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# Antitumor Effects in Gas Plasma-Treated Patient-Derived Microtissues—An Adjuvant Therapy for Ulcerating Breast Cancer?

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**Abstract:** Despite global research and continuous improvement in therapy, cancer remains a challenging disease globally, substantiating the need for new treatment avenues. Medical gas plasma technology has emerged as a promising approach in oncology in the last years. Several investigations have provided evidence of an antitumor action in vitro and in vivo, including our recent work on plasma-mediated reduction of breast cancer in mice. However, studies of gas plasma exposure on patient-derived tumors with their distinct microenvironment (TME) are scarce. To this end, we here investigated patient-derived breast cancer tissue after gas plasma-treated ex vivo. The tissues were disjoint to pieces smaller than 100 µm, embedded in collagen, and incubated for several days. The viability of the breast cancer tissue clusters and their outgrowth into their gel microenvironment declined with plasma treatment. This was associated with caspase 3-dependent apoptotic cell death, paralleled by an increased expression of the anti-metastatic adhesion molecule epithelial (E)-cadherin. Multiplex chemokine/cytokine analysis revealed a marked decline in the release of the interleukins 6 and 8 (IL-6, IL-8) and monocyte-chemoattractant-protein 1 (MCP) known to promote a cancer-promoting milieu in the TME. In summary, we provide here, for the first time, evidence of a beneficial activity of gas plasma exposure on human patient-derived breast cancer tissue.

**Keywords:** CAP; cold physical plasma; oncology; plasma medicine; reactive nitrogen species; reactive oxygen species

## 1. Introduction

Cancer is considered one of the significant public health problems [1]. Breast cancer is the most common cancer and the second leading cause of death among women in developed and developing countries [2,3], necessitating research to find new methods to treat this disease [4]. Cancer cells often have a dysregulated metabolism, and it has been found that redox regulation and metabolic alterations are critical determinants in the therapy and resistance of breast cancer [5,6]. This suggests that reactive oxygen and

nitrogen species (ROS/RNS) might be harnessed as a therapeutic adjuvant breast cancer treatment. For instance, singlet oxygen has been shown to promising tool in this regard [7], and redox-active nanomedicines are promising agents of modern oncology [8] that can be also be combined with classical therapies such as radiation [9].

For a few years, a novel principle of generating therapeutic ROS/RNS levels has emerged in experimental oncology: plasma technology [10]. This partially ionized gas is operated at body temperature and does not convey thermal harm to cells and tissues [11]. This topical treatment modality dwells on a principle of physics for the generation of vast amounts of ROS/RNS simultaneously from reactive noble gas ions and electrons. Several in vitro studies have shown promising antitumor effects in gas plasma-treated breast cancer cells [12–21]. Additional work has also provided evidence of reduced growth [22–25] and metastasis [26,27] in three-dimensional tumor spheroids. In addition, we have previously shown that gas plasma treatment of breast cancer in mice modulated the inflammatory milieu within the tumor microenvironment (TME) in terms of leukocyte infiltration [28]. Similar findings were made in murine melanoma models [29,30].

However, studies on plasma treatment of patient-derived tumor tissues are scarce. It is understood that 2D and 3D cultures of homogenous cell lines are of limited use for illustrating the complexity of the TME [31], particularly in the case of intercellular interactions and extracellular matrices reactions [32,33]. Therefore, finding an appropriate platform for this type of culture is significant. Due to advantages, such as similarity to the extracellular matrix structure of body tissues, cell–matrix interaction, cell contacts, water, nutrient transfer, and growth and prolonged survival of encapsulated cells, microgels are suitable options for simulating the body environment [34,35]. Besides, the third dimension of cell growth provides more spatial communication for mechanical inputs and cell adhesion, which is necessary for connecting integrins, cell shrinkage, and even intracellular signaling [36]. However, cultured 3D unicellular organisms lack immune and angiogenic cells being important for tumor growth in vivo. Hence, the direct culture of tissue extracted from the body, due to having immune cells, angiogenesis, and growth in microgels, can be a more acceptable simulator for examining the toxicity effect of drugs and treatments [37].

