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Cold plasma-mediated toxicity in cutaneous and ulcerating cancer tissues

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List of abbreviations

TME	Tumor microenvironment
CBMN	Cytokinesis-block micronucleus
MN	Micronuclei
ROS	Reactive oxygen species
SCC	Squamous cell carcinoma
BCC	Basal cell carcinoma
BC	Breast cancer
OSCC	Oral squamous cell carcinoma
OECD	Organisation for economic cooperation and development

1. Introduction

1.1 Cancer, breast cancer, and ulcerating breast tumors

Cancer refers to a group of diseases characterized by abnormal cell proliferation. When cell proliferation in an organ becomes out of control, a tumor forms^{1,2}. Breast cancer is one of the most frequently diagnosed cancers and a leading cause of cancer-related death. According to the World Health Organization, it accounts for approximately one-eighth of all new annual cancer cases worldwide. In 2020, about 2.3 million new cases of breast cancer were reported worldwide, leading to approximately 685,000 deaths³. Approved therapies such as surgery, chemotherapy, radiotherapy, hormone therapy, and immunotherapy remain the primary treatment options for breast cancer, and have significantly contributed to increasing patients' overall survival⁴. If the cancerous tumor is not controlled by these therapies, cancer cells continue to proliferate. The major challenges in this disease are the resistance of tumor cells to programmed cell death and their ability to invade host tissues^{5,6}. Early diagnosis often enables proper control and effective treatment of this disease. However, limitations such as the inability to diagnose early and a high proliferation rate of cancerous cells contribute to treatment failure, metastasis, and unfavorable outcomes^{7,8}. Another major challenge faced by cancer patients is the resistance of cancer cells to treatment. A variety of mechanisms may contribute to drug resistance and promote breast cancer metastasis. The metastasis of breast cancer cells involves a complex series of steps, including invasion, intravasation, circulation, extravasation, and colonization. Invasion requires modifications in cell-cell and cell-matrix adhesion, often accompanied by changes in cadherin family molecules associated with mesenchymal cells. During epithelial-mesenchymal transition (EMT), matrix metalloproteinase (MMP) enzymes break down the extracellular matrix, allowing cancer cells to enter the bloodstream or lymphatic vessels. Metastatic cancer indicates an advanced disease stage, suggesting that cancer cells have already broken away from the primary tumor^{9-11,12}. These mechanisms include genetic mutations, activation of different signaling pathways, EMT, increased DNA repair capacity, and the presence of cancer stem cells¹³. A secondary problem is the development of ulcerating tumors, which complicates the situation further. An ulcerative breast tumor occurs when tumor cells breach the skin's surface and cause a wound to form. This results in non-healing lesions at the site of the primary tumor or at metastatic sites such as the lymph nodes. A tumor that becomes ulcerative manifests a number of symptoms, including pain,

itching, oozing or bleeding, and infection. Multiple factors can lead to bacterial infection of these wounds, which can lead to significant complications for patients¹⁴⁻¹⁶. Ulcerating tumors behave in a similar manner to primary tumors. Consequently, any modifications in these tumors can stimulate antitumor activity and induce systemic effects in tumors located in other parts of the body¹⁷. Within cancer treatment, the abscopal effect is characterized by the induction of programmed cell death in tumor cells that have not been directly exposed to therapeutic interventions. The abscopal effect has considerable therapeutic potential, particularly when it comes to metastatic diseases. As an example, radiation therapy produces therapeutic effects when the radiation is applied to a tumor site, but the effects extend beyond the irradiated area and indirectly affect other tumors. The underlying mechanism involves complex communication between cells within the tumor microenvironment (TME) and immune cells that migrate through the body¹⁸⁻²². An important aspect of the abscopal effect is its ability to affect ulcerating tumor cells. Therefore, applying targeted local therapy for ulcerative tumors on the skin surface can yield systemic effects. It might affect tumor cells in deeper tissue layers that are otherwise inaccessible. Hence, the immune response triggered by local therapy of superficial tumors can affect cancer cells at sites distant from the treated area²³⁻²⁵.

1.2 Tumor microenvironment

A tumor represents more than just an aggregation of cancer cells. It consists of a diverse mixture of infiltrating and resident host cells, secreted factors, and extracellular matrix components. Tumor cells affect their surrounding tissues at multiple molecular, cellular, and physical levels. This provides essential support for tumor growth and progression. This complex and continually changing milieu is known as the TME²⁶. The TME composition varies between different types of tumors. It consists of immune cells, stromal cells, blood vessels, and an extracellular matrix²⁷⁻²⁹. The extracellular matrix is a highly specialized three-dimensional scaffold in which cells reside within tissues. It consists primarily of collagen, fibronectin, laminin, and elastin³⁰⁻³². In solid tumors, the extracellular matrix makes up about 60% of the mass. It is increasingly recognized that the TME is not only a passive observer but also an active promoter of cancer progression³³. A dynamic relationship develops between cancer cells and other components in the TME in the early stages of tumor development. This relationship facilitates cancer cell survival, local invasion, and metastasis to distant sites. Having a hypoxic and acidic microenvironment is one of the challenges tumor cells face. To overcome these unfavorable conditions, the TME initiates processes that promote

angiogenesis. This process helps restore sufficient oxygen and nutrients to the tumor, ensuring its survival and growth^{32,34-36}. A key component of the TME is the presence of immune cells. Tumors become infiltrated with diverse adaptive and innate immune cells that have both pro- and anti-tumorigenic effects. These include T cells, B cells, natural killer (NK) cells, macrophages, and dendritic cells, among many others. These immune cells infiltrate the tumor site and engage in complex interactions with cancer cells and other components of the environment. The balance between these opposing functions determines the overall immune response within the TME³⁷⁻³⁹.

1.3 Apoptosis and cancer therapy

Various mechanisms of programmed cell death are employed to eliminate cancer cells. One such mechanism is apoptosis, which naturally occurs in the body when cells are no longer needed. When apoptosis is initiated, cells experience noticeable changes in their physical structure and chemical composition. These changes include cell shrinkage, chromatin condensation, nuclear fragmentation, and the formation of membrane blebs. Targeting apoptotic pathways in tumor cells represents an attractive therapeutic strategy because it is not limited to a specific type of cancer^{40,41}. Apoptosis depends significantly on caspases, a family of cysteine proteases⁴². The caspase family plays a key role in programmed cell death by cleaving specific proteins. They can effectively degrade a wide range of proteins through their protease activity. Caspase-3 is recognized as the final effector of the caspase cascade. It is responsible for cleaving a wide range of downstream substrates that contribute to the morphological alterations in apoptotic cells. The activation of caspase-3 can be identified through various techniques, including immunohistochemistry employing specific antibodies targeting cleaved caspase-3^{43,44}. Apoptosis has emerged as a highly effective approach for targeting cancer⁴⁰. However, cancer cells have developed various mechanisms to evade apoptosis. One such strategy involves inhibiting caspase function. Additionally, cancer cells can disrupt apoptotic signaling pathways, further enhancing their survival. A significant evasion mechanism involves the upregulation of anti-apoptotic BCL-2 proteins while the expression of pro-apoptotic BAX proteins is reduced⁴⁵. Therefore, focusing on apoptosis induction opens up opportunities for treating a wide range of cancers^{44,46}.

1.4 Physical plasma generated reactive oxygen and nitrogen species as therapy

Physical plasma, which is known as the fourth state of matter, has gained substantial attention across diverse fields, including in medicine, due to its exceptional

properties⁴⁷. The most commonly used device for applying physical plasma in biomedical applications is the plasma jet. It is a portable device that emits a focused stream of plasma directly onto the intended target⁴⁸⁻⁵⁰. The physical plasma jet apparatus has two main components, namely the inner electrode and the outer electrode. A high-voltage electrode is typically used as the inner electrode, while a grounded electrode is used as the outer electrode. The physical plasma configuration uses dielectric barriers to prevent direct current flow between electrodes and to facilitate plasma generation. Gas or gas mixtures are ionized through a gas supply system to produce plasma. The gas can be a noble gas, such as helium or argon, or a mixture of noble gases with other gases such as oxygen or nitrogen. Plasma is formed when gas flows around electrodes and then exits through a nozzle into the surrounding atmosphere. Simultaneously, the power supply plays a crucial role in supplying the required electrical energy to initiate plasma generation.

This power supply provides a high-frequency alternating current voltage or pulsed voltage to the electrode assembly, allowing for adjustable parameters such as frequency and voltage. These adjustments effectively control the characteristics and discharge of the physical plasma. Consequently, physical plasma jets emit an elongated plasma plume from the nozzle, with the length varying based on the specific design. The plasma plume is surrounded by effluent containing a wide variety of components such as ions, electrons, reactive species, and UV photons⁵¹. Plasma medicine is founded on the generation of reactive oxygen and nitrogen species by physical plasma⁵²⁻⁵⁴. Plasma-derived reactive species include atomic oxygen ($O\cdot$), singlet oxygen (O_2), ozone (O_3), superoxide radicals ($O_2\cdot^-$), hydroxyl radicals ($OH\cdot$), hydrogen peroxide (H_2O_2), peroxy radicals ($ROO\cdot$), peroxynitrite anion ($ONOO^-$), hypochlorous acid ($HOCl$), nitric oxide (NO), nitrogen dioxide (NO_2), and nitrous oxide (N_2O). Physical plasma-induced reactive species can lower the antioxidant capacity of target cells and increase intracellular reactive species levels⁵⁵. While oxidative stress can cause imbalances and damage, controlled ROS production can have therapeutic advantages. In cancer cells, these reactive species can cause DNA damage, protein degradation, and membrane disruption^{55,56}.

Physical plasma has been tested in various types of cancers, such as melanoma, glioblastoma, and pancreatic cancer in pre-clinical models⁵⁷⁻⁶¹. Local plasma therapy primarily impacts the superficial layers of tissue. This is due to the limited penetration depth of plasma-derived reactive species and UV photons. Therefore, it can be employed in clinical trials specifically to target superficial tumors^{62,63}. Consequently, it is commonly employed for superficial tumors in clinical trials⁶⁴⁻⁶⁸. Nevertheless, the

utilization of physical plasma may facilitate the modification of the TME, offering potential benefits for ulcerative and metastatic breast cancer therapy⁶⁹. Tumor cells release various signaling factors, such as cytokines, chemokines, and growth factors. These signals attract immune cells to the tumor site. When immune cells are present in the TME, they are exposed to signals from tumor cells. This results in specific changes in their phenotype and function. These cells support tumor growth and progression rather than showing an anti-tumor response. Therefore, replacing the local immune cells that promote tumor growth can be advantageous as it can alter the TME and prompt the entry of new immune cells, providing a valuable opportunity to eliminate tumor cells^{25,70-72}.

1.5 Direct and indirect physical plasma treatment regime

When it comes to biological applications, direct and indirect physical plasma treatment approaches are commonly utilized⁷³. In the case of direct physical plasma treatment, plasma sources are directly applied to cells or tissues for treatment. This approach involves short and long reactive species, radiation (ultraviolet, visible light, near-infrared radiation), and electromagnetic fields, which can influence the biological target. Reactive species are known to be the most effective among them⁷⁴. Short-lived reactive species are eliminated very quickly, but they play a crucial role in inducing cell death in cancer cells^{75,76}. Indirect physical plasma treatment involves exposing a biological substance, such as cell culture medium or another biological solution, to physical plasma, resulting in the generation of long-lived reactive species such as hydrogen peroxide (H_2O_2), nitrite (NO_2) and nitrate (NO_3)^{55,77}. This method focuses exclusively on delivering long-life reactive species to cancer cells. Both direct and indirect physical plasma treatments have their own advantages and limitations in biological applications⁷⁸. Reactive species can be delivered to a tumor in a more targeted and efficient manner through direct plasma treatment. However, indirect exposure is more feasible when the tumor is inside the patient's body. Studies show that direct plasma treatment is more toxic than indirect treatment. However, indirect plasma treatment can be more widely used in clinical applications with fewer side effects^{79,80}. Both direct physical plasma and plasma-treated media have shown effectiveness in treating various cancer types, including brain^{81,82}, lungs^{83,84}, gastric⁸⁵, liver⁸⁶, pancreas^{87,88}, colon^{89,90}, and skin⁷⁸.

1.6 Physical plasma-mediated toxicity in cutaneous and ulcerating cancer tissues

One challenge in applying physical plasma for tissue treatment is its limited penetration depth. Physical plasma therapy primarily affects the superficial layers of tissue due to the limited penetration depth of plasma-derived ROS. It is often used for superficial tumors or wound healing applications^{63,91,92}. However, researchers are actively exploring strategies to overcome this limitation and enhance the efficacy of physical plasma therapy. One approach being investigated is combining physical plasma-treated media with conventional treatments. By using physical plasma-treated media in conjunction with existing clinical treatments, such as chemotherapy, radiation therapy, or immunotherapy, synergistic effects can be achieved. This may also increase the efficacy of conventional therapies, allowing for reduced dosing and fewer associated side effects^{78,93}. The local treatment of superficial micrometastases or ulcers on the skin can represent another promising application of physical plasma therapy. Preclinical studies have demonstrated the potential of physical plasma in enhancing the immunogenicity of cancer cells and improving the efficacy of immunotherapies⁹⁴⁻⁹⁸. Physical plasma treatment has been shown to increase the expression of immunogenic cell death markers, such as calreticulin, heat shock proteins, and high mobility group box 1 protein, in cancer cells^{99,100}. This enhances their recognition by immune cells and promotes the activation of an antitumor immune response^{101,102}. Furthermore, our research on BC tumors in mice found that physical plasma not only induces toxicity and inflammation in the irradiated tumor but also reduces tumor growth and promotes immune cell infiltration in non-irradiated tumors through systemic immune responses of the host⁹⁹. Hence, physical plasma can be precisely administered to the skin's surface, enabling the targeted eradication of cancer cells within these visible lesions. Consequently, the immune system can recognize and eliminate cancer cells at distant sites, potentially providing a systemic antitumor effect. As a result, patients with ulcerating and infected breast tumors may benefit from physical plasma therapy, including wound disinfection and both local and systemic anticancer effects. Once physical plasma-derived ROS have entered the ulcerating tumor on the skin's surface, the tumor cells sense the oxidative stress and respond via specific signaling pathways, leading to altered transcriptional and translational profiles. This induction of systemic effects can impact tumor cells located elsewhere in the body.

