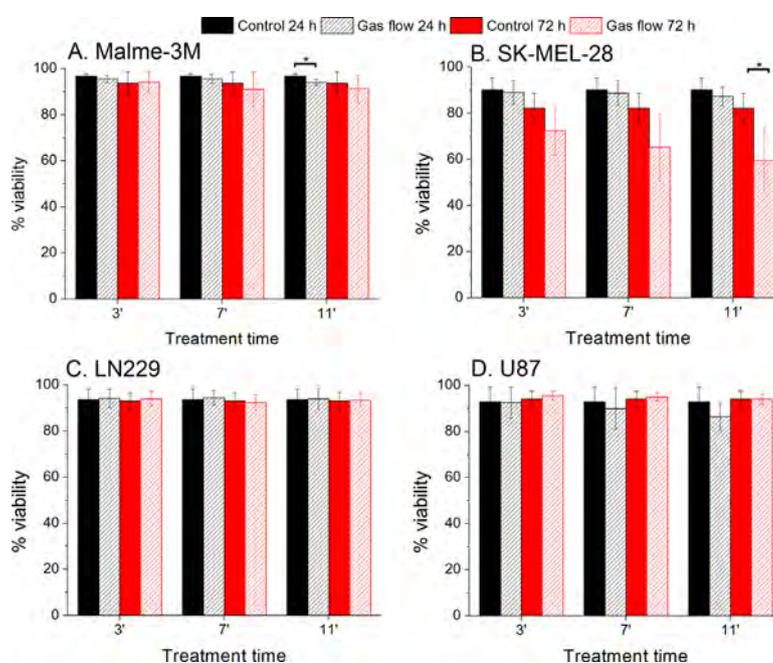
## 2.5 | Statistics

Statistical analyses were performed using RStudio (RStudio, Boston, USA) and SPSS 23 (IBM, Armonk, USA). Outliers in the datasets, based on at least 3 setups ( $n \geq 3$ ), were omitted using boxplots. Analysis of multiple groups (treatment times) was performed using a Kruskal-Wallis test, followed by a post-hoc Dunn's test. One-on-one analyses were performed using a Mann-Whitney *U*-test. Significance was set from  $p < 0.05$ . Graphs were made using OriginPro8.5 (OriginLab, Northampton USA).

## 3 | RESULTS AND DISCUSSION

### 3.1 | Effect of gas flow on cell viability

The gas flow only was investigated because it might induce mechanical stress during direct treatment of the cells. Figure 2 shows that treatment with only gas flow had no biological effect on the viability of Malme-3M, LN229, and U87 cells, which is in accordance with observations by others.<sup>[15,17,30,31]</sup> However, the gas flow negatively affected the viability of SK-MEL-28 cells significantly ( $p < 0.05$ ). The decrease in viability was dependent on the duration of the treatment, but independent of the gas mixture and the treatment method, i.e., CAP and PAM treatment (Supplementary Table S1). Hence, the effect cannot be explained by an induction of mechanical stress due to direct treatment of the cells. Possible explanations for this effect could be changes to the medium conditions. Therefore, the pH of the medium was measured before and directly after treatment, as well as at the time points of analysis (24 and 72 h). No differences in pH between the different conditions (untreated, gas flow and plasma treated) could be observed (Supplementary Figure S1), which is in line with literature.<sup>[32]</sup> Tonicity of the medium is another parameter that could explain the gas effect. Evaporation of the medium during treatment can lead to increased osmolality, which can influence cell behavior.<sup>[33]</sup> In this context, we also observed evaporation of the medium, but proportional to treatment time. Although osmolality was not analyzed, tonic effects were minimized by adding 300  $\mu$ L fresh medium to the 500  $\mu$ L treated medium in either treatment method. In conclusion, our results show that gas



**FIGURE 2** Effect of gas flow only on the cell viability, using a He/O<sub>2</sub> gas mixture, analyzed 24 and 72 h after treatment. \* $p < 0.05$