To this end, we here extracted human-derived primary breast cancer tissues from 20 patients and developed a model to study the effects of gas plasma treatment of engrafted microtissues in collagen matrix and using microscopy. We found promising antitumor effects of the plasma exposure that increased cell death responses and modulated the release of several inflammatory mediators important in the TME.

## 2. Material and Methods

### *2.1. Breast Cancer Patient Tumor Material and Preparation*

The local ethics committee approved the study (approval number IR.SBMU.RETECh. REC.1398.072). Twenty breast cancer patients at the Motamed Cancer Institute agreed to participate in the study with informed consent and according to the guidelines of the Declaration of Helsinki. The studied patients had no treatment of any kind (e.g., chemotherapy, hormone therapy, or radiotherapy) before surgery. The breast cancers were of stage I-II, and since the tumors have not been exposed to any form of therapeutic intervention, the cancer tissue was unlikely to have developed any resistance against standard therapies. All patients were female. The tumor samples were transferred to glass Petri dishes containing RPMI culture medium supplemented with 10% fetal bovine serum in a laminar flow hood under sterile conditions. Then, the tumor sample was cut into tiny pieces by a scalpel. During this process, it was ensured that the culture medium on the tissue was sufficient for proper nutrition. After the tissue was cut into proper sizes, 500 µL of type IV collagenases was poured onto the samples, followed by incubation at 37 °C for up to 30 min until the tissue lost its rigidity. The samples were filtered using a 100 µm cell strainer, and the eluent was filtered using a 40 µm cell strainer. The eluent containing single breast cancer cells and debris was discarded while the material on the filter, being 40–100 µm in size, was transferred to microtubes and washed. Subsequently, the tissue was

mixed with type I collagen at a concentration of 2.8% and placed in an incubator for 30 min to form a gel. Fully supplemented cell culture medium was then added, and cultured tissues were placed in the incubator for 24 h and prior to plasma exposure.

### 2.2. Plasma Source, Characterization, and Treatment

A sinusoidal power supply was used to generate the cold plasma. Helium with five standard liters per minute was used as feed gas supplemented with ten standard cubic centimeters per minute of molecular oxygen as carrier gases. The plasma jet was operated at 8 kV peak-to-peak voltage and 6 kHz frequency, resulting in a total dissipated power of 1 W. The distance between the plasma jet and the treated target was fixed to 10 mm. Optical emission spectroscopy (AvaSpec-ULS3648-USB2 with diffraction grating of 300 lines per mm, ten μm gap, and a wavelength range of 200 to 1100 nm) was employed to analyze the plasma gas. In plasma-treated liquids, hydrogen peroxide ($H_2O_2$) and nitrite ($NO_2^-$) were measured using spectrophotometry as described before [38]. The pH of plasma-treated cell culture media was measured using a pH meter. The temperature of plasma-treated cell culture media was measured using a thermal infrared camera (FLIR E4 camera 80 by 60 pixels). For the treatment of collagen tissues, either direct or indirect plasma exposure was performed. In the direct treatment, the tumors embedded in collagen were directly exposed to physical plasma for 4 min while, in the indirect treatment, 1 mL of fully supplemented culture medium was exposed to plasma for 4 min and then transferred to collagen-tumor tissues. The control group remained untreated without any intervention and therapy. The plasma treatment was repeated 24 h later, and the collagen tissues were incubated for at least another 24 h after that. The plasma treatment time was identified to be suitable based on results obtained in pilot experiments.

### 2.3. Live Dead Assay

Twenty-four hours after the second plasma treatment, the culture medium was removed, and acridine orange/propidium iodide (AO/PI) staining solution was added and incubated for 20 min at 37 °C with the samples. AO enters all cells and stains their DNA, with a fluorescence emission at 520 nm. PI only enters dead cells and stains their DNA, with a fluorescence emission at 600 nm. Fluorescence quantification was conducted using MATLAB.