2. Goals of the study

Despite the emergence of numerous innovative and progressive treatment approaches in recent decades, cancer continues to be a deadly disease. Through this comprehensive research, we aimed to determine how cutaneous and ulcerative cancers can benefit from physical plasma treatment. The study also addressed the following research questions:

- a) Can plasma induce cancer cell death in vitro, in vivo, and in patient-derived cancer tissue ex vivo?
- b) What are the inflammatory and immunological consequences of plasma treatment in cancer cells?
- c) Is plasma treatment associated with genotoxic effects such as micronucleus formation?

3. Methods

In this thesis, we employed different approaches and combined various techniques to investigate the potential toxicity of physical plasma in cutaneous and ulcerating cancer tissues. All methods used in this research are described in detail in the corresponding publications. However, we present a brief summary of these methodologies below:

3.1 In vitro study

We utilized both 2D and 3D cell cultures to explore the effects of physical plasma treatment in vitro. The following cell lines were used in this study: murine breast cancer (4T1), human breast cancer (MCF-7, MDA-MB-231, T-47D), human squamous cell carcinoma (SCC-4, SCC-9, SCC-13), human epidermoid carcinoma (A431), human melanoma (A375), and the non-malignant human keratinocyte cell line HaCaT.

3.2 In vivo study

To investigate the effects of physical plasma treatment in an in vivo setting, we injected breast cancer cells into both flanks of mice to establish the mouse tumor model. The animal experiments conducted in this study received ethical approval from the Shahid Beheshti University of Medical Sciences Animal Ethics Committee (approval number IR.SBMU.RETECH.REC.1397.1212) and were carried out at the Shahid Beheshti University, Tehran, Iran. The animals were kept under standard laboratory conditions, with a room temperature of 23°C, and provided with unlimited access to food and water. Their environment was controlled with a 12-hour light-dark cycle. All animal procedures were carried out under ketamine-xylazine anaesthesia.

3.3 Ex vivo studies

Ex vivo experiments refer to investigations or measurements conducted in or on tissue from an organism in an external environment with minimal alteration¹⁰³.

3.4 Tissue culture regimen in collagen

To mimic the nature of tissues and organs within the human body and better represent the complexity of the TME, a type 1 collagen matrix was used as a scaffold to culture patient-derived tissues. The ex vivo study on the patient samples was ethically approved by the Laser Application in Medical Sciences Research Center of Shahid Beheshti University of Medical Sciences Ethics Committee (approval number IR.SBMU.RETECh.REC.1398.072). For the clinical aspect of the study, twenty breast cancer patients at the Motamed Cancer Institute willingly participated after providing

informed consent, adhering to the principles outlined in the Declaration of Helsinki guidelines.

3.5 Patient tumor punch biopsies

To ensure the accuracy of our findings from the in vitro and in vivo experiments, we decided to use patient biopsy samples without any manipulation. These biopsy samples represented the actual tumors in the patients, making our research more clinically important. The research involving breast and tongue samples received approval from the ethics committee of the Motamed Cancer Institute (approval number: IR.ACECR.IBCRC.REC.1400.007) and the study on basal and cutaneous squamous cell carcinoma tissues was approved by the University of Duisburg-Essen's ethical committee (Institutional Review Board protocol number 12-4961-BO). Before participating in the study, each patient provided written informed consent.

3.6 Genotoxicity and the cytokinesis-block micronucleus assay

The cytokinesis-block micronucleus (CBMN) assay is considered one of the methods to determine the potential genotoxic effects of the treatment on mammalian cells. The existing literature on genotoxic properties often presents conflicting findings, primarily due to technical limitations. Genotoxicity refers to a substance's ability to damage genetic information in cells potentially resulting in genetic mutations and chromosomal abnormalities. Genotoxic substances can either directly bind to DNA or act indirectly by affecting enzymes involved in DNA replication¹⁰⁴⁻¹⁰⁶. The CBMN assay is a mutagenicity testing system to identify chemicals that cause the formation of small DNA aggregates known as micronuclei (MN) in the cytoplasm of cells during the interphase¹⁰⁷. These MN can originate from chromosome fragments of the primary nucleus that fail to migrate to the poles during the anaphase of cell division. The method involves using cytochalasin B to inhibit actin filaments during cytokinesis. It provides an effective way to differentiate between cells that did not divide and those that completed nuclear division during the exposure to a genotoxic agent^{108,109}. Any medical treatment must satisfy both efficacy and safety standards. Multiple studies were conducted following Organisation for Economic Co-operation and Development (OECD) protocols to investigate the genotoxicity of physical plasma exposure on cells. Assessing the genotoxicity of physical plasma involves determining whether physical plasma exposure can cause DNA damage. The findings from these studies consistently demonstrated the absence of any genotoxic effects across various types of physical plasma devices¹¹⁰⁻¹¹³.

3.7 High content imaging and algorithm-based image analysis

In our study, we used high-content imaging, which is an advanced microscopy technique that captures multiple cellular parameters information. The algorithm-based image analysis system enabled us to extract quantitative and qualitative information from the images.

3.8 Flow cytometry

In our study, we employed flow cytometry as a quick and efficient method to analyze individual cells. We labeled the cells with monoclonal antibodies targeting 8-OHdG (8-Hydroxy-2'-deoxyguanosine), 3-NT (3-nitrotyrosine), and γ H2AX (phosphorylated histone 2A family member X) fluorescent markers. This allowed us to examine the cellular response to oxidative and nitrosative stress, as well as the DNA damage and repair mechanisms within the cells. Cell cycle status was also checked using this technique.

3.9 Multiplex cytokine analysis

To detect inflammatory mediators in the cells or tissues, we employed multiplex analysis to simultaneously measure the levels of multiple cytokines.

3.10 Tissue and cell staining

We used a variety of staining techniques, including immunofluorescence and TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labeling), to detect specific molecular targets. A TUNEL assay is a technique commonly used to detect apoptotic cells. This assay allows for the visualization of DNA fragmentation, a hallmark of apoptosis. In this assay, the free 3'-OH ends of DNA fragments generated during apoptosis are labelled with modified nucleotides, usually incorporating fluorescein or other fluorophores. The labeled DNA fragments can be visualized and quantified using high-content imaging and algorithm-based image analysis system¹¹⁴.

4. Result and discussion

4.1 Plasma treatment-induced breast cancer growth reduction and altered immune profile in vivo

Through an extensive literature review, we identified a series of proof-of-concept studies conducted on various tumor types in patients and in animal models of breast cancer. With this focus, we aimed to enhance our understanding of the potential effects of physical plasma on breast cancer treatment (**Article I**). The review highlights the effectiveness of physical plasma technology in targeting breast cancer and provides a comprehensive overview of device function, mechanisms, and benefits. It focuses on the impact of ROS, signaling pathways, and explore their influence on breast cancer cells. Additionally, the article discusses the diverse mechanisms of cell death, the potential of combination therapies, and the immunological effects resulting from exposure to physical plasma in breast cancer cells. Although physical plasma therapy generally targets the superficial layers of tissue due to the limited penetration depth of the reactive species, it still has therapeutic potential. For the first time, we proposed that patients with ulcerating and infected breast tumors could still benefit from physical plasma therapy. This is because the tumors in these particular patients have ruptured the layers of the skin, which has enabled tumor cells to infiltrate the skin surface surrounding the breast. This accessibility allows physical plasma to enhance tumor immunogenicity effectively. Consequently, we conclude that utilizing physical plasma-induced ROS could be an extremely valuable complementary treatment modality.

After conducting a comprehensive analysis of various methodologies and scholarly articles relevant to the utilization of physical plasma in breast cancer, our preliminary objective was to investigate the effects of physical plasma exposure on the proliferation of 2D and 3D in vitro tumor models. For this purpose, breast cancer cell lines MCF-7 and MDA-MB were cultured and exposed to physical plasma (**Article II**). The treatment resulted in a significant increase in terminal cell death for both MCF-7 and MDA-MB-231 cells, accompanied by a subsequent decrease in cellular metabolic activity. However, MDA-MB cells exhibited higher terminal cell death than MCF-7 cells, while metabolic activity reduction was similar between the two cell lines at the specified time points. To further verify the validity of our findings, we used a more realistic in vitro model by generating 3D tumor spheroids within ultra-low attachment plates and irradiating them to physical plasma. The 3D tumor spheroid models of the MDA-MB cells exhibited a significant increase in terminal cell death, accompanied by a notable increase in intracellular oxidation. Conversely, the effects observed in the MCF-7 cells

were comparatively moderate. These differences in cytotoxic effects between two cell lines may be attributed to the variations in the genetic, antioxidant enzyme levels, DNA repair mechanisms, cellular adaptations, and ROS-scavenging molecules involved in cells. Several studies reported that physical plasma inhibits growth of MCF-7 and MDA-MB-231 cells by activating multiple cell death pathways, including apoptosis, autophagy, and necrosis. Each of these cell death types exhibits specific morphological features. One important pathway caused by physical plasma is apoptosis. This effect is achieved through the generation of ROS, which cause damage to the cancer cells and reduce their proliferation¹¹⁵⁻¹¹⁷. Apoptosis induced by physical plasma is mediated through several signaling pathways. Activation of p53 is one of the key pathways involved in this process. This causes BAX to be expressed, which is a proapoptotic protein. When BAX is activated, it blocks BCL2 (an anti-apoptosis protein) and promotes mitochondrial membrane permeabilization^{118,119}. The permeabilization results in the release of cytochrome c from the mitochondria. The release of cytochrome c triggers the formation of the apoptosome with apoptotic protease-activating factor 1 (Apaf-1). This complex formation activates caspase-9, an enzyme critical for initiating apoptosis. Caspase-9 then activates caspases 3 and 7, which cleave various cellular substrates and eventually lead to apoptosis^{120,121}. The MAPK pathway, which includes various subfamilies such as ERK, JNK, and p38 MAPK, is involved in cell survival, proliferation, and apoptosis. It has been reported that JNKs are activated in response to oxidative stress induced by physical plasma. Upon activation, JNK is phosphorylated (JNK-P) by upstream kinases and translocates to the nucleus, where it regulates transcription of apoptosis genes¹²².

Depending on cellular conditions and signals, MAPK pathway activation can also activate JNK or ERK. The interplay between MAPK signaling and ERK1/2 phosphorylation is reported after cells are exposed to physical plasma, resulting in the activation of caspases in cancer cells¹²³. Furthermore, Xia et al. reported that physical plasma treatment enhanced intracellular NO levels in cancer cells, leading to MAPK p38 activation as a subfamily of MAPK. Thereafter, p38 MAPK regulates various downstream targets involved in apoptosis¹²⁴.

Signal transducer and activator of transcription 3 (STAT3) is involved in proliferation, survival, migration, invasion, and angiogenesis. Physical plasma therapy demonstrated the initiation of the apoptotic pathway by suppressing phosphorylation in the AMPK or STAT3 pathways, which had an inhibitory influence on cancer cell proliferation¹²⁵. Subsequently, our research was directed towards examining the capacity of physical plasma irradiation to elicit cytotoxic responses not only in vitro but

also in xenograft tumor models in vivo (Article III). Additionally, we were curious about the possible immune-mediated effects of the treatment on tumors distant from the area exposed to physical plasma. To this end, tumor cells were injected into both flanks of the mice. The left tumor was treated with physical plasma irradiation while the right tumor remained untreated. We found that plasma therapy prolonged the survival rate and overall lifespan of tumor-bearing mice. Furthermore, both the treated and untreated tumors on the opposite flank were significantly reduced in growth and weight. We performed immunofluorescent staining of the tumor tissues and found apoptotic cell death and immune cell infiltration, particularly CD4⁺ T-helper cells and CD11c⁺ dendritic cells, in both treated and untreated tumors on the opposite flank. Based on our experimental model, the data suggest that physical plasma irradiation induces an abscopal effect, meaning that not only does the local therapy lead to inhibited growth of the treated tumor, but it also enhances immune cell infiltration in the treated tumor and untreated distant tumor lesions. In summary, this technology not only restricted tumor growth in 2D and 3D in vitro and in vivo platforms but also demonstrated an immunological aspect. Several studies showed that plasma therapy has a significant impact on immune cell activation and antitumor immunity. The immune system can both promote and restrict cancer progression through various mechanisms. Nguyen et al. showed that physical plasma treatment induces immunogenic cancer cell death (ICD) by triggering tumor cells to release damage-associated molecular patterns (DAMPs) such as adenosine triphosphate (ATP), high-mobility group box 1 (HMGB1), calreticulin (CRT), and heat shock protein 90 (HSP90)¹²⁶. DAMPs facilitate immune cell recruitment, promote dendritic cell (DC) maturation, and antigen presentation, and result in antitumor T-cell activation. In the activation of T-cell responses, DCs play a crucial role as antigen-presenting cells which present antigens to the T-cells. DCs phagocytose and process tumor antigens, and then present these antigens to CD4⁺ and CD8⁺ T cells. Activation of T-cell responses is crucial for the direct killing of cancer cells and the generation of long-lived memory cells in the TME. Several studies demonstrated that physical plasma treatment promotes DC maturation and T-cell activation, including CD8⁺ cytotoxic T-cells^{25,125,127,128}.

Moreover, several studies showed that physical plasma treatment increases the immunogenicity of tumor cells by upregulating cell surface molecules and enhancing the expression of immune cell markers^{129,130}. Macrophages are another crucial immune cell in the TME and can polarize into different phenotypes, M1 and M2¹³¹. M2 macrophages support tumor growth, while M1 macrophages can control tumor progression. It has been demonstrated that exposure to physical plasma can induce

polarization of monocyte-derived macrophages towards an M1 phenotype, leading to enhanced cytotoxic activity against tumor cells in the TME. Furthermore, physical plasma treatment promotes the differentiation of monocytes into macrophage-like cells when co-cultured with tumor cells^{38,132-134}.