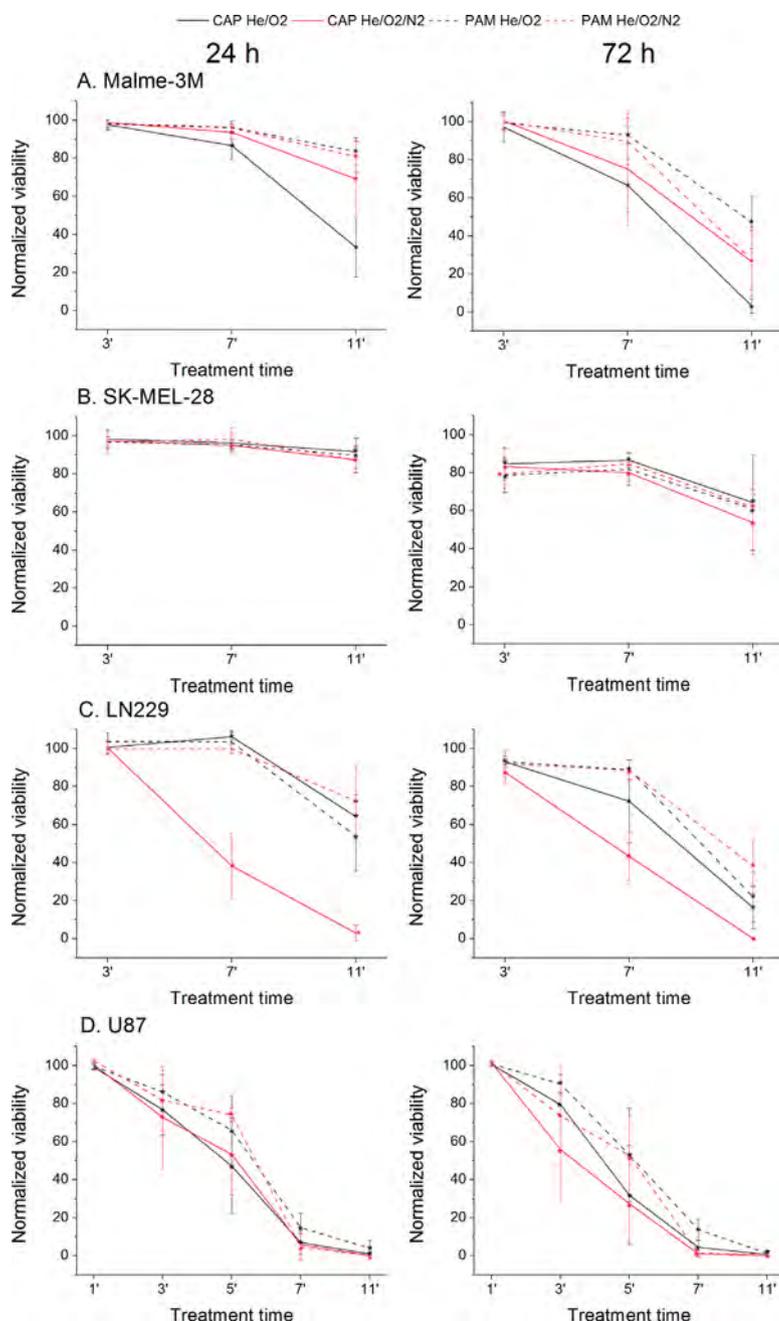
flow only affects the viability of SK-MEL-28 cells, independent of mechanical stress or changes in the pH. We speculate that gas flow might change one or more medium parameters for which only SK-MEL-28 cells are sensitive. Of note, SK-MEL-28 cells acquired an enhanced proliferative capacity following culturing in RPMI instead of Minimal Essential Medium (MEM), possibly contributing to its sensitivity. Since the gas flow affected the cell viability of SK-MEL-28 only, it was decided to no longer include this condition for the other cell lines in further experiments.

### 3.2 | Effect of plasma treatment on cell viability

Plasma treatment induces cell death in cancer cell lines. Figure 3 illustrates the effects of gas mixture, treatment time, and treatment method on the cell viability at different time points after treatment, normalized to the untreated conditions. In general, the induction of cell death is correlated with the treatment duration and time until analysis, while the effect of treatment method (CAP vs. PAM) and gas mixture differs between conditions. In the next paragraphs, we will present and discuss the effects of plasma on the cell viability with respect to the different treatment and analysis parameters (see Figure 3 and Table S1).

#### 3.2.1 | Influence of treatment time

In all four cell lines the viability decreased statistically significantly with longer treatment times, although the rate of decline was different. SK-MEL-28 appears the most resistant cell line, because only the 11 min treatment neared the half maximal inhibitory concentration (IC<sub>50</sub>) threshold when analyzed after 72 h. Nevertheless, the portions of viable cells dropped statistically significant in comparison to the untreated controls ( $p < 0.05$ ). Furthermore, no difference in treatment effect was observed with either method or gas mixture between 3 and 7 min plasma for SK-MEL-28. However, gas flow treatment only results in a similar, statistically non-different decrease in viability (Figure S2), which means SK-MEL-28 is resistant to plasma treatment. U87 was the most sensitive cell line, as already a 7 min treatment killed most cells. Since also a 3 min treatment decreased the cell viability significantly, we additionally analyzed U87 following 1 and 5 min treatment. Whereas 1 min treatment did not affect cell viability ( $p > 0.05$ ), 5 min treatment showed an effect intermediate to 3 and 7 min. This suggests that nearly all U87 cells die within a treatment window between 5 and 7 min.