### 2.4. Immunofluorescence

Twenty-four hours after the second plasma treatment, the tissues were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS. Then, the samples were incubated with 3% BSA for 30 min to block nonspecific binding. Caspase-3 and E-cadherin antibodies were added to the samples and incubated at 4 °C overnight. The next day, the primary antibodies were washed away, and the samples were incubated with FITC-conjugated secondary antibodies for 2 h at room temperature in the dark. Finally, DAPI was added to counterstain the nuclei of the cells, and the images were recorded using fluorescence microscopy. The results were quantified using MATLAB.

### 2.5. Multiplex Supernatant Analysis

Supernatants from untreated and directly plasma-treated samples were collected and stored at −20 °C until analysis. Multiplex chemokine and cytokine analysis were performed as previously described [39]. Briefly, the supernatants were incubated with capture beads (LEGENDplex; BioLegend, Amsterdam, The Netherlands) according to the manufacturer's instructions and measured using flow cytometry (CytoFLEX S; Beckman-Coulter, Krefeld, Germany). For quantification, data analysis LEGENDplex software (BioLegend, Amsterdam, The Netherlands) was used. For each analyte, separate standard curves were calculated using fifth-degree polynomials, paying attention to each analyte's specific detection limits.

### 2.6. Statistical Analysis

Graphing and statistical analysis (*t*-test, one-way ANOVA, or two-way ANOVA) were conducted using prism 9.1 (GraphPad Software, San Diego, CA, USA).

## 3. Results

In this study, direct and indirect plasma treatment was conducted (Figure 1a). The helium plasma jet emitted several reactive species, including ozone, atomic oxygen, hydroxyl radicals, and species of the second positive system of nitrogen (Figure 1b). In liquids, these species deteriorate to more stable oxidants such as hydrogen peroxide ($H_2O_2$; Figure 1c) and nitrite ($NO_2^-$; Figure 1d) found in plasma-treated liquid but not helium gas-treated or untreated liquids. The plasma jet was engineered to be operated at about body temperature, leading to a surface heating of about 37 °C (Figure 1e). Exposure of fully supplemented cell culture medium led to a degassing effect of carbon dioxide and subsequent increase of the pH (Figure 1f), which quickly normalized after incubation in the humidified and $CO_2$-containing incubator.