4.2 Effects of plasma treatment on the tumor microenvironment

Although 3D cell growth has many benefits, such as spatial communication, a physiologically relevant environment, cell-cell interactions, and molecular signaling, they cannot fully replicate the complexity of the TME with 3D cultures of homogeneous cell lines. The TME involves dynamic interactions between cells, the extracellular matrix, and the presence of immune and angiogenic cells, all of which play pivotal roles in tumor growth in vivo. Unfortunately, these complex components cannot be faithfully recapitulated in cultured 3D monocellular systems. To overcome these limitations, we employed a promising approach that involves the direct culture of tissue extracted from the body. This methodology incorporates immune cells, angiogenesis, and growth within microgels, which more realistically mimics the natural conditions found in tissues and organs within the human body. An extensive investigation was conducted to explore the implications and potential advantages of this innovative approach in patient-derived breast cancer microtissues (Article IV). The study aimed to address the gap between simplistic monocellular cultures and the complex in vivo TME by employing tissue culture techniques that incorporated multiple cell types. A key objective of the research was to ascertain whether the effects observed in 2D and 3D in vitro models and mouse tumors would also be replicated in human breast tumors. To investigate this, the study employed cultured tissue to evaluate the impact of physical plasma treatment on engrafted microtissues within a collagen matrix. Primary breast cancer tissues were obtained from 20 patients who had not received any prior treatment before surgery. The tumor samples were handled in a sterile environment within a laminar flow hood and tumor microtissues ranging from 40-100 μm in size were produced. The microtissues were combined with type I collagen, and the collagen-embedded tumors were treated directly and indirectly with physical plasma. Direct plasma therapy involves administering physical plasma directly to collagen-encrusted microtissues. By indirect treatment, complete cell culture media are treated with physical plasma and subsequently applied over the collagen-encapsulated microtissue. Both direct and indirect plasma treatments reduced tumor growth and increased programmed cell death in the microtissues. Moreover, both treatment approaches also significantly increased E-cadherin on the cell surface. Low expression

of E-cadherin is closely associated with EMT. During EMT, E-cadherin is downregulated in mesenchymal cells, while N-cadherin expression is upregulated to facilitate invasion. When an epithelial cell transforms into a mesenchymal cell, it uses MMP enzymes to break down the matrix surrounding it. Several studies showed that E-cadherin down-regulation is linked to aggressive and advanced tumor stages in various cancers such as lung¹³⁵, pancreatic¹³⁶, colorectal¹³⁷, and breast cancer¹³⁸. E-cadherin analysis in the 3D microtissue system suggests that plasma irradiation may suppress tumor cell migration while strengthening cell-cell adhesion^{139,140}.

As expected, direct treatment showed a more pronounced effect than indirect treatment. For direct treatment, there are at least three factors that can impact cancer cells such as short-lived reactive species, long-lived reactive species, and physical factors. In contrast, for indirect physical plasma treatment, only the long-lived reactive species need to be considered. Therefore, either short-lived reactive species or physical factors must be responsible for the stronger anti-cancer effect observed in direct physical plasma treatment^{80,141}. This means that the in vitro plasma treatment cannot be simply explained as the treatment with a specific long-lived reactive species, like H₂O₂, NO₂⁻, NO₃⁻, or ONOO⁻¹⁴¹⁻¹⁴⁴.

A comprehensive assessment of the inflammatory profile of breast cancer microtissues was performed through the multiplex analysis of chemokines and cytokines. The results revealed notable activity of several key cytokines, including interferon (IFN)- α 2, interleukin (IL)-17A, IL-18, IL-33, IFN- γ , IL-6, IL-8, and monocyte chemoattractant protein-1 (MCP)-1. The presence and activity of these secreted factors with chemotactic properties can signify an anti-tumor response in an in vivo setting.

4.3 Analysis of micronuclei formation potential in plasma-treated skin cells

As with any emerging medical technology, continuous research and safety evaluation are essential to ensure the safety of physical plasma devices. Preliminary studies and clinical trials have shown that although physical plasma therapy can trigger an inflammatory response, its side effects are minimal compared to conventional cancer treatments. Safety evaluation must continue to refine the protocols and guidelines for effective use. The assessment of genotoxicity plays a critical role in ensuring the safety of medical devices and therapeutic treatments. It refers to the capacity of a substance or treatment to cause damage to genetic material, resulting in mutations or other harmful effects. In contrast, automated analysis of MN formation in non-malignant HaCaT skin cells has been unavailable due to the clustered growth

pattern exhibited by these cells, particularly after cytochalasin B treatment. These challenges arise from difficulties in accurately detecting individual cells and nuclei through image segmentation. To overcome these challenges, we developed an innovative approach to evaluate genotoxicity based on the OECD-CBMN protocol in vitro using high-content imaging and algorithm-driven quantitative image analysis to minimize technical artifacts (Article IV). In these protocols, cells were seeded and treated with standard positive and negative controls for genotoxicity assessment. Actin polymerization and cytokinesis were inhibited by cytochalasin B treatment, to allow the identification of MN in cells that had undergone one cell division post-treatment. The treatment with cytochalasin B resulted in the deformation of the cell, making it challenging for the software to determine cell type or morphology. This clustering effect is attributed to cytochalasin B's reported role in cluster formation and cell malformation. To resolve this, we treated the cells with accutase enzyme before fixation. Microscopic examination during accutase treatment ensured cell separation and prevented detachment from the plate. Any possible harmful reactions or detachment during accutase therapy were measured. Although the software recognized the cells, the MN were not easily visible on the 2D surface due to cell compression after accutase treatment. Therefore, we incubated the cells for 1 hour after the accutase treatment to ensure they flattened on a 2D screen. In this study, we easily visualized changes in the number of bi-nucleate cells with MN using the high content imaging system and quantified them using the Harmony software. Images were segmented into nuclei and cytoplasm, and parameters such as nuclear area, roundness, and width-to-length ratio were measured. Consequently, we could classify the cells as mononucleate, binucleate, or multinucleate. To specifically detect binucleate cells with MN, we applied a function to identify small, round DAPI objects. We then selected only MNs close to binucleated cells with specific intensity.

Using the well-established CBMN assay, we assessed the genotoxic effects of physical plasma on non-malignant HaCaT cells (Article V). Our results indicated that physical plasma treatment has no effect on MN formation in human HaCaT keratinocytes. The absence of genotoxic effects observed in non-malignant cells suggests the potential safety of physical plasma treatment. This research provides a foundation for future research and development of physical plasma-based therapies, ensuring their safe implementation in clinical and biomedical settings. Our findings is in perfect agreement with established data, which reported that there was no evidence of genotoxicity in cells exposed to physical plasma^{110,112,145,146}.

4.4 Plasma treatment increased apoptosis in ex vivo study

We aimed to expand our research by exploring whether similar toxic effects can be observed in patient-derived tissues, without any additional manipulation. We also assessed inflammatory markers to detect potential changes. We therefore examined the effects of cold physical plasma on ex vivo-generated punch biopsies from 20 cancer patients without prior treatment (Article VI). Ex vivo tumor tissue biopsy samples were generated and exposed to cold physical plasma. The tissue was sectioned, stained, and imaged to quantify apoptotic cells and immune cell status. Through our quantitative analysis, we observed a significant increase in apoptotic cell death within the BC samples that underwent treatment with cold physical plasma. This effect was particularly prominent at the boundaries of the tissue where the treatment was administered. Treatment also significantly reduced the population of tumor-associated macrophages in BC tissue samples. However, exposure to physical plasma did not produce a substantial effect on the total count of leukocytes or regulatory T cells within the BC tumor samples. We next included another cancer type called oral squamous cell carcinoma (OSCC). Interestingly, the application of physical plasma demonstrated a similar toxic effect when applied to OSCC samples. Nonetheless, in agreement with the BC samples, physical plasma exposure did not elicit any significant effects on the total count of leukocytes in the OSCC tumor samples. Although ex vivo systems present challenges, such as maintaining isolated tissues and their limited viability outside the body, they offer unique advantages, such as accessibility, use of real patient samples, and a controlled environment. Tumor cells can reprogram immune cells within the TME. As a result, these immune cells become tumor-suppressive cells and instead of attacking the tumor cells, adopt an anti-inflammatory phenotype. This immune suppression allows the tumor to evade immune surveillance and facilitates its ability to grow and metastasize. Targeting the local immune cells that protect the tumor cells not only disrupts their tumor-promoting effects but also establishes a fresh and favorable environment for the infiltration of new immune cells. To the best of our knowledge, this is the first time such an investigation has been undertaken with patient-derived samples. Our findings are consistent with earlier research focused on cancer cell lines derived from these cancer types. Almeida-Ferreira et al. investigated the effects of physical plasma on several adherent human cancer cell lines, including BC. They reported that plasma reduced metabolic activity and induced apoptotic and oxidative effects in human breast cancer cells in a time-dependent manner^{147,148}. Pansare et al. conducted a study investigating the effects of different plasma doses on BC and SCC cells. Their findings revealed that cell viability

assay and Raman spectroscopy demonstrated a potent effect of the plasma jet on both SCC and BC cells¹⁴⁹.

Due to the limited penetration of physical plasma, clinical trials are restricted to superficial tumors. We therefore investigated whether its toxic effects extend to dermatological tumors. We furthered our study by investigating the patient-driven ex vivo model in SCC and BCC. This study is detailed in Article (VI). Briefly, three malignant and one non-malignant skin cell lines were exposed to varying doses of physical plasma irradiation. When compared to skin cancer cell lines, non-malignant HaCaT keratinocytes did not show a significant reduction in metabolic activity when exposed to physical plasma. However, exposure to physical plasma resulted in similar outcomes by inducing terminal cell death in all the cell lines examined. Similar toxic effects of physical plasma can arise from genetic variations, cell culture conditions, and other factors that may influence cellular behavior when using normal and cancer cells from different sources. Obtaining cells from the same sources allows for a more accurate assessment of the selectivity and efficacy of physical plasma treatment on cancer cells compared to normal cells. There have been several studies that demonstrate plasma's selective ability to target SCC cells over healthy cells^{150,151}. Lee et al. found degradation and dysfunction of epidermal growth factor receptors (EGFRs) in SCC overexpressing EGFRs but not in normal human fibroblasts treated with CAP¹⁵². Similarly, Pasqual-Melo et al. confirmed plasma's selectivity in vivo, and found that plasma limited the progression of SCC without compromising non-SCC skin, with the expression of antioxidant transcription factor Nrf2 reduced in the lesion and increased in the non-lesion tissue¹⁵³.

After being exposed to physical plasma, the G1/G2 ratios of tumor cell lines were calculated. The cells were unable to progress through the cell cycle, resulting in a relative accumulation of cells in the G2 phase. Oxidative stress contributes to plasma-induced cancer cell death. This implies that the presence of reactive species in the physical plasma induces a state of oxidative stress within the cells. Furthermore, flow cytometry analysis suggested the potential occurrence of cell death resulting from oxidative stress.

In the following, SCC and BCC skin cancer samples were tested ex vivo, similar to BC and OSCC samples. The results revealed that exposure to physical plasma led to increased levels of apoptosis in BCC and SCC samples, indicating its cytotoxic effects on tumor cells. Changes in immune cell expression suggest that plasma treatment might affect the immune profile, despite the fact that our ex vivo model does not fully reflect realistic immunodynamics, including new leukocyte extravasation and

infiltration. Nevertheless, our data suggest that physical plasma treatment may create a favorable environment for immune cell infiltration and activation. This could potentially enhance the therapeutic response in SCC and BCC samples. The release of chemokines, cytokines, and growth factors was investigated to gain a deeper understanding of physical plasma effects on TME. The decrease in chemokine ligand (CCL) 5, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-1 β levels in BCC samples indicates the potential for an inflammation and immunomodulatory role of physical plasma treatment.

Additionally, the significant elevation in granzyme A and IL-17A release suggests a favorable prognosis in BCC samples following physical plasma treatment. In SCC samples, we found that physical plasma treatment caused the upregulation of CCL5 and GM-CSF. This may suggest the potential recruitment of immune cells to the site of treatment in an in vivo regime. Additionally, platelet-derived growth factor (PDGF)-aa was reduced in SCC samples, indicating a potential suppression of growth factors that promote cell proliferation. This reduction in PDGF-AA may contribute to the overall anti-tumor effects of physical plasma treatment in SCC. Yang et al. demonstrated that plasma treatment of phosphate-buffered saline (PBS) and Dulbecco's modified Eagle's Medium (DMEM) could decrease cell viability and induce cell apoptosis in BCC cells in vitro. Through next-generation RNA sequencing, the researchers observed an upregulation of MAPK, TNF, and IL-17 signaling pathways, which appeared to be closely associated with the apoptosis induced by the plasma treatment. This validates the outcomes and discoveries we have obtained in our own research¹⁵⁴. Moreover, Kang et al. showed that nitrogen plasma exhibited the most potent inhibition of SCC cell migration and invasion, primarily achieved by downregulating the expression of focal adhesion kinase (FAK) and MMPs (matrix metalloproteinases)¹⁵⁵. Overall, the findings suggested that physical plasma treatment induces toxicity in patient-derived skin samples through ex vivo investigation, while exhibiting immunomodulatory effects. The treatment results in a decrease in certain pro-inflammatory molecules and an increase in molecules associated with enhanced cytotoxic activity. These effects likely contribute to the therapeutic efficacy of plasma in both skin cancer types.

In conclusion, our findings strongly indicated that the treatment of patient-derived tumor tissue with physical plasma leads to the induction of programmed cell death in an ex vivo setting. Moreover, our study highlights the presence of both toxic effects and specific modifications in the inflammatory profile, notably changes in immune cell composition. The decision to use different physical plasma sources in this thesis

stemmed from the need to work within the constraints we faced during sample collection, which made device transfer unfeasible. However, we ensured that all physical plasma jets utilized in our study had optical emission spectrum profiles consistent with the patterns observed in the kINPen device. This adjustment allowed us to maintain consistency and effectively replicate the key spectral characteristics of the kINPen in our experimental setup.