**FIGURE 3** Viability of the different cell lines analyzed 24 and 72 h after plasma treatment. The data were normalized to the untreated cells. \* $p < 0.05$

Malme-3M and LN229 show intermediate sensitivity with different response patterns to plasma treatment ( $p < 0.05$ ). This is in line with Körtzner et al., who observed a higher sensitivity of U87 compared to LN229 in a plasma-chemotherapy setup.<sup>[20]</sup> Moreover, these authors demonstrated restoration of sensitivity to chemotherapy by CAP, underlining the potential applicability of CAP to tackle current hurdles in standard oncological treatment regimens.<sup>[20]</sup>

The dose dependency of the plasma effect observed in this study is in accordance with literature.<sup>[7,13,18,31,34–36]</sup> This is not surprising, as longer treatment results in the generation of more reactive species, for which Cheng et al. include

treatment time in their equation for plasma dose.<sup>[37]</sup> The inefficacy of 3 min treatments, except for U87, may partially be explained by the lower ROS concentrations generated, which can actually stimulate cell proliferation.<sup>[34]</sup> In addition, when treatment times are too short, cells can overcome the plasma-induced cell cycle arrest and cell death.<sup>[38]</sup> Finally, the effective treatment times in our study are generally longer than those described by others in the literature, i.e., less than 3 min to reach the IC50 for melanoma and GBM cell lines.<sup>[8,9,11–13,20,21,26,30,37,39,40]</sup> Possible explanations for this observation will be discussed in Section 3.5.

### 3.2.2 | Influence of time of analysis

The cell viability was significantly lower 72 h following treatment compared to 24 h ( $p < 0.05$ ), which has also been observed with U87 by others.<sup>[26,37]</sup> Cheng et al. explain this effect by the generation of peroxynitrite in the medium after the plasma treatment has finished.<sup>[37,41]</sup> They suggest that the generated amount is insubstantial to induce cell death after 24 h, but increases over time to induce oxidation and potentially disruption of the antioxidant system, eventually leading to cell death.<sup>[37]</sup> Figure S3 demonstrates such a statistically significant increase of peroxynitrite and  $\text{OH}^\bullet$  over time, however after 24 h a statistical substantial amount was already detected ( $p < 0.05$ ). Similar processes possibly happen with other reactive species as well.

### 3.2.3 | Influence of treatment method

We investigated the killing efficiency of CAP and PAM treatment. Little to no differences in efficiency between CAP and PAM treatments were observed for cell lines SK-MEL-28 and U87, which is in line with others.<sup>[18,38]</sup> In contrast, PAM showed a lesser capacity of killing Malme-3M and LN229 compared to CAP treatment. Of note, this difference was dependent on the gas mixture used. In Malme-3M, only PAM generated using  $\text{He}/\text{O}_2$  was significantly less cytotoxic than CAP ( $p < 0.05$ ), whereas in LN229 only  $\text{He}/\text{O}_2/\text{N}_2$ -generated PAM was significantly less cytotoxic than its CAP counterpart ( $p < 0.05$ ). A possible explanation for the differences by PAM treatment may be the absence of the direct effect of the reactive species to the cells.<sup>[34]</sup> On the one hand, part of the reactive species will be directly taken up by the cells, where they will interact with oxidizable substrates, such as DNA and lipids. Meanwhile on the other hand, another part of the reactive species will dissolve in the medium, where it will interact with its components, such as amino acids, producing even more reactive species in the liquid, which will then diffuse into the cell and will cause internal DNA damage.<sup>[34]</sup> The lack of the direct effect of the reactive species to the cells with PAM may offer an explanation for the observed difference between the CAP and PAM treatments. Nevertheless, in this scenario one might suspect a consistent lesser effect of PAM, disregarding

the gas mixture used and cell line applied to. Furthermore, differences in treatment efficiency may be explained by the transfer of PAM. Although transfer of PAM was immediate, i) it has been shown in the literature that the killing factors in PAM decay in time<sup>[42]</sup>, and ii) it is not possible to transfer the exact total PAM volume. Nevertheless, the applicability of PAM instead of direct plasma therapy offers opportunities with regard to clinical and research applications as an off-the-shelf product, as it has been shown that PAM frozen at  $-80^\circ\text{C}$  retains its killing capacity.<sup>[43]</sup> Our data suggest that this has to be validated for each gas mixture and target cell.