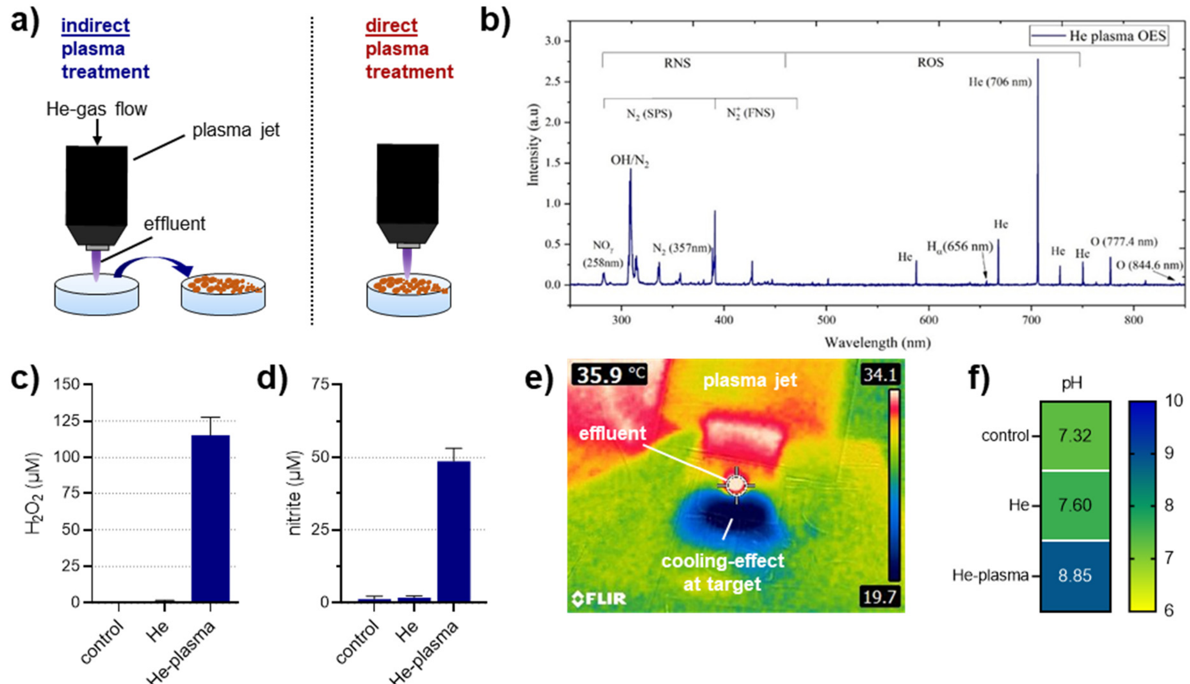


**Figure 1.** Characteristics of the gas plasma jet used in this study. (**a**) scheme of the indirect vs. the direct treatment regimens; (**b**) optical emission spectroscopy of the plasma jet; (**c**) hydrogen peroxide ($H_2O_2$) production of the gas plasma jet; (**d**) nitrite production of the gas plasma jet; (**e**) thermal camera image of the gas plasma jet; (**f**) pH of cell culture medium exposed to the gas plasma jet. Data are representative or mean and standard deviation (**c**,**d**) of three experiments.

Human patient-derived breast cancer tissues were received after informed consent and processed in the laboratory (Figure 2). After dissection of the material, it was filtered to remove material larger than 100 µm followed by filtering through a 40 µm filter. The eluent of the later step including single cells and debris was discarded, and tumor microcolonies between 40–100 µm in size were mixed with collagen and added to multiwell dishes. The collagen-tumor matrix was plasma-treated directly or indirectly (Figure 3a), followed by allowing the colonies to grow through the course of incubation over several days. During this time, the colonies extended their volume due to the proliferation of individual cancer cell clones within the colonies. Non-malignant cells such as fibroblasts and immune cells of the TME do not proliferate because contact inhibition provided by the matrix inhibits their growth. Colony quantification revealed a significant decline of tumor growth in the collagen matrix for both direct and indirect plasma treatment (Figure 3b).

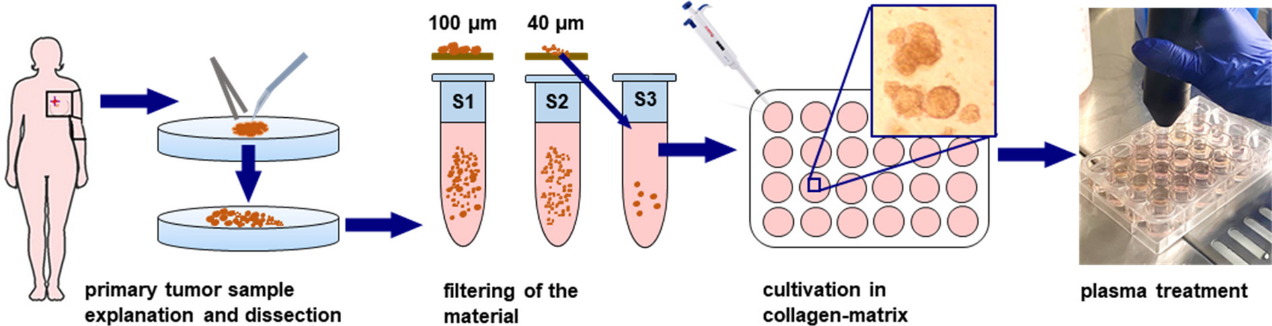


**Figure 2.** Scheme of sample preparation and treatment. Human patient-derived breast cancer tissue was surgically removed and dissected ex vivo. Subsequently, the material was filtered several times to achieve microtissues with a size < 100 µm and >40 µm, prior to mixing with collagen. The patient-derived cancer tissue-collagen-matrix was cultured in microtiter plates and exposed to the gas plasma jet in a direct or indirect fashion.