5. Summary

Breast cancer is the most frequently diagnosed cancer worldwide. The high annual number of people dying from breast cancer and experiencing various therapeutic side effects indicates that more research is needed to find alternative or complementary therapies. Physical plasma technology has recently gained attention as a potential anticancer therapy. Physical plasma generates reactive oxygen and nitrogen species (ROS/RNS), inducing oxidative stress and modulating cellular signaling pathways. Through an extensive literature review, we identified studies that explored the potential cytotoxic effects of physical plasma on breast cancer. Our review paper consolidated existing findings to understand potential effects of plasma on breast cancer and highlighted the effectiveness of physical plasma in targeting breast cancer, and its mechanisms of action^{156,157}. In this thesis, we build upon existing research to examine how physical plasma can be used to treat ulcerative breast cancer. We investigated whether local therapy of ulcerating breast cancer tumors effectively targets both tumor cells and tumor-promoting immune cells to enhance antitumor responses. This study employs physical plasma to induce cytotoxicity in cutaneous and ulcerating breast cancer tissues, modulate the inflammatory in the TME, and explore the potential of this strategy to enhance various cancer therapy outcomes. In order to accomplish our research objectives, we implemented a comprehensive experimental strategy using multiple tumor models. These models provided representative platforms that replicated breast cancer growth complexities. This allowed us to examine the effects of physical plasma exposure in a controlled environment. Physical plasma effects were evaluated in 2D and 3D in vitro breast cancer models, in vivo mouse xenograft models, patient-derived human breast cancer microtissues, and ex vivo breast and dermatology tumor biopsies. Across these systems, physical plasma treatment consistently reduced tumor growth and metabolic activity and induced programmed cell death. In vivo studies further demonstrated reduced growth of both treated and untreated distant tumors, indicating the induction of systemic and immune-mediated abscopal effects. Furthermore, physical plasma treatment significantly changed the inflammatory profile of the TME, modulated cytokine and chemokine secretion, and increased markers associated with immunogenic cell death. Finally, the safety of the plasma jet was evaluated using self-developed automated methods following OECD genotoxicity guidelines. Importantly, plasma treatment did not induce micronucleus formation in non-malignant keratinocytes and showed no genotoxic effects.

In conclusion, this thesis support physical plasma as a safe and effective complementary therapeutic approach for ulcerating and superficial cancers and provide a strong experimental basis for future clinical translation and therapy development.

7 References

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REVIEW

The potential of gas plasma technology for targeting breast cancer

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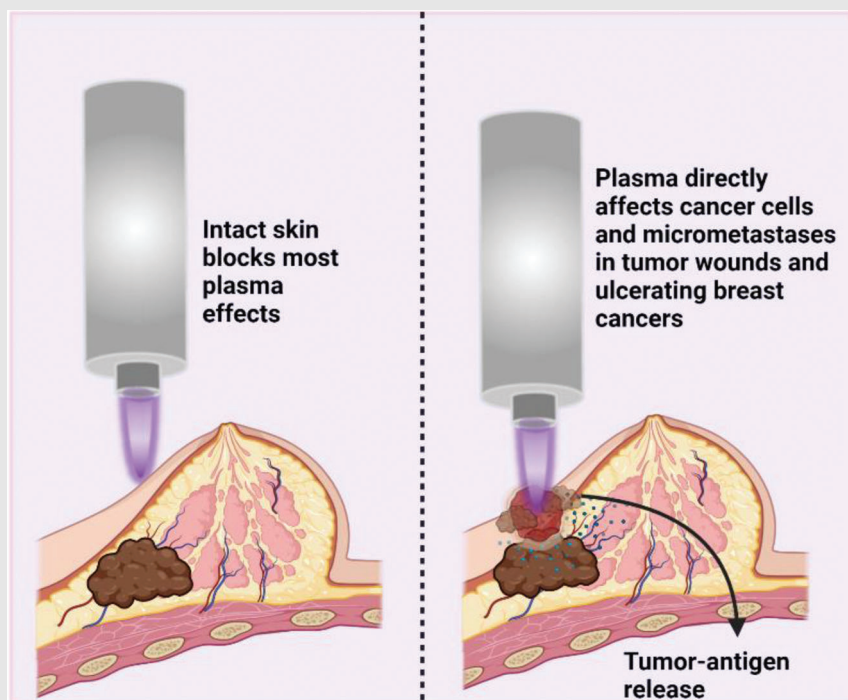
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Graphical Abstract

- Gas plasma is a multi-ROS/RNS generating technology with potent antitumour activity.
- Gas plasma exposure elicits immunogenic cancer cell death (ICD) and provides immunostimulation against breast cancer in vivo.
- Gas plasma technology has considerable potential to be used as novel neoadjuvant or adjuvant physical treatment modality for targeting ulcerating breast cancer within in situ vaccination schemes.

REVIEW

The potential of gas plasma technology for targeting breast cancer

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Despite therapeutic improvements in recent years, breast cancer remains an often fatal disease. In addition, breast cancer ulceration may occur during late stages, further complicating therapeutic or palliative interventions. In the past decade, a novel technology received significant attention in the medical field: gas plasma. This topical treatment relies on the partial ionization of gases that simultaneously produce a plethora of reactive oxygen and nitrogen species (ROS/RNS). Such local ROS/RNS overload inactivates tumour cells in a non-necrotic manner and was recently identified to induce immunogenic cancer cell death (ICD). ICD promotes dendritic cell maturation and amplifies antitumour immunity capable of targeting breast cancer metastases. Gas plasma technology was also shown to provide additive toxicity in combination with radio and chemotherapy and re-sensitized drug-resistant breast cancer cells. This work outlines the assets of gas plasma technology as a novel tool for targeting breast cancer by summarizing the action of plasma devices, the roles of ROS, signalling pathways, modes of cell death, combination therapies and immunological consequences of gas plasma exposure in breast cancer cells in vitro, in vivo, and in patient-derived microtissues ex vivo.

KEYWORDS

adenocarcinoma, adjuvant therapy, ICD, immunogenic cell death, palliation, plasma medicine, reactive oxygen species, ROS

1 | INTRODUCTION

Breast cancer is the most common cancer diagnosed in women worldwide. It is assumed that breast cancer cells derive from epithelial tissue of the breast,¹ while bipotential (mammary epithelial) stem cells are also discussed as tumour-initiating events.² Approved therapies such as surgery, chemotherapy, radiotherapy, hormone therapy and immunotherapy have contributed significantly to

breast cancer treatment and increased the patients' overall survival. The use of a specific breast cancer therapy depends on the origin, stage and other breast tumour characteristics. Surgery is the most common type of treatment due to the lack of systematic side effects and complications. In addition, other therapeutic techniques are used to prevent the recurrence of the disease.³ However, the resistance of tumour cells to induced programmed cell death by such treatments and the ability to invade host tissues and metastasize to distant sites is one of the most critical challenges in this disease. The annual number of people

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dying from breast cancer and suffering from various therapeutic side effects shows that more research is needed to find alternative or complementary therapies.^{4,5} Ulcerating breast tumours occur when tumour cells under the skin break through the skin surface and appear as an ulcer in various areas such as the primary tumour site or metastasis site such as lymph nodes. These ulcers can become infected due to various factors, which causes severe problems for patients. Symptoms of these ulcers include foul odor, unpleasant discharge, pain, bleeding and itching.⁶

Cold physical plasma, an ionized gas generated near room temperature, demonstrated impressive capabilities in cancer therapy.⁷ Many pre-clinical models have been employed to evaluate the efficacy of gas plasma on different types of cancers. The central concept in the field of plasma medicine is the generation of reactive oxygen and nitrogen species (collectively referred to as reactive oxygen species (ROS), as and reactive nitrogen species (RNS) also contain reactive oxygen in most cases) by gas plasma, referred to hereafter as 'plasma'. Extensive reactivity, damage to cells and tissue at higher concentrations, and generation of new ROS by chain reactions are the known characteristics of ROS. ROS are involved in cellular regulation and signalling at low concentrations, while at higher concentrations, their effect can be deleterious⁸ but simultaneously also therapeutic in the context of, for example, cancer.⁹ Accordingly, gas plasma technology is complementary to existing local physical treatment modalities that were shown to partially or fully rely on the generation of high ROS levels, such as photodynamic therapy,¹⁰ radiotherapy,¹¹ electrochemotherapy¹² and hyperthermia.¹³ Endogenously generated ROS and oxidative stress in the breast cancer tumour microenvironment (TME) have received increasing attention recently,¹⁴ and multi-species gas plasma ROS applications may redox modulate the TME potentially in favor of breast cancer therapy, which is the focus of this review, complementing previous summaries on the prospects of gas plasma treatment in, for example, melanoma,¹⁵ glioblastoma,¹⁶ osteosarcoma¹⁷ and pancreatic¹⁸ and head and neck cancer.¹⁹

1.1 | Breast cancer

Breast cancer is the most common and second leading cause of cancer death in women. In 2020, 2.3 million women were diagnosed with breast cancer, and 685 000 breast cancer deaths were recorded globally.²⁰ These numbers are striking and motivate a better understanding of breast cancer and its existing and potential therapies. Breast cancer is a complex and multifactorial disease affected by genetic and environmental factors. Breast can-

cer is generally divided into two categories based on the site from which the tumour originated. Lobular breast cancer instigates in the breast's milk-producing glands, while ductal breast cancer presents in the gland's ducts.²¹ Several risk factors are associated with breast cancer, such as family history, aging, early menstruation, late menopause, overuse of alcohol and a high-fat diet.²² On the molecular levels, epigenetics (e.g., altered DNA methylation and histone modifications) and mutations of oncogenes and tumour suppressor genes such as BRCA1, BRCA2, P53, RB1, PTEN, NME1, CCND1, PIK3CA, CDH1, ATM, FHIT and Mapsin can trigger this disease.^{23,24} Different kinds of systemic and non-systemic treatment are selected for breast cancer therapy based on the tumour's molecular subtype, stage and location. Surgery, radiotherapy, chemotherapy, hormone therapy, immunotherapy and targeted therapy alone or in combination are the most common breast cancer treatment methods (Figure 1). Classical multimodal breast cancer treatment schemes include surgery for tumour removal or mastectomy followed by chemotherapy or radiotherapy.²⁵ The adjuvant or neoadjuvant chemotherapy is often comprised of taxanes and anthracyclines, together with adjuvant hormonal suppression treatments (e.g., tamoxifen) in the case of progesterone or estrogen receptor positivity.²⁶ For radiotherapy, there are different modalities recommended in curative or palliative treatment intentions, and specific details of target tissue exposure (e.g., ductal carcinoma in situ, accelerated partial breast irradiation, and regional nodal irradiation) and timing are still a matter of active research.²⁷ Compared to other types of cancers, the 5-year survival is relatively high but nevertheless dramatically impacts the overall number of cases because of the high prevalence of breast cancer.

Many therapies come with various adverse and severe side effects such as organ damage and drastically reduced overall quality of life.²⁸ For instance, rehabilitation needs already in early-stage breast cancer patients include focal problems due to local therapies (e.g., pain, lymphedema, arm and shoulder problems), systemic issues related to treatment (e.g., neuropathy and fertility), and psychosocial illness (e.g., depression, sleep disturbances, and fatigue) that require attention.²⁹ Disease recurrence is a significant issue after surgical excision of the primary tumour that is propelled by tumour micrometastases and breast cancer stem cells.³⁰ In case of evident or suspected tumour metastases, the main goal of chemotherapy is to eradicate the breast cancer microcolonies systemically and loco-regionally via, for instance, interference in cell cycle progression.³¹ Unfortunately, intrinsic or acquired resistance of breast cancer cells to chemotherapy, especially in the aggressive triple-negative breast cancer (TNBC) molecular type, hinders therapeutic success in many cases.³²

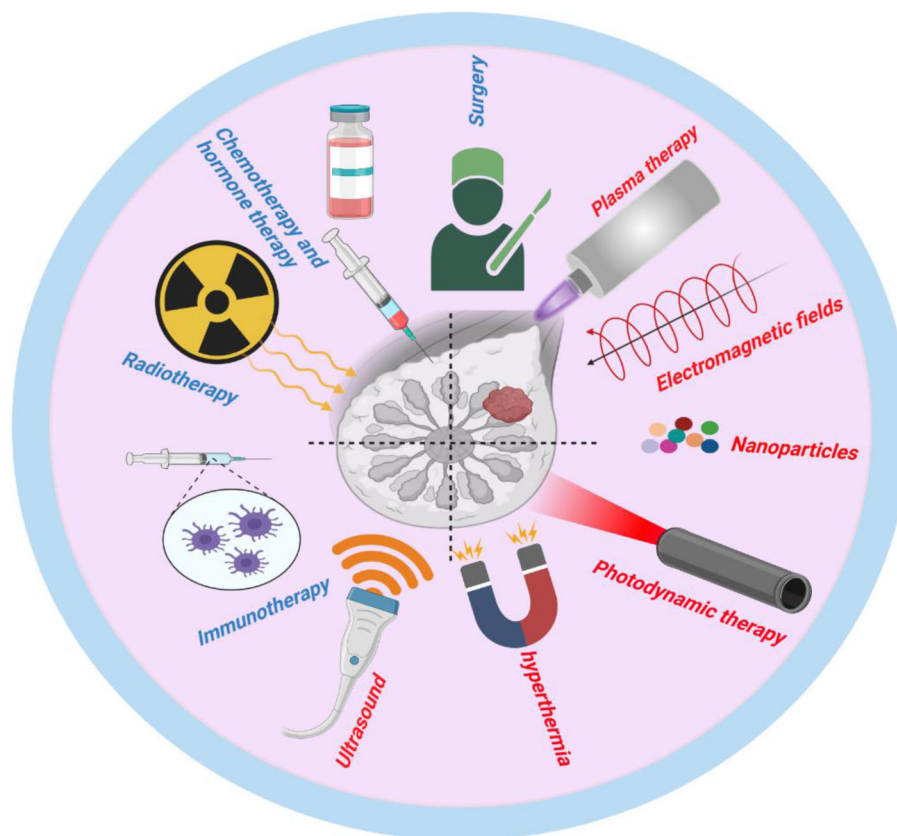


FIGURE 1 Currently approved (blue) and experimental (red) treatment modalities for breast cancer treatment

Patients undergoing radiotherapy suffer from irreversible skin damage and significant weight loss following non-malignant cell damage.³³ Hormone therapy imposes high medical costs for patients and prolongs treatment, which can promote invasive growth of malignant tumours.³⁴ Altogether, tumour immune evasion, heterogeneity, resistance to treatment and apoptosis, invasion and metastasis are hallmarks of tumour progression that enable tumour growth and promote treatment failure, causing disease fatality.³⁵ Hence, there is a constant need to investigate novel research lines in cancer therapy in general and breast cancer in particular. This includes not only systemic treatments but also local therapy modalities that might be adjuvant or neoadjuvant to approved and first-line treatments.