### 3.2.4 | Influence of gas mixture

The effect of plasma on cancer cells is a direct consequence of the generation of RONS, which leads to intracellular oxidative stress and ultimately cell death.<sup>[5]</sup> We compared plasma generated from two different gas mixtures:  $\text{He}/\text{O}_2$  (99.4/0.6 mol%) and  $\text{He}/\text{O}_2/\text{N}_2$  (99.59/0.35/0.06 mol%). Whereas the different gas mixtures gave comparable results in SK-MEL-28 and U87 cells,  $\text{He}/\text{O}_2$ -generated plasma was more effective in Malme-3M but less effective in LN229 in comparison to  $\text{He}/\text{O}_2/\text{N}_2$ . These results suggest that the sensitivity for ROS and RNS can vary within one cell line. A first difference between the gas mixtures is the presence of  $\text{N}_2$  in  $\text{He}/\text{O}_2/\text{N}_2$ , which allows for the generation of higher amounts of RNS compared to  $\text{He}/\text{O}_2$ . Depending on the concentration,  $\text{NO}^\bullet$  can either strengthen or weaken protection against oxidative stress.<sup>[44]</sup> Furthermore,  $\text{NO}^\bullet$  can react with  $\text{O}_2-\bullet$  to produce peroxynitrite, which the cell cannot neutralize and which can ultimately lead to cell death via a myriad of effects (reviewed by Pacher et al.).<sup>[45]</sup> Due to the treatment being performed in open air, peroxynitrite is formed in both gas mixtures (Figure S3). As one might expect it is formed to a larger extent in the nitrogen-containing gas mixture ( $p < 0.05$ ), except with Malme-3M after 72 h ( $p > 0.05$ ). A second difference is the lower  $\text{O}_2$  concentration in  $\text{He}/\text{O}_2/\text{N}_2$  which may alter the amount of generated ROS. However, data published by Cheng et al. suggest that the mere addition of  $\text{O}_2$  rather than its percentage in the gas mixture determines the cytotoxic effect.<sup>[37]</sup> Overall, these results suggest that the optimal gas mixture for plasma medicine applications will need to be determined by comparison of different gas mixtures.

## 3.3 | Types of cell death following plasma treatment

A first step toward understanding the mechanism of plasma treatment of cancer cells is to take a closer look at the type of cell death. AnnV/PI staining allows for a distinction between four different groups: AnnV-/PI- (viable cells); AnnV+/PI- (early apoptotic cells); AnnV+/PI+ (late apoptotic and necrotic cells); and AnnV-/PI+ (necrotic