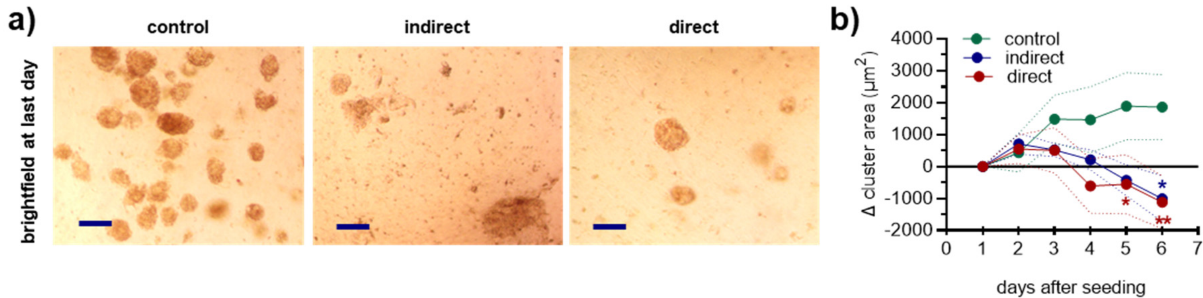


**Figure 3.** Gas plasma-treated human breast cancer tissue. (**a**) representative images of human patient-derived breast cancer tissue colonies in collagen 120 h after seeding and being either untreated or exposed to direct or indirect plasma treatment; (**b**) quantification of colonies with plasma treatment at day 2 and show mean and standard error of 7–10 patients, statistical analysis was performed using two-way ANOVA with (*) being $p < 0.05$ and (**) being $p < 0.01$. Scale bar is 250 µm.

Exposure of the microtissue to helium gas alone did not give overall different results from untreated tissue in pilot experiments and hence was not included in the study. At 24 h after plasma treatment, the collagen-tumor-matrices were stained with the live/dead fluorescence dyes AO/PI (Figure 4a). Quantitative image analysis demonstrated a significantly increased presence of dead cells for both direct and indirect plasma treatment regimens (Figure 4b), showing significantly enhanced effects of the former over the latter. Next, the microtissues were fixed, and ultrathin sections were conducted. Staining was performed against active caspase or E-cadherin together with DAPI as nuclear counterstain (Figure 4c). The direct and indirect plasma treatment significantly increased the number of caspase 3-positive cells indicative of apoptosis (Figure 4d). Again, the direct treatment showed a more substantial effect compared to the indirect treatment. For the cell surface molecule E-cadherin, which is increasingly expressed in low-metastatic breast cancer tissues [40], a significantly increased presence was found for direct and indirect plasma treatment. These data suggested the indirect and especially the direct plasma treatment procedure to reduce tumor colony formation, increase apoptosis and terminal cell death, and elevated the expression of a marker associated with a more epithelial phenotype. Chemokine and cytokine release is another critical effector in the tumor microenvironment (TME). To better understand the effects of direct plasma treatment on the inflammatory profile of our collagen breast cancer microtissues, supernatants were collected 24 h after plasma treatment, and 13 different cytokines and chemokines were quantitatively assessed in these samples. Significantly increased levels were found for interferon (IFN)-α2, interleukin (IL)-17A, IL18, and IL33. Despite the significant increases, the absolute changes were of these targets were modest, however. For IFN-γ, IL-6, IL-6, and MCP-1, significantly decreased levels were determined, which was especially pronounced for IL-6 and IL-8. These data suggested that

direct plasma treatment reduced cell viability and the expression of several cytokines and chemokines known to modulate the inflammatory profile of the TME critically.