1.2 | Gas plasma technology

Physical plasma is described as the so-called fourth state of matter. These plasmas are generated by partial ionization of a gas, usually a noble gas in the case of plasma jets for medical applications. The partially ionized gases exhibit different chemical and physical properties, including the generation of ROS and RNS when in contact with

ambient air's oxygen and nitrogen, low-dose UV radiation, visible light, electromagnetic fields and electrons and ions (Figure 2). The leap innovation in using this technology was the production of so-called cold physical plasmas at atmospheric pressures. Before, plasmas were easily ignited in many technical processes, such as halogen lamps; however, usually under lower-than-atmospheric pressure. At the same time, hotter plasmas are known from other technical processes, such as welding and discharges during switching. Generating a highly reactive gas at about room temperature enabled several putative applications, including the treatment and functionalization of heat-sensitive materials and surfaces as well as medical treatments. This was the birth hour of the research field of plasma medicine.³⁶

Today, it is consent that the majority of biomedical plasma effects observed are based on the plasma sources' ROS/RNS generation.³⁷ At the same time, several studies revealed that the effects of other physical plasma components, such as low-dose UV-radiation and electric fields, especially in terms of cellular toxicity, are negligible. This is especially true for in vitro studies where excess liquid surrounds the treated target in question, such as microorganisms and eukaryotic cells in 2D monolayers. It is also clear that in those situations, the effect of

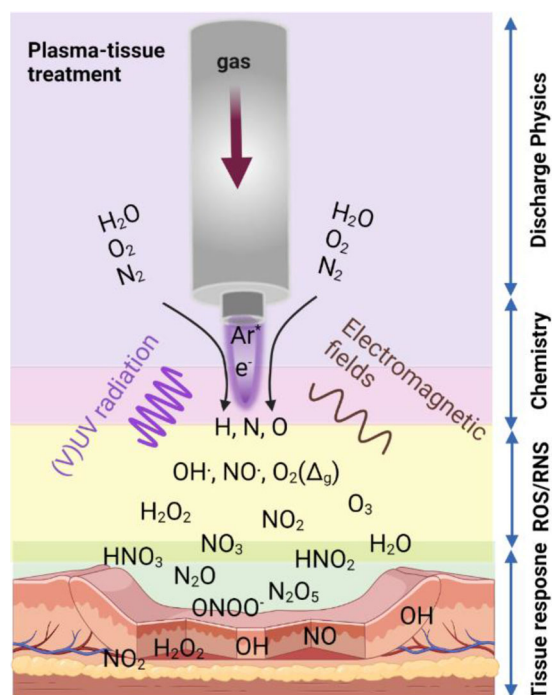


FIGURE 2 Schematic of gas plasma tissue treatment and potential mediators and effectors

the long-lived oxidants—chemical reaction products of the short-lived ROS/RNS produced by plasma—dominates the biomedical effects observed. In the absence of excessive amounts of water, such as the clinical application of dry but infected skin,³⁸ short-lived molecules will directly oxidize proteins and lipids without deteriorating to less reactive but more long-lived species as in liquids. In the case of wet tissues or wounds without protecting keratin layers, both short- and long-lived species will probably be generated and effective. A unifying model for in situ redox chemistry of plasma-treated tissues does not exist. This is because the experimental proof and unambiguous identification of ROS/RNS in tissues, potentially spatiotemporally resolved, is technically not possible until today.³⁹ The lack of information on ROS types and concentrations in tissues hampers the understanding of ROS-generating treatments, including certain chemotherapeutics,⁹ as well as pinpointing clear roles of ROS in the pathophysiology of the TME in breast cancer). Major potential mechanisms of gas plasma effects in cells have been summarized in Figure 3.

Currently, only a handful of devices have been approved based on clinical results and thorough scientific investigations in the EU as medical device class IIa for application in dermatology.⁴⁰ Clinical plasma applications can be divided into experimental and guideline-directed applications. Plasmas have potent antimicrobial effects evident in vitro and in wounds in vivo^{41,42} and support the healing of chronic wounds and ulcers. The appropriate use of gas

plasma technology in medicine has been implemented in an S2k medical guideline by the Association of the Scientific Medical Societies in Germany for the first time worldwide in early 2022. The consent document was produced by over 10 medical societies, such as the German Societies for Dermatology, Periodontology, Ophthalmology, General and Visceral Surgery, and Head and Neck Surgery. At the same time, clinically approved plasma technology is currently observed by German institutions responsible for health insurance reimbursements. Interestingly, a recent randomized clinical trial indicated improved wound healing with plasma jet application independent of antimicrobial effects.⁴³ Therefore, plasma technology has solidly entered daily medical practice for some indications. Experimental clinical plasma applications include several dermatology diseases, such as fungal and viral skin infections and actinic keratosis (carcinoma in situ) treatment.^{44,45} Antiviral effects of gas plasma have been described for long and re-gained importance during the COVID19-pandemic in the form of room air and water disinfection devices and antiviral plasma treatment of abiotic and biotic surfaces.^{46,47} In addition, plasma cancer applications have already been suggested for more than 15 years. Often, the question arises how these two seemingly distinct processes, that is, healing and toxic action, could be achieved with the same method. The answer is hormesis, a well-described phenome in pharmacology describing opposite effects of the same compound in the host, depending on its low or high concentration.⁴⁸ It has been known in the field of redox biology and medicine for long that ROS and RNS perform hormetic actions. At low concentrations, hydrogen peroxide acts as a signalling molecule, intertwined in redox signalling cascades, even providing growth stimulation under certain circumstances. However, redox signalling is disrupted at higher concentrations, and toxic effects dominate.⁴⁹ Another example is nitric oxide (NO), which stimulates vasodilation at lower concentrations, and is intentionally produced by macrophages at higher concentrations for antimicrobial defense purposes. Since gas plasma devices produce ROS, and ROS are the biochemical mediator of biomedical gas plasma exposure effects, the hormesis concept of ROS also applies to this technology, inevitably making the field of plasma medicine a part of the redox biology and medicine field: applied redox medicine.⁵⁰ Accordingly, short plasma treatment times or energies have stimulating properties beneficial in, for example, defective wound healing, while extended treatment times or higher energies provide tumour control.³⁷

Its superior safety profile is a prime hallmark of gas plasma technology in medicine. Several clinical studies have concluded a lack of severe adverse events or notable side effects.^{41,43,51–59} Besides occasional mild stitching and

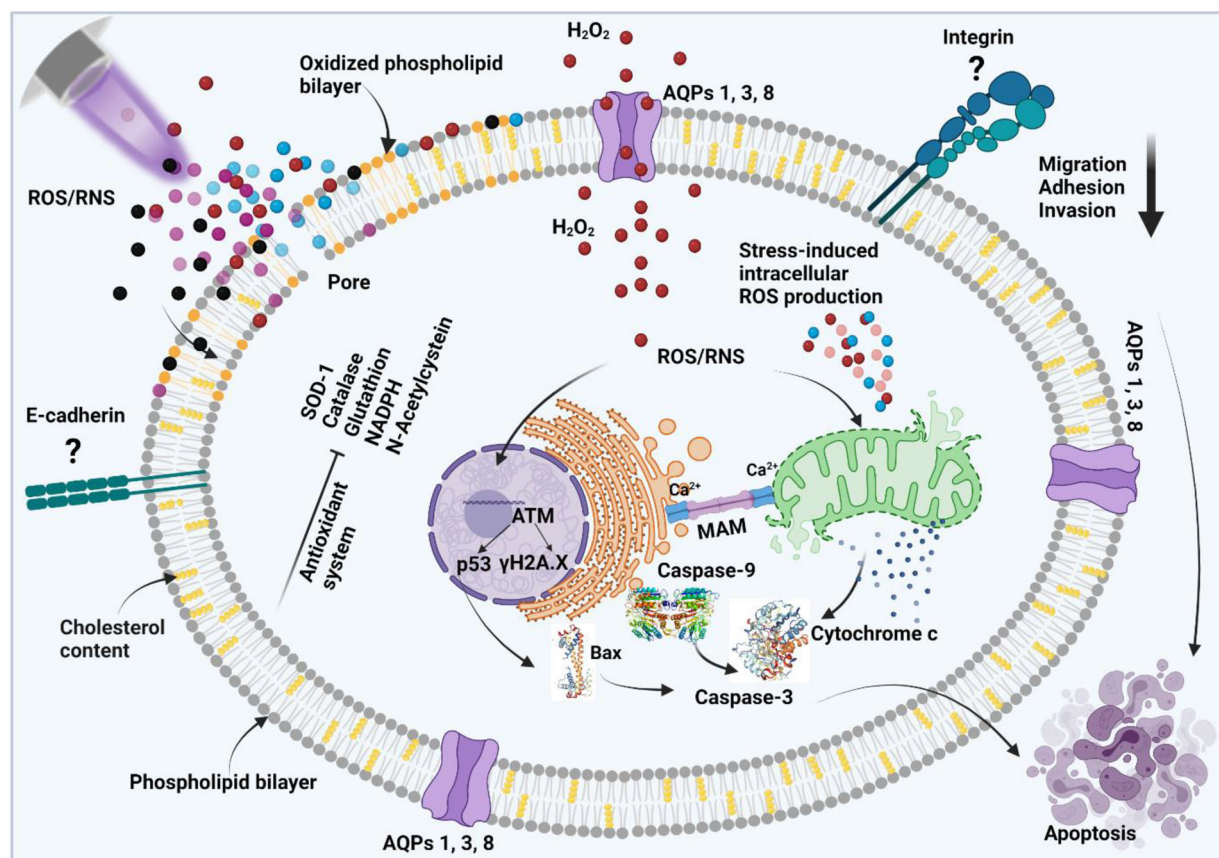


FIGURE 3 Illustration shows selected effects proven or hypothesized to be important in gas plasma-mediated breast cancer cell demise

modest ozone generation, gas plasma exposure is exceptionally well tolerated. No local anesthesia is required. Two long-term follow-up studies of 1-year and 5-years demonstrated an absence of abnormal effects in gas plasma-treated tissues.^{60,61} Similar conclusions were reached in two long-term in vivo studies. One is on gas plasma-treated ear wounds and animal analysis by pathology, PET-CT, MRI and molecular analysis 1 year later, reporting an absence of abnormal development or tumour formation in more than 80 animals.⁶² The second study included over 400 animals exposed to gas plasma alone or in combination with the carcinogenic agent DBP in the oral mucosa monthly for 1 year. Gas plasma did not lead to negative long-term effects. It did not promote the pro-carcinogenic action of DBP exposure to accelerate lesion or invasive tumour formation, as confirmed by thorough macroscopic and pathology investigations as well as molecular profiling of 140 transcripts.⁶³ It should be noted that 1 year in mice translates to about 60 years of life in humans. On the molecular level, several studies were performed based on OECD protocols to analyze the genotoxic potential of gas plasma exposure of cells. Several studies found no genotoxic effects for different types of gas plasmas devices.^{64–69} Many studies,^{70,71} including our own,^{72,73} reported exten-

sive histone 2A.X phosphorylation following gas plasma exposure, which is a biomarker for double-strand breaks in radio-biology. However, the effects observed with gas plasma exposure are related to the pleiotropic roles of this molecule, namely in response to oxidative stress, apoptosis and DNA-damage response initiation, that in turn is also intertwined with oxidative stress. Therefore, γ H2A.X is not a suitable biomarker for genotoxic events in gas plasma-treated cells and tissues.⁷⁴

1.3 | ROS

ROS are small, unstable, highly reactive molecules formed by electron addition or subtraction of oxygen to gain stability due to unpaired electrons in its orbitals. The ROS abbreviation sometimes also covers RNS, as the latter often contain reactive oxygen. Upon contact with biomolecules, such as lipids, proteins and nucleic acids, ROS mostly oxidize these by transferring electrons.⁷⁵ The body is frequently exposed to exogenous ROS from environmental stressors, such as UV radiation, smoking and air pollution. In addition to exogenous factors, endogenous sources like mitochondrial, microsomal and peroxisomal activity

in the electron transport chain and exacerbated activity of ROS/RNS-generating enzymes in phagocytes, such as NOX and MPO, can elevate oxidative stress locally.⁷⁶ Simultaneously, cells have various enzymatic and non-enzymatic antioxidants to detoxify ROS, such as glutathione, thioredoxins, superoxide dismutases, catalase and peroxidases.⁷⁷ It is well known that excessive and/or chronic oxidative stress can induce redox status imbalance, lipid peroxidation and ultimately apoptosis.⁷⁸ Several chemotherapeutics, such as anthracyclines, are related to oxidative stress induction.⁷⁹ ROS production is also the basis of photodynamic therapy⁸⁰ and part of the action of radiotherapy, also in breast cancer.⁸¹ Importantly, oxidative stress can also be combined with existing oncological therapies. Ogawa and colleagues reported that applying H₂O₂ before radiotherapy renders cancer cells more sensitive to radiotherapy.⁸²

Gas plasma-induced ROS/RNS can increase intracellular reactive species levels and decrease the antioxidant capacity of the target cells.⁸³ Common ROS/RNS produced by gas plasmas are hydroxyl radicals, atomic oxygen, superoxide radical, NO, peroxyxynitrite, singlet delta oxygen, ozone and others. The ROS/RNS redox chemistry in the plasma gas phase is highly dynamic and also depends on the plasma device in question, with hundreds of different reactions taking place in a concise time frame.^{84,85} The species being deposited in liquid matrices are less plentiful but still more than a dozen.⁸⁶ The plasma-derived ROS can be delivered via two modalities, direct and indirect plasma treatment. Direct plasma treatment refers to the plasma source producing a gas plasma that is in direct contact with or slightly above the treatment target to unleash its active cocktail of components for therapeutic purposes. This treatment contains all plasma effectors, including ROS, and has the most potent activity per treatment time.⁸⁷ By contrast, indirect treatment involves plasma treatment of a liquid that can be used for treatment immediately or stored for later use. A promising application is the combination with existing chemotherapy in the approved hyperthermal intraperitoneal chemotherapy or pressurized intraperitoneal aerosol chemotherapy for targeting peritoneal carcinomatosis or for treating bone cancer.⁸⁸ These liquids, however, mainly contain long-lived species that could also be generated independently of plasma processes.