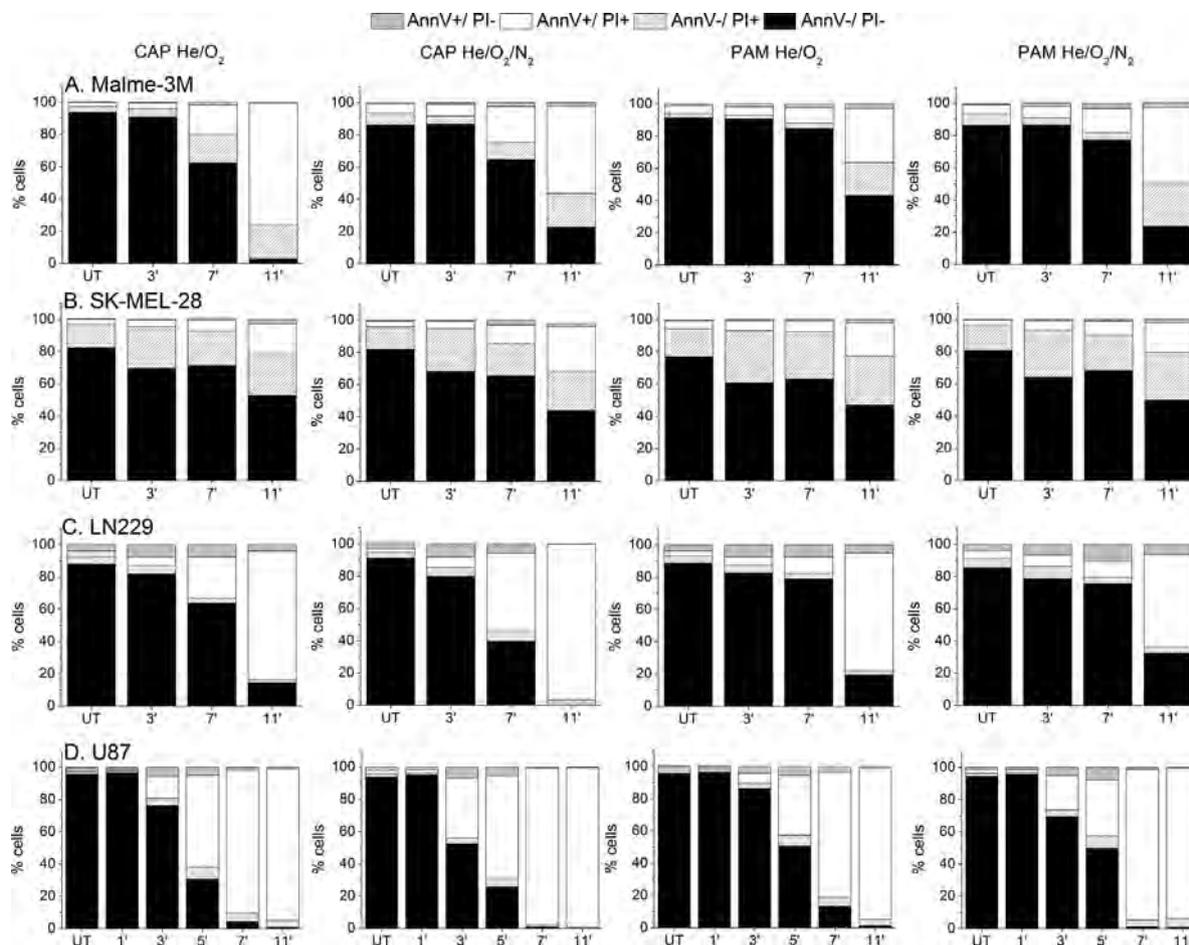


FIGURE 4 Overview of cell death, 72 h after CAP and PAM treatment

cells). Figure 4 presents an overview of the different types of cell death for the different cell lines, considering CAP and PAM treatment for both gas mixtures, 72 h after plasma treatment. Supplementary Table S1 additionally shows all data for each time point and treatment duration. The general trend indicates that a longer treatment time decreases cell viability, mainly by the growing portion of AnnV+/PI+ cells, which is in accordance to Keidar et al.,<sup>[7]</sup> and irrespective of gas mixture and treatment method, which corresponds to earlier studies on plasma-induced cell death in general.<sup>[13,17,46]</sup> SK-MEL-28 appears to be the exception as most dead cells are AnnV-/PI+. Interestingly, this is also the most plasma-resistant cell line and SK-MEL-28 responds to a similar extent to treatment with only gas flow as well. This indicates that other parameters affect the cell viability in SK-MEL-28. The increased proliferation rate observed by culturing SK-MEL-28 in RPMI instead of MEM indicates that these cells adjusted to their new environment by modulating some processes, which might be correlated to confluency. In comparison to the GBM cell lines, also the number of AnnV-/PI+ Malme-3M cells increases following 11 min treatment. Possibly, this type of cell death

is more amenable in melanoma cells. It is apparent that the portion of AnnV+/PI- cells remained small for each condition in each cell line. As AnnV/PI staining can be indicative yet inconclusive to determine apoptotic or necrotic cell death, these results warrant further investigation on these processes. Indeed, AnnV binds to phosphatidylserine (PS), a phospholipid located at the inner side of the cell membrane. Early in apoptosis, a flipflop to the outer side exposes PS, enabling binding to AnnV (Ann+/PI-). Later in apoptosis, loss of membrane integrity will allow PI to intercalate to DNA (Ann+/PI+). In the necrotic process, the membrane integrity is lost and PI will intercalate to the DNA (Ann-/PI+). However, AnnV will enter this permeated cell membrane in necrosis in order to bind to PS on the inner cell membrane (Ann+/PI+).<sup>[47,48]</sup> Therefore, in the current experimental setup, Ann+/PI+ cells, which represented the major phenotype of dead cells, could not be distinguished as being either apoptotic or necrotic cells. Regardless of this, the effect of plasma treatment on cancer cell viability, our foremost experimental objective, could easily be explored by analyzing the viable cells, being Ann-/PI-. In this context it is, however, interesting to note that other studies have also

shown that altering plasma treatment parameters can steer the type of cell death.<sup>[49–51]</sup>