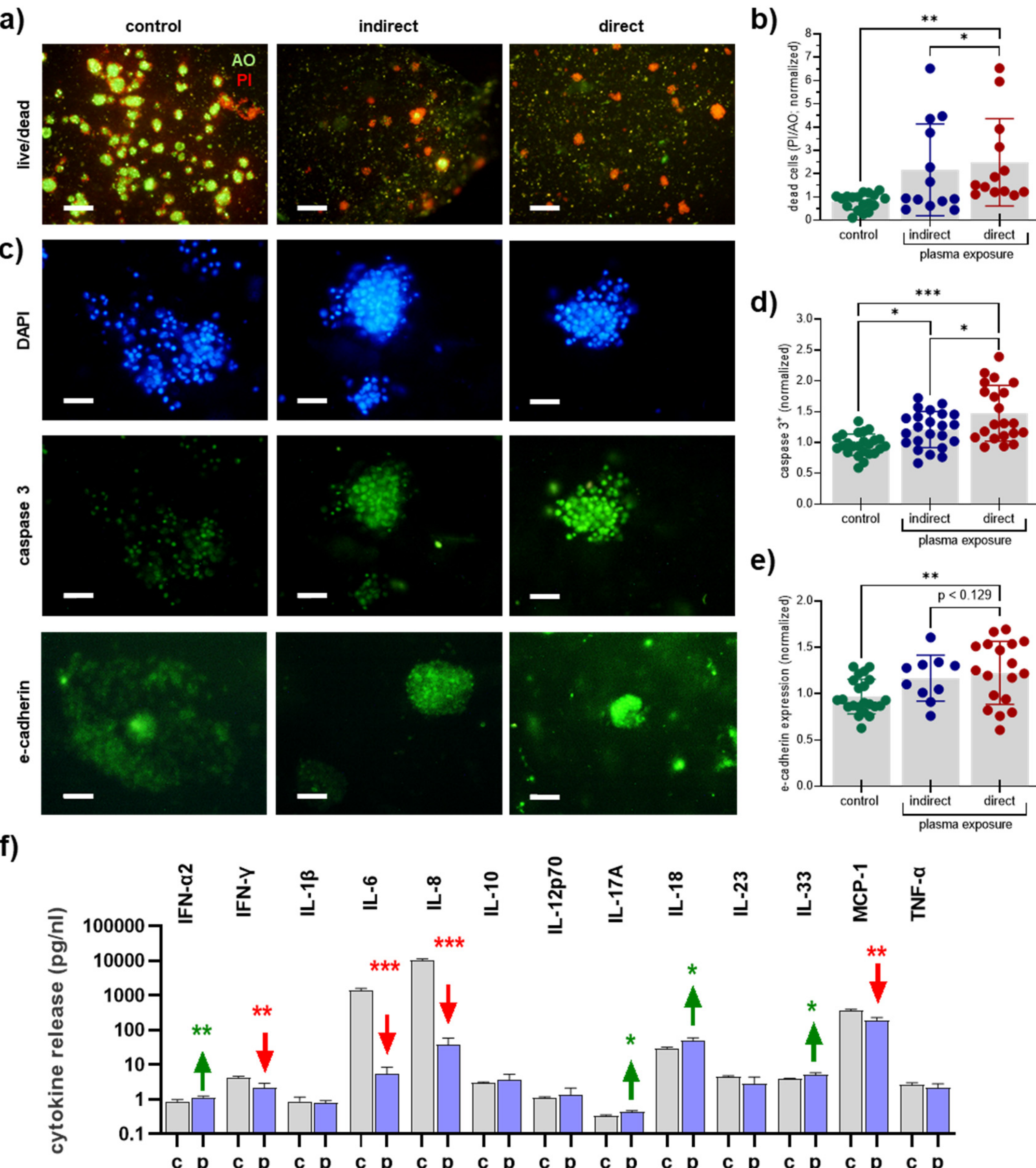


**Figure 4.** Microscopy and supernatant analysis. (**a**) representative fluorescence microscopy images of human patient-derived breast cancer tissue 24h after the second plasma treatment followed by staining with acridine orange (AO) and propidium iodide (PI); (**b**) quantification of dead cells; (**c**) representative fluorescence microscopy images of thin-sectioned patient-derived breast cancer tissues and staining with DAPI and anti-activated caspase 3 or anti E-cadherin antibodies; (**d**,**e**) quantification of caspase 3 positive cells (**d**) and E-cadherin expression (**e**) in untreated and direct or indirect plasma-treated human patient-derived breast cancer tissues; data (**b**,**d**,**e**) are mean and standard deviation of at least ten patients per group; (**f**) multiplex chemokine/cytokine analysis of supernatants from untreated and direct plasma-treated samples showing absolute levels in pg/mL of 13 targets. Statistical analysis was performed using one-way ANOVA (**b**,**d**,**e**) or *t*-tests (**f**), all with (*) being $p < 0.05$, (**) being $p < 0.01$, and (***) being $p < 0.001$. Scale bars are 200 µm (AO/PI and E-cadherin) and 100 µm (DAPI and caspase 3). c = control, *p* = plasma.

## 4. Discussion

Gas plasma treatment is a promising and novel avenue to target cancer cells, including breast tumors. Albeit an array of in vitro and in vivo studies, data on primary tumor material from patients is scarce, hampering the translational value of this treatment and technology in oncology. To this end, we here investigated 20 clinical breast cancer samples using matrix-microtissues along with the functional consequences of gas plasma exposure in this tumor model of primary patient material.

We found an increase of apoptotic cells in our collagen-microtissue-matrices after plasma treatment accompanied by an increase of the adhesion marker E-cadherin associated with a less migratory activity of tumor cells [41]. Apoptosis and cell migration are two known effects of cold plasma in cancer therapy accompanied by consequences such as growth retardation and metastasis [42]. Tumor invasion and metastasis is the leading cause of death in cancer patients, including breast cancer [43]. The metastasis process is controlled and regulated by microenvironmental and systemic processes, including