Plasma-derived ROS for therapy can be produced by basically two categories of plasma devices. Plasma jets require a gas, usually a noble gas (due to its easy excitation), but ambient or pressurized air is also used in some devices, actively fed into the plasma zone.⁸⁹ There, the gas becomes partially ionized (reactive) and is expelled into the ambient air, where chemical processes produce reactive oxygen and nitrogen species from ambient oxy-

gen and nitrogen. The advantage of plasma jets is the scalpel-like application in medical practice, helping the health care staff visually track the target's treatment. Also, plasma jets are very focused and efficiently enter even the smallest cavities, such as hair follicles and tooth-root channels.^{90,91} Yet, their treatment area is limited. By contrast, dielectric barrier discharges can cover larger areas to treat simultaneously.⁹² However, their geometry is flat, leading to uneven treatment of non-flat surfaces as frequently the case in many medical fields. Moreover, the plasma treatment of the target cannot be tracked visually, as the device covers the area on which it expels its active cocktail. So-called plasma multi-jets, devices with multiple single plasma jets combined to cover larger areas, may combine the benefits of both systems but have only started to be explored.^{93–95} A frequent debate is whether plasma therapy can be 'dosed'. Unfortunately, there is no consensus yet on how such dose would be defined in such a multi-component and multi-ROS technology with less clear individual contributions of each component and supposedly different therapeutic needs across several diseases.

A future dosing concept based on ROS generation is likely, however. By contrast, in radiotherapy, dosing is already a long-standing concept measured in Gray (Gy). The treatment modality is mediated by either electromagnetic (e.g., X- and gamma rays) or particulate radiation (electrons, protons, neutrons). The biological principle of this ionizing radiation in the treated target tissue is the dislodgment of electrons from molecules. Ultimately, this produces free radicals in all cellular microenvironments,⁹⁶ including extracellular, intracellular, intravesicular and intranuclear compartments. This starkly contrasts with gas plasma technology, where ionization occurs in the active plasma zone but not in the target tissue since the electromagnetic energies in plasmas are too weak. Yet, the similarity with both technologies is ROS generation, with gas short-lived plasma-derived ROS only reaching extracellular targets and cell membranes at sufficient concentrations, as they are transported to the cells and not generated within the cells as the case with radiotherapy. This explains the perhaps greatest difference between both modalities, the penetration depths, which is not an obstacle in radiotherapy. On the contrary, gas plasmas only act topically on the target tissue within the first dozen micrometers to confer primary effects,^{97,98} while secondary effects can occur up to several millimeters in the tissue.^{99,100} Radiotherapy is usually applied in fractionated regimens; breast cancer frequently receives hypofractionation.¹⁰¹ Also gas plasma therapy, within its current indications (e.g., chronic wound healing⁴⁰), is applied several times. By definition, this is not necessarily fractionation, as a total therapeutic dose for the disease or target-ot-treat is not definable (yet).

TABLE 1 Brief comparison between radiotherapy and gas plasma technology

Category	Radiotherapy	Gas plasma therapy
Mode of action	Unimodal; ionizing radiation unleashes electrons and generates ions, producing reactive species and direct defects on biomolecules	Multimodal; gas plasma ionization generates several components, such as electric fields, ions, electrons, ultraviolet and visible light, and dozens of reactive oxygen and nitrogen species simultaneously
Potentially mutagenic	Yes	No
Penetration depth	High, not a limitation in clinical application	Low, only surface or near-surface primary (a few dozen micrometres) or secondary (millimetres) effects
Cancer therapy approval	Yes	No, but approved if tumour wound antiseptics is the goal, e.g., ulcerating and infected breast and head and neck cancer wounds
Fractionation and dose	Yes, usually the case based on dose	Yes, usually therapeutic effects in clinics always require multiple treatment cycles that are however continued based on objective responses and not on overall dose; no overall dosing concept available yet
Effectors	Radiations effects all biomolecules, regardless of their localization being intracellular or extracellular	Extracellular ROS/RNS generation and delivery to cells, only membrane oxidation or passive ROS/RNS delivery to intracellular sites
Electron energies	Very high (>50 000 electron volts)	Very low (~1–10 electron volts for clinical plasma devices)

Abbreviation: ROS/RNS, reactive oxygen and nitrogen species.

Usually, gas plasma treatment is endorsed as long as there is an objective therapeutic benefit. This was also the case for the small cohort of palliative head and neck cancer patients receiving a few to several dozen gas plasma treatment cycles in the clinical setting, with partially remarkable results, before tumour progression continued.^{102,103} Similarities and differences between radiotherapy and gas plasma technology are briefly summarized below (Table 1).

1.4 | Tumour immunology and immunogenic cancer cell death

According to McFarlane's immune surveillance theory, the immune system can recognize and destroy tumour cells.¹⁰⁴ In brief, tumours can stimulate their own demise by stimulating anticancer immunity via the expression of tumour-associated antigens or neoantigens considered foreign to the immune system.¹⁰⁵ The efficiency of this process is also related to the tumour's mutational burden, which differs considerably between cancer types.¹⁰⁶ At the same time, the lack of expression of immunogenic antigens, decreased expression of MHC class I molecules, inadequate stimulation of T lymphocytes, production of immunosuppressive or immune cell-killing factors, and immunosuppressive cells such as regulatory T-cells are among the most critical mechanisms of tumour escape from immune responses.

This is because immune-mediated tumour cells removal involuntarily puts evolutionary pressure on tumour cells to mutate further and generate variants efficient in evading immune responses by either down-regulating pro-immunogenic features (such as expression of HLA molecules) or up-regulating immunosuppressive factors, for example, immune checkpoint receptors and ligands, such as PD-L1 and CTLA4. The discovery of the therapeutic blockade of immune checkpoints via antibodies was acknowledged with the Nobel Prize in Physiology or Medicine in 2018.¹⁰⁷ Inhibitory immune receptors, which have been identified in breast cancer, are PD-1, CTL-4, PD-L1, TIM3, TIGIT, BTLA and LAG3.¹⁰⁸ The most common immune checkpoint antibodies approved for breast cancer treatment are atezolizumab, pembrolizumab and dostarlimab, which target the PD-1/PD-L1 pathway^{109,110} (Table 2). Clinicians focused on combining standard of care treatment with immunotherapy to target breast cancer more efficiently. The general concepts of empowering antitumour immunity have been reviewed thoroughly before.^{111,112} Several cell types participate in this process, and especially cytotoxic T-cells are viewed as the most efficient effectors targeting cancer cells.^{113,114}

One bottleneck of generating antitumour T-cells is the supply of tumour antigens in a pro-inflammatory fashion. The concept of immunogenic cancer cell death (ICD) predicts this form of sterile and pro-inflammatory cell death

TABLE 2 FDA-approved checkpoint antibody immunotherapies for breast cancer

Drug name	Target protein	Mechanism of action
Atezolizumab (Tecentriq)	PD-L1	Humanized IgG1 κ monoclonal antibody against PD-L1 expressed on tumour and non-tumour cells; inhibits the binding of PD-L1 to PD-1 predominantly expressed on T-cells and thereby blocking a major immunosuppressive pathway.
Pembrolizumab (Keytruda)	PD-1	Humanized IgG4 monoclonal antibody against PD-1 expressed predominantly on T-cells; inhibits the binding of PD-L1 expressed on tumour and non-tumour cells to PD-1 and thereby blocking a major immunosuppressive pathway.
Dostarlimab (Jemperli)	PD-1	Humanized IgG4 monoclonal antibody against PD-1 expressed predominantly on T-cells; inhibits the binding of PD-L1 expressed on tumour and non-tumour cells to PD-1 and thereby blocking a major immunosuppressive pathway; approved to treat mismatch repair deficient (dMMR) advanced-stage breast cancer that had emerged during or after therapy.

to promote the generation of T-cells targeting cancer. The concept has been reviewed several times.^{115,116} The idea is that a given agent (e.g., anthracyclines chemotherapy) or process (e.g., photodynamic therapy or radiotherapy) promotes local tumour cell death concomitant with the release of damage-associated molecular patterns (DAMPs), such as ATP, HSP70 and HMGB1,¹¹⁷ together with endoplasmic reticulum stress that promotes externalization of eat-me signals, such as calreticulin (CRT).¹¹⁸ Dendritic cells (DCs) in TME phagocytose the tumour material, and the DAMPs lead to their maturation and activation. Subsequently, the DCs migrate to sentinel (draining) lymph nodes where they (cross-) present tumour antigen to T-cells. Insufficient co-stimulation during this process can lead to T-cell anergy or failure of T-cell activation. Since DCs have phagocytosed the tumour antigen in a pro-inflammatory (ICD) environment in the context of DAMPs, antigen presentation to T-cells is above threshold and cognate T-cells proliferate. The clonal expansion provides the body with systemic anticancer immunity, also targeting metastases not treated initially. It is later described that gas plasma breast cancer treatment was capable of eliciting such a cancer-immunity-cycle.¹¹⁹ This concept is generally attractive for local therapies and can be combined with checkpoint immunotherapy to spur further the quality or quantity of the newly generated T-cells' activity.

2 | PLASMA BREAST CANCER TREATMENT AND MECHANISM OF ACTION

Many studies have investigated the effects of gas plasma treatment on breast cancer cells in vitro (Table 3) and in vivo (Table 4); clinical gas plasma breast cancer treatments have not been reported so far. The reports addressed various questions, ranging from cell death modalities, over combination treatments with existing cancer treatment

regimens, to drug re-sensitization and immunological consequences. Moreover, our recent studies investigated for the first time gas plasma toxicity in patient-derived breast cancer tissues exposed ex vivo.¹²⁰ Markedly, overall similar effects were noted across most studies, independent of the design and engineering of the plasma device used, and dependent on ROS generation of the plasma device. The majority of studies used the cell lines MDA-MD-231 and MCF-7, apart from about a dozen other cell lines utilized to a lesser extent. MCF-10A was often referenced as normal (non-cancerous) breast cell line. All treatments showed overall dose- or treatment time-dependent effects regarding gas plasma-derived ROS production and cytotoxic or growth-inhibiting effects in the breast cancer cell lines.

2.1 | Cytotoxicity and ROS production

An extensive array of plasma devices and feed gas settings was used across different studies to investigate the toxicity in breast cancer cell lines in vitro. Cytotoxic effects were used in the direct treatment regimen with plasma jets driven with helium (He),^{121–133} which was the setting mostly applied in the literature. In some studies, the direct He plasma treatment was compared with Helium-oxygen (He/O₂) plasma treatment,^{123,126,129,132,134} and generally, the He/O₂ conditions were found to be more toxic than the He condition. This is because oxygen addition, especially to helium, increases the presence of atomic and singlet delta oxygen in the plasma gas phase.¹³⁵ If not quenched by molecular oxygen to generate ozone, oxygen addition can lead to hypochlorous acid formation, regardless of whether helium or argon (Ar)¹³⁶ is used as carrier gas. Also other cell lines were found before to be more sensitive to He/O₂ than He direct plasma jet treatment.¹³⁷ Direct Ar plasma jet treatment of breast cancer cell lines was also reported in various studies.^{72,138–146} With regard to plasma jets, one study compared nitrogen

TABLE 3 In vitro studies and findings in gas plasma breast cancer treatment. BC = breast cancer

Cell line (s)	Gas plasma device	Exposure modality	Gas plasma treatment effects and mechanisms of action in breast cancer cells	Ref.
MDA-MD-231	Plasma jet (He) direct treatment	5–120 s, 24 h and 48 h incubation	- Gas plasma treatment time-dependent increase of superoxide, H_2O_2, OH, NO_2^-, and NO_3^- in treated cell culture medium - Treatment-time dependent toxicity greatest at 48 h	121
MCF-7, MJ1, MN3, HBL	DBD (air) direct treatment	5–15 s, 72 h incubation	- The three BC cell lines demonstrated different responses in a dose-dependent manner via caspase 9-induced apoptosis, while gas plasma did not show toxic effect in HBL non-malignant cells. - p53 played no significant role in the apoptosis pathways	163
MCF-7, T-47D, SK-BR-3, BT-474, MDA-MB-231, Hs578T, HCC1806	Plasma jet (He) direct treatment	120–360 s, 48 h incubation	- Seven BC cell lines with differing molecular profiles were analyzed via MTT assay 48 h after gas plasma exposure - ER+/PR+/HER2+ cells were the most resistant to gas plasma treatment - ER+/PR+/HER2+ cells were most sensitive to gas plasma treatment	122
MCF-7, T-47D, ZR-75-1, BT-549, MDA-MB-231, Hs578T, HCC1569, MDA-MB-157, MDA-MB-175VII, HCC1954, MDA-MB-361, HCC1428, MDA-MB-468, AU-565	Plasma jet (He or He/O ₂) direct treatment	Five different modes compared with treatment times from 10–240 s; 144 h incubation	- Additive toxicity of gas plasma and radiotherapy observed in the cell lines tested - Cell lines sensitive to gas plasma exposure were also sensitive to radiotherapy (high correlation). - The addition of O₂ into the plasma gas inlet increased the cytotoxicity of gas plasma - Gas plasma in combination with the drug olaparib has higher toxicity, hence DNA repair inhibitors like olaparib may increase the effectiveness of gas plasma. - Gas plasma increased phosphorylation of H2A.X associated with increased DNA damage responses.	123
MDA-MB-453, MDA-MB-231, MCF-10A	DBD (air) direct treatment	60–120 s, 48 h incubation	- Treatment time-dependent cytotoxicity higher in MCF-10A than MDA-MB-231 and MD-MB-453 cells. - NAC and catalase rescued viability only partially in MCF-10A and not at all in MDA-MB-231 cells - Treatment inhibited IL-6R/pSTAT3 signalling pathway, leading to increased PTEN and decreased AKT phosphorylation. - Gas plasma resistance is mediated by HER2 increase together with ROS scavenging.	164
MCF-7, T-47D, MCF-10A	DBD (Ar) direct treatment	30 s each hour 10 times and single 600 s, 24 h incubation	- Gas plasma suppressed, depending on treatment condition, BC cells proliferation by down-regulation of ZNRD1 and its antisense long noncoding RNA ZNRD1-AS1 expression - Modified the methylation status of CpG sites - Inhibited growth rate and colony formation in ZNRD1 and ZNRD-AS1 transfected BC cells	165
MCF-7, MDA-MB-231, MCF-10A, MCF-12A	DBD (Ar) direct treatment	30 s each hour 10 times and single 600 s, 24 h incubation	- Increased intracellular reactive oxygen species (ROS) - Induced apoptosis preferentially in BC cells - Changed methylation status in some of the CpG islands in BC cells (DNAJC8, POTED, and EIF1YA) and estrogen receptor-positive BC cells (ESFR1, PRR7, CD86, DHRS7B, FDX1, CREB3, BCL-2, and BDNF)	166