### 3.4 | Differences between cell types in response to plasma therapy

Our data show that SK-MEL-28 and LN229 are more resistant to plasma treatment compared to their investigated cancer type counterparts Malme-3M and U87, respectively. Interestingly, these more resistant and more sensitive cancer cell lines each share common traits, based on the literature: whereas Malme-3M and U87 have a wild-type (wt) p53 gene and a mutated (mut) cyclin-dependent kinase inhibitor 2A (CDKN2A) gene, both SK-MEL-28 and LN229 bear p53<sup>mut</sup> and CDKN2A<sup>wt</sup>.<sup>[52,53]</sup> Ma et al. have demonstrated in several cancer cell lines that p53-deficient cells are more sensitive to CAP than p53<sup>wt</sup> cells.<sup>[54]</sup> This could partly be explained by the relatively high concentrations of endogenous ROS present in p53-deficient cells, which, therefore, will be more sensitive to damage induced by ROS-generating plasma.<sup>[55]</sup> p53 also plays a role in the repair of oxidative stress to DNA by, e.g., H<sub>2</sub>O<sub>2</sub>, one of the most reactive species generated during plasma treatment.<sup>[21,56,57]</sup> Furthermore, upregulation of p53 during plasma treatment has been described, while introduction of p53 in p53-negative cells partially protected against the pro-apoptotic effect of plasma therapy.<sup>[54,58,59]</sup> CDKN2A encodes for tumor suppressor protein p16. Jenkins et al. reported that loss of p16 dysregulates intracellular ROS leading to DNA damage, which could be neutralized by restoration of p16.<sup>[60]</sup> Whereas our data do not accord with literature on the plasma response related to p53 data, our results corroborate the findings of Jenkins et al. as the CDKN2A<sup>mut</sup> cell lines were more sensitive to plasma treatment than their CDKN2A<sup>wt</sup> cancer-type counterparts. As cancer cell lines bear many other mutations and are genomic unstable, it is conceivable that the overall effect of mutational burden on ROS and the antioxidant systems will dictate the response to plasma treatment. This remains an intriguing question to be answered, which may allow patient stratification for plasma therapy in the future. Therefore, we suggest further research on the influence of mutated cancer genes on cellular response to plasma treatment.

Next to mutational burden, other explanations have been suggested to account for differences in response to plasma treatment between cell lines. Naciri et al. postulated that the anticancer capacity of CAP is proportional to the proliferation rate of the cell line.<sup>[61]</sup> However, it should be noted that in their experiments proliferation was examined using the Alamar Blue assay, which actually measures mitochondrial metabolic activity.<sup>[62]</sup> Therefore, it is possible that the observed effects were due to variations in metabolic activity rather than proliferation rate. Nonetheless, a rationale behind this postulation might be analogue to that behind chemo- and

radiotherapy in oncological treatments: more proliferative cells have less time to repair damage and therefore undergo cell death more rapidly.<sup>[63]</sup> Although we observed an increased proliferation rate in SK-MEL-28 following culturing in RPMI instead of MEM, this melanoma cell line proved to be the most resistant of all investigated cell lines. However, the proliferation rates of the different cell lines were not investigated, hence no conclusions can be drawn on this matter. In future experiments with different cell lines, proliferation rates will be determined in order to investigate this parameter. Also, Yan et al. showed that the rate of absorption or elimination of reactive species from the extracellular environment determines the resistance to plasma therapy. U87 showed a higher consumption rate of reactive species in the medium and a higher viability after treatment, whereas the opposite was observed in breast cancer cell lines.<sup>[21]</sup>

Furthermore, as antioxidant systems can explain the selectivity of plasma therapy on cancer cells over normal cells,<sup>[10,24,25]</sup> differences in the antioxidant capacity between cell lines, inherently or caused by mutations, can affect their sensitivity to plasma. In this context, melanoma cells have been described to harbor an extensive antioxidant system, compared to other cell types, that will react and adapt to different ROS concentrations.<sup>[64,65]</sup> Our results suggest that SK-MEL-28 may have a greater antioxidant capacity than Malme-3M, but further work is needed to study this more in detail.

### 3.5 | Influence of parameters on treatment outcome

Effective treatment times in literature are generally shorter than observed in our study, i.e., between 1 and 3 min.<sup>[8,9,11–13,20,21,26,30,37,39,40]</sup> The numerous parameters involved in experimental plasma medicine may each alone or in combination offer explanations to this observation, as well as to the variation encountered between replicates. In this section, we discuss the various experimental and analysis parameters (numbered in brackets) used in our study. Our experimental design employed a cross-field (i)  $\mu$ -APPJ (ii); with 230 V AC voltage (iii); 13.56 MHz frequency (iv); and 1.4 L · min<sup>-1</sup> flow rate (v). A different plasma source or different settings obviously would change the experimental outcome. Dielectric barrier discharges (DBDs) and APPJs are the most commonly used sources in plasma medicine. Daeschlein et al. report that DBDs are more potent in killing bacteria than APPJs.<sup>[66]</sup> This is expected, as there is a higher electrical field between the DBD and the substrate, which will lead to more ionized particles reaching the substrate. Moreover, this high electrical field can induce electroporation, facilitating the uptake of RONS by the cell. In contrast, with the APPJ only the flowing afterglow will reach the substrate.<sup>[67]</sup> Furthermore, linear field plasma jets have been