intercellular adhesion molecules. Weakening cell–cell and cell–matrix connections are essential for metastasis. Numerous families of adhesion molecules, including cadherins, integrins, adhesion molecules of the immunoglobulin family, selectins, and CD44 are identified [44]. The latter has been recently suggested to be affected by plasma treatment as well [45]. According to this hypothesis, where the lack or decreased expression of these molecules increases the rate of metastasis, these molecules can be used as markers of metastasis. E-cadherin is a transmembrane glycoprotein mediating calcium-dependent intracellular adhesion and is involved in epithelial cell–cell adhesion [46]. We had previously investigated metastasis on various pancreatic cancer cell lines by examining E-cadherin, EpCam, integrin α-2, integrin α-4, and ZEB-1, and concluded that the plasma treatment reduced metastasis in cancer cells [47], which is consistent with the results of cadherin expression in this study.

Initiation of apoptosis is a frequently described process in plasma-treated cancer cells. In vitro, it was found for several cancer types, such as colon [48–50], pancreas [51–53], head and neck [54–56], leukemia [57–59], glioblastoma [60–62], and melanoma [63–65]. For stage IV malignant melanoma patient-derived samples, we also have previously found augmented apoptosis within tissues after gas plasma exposure with the kINPen jet [66]. It is understood that this is a consequence of supraphysiological amounts of ROS/RNS delivered by the plasma system [67]. Nevertheless, there is a sensitivity gradient, as we have previously reported across 35 different tumor cell lines that have individual capacities to cope with the ROS/RNS [68]. The tumor-toxic activity of some of the long-lived species is retained in cell culture medium and other types of liquids, which can be subsequently used for cancer therapy [69]. Our present findings in primary human breast cancer tissue underline this notion, albeit the plasma-treated medium—called "indirect" in this work—was slightly less active than the direct gas plasma exposure. With plasma-treated cell culture medium, it should also be kept in mind that its utilization in preclinical cancer research is of limited translational value, as these solutions have not been accredited for use in humans as certified medical products [70].