(Continues)

TABLE 3 (Continued)

Cell line (s)	Gas plasma device	Exposure modality	Gas plasma treatment effects and mechanisms of action in breast cancer cells	Ref.
MDA-MB-231	Plasma jet (He) direct treatment	60–300 s, 48 h incubation	■ Viability decreased in a treatment time-and input power-dependent manner ■ Significant temperature increase in the gas plasma-treated in vitro cultures	124
MCF-7, BT-474, MDA-MB-231, SK-BR-3	Plasma jet (He) direct treatment	120–360 s, 6 h–48 h incubation	■ Reduced proliferation (Ki-67) and increased apoptosis and cell cycle arrest in a caspase 3 and 7 and treatment time-dependent manner ■ Toxicity varied between different BC cell lines ■ DNA damage response induced (ATF3, EGR1, ID2) ■ Histone RNA oxidation proposed to mediate gas plasma toxicity ■ DNA damage is not the primary mode of BC cell death by gas plasma exposure	125
AMj13	DBD (air) direct treatment	5–15 s, 72 h incubation	■ Gas plasma reduced viability and colony formation in long-term observation	167
MDA-MB-231	Plasma jet (Ar) direct treatment	5–25 s, 24 h incubation	■ Gas plasma reduced BC cell viability in an exposure time-and input power-dependent manner	138
MDA-MB-231	Plasma jet (Ar) direct treatment	5–30 s, 24 h incubation	■ Gas plasma in combination with a static magnetic field (SMF) and vitamin C decreased cell viability and migration, while SMF alone had no effect	139
MCF-7	Plasma jet (He, He/O ₂ , or Ar/O ₂) direct treatment	5–30 s, 48 h incubation	■ ROS derived from gas plasma treatment increased apoptosis in BC cells ■ He/O₂ plasma showed the highest toxicity	126
MDA-MB-231	Plasma jet (He) direct treatment	5–300 s, 144 h incubation	■ Exposure time-dependent toxic effects ■ Different discharge modes of gas plasma have different anti-proliferation effect. ■ Gas plasma toxicity was different in a dose-dependent-manner, and apoptotic cells showed regular DNA fragmentation behaviour	127
4T1	Plasma jet (He) direct treatment	1 min, 24 h and 36–38 h incubation	■ Decrease of metastatic behavior ■ Augmented apoptosis ■ Delayed DNA fragmentation in sub G1 phase compared to drug-controls	128
SK-BR-3, HaCaT	Plasma jet (N ₂ or N ₂ /H ₂ O) direct treatment	1–5 min, 48 h (SK-BR-3) and 72 h (HaCaT) incubation	■ Humidified gas plasma exposure led to higher ROS production, cellular oxidation and oxidative stress, and caspase 8-dependent cell death mediated by p38-MAPK phosphorylation and ERK inhibition together with PARP-1 cleavage. ■ ATM and p53 DNA damage response activated ■ HaCaT cells are overall less affected	147
MDA-MB-231, MCF-10A	Plasma jet (Ar) direct treatment	30 s, 6 h incubation	■ Up-regulation of the chemotherapy cationic transporter SLC22A16 gene expression in MDA-MB-231 but not MCF-10A	72
MCF-7, MCF-10A	Plasma jet (He or He/O ₂) direct treatment	30–300 s, 24 h incubation	■ Gas plasma selectively induced toxicity on BC cells with negligible effect on low malignant MCF-10A cells ■ Gas plasma increased BC cell death through activation of caspases 3 and 7	129

(Continues)

TABLE 3 (Continued)

Cell line (s)	Gas plasma device	Exposure modality	Gas plasma treatment effects and mechanisms of action in breast cancer cells	Ref.
MCF-7	Plasma jet (Ar) direct treatment	30 s each hour 10 times, 24 h incubation	- Gas plasma inhibited BC cell growth and recovered drug sensitivity of Tx-resistant MCF-7 (MCF-7/TxR) - Gas plasma-induced cell sensitivity to drug (Paclitaxel) not related to changes in drug uptake but modification of oncogene and tumour suppressor gene expression (KIF13B, CEACAM1, GOLM1, TLE4, PHKA1, DAGLA) - Gas plasma-induced up-regulation of tumour suppressor DAGLA and down-regulation of tumourigenic CEACAM - Gas plasma decreased drug resistance and toxicity by altering colony formation and tumourigenesis in siDAGLA and siCEACAM transfected BC cells	140
MCF-7	Plasma jet (Ar) direct treatment	30 s each hour 10 times, 2–6 days incubation	- Increased toxicity and reduced tumourigenesis in drug (Tamoxifen) resistant BC cells - Gas plasma re-sensitized BC cells to Tamoxifen by reversing expression of BAG1, CD24 and HDAC4 genes - Decreased colony formation, and tumourigenesis in siMX1 and HOXC6 ORF transfected cells - Altered drug resistance status in BC cells by modification of XRCC, SOX9 and SULT1A1 expression - ROS scavenger inhibited the gas plasma effects	141
MDA-MB-231, MSC	Plasma jet (He) direct treatment	30–120 s, 24 h incubation	- BC cell viability and invasion were inhibited - Non-malignant MSC cells were less affected	130
MDA-MB-231, MSC, MCF-7	Plasma jet (air) direct and nanoparticle treatment	60–90 s, 24h incubation	- Increased nanoparticles uptake in BC cells - Higher permeabilization and toxicity when combined with drug-loaded nanoparticles - Gas plasma down-regulated MTDH, MMP2, MMP9 and VEGF as indicators of cancer progression	191
MCF-7, HF	Plasma jet (He/O ₂) direct treatment	15–45 s, 24 h incubation	- Gas plasma combined with iron NPs changed morphology and activated programmed cell death through BAX/BCL-2 but not β2-microglobulin - Less damage in non-malignant HF cells	134
MDA-MD-231, MCF-7	DBD (Ar) direct treatment	30 s each hour 10 times; 100 s and 600 s six times per day; up to 6 days incubation	- Tumour suppressor and antitumour properties by increased ABCA1, PTEN, HBP1 and GJA1 expression in miR-19 transfected and non-transfected BC cells - Modified methylation status of promoter CPG sites - Decreased cell colonies and proliferation (by increasing ABCA1, PTEN, HBP1, and GJA1 genes expression) in miR-19 transfected cells - ROS scavenger suppressed gas plasma effects on miR-19a cells and its target genes	189
4T1	DBD (air) direct treatment	10–40 s, 24 h incubation	- Treatment time-dependent ROS formation and toxicity - Activation of ICD pathways (calreticulin - Treated BC cells activated DCs (CD80 and CD86)	196
MDA-MD-231	Plasma jet (He)-treated liquid (indirect) and direct treatment	1 min (direct), 2–10 min (indirect), 24 h incubation	- Treatment time-dependent extracellular and intracellular H₂O₂ increase for direct treatment while indirect treatment only increased extracellular H₂O₂ - Direct gas plasma treatment shows higher toxicity than indirect treatment	160

(Continues)

TABLE 3 (Continued)

Cell line (s)	Gas plasma device	Exposure modality	Gas plasma treatment effects and mechanisms of action in breast cancer cells	Ref.
MDA-MD-231, MCF-7	Plasma jet (He) direct treatment	3 and 5 min, 24 h and 48 h incubation	■ Mitochondria oxidation and metabolic activity decline in 2D BC cells and 3D BC spheroids ■ DAMPs and ICD-associated molecules (HSP70, HSP90, calreticulin, PD-L1, MHC-I, ATP, IFN-α2, IFNγ, IL-6) observed in treated cells	131
MDA-MB-231, MDA-MB-468, MCF-7, MCF-10A	Plasma jet (He)-treated liquid (indirect) and direct treatment	10–50 s (direct), 1–5 min (indirect), 24 h incubation	■ Indirect exposure in TNBC cells was dose-dependent ■ Apoptosis and migration were different in treated TNBC compared to non-malignant and non-TN BC cells	150
MDA-MB-231, human fibroblasts	Plasma jet (He or He/O ₂)-treated liquid (indirect) and direct treatment	1–5 min, 0 h and 48 h incubation	■ Direct and indirect exposure selectively decreased BC cell viability due to an increase of ROS and programmed cell death through BAX, BCL-2, caspase 8 and caspase 3 pathways	132
MCF7, HCC1806	Plasma jet (air)-treated liquid (indirect) and direct treatment	15–120 s (direct), 60–120 s (indirect), 24 h incubation	■ Reduced BC cell protein content and metabolic activity in a treatment time-dependent manner	151
MCF-7, MDA-MB-231, MCF-10A	DBD (He)-treated cell culture medium (indirect)	45–240 s, 24 h and 48 h incubation	■ Long but not short treatment times affected mitochondrial activity and BC cell growth and migration ■ Presence of FBS supposedly increased OH radical and H₂O₂ production ■ Metastatic BC cells are more sensitive	169
MDA-MB-231, primary murine fibroblasts	DBD (Air)-treated DI-water (indirect)	18 min, 24 h incubation	■ More toxic in BC cells over fibroblasts ■ Less toxicity the longer time difference from gas plasma-treated liquid generation to application ■ Induced cell death was not related to higher acidity	170
SUM149PT, SUM159PT, MDA-MB-231, MDA-MB-436, MCF-7, SK-BR-3, MCF-10A	Plasma jet (He)-treated liquid (indirect)	1–2 min, 24 h incubation	■ Toxicity depends on cell density, treatment time, gas flow rate, plasma device output voltage, well size, and plasma effluent distance to the liquid ■ Gas plasma induces higher toxicity in triple-negative (TN) cancer cells compared to non-TN cells may result in more AQPs on their cell surface ■ Gas plasma is a promising treatment method for cancer stem cells and can inhibit metastasis	152
MCF-7, MDA-MB-231, MCF-10A	Plasma jet (Air)-treated liquid (indirect)	30–120 s, every week for 10 week	■ Increased oxidative stress and cell cycle arrest, and decreased proliferation ■ Programmed cell death through activation of BAX and PUMA, cytochrome C release, and caspase activation ■ Elevated H2A.X phosphorylation and p53 activation	153
MDA-MD-231, MCF-7	Plasma jet (He)-treated liquid (indirect)	.5–2 min, 24 h incubation	■ Exposure time, well plate size, cell density, volume of media and distance of gas plasma with treated surface affect relative ROS levels and cytotoxicity toxicity ■ H₂O₂ levels and RNS affected by host cell amino acids, especially cysteine and tryptophane, and weakened intracellular antioxidant system ■ Absorption and elimination of gas plasma-produced ROS varied among different BC cell lines	154

(Continues)

TABLE 3 (Continued)