described as more capable of active plasma chemistry and larger electron mean energy in the downstream region in comparison to cross-field plasma jets, such as the  $\mu$ -APPJ we employed.<sup>[68]</sup> The output voltage has been correlated to the intensities (proportionally) and saturation time of generated species.<sup>[37,42]</sup> Our output voltage of 230 V is low in comparison to many other studies, reporting a higher order of magnitude (kV).<sup>[10,20,37,40]</sup> Higher flow rates have reported to result in more generation of RONS and lower cell viability.<sup>[49]</sup> The longer treatment time in our work compared to other studies is based on a flow rate of only  $1.4 \text{ L} \cdot \text{min}^{-1}$ . Studies reporting shorter time generally have a higher flow rate.<sup>[20,21,26,30,37,39]</sup> We hypothesize that the product of treatment time and flow rate might be a determining factor rather than the individual parameters. Indeed, extracellular ROS has been shown to increase in function of treatment time and flow rate.<sup>[69]</sup> In addition, Cheng et al. include the output voltage in this equation to calculate plasma dosage.<sup>[37]</sup>

The plasma jet was positioned 8 mm above the treated surface (vi). This was experimentally determined in combination with the gas flow rate in order to avoid the cells being blown away by the plasma jet (too close to the well surface or too high flow rate) or not being affected at all (too far away from the well surface or too low flow rate). Yan et al. state that the anticancer capacity varies with the gap between plasma source and media.<sup>[21]</sup> Indeed, the need to experimentally derive the gap in our experimental design confirms it affects the outcome. Our gap is different from others, reporting both smaller and bigger gaps.<sup>[11,20,21,61]</sup> Despite these gap differences, most studies also employed a higher gas flow rate.<sup>[10,11,13]</sup> However, this was not an option in our experimental setup without possible interference of the treatment effect. The use of PAM as treatment modality would circumvent this issue.

Our experimental design was suited for the use of 24-well plates, 96-well plates proved to be incompatible with the plasma jet nozzle. The recipient type is associated with well size (vii) as well as medium volume (viii) and the number of cells or well confluency (ix). Indeed, Yan et al. demonstrated that well size is proportionally related to plasma-generated anticancer effects.<sup>[21]</sup> A bigger well size leads to a bigger media surface, allowing more reactive species to diffuse over the liquid surface. This was observed for  $\text{NO}\bullet$  and  $\text{H}_2\text{O}_2$ , but not for  $\text{OH}\bullet$ , which probably is related to the longer and short half-life of those species, respectively.<sup>[21]</sup> This is particularly interesting when using PAM as treatment method. The well size determines the volume of medium. We treated 500  $\mu\text{L}$  medium in a 24-well for both treatment modalities, however, immediately thereafter 300  $\mu\text{L}$  of fresh medium was added to account for evaporation of medium and hence, to minimize hypertonicity. This addition diluted the generated reactive species, and therefore also the anticancer effect (data not shown). Indeed, it has been reported that medium volume is inversely correlated to plasma treatment

efficiency, which has been ascribed to a volume-dependent dilution effect, as reactive species are only generated at the medium surface.<sup>[21]</sup> Regarding the number of cells, we opted to use well confluency as density parameter, which explains the different seeding densities between the cell lines. On the one hand Yan et al. suggest that the amount of RONS per cell unit influences the outcome, while on the other hand confluency is a measure for the total cellular surface.<sup>[21]</sup> We decided to use confluency instead of cell number in our experimental design in order to minimize differences due to, e.g., proliferation rate and cell size. Nonetheless, both parameters are obviously interconnected, as more proliferative or bigger cells will result in a higher confluency. Either density parameter affects the response to plasma. Indeed, seeding density and well confluency were inversely correlated to the response on plasma treatment (data not shown). As mentioned earlier, proliferation rate (x) has been reported to be correlated to plasma treatment efficiency, which adds a complicating factor when comparing cell lines differing in this parameter.<sup>[61]</sup> To this end, we will examine proliferation rate in future experiments as well. Moreover, plasma has been shown to alter proliferative behavior.<sup>[69]</sup>

The composition of the medium (xi) also determines the outcome. Reactive species react with medium constituents such as amino acids, of which cysteine and tryptophan are stated to be the most reactive, leading to consumption of effective reactive species. Indeed, cysteine-rich Dulbecco's MEM (DMEM) has lost nearly its entire anticancer effect.<sup>[21]</sup> We also observed different responses to plasma when treating the same cells in different medium (RPMI, DMEM, MEM; data not shown). This emphasizes the importance of accurately reporting treatment media and supplements for plasma medicine studies. Although each cell line has its unique medium for optimal culture, most cell lines can be grown in other media as well. In order to standardize this variable, we selected the RPMI1640 with 10% FCS as universal culture medium. However, altering culture medium may also alter cell characteristics, which has to be kept in mind. Indeed, we observed an elevated proliferation rate for SK-MEL-28 compared to when it was grown in MEM. In future research we will explore the effect of plasma treatment using a physiological saline solution in order to obtain more clinically translational results.