In this study, 3D tissue culture technology has been used inside collagen to monitor plasma function on the tumoral tissue growing outside the body. Gel-like substances such as agarose have been used in other studies before the monitor the plasma-mediated ROS/RNS diffusion through different matrices [71–73]. Collagen has the added value that it is part of the in vivo TME of the tumors, making our microtissue model more realistic. A critical part of the TME is the release of various chemokines and cytokines, often rendering the micromilieu more tumorigenic. For instance, IL-6 has been reported to participate in a positive feedback loop in the breast cancer TME to fuel tumor growth by promoting immunosuppression [74]. IL-6 has also been found to increase the expression of the hyaluronic acid receptor CD44 that promotes breast cancer stem cell metastasis [75]. Along similar lines, the chemokine IL-8 (CXCL8) is linked with enhanced breast cancer stem cell activity and chemoresistance [76] and promotes the growth of patient-derived breast cancer

growth in ex vivo models [77]. In our study, gas plasma exposure reduced levels of both IL-6 and IL-8 by a factor of 100, exemplifying the potency of this technology. Interestingly, we have recently identified a role of the CXCL8/HO-1 axis as critical mediators of plasma-induced cell death in leukemia cells [78]. Moreover, the chemokine MCP1 (CCL2) that was decreased with plasma treatment by a factor of two has been implemented in shaping a pro-tumorigenic TME as well [79]. More significantly changed soluble mediators were identified for the plasma treatment condition, but these were less pronounced in terms of absolute changes.

Nevertheless, gas plasma exposure was promising in elevating tumor cell death and E-cadherin expression and modulating inflammatory mediators in a favorable manner. Plasma technology is a surface treatment technology, and patients suffering from ulcerating breast cancers that are often infected and different to manage might benefit from repeated plasma exposure. The group of Philipp Wong has recently been able to demonstrate additive toxicity of plasma treatment with radiotherapy [16], which we could confirm for melanoma cells [80]. Electrochemotherapy (ECT) is another reported therapy for ulcerating breast cancer [81], and first reports have reported combined toxicity with plasma and ECT in vitro [82,83]. There is ample evidence that plasma treatment also combines with chemotherapy to promote cytotoxicity [84–89]. These considerations are both promising and guiding in advancing the therapeutic concept of adjuvant gas plasma therapy in breast cancer management.

## 5. Conclusions

Gas plasma exposure augmented cell death in three-dimensional microtissue-matrices of 20 patient-derived breast cancer tissues. These findings provide a translational value that complements several earlier and promising in vitro and in vivo studies on the tumor-toxic effects found in plasma-treated breast cancer cells. Despite the promising nature of the results, large-scale studies are needed to confirm the findings. Subsequent studies should use patient-derived cancer tissue models and clinically useful combination regimens to promote gas plasma-based cancer therapies in the future.

**Author Contributions:** Conceptualization, F.S., H.Z. and S.B.; methodology, Z.A., F.S., E.F., F.A. and S.B.; software, F.S., H.M., E.F. and S.B.; validation, Z.A. and F.S.; formal analysis, F.S.; investigation, Z.A. and F.S.; resources, F.S., B.S., H.Z. and S.B.; data curation, F.S.; writing—original draft preparation, F.S. and S.B.; writing—review and editing, S.B.; visualization, F.S., E.F. and S.B.; supervision, H.Z. and S.B.; project administration, H.Z. and S.B.; funding acquisition, H.Z. and S.B. All authors have read and agreed to the published version of the manuscript.

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**Institutional Review Board Statement:** The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Ethics Committee of Motamed Cancer Research Institute (protocol code IR.SBMU.RETECh.REC.1398.072 in April 2020).

**Informed Consent Statement:** Informed consent was obtained from all subjects involved in the study.

**Data Availability Statement:** Data are available from the corresponding author upon reasonable request.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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