Cells were plated and allowed to grow for 3 days prior treatment (xii). As we used biological replicates of cell lines by culturing the same cell line in different flasks, variation due to acquired mutations in given replicates might be amplified during the growth period. In future work, we plan to use a 1 day growth period for more control over the seeded cell numbers.

Variation in relative humidity (xiii) has a great impact on the production of reactive species since water molecules in the air will be converted to ROS, while also ambient

temperature (xiv) is a fluctuating parameter.<sup>[70–72]</sup> Both parameters depend on the weather conditions but cannot be accounted for without a workstation with a controllable atmosphere. Evaporation of treated medium (xv) is affected by these environmental parameters and treatment time, which may partially explain the variance between replicates sometimes observed.

As indicated earlier, the treatment method (xvi), time of analysis (xvii), and gas composition (xviii) all affect the outcome. PAM has been postulated to be at least as effective as direct CAP treatment.<sup>[30,73]</sup> We observed comparable effects between PAM and CAP in 6/8 cases, which corroborates with others,<sup>[18,38]</sup> whereas 2/8 showed a reduced anticancer effect of PAM. The actual reason warrants further research, but possible factors may be cell line-specific sensitivities to certain RONS and operator-induced variation. Nonetheless, PAM simplifies translation to the clinic over CAP. Nevertheless, standardizing PAM parameters will be of major importance to minimize variation for both clinical and research applications, e.g., transfer time, freezing method. Time of analysis after treatment influences the magnitude of the outcome, probably due to the time-dependent increase in peroxynitrite, as stated earlier and shown by others.<sup>[37]</sup> The gas composition is of major influence on the generation of reactive species and more specifically, the types and intensities of RONS. For example, Cheng et al. report that the supplementation of O<sub>2</sub> to the plasma gas impedes ionization, leading to a quickly dampened plasma plume. This resulted in decreased intensities of RONS, followed by less peroxynitrite generation and eventually less anticancer plasma effect. This effect already reached drastic measures with the addition of only 0.21% O<sub>2</sub>.<sup>[37]</sup> Our gas mixtures contain 0.35 and 0.6% O<sub>2</sub>, which makes a plausible explanation for the longer treatment times observed. As gas mixtures can be composed by unlimited recipes, this parameter is a major factor in complicating the comparison of different data sets.

Finally, also the method of analysis (xix) plays a role. Different techniques to investigate the same parameter may be analyzing different factors or have different sensitivities. We and others used the flow cytometric AnnV/PI assay, which considers both apoptotic and necrotic cell death.<sup>[61]</sup> This assay has also been performed using 7-aminoactinomycin D (7-AAD) instead of PI.<sup>[7]</sup> However, this test is often used with only AnnV, PI, or 7-AAD as well<sup>[18,38]</sup>, resulting in a loss of sensitivity to detect cell death. Another commonly used viability assay in plasma medicine is the MTT test, which actually investigates metabolic activity rather than viability, and hence a different read-out, although these assays are widely accepted as cytotoxic methods.<sup>[37,74,75]</sup> Lastly, a basic but time-consuming method to investigate cell death is microscopy. While trypan blue exclusion detects cells with an intact cell membrane, morphological studies on higher magnification allow to differentiate between live,

apoptotic, and necrotic cells.<sup>[47,73]</sup> There are many other techniques available to investigate cell death, but this list already indicates that the choice of method can alter the outcome.

The exhaustive list of influencing parameters we present here emphasizes the complexity which accompanies the comparison of plasma treatment of cancer cells. As the options are nearly infinite, it also advocates for guidelines on how to perform and to report studies, and, when more clinical applications will be approved in the future, for establishing reference settings.

## 4 | CONCLUSIONS

This study demonstrates that the  $\mu$ -APPJ with He/O<sub>2</sub> or He/O<sub>2</sub>/N<sub>2</sub> has an anticancer effect that increases with treatment duration and continues in time. Both CAP and PAM treatment are effective, but PAM is more applicable to obtain clinically translational results. Within a given cancer type, variations in sensitivity between different cell lines may be related to specific mutations, a future research topic which would allow for prognostic biomarker screening. The study underlines that plasma settings and experimental design will affect the plasma effect. In further research, we will compare cytotoxicity results with normal fibroblast and astrocytes, and explore physiological salt solutions in order to enhance clinical translation.

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