Cell line (s)	Gas plasma device	Exposure modality	Gas plasma treatment effects and mechanisms of action in breast cancer cells	Ref.
MCF-7, MDA-MB-468, MDA-MB-231	Plasma jet (Ar)-treated cell culture medium (indirect)	10 min, 24 h incubation	■ Mesenchymal BC cell lines showed more susceptibility than epithelial counterparts ■ Sensitivity of luminal subtypes lower than basal subtypes ■ ROS levels were dramatically higher in mesenchymal compared to epithelial breast cancer cells ■ Cells with a higher level of epithelial-mesenchymal transition (EMT) score are more sensitive, as evident by a weighted analysis of 76 EMT-related genes ■ 10% fetal bovine serum in gas plasma-treated media decreased cytotoxic treatment efficacy BC cells from being damaged after gas plasma-activated media ■ Exposure modified epithelial and mesenchymal gene expression correlating with metastasis and invasion inhibition	155
MDA-MD-231	Plasma jet (He)-treated DI-water (indirect)	5–30 min, 24 h and 48 h incubation	■ ROS produced by gas plasma in DI water decreased metabolic activity of BC cells with increasing exposure time	156
MDA-MD-231	Plasma jet (Ar, N ₂ , He)-treated DI-water (indirect)	Not stated treatment time, 24 h and 48 h incubation	■ Ar-treated liquids produced more ROS and were more cytotoxic than N₂- and He-treated liquids ■ Effects in gas plasma-treated cell culture media were greater than in non-media liquids	218
MDA-MB-231	Plasma jet (Ar)-treated liquid (indirect)	180 s, 24 h incubation	■ Gas plasma alone and combined with FK866, a Nicotinamide phosphoribosyltransferase (NAMPT) inhibitor, increased intracellular ROS and cytotoxicity ■ PARP-1 activation and cell death associated with energy constraints by NAD⁺ and ATP level depletion ■ ΔΨ_m disruption and mitochondrial dysfunction ■ Weakened antioxidant defense system (reduction of GSH and NADPH) ■ siRNA-mediated NAMPT increased plasma cytotoxicity	157
MDA-MB-231, MCF-7	Plasma jet (Ar) direct treatment	20 s, 24h incubation	■ Non-cytotoxic treatment condition ■ Increased expression of the immune checkpoints and inflammation-related surface molecules CD40 and CD112, as well as CD273 and Gal-9 in MDA-MB-231 and CD271 in MCF-7 cells	142
MDA-MB-231, MCF-7	Plasma jet (Ar) direct treatment	60–180 s, 6 h and 24 h incubation	■ Increased intracellular ROS ■ Cleaved PARP-1 and HSP90β and PKD2 degradation, and affect enhancement via HSP90 inhibitor PU-H71	143
MDA-MB-231, MCF-7	Plasma jet (Ar) direct treatment	30–120 s, 20 h incubation	■ In a 36 cancer cell line comparison, MCF-7 and MDA-MB-231 were of high and low resistance, respectively, to gas plasma treatment ■ Both cell lines showed modestly enhanced NOX3 and AQP1 expression that had a good correlation with gas plasma-induced cytotoxicity	144
MDA-MB-231, MCF-7	Plasma jet (Ar)-treated cell culture medium (indirect)	60 min treatment of 50 ml, exposure to 100 μl	■ Modest cytotoxic effects ■ No major change in the expression of 26 redox-related transcripts (peroxiredoxins, glutaredoxins, etc.) except for HMOX1, especially in MCF7 ■ No major effect on ICD and immunology-related molecules (CD47, CRT, HLA-ABC, HSP70, HSP90) in modest treatment conditions	158

(Continues)

TABLE 3 (Continued)

Cell line (s)	Gas plasma device	Exposure modality	Gas plasma treatment effects and mechanisms of action in breast cancer cells	Ref.
MDA-MB-231	Plasma jet (Ar) direct treatment	30–60 s, 6 h and 24 h incubation	■ Relatively low resistance to gas plasma-induced toxicity when compared to 10 other cell lines ■ Exceptionally high oxidized GSH after exposure	145
MDA-MB-231, MCF-7	Plasma jet (He) direct treatment	120–300 s, 6 h–48 h incubation	■ Treatment time-dependent generation of H_2O_2, NO_2^-, and HOCl with modest pH increase in culture medium ■ Treatment time-dependent cytotoxicity and decline in metabolic activity in 2D cultures and 3D tumour spheroids ■ At 24 h and 48 h, increased surface expression of CRT, HSP70, HSP90, MHC I, and PD-L1 in both cell lines ■ LC3 (autophagy) increase in MCF-7, and elevated ATP and HSP70 secretion in both cell lines	131
MDA-MB-231, T-47D, SK-BR-3, MCF-7, BT-474, HS578T	Plasma jet (He) direct treatment	300 s, 3–24 h incubation	■ Analysis of 48 apoptosis and 35 oxidative stress genes after plasma treatment ■ BCL2A1 increased only in plasma-treated TNBC lines ■ Silencing BCL2A1 augmented plasma-induced cytotoxicity in breast cancer cells ■ BCL2A1 was described before to contribute to malignancy and chemoresistance	133
MCF-7	Plasma jet (Ar)-treated culture medium (indirect)	60–240 s, 48 h incubation	■ Plasma-treated medium combined with doxorubicin treatment ■ Intracellular ROS generation and metabolic activity decline ■ Combination effect observed	159
MDA-MB-231	DBD (air) direct treatment	60–120 s, 24 h incubation	■ Increased ROS and decreased viability in plasma treatment time-dependent manner ■ Correlated with decreased HIF-1α and VEGF expression	168
BT-474	Plasma jet (Ar/O ₂) direct treatment	60–150 s, 24–72 h incubation	■ Nearly 90% reduced cell viability already with 60 s and at 24 h, with not much increase of cell death with longer gas plasma treatment or incubation times	146

(N₂) against humid nitrogen (N₂/H₂O) and found the latter to be significantly more toxic than the former.¹⁴⁷ This can be explained based on earlier findings identifying a critical role of humidity in the feed but not in ambient air. Specifically, humidity in the feed gas increases the production of H₂O₂ and one of its precursors, OH radicals, directly in the plasma gas phase.¹⁴⁸ Accordingly, treated liquids show several-fold increased ROS production and much higher cytotoxic effects if humidified feed gas is used.¹⁴⁹ As stated above, the ROS produced by plasmas in liquids can also be used in the so-called indirect plasma treatment, where plasma-treated liquids are added to tumour cells to perform cytotoxic action.⁸⁸ Several studies used such an approach using plasma jets operated in different feed gas settings and utilizing different types of treated liquids to successfully induce cytotoxicity in breast cancer cell lines in vitro.^{132,150–159} On average, indirect plasma treatment was comparable to direct treatment

effects in those studies where a side-by-side comparison was performed. However, one study found indirect treatment to only increase extracellular but not intracellular ROS and induced remarkably less cytotoxic, than direct treatment.¹⁶⁰ This can be explained by the cell culture medium used to perform the plasma treatment. Earlier reports already pointed to a pivotal role of the composition of liquid to be plasma-treated on the latter effects in the cells exposed to these liquids.¹⁶¹ Specifically, some media types are enriched with greater levels of antioxidants such as pyruvate, which scavenge ROS and thereby reduce the plasma treatment effect. On a relative scale, the scavenging role of fetal bovine serum often supplemented to cell culture media to provide growth factor stimulation is less pronounced, at least in terms of eukaryotic plasma treatment in vitro.¹⁴⁹ Hence, plasma-treated medium containing high levels of ROS scavengers quickly lose their cytotoxic effects after being generated (within

TABLE 4 In vivo and ex vivo studies and findings in gas plasma breast cancer treatment. BC = breast cancer

Model	Gas plasma device	Exposure modality	Gas plasma treatment effects and mechanisms of action in breast cancer cells	Ref.
AN3 cells, female Swiss albino mice (6–8 weeks), xenograft	DBD (air) direct treatment	20–50 s, three times, every 48 h, 21 days follow-up	■ Decreased tumour growth ■ Modestly increased survival ■ Decreased body weight gain	163]
4T1 cells, female BALB/c mice (4–6 weeks), syngeneic	DBD (He) direct treatment	180 s every 48 h, 25 days follow-up	■ Reduced tumour volume and enhanced survival ■ Increased BC tumour apoptosis and altered BAX and BCL-2 as well as p53 expression	128
MDA-MB-231 or MCF-7 cells, in female BALB/c (4–6 weeks), xenograft	Plasma jet (He)-treated cell culture medium for injection	100 µl 15-min plasma-treated medium, daily 29 days	■ No effect in MCF-7 tumour xenografts ■ 80% weight and tumour volume reduction in MDA-MB-231 xenografts	150
4T1 cells, female BALB/c mice (4–5 weeks), syngeneic	DBD (air) direct treatment	1–4 min tumour wound plasma treatment immediately after surgery, 60 days follow-up	■ Decreased relapse of tumour growth from surgically removed tumour wounds with gas plasma treatment, and enhanced survival ■ The effect was treatment-time dependent ■ Gas plasma-induced ICD locally and enhanced DC maturation (CD80, CD86)	196
4T1 cells, female BALB/c mice (6–7 weeks), syngeneic	Plasma jet (He) direct treatment	5 min, daily, for 21 days	Tumour growth and volume reduction True abscopal effect with tumour reduction on the plasma-treated flank and in parallel reduced growth of an untreated tumour of the opposite flank in the same animal Plasma-induced ICD locally, enhanced apoptosis and CRT expression, increased leukocyte infiltration (DC, CD4⁺ and CD8⁺ T-cells) in both the untreated and gas plasma-treated tumour flank	131
Breast cancer patient-derived tumour tissue minced and gas plasma-treated ex vivo	Plasma jet (He) direct treatment and plasma-treated medium	5 min, two times on two consecutive days, 24 h incubation	■ Gas plasma elevated tumour cell death in microtissues by activation of caspase 3 and reduced microsatellite growth in a 3D model system ■ Reduced migratory activity of Tumour cells in microtissues ■ Gas plasma exposure modified the release of several inflammatory agents in the TME, such as IL-6, IL-8, IL-18, IL-33, IL-17A, IFN-α2 and MCP1	120

minutes to hours, depending on storage), while during direct plasma treatment of cells immersed in those media, the scavenging kinetic might be too slow to shield all cells from the effects of plasma-derived ROS. In general, the generation of plasma-treated cell culture media is an academic endeavor rather than of clinical significance.

Similar to the need for medical devices' approval in order to be used in clinical applications, only approved types of liquids can be used in patients. Such liquids include, for instance, .9% sodium chloride and Ringer's lactate, both showing favorable effects as plasma-treated liquids in a broad comparison on the utilization of plasma-treated

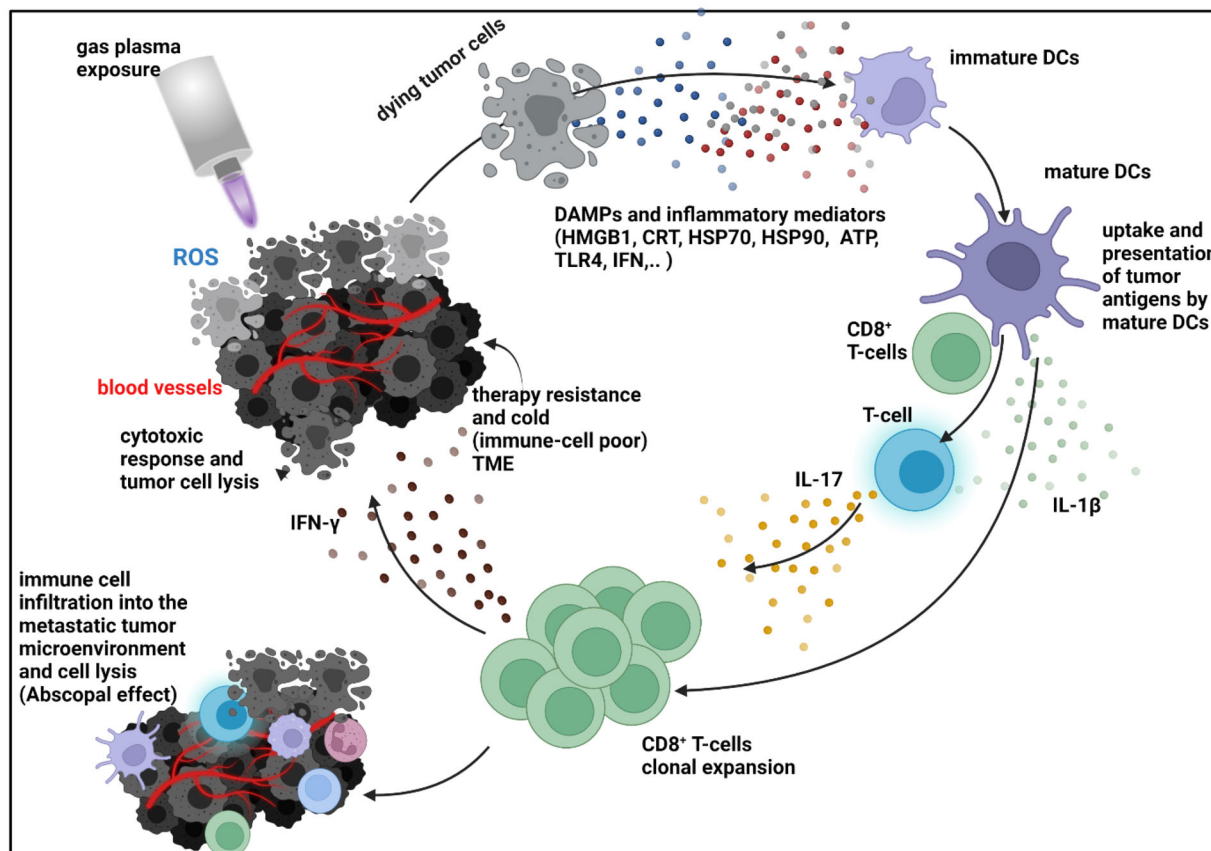


FIGURE 4 The cancer-immunity cycle involves immunogenic cell death (ICD) and release of damage-associated molecular patterns (DAMPs), spurring dendritic cell (DC) maturation and cognate antigen effector T-cell activation and clonal expansion contributing to anticancer immunity. Gas plasma exposure is hypothesized to kick-start this cycle, as shown in two published in vivo studies.^{131,196} The image is adapted based on established ICD concepts.^{116,217}

liquids for anticancer effects.¹⁶² In addition, there are many studies on direct^{128,163–168} and a few on indirect^{169,170} DBD plasma breast cancer treatment. Overall, results did not differ profoundly in terms of a principle induction of cytotoxic effects as found in the plasma jet studies. The DBDs were operated mostly in ambient air or a feed gas, such as argon, was used. Unfortunately, none of the studies using jet or DBD plasma-treated liquids (indirect approach) used a type of liquid approved for clinical use. This gap might be filled by future studies.

2.2 | Mode of action and selectivity

Since gas plasmas produce sufficient amounts of ROS, it is understood that gas plasma-mediated (breast) cancer cell death is in line with many findings in redox biology and ROS-dependent initiation of cell death.³⁷ Most of these pathways lead to the initiation of apoptosis (Figure 4). However, it should be mentioned that the involvement of other forms of regulated cell death has been mentioned in tumour cells subsequent to gas plasma exposure, such as

autophagy and lysosomal cell death,^{171–175} ferroptosis^{176–179} and necroptosis.¹⁷²

In contrast to other physical modalities, such as ionizing and UV radiation, where ROS are generated intracellularly and extracellularly, gas plasma produces majorly extracellular ROS because this modality is void of extensive radiation. Therefore, extracellular biomolecules and cell membranes are the first targets of these ROS. Simulation studies on gas plasma-treated membranes predicted the possibility of short-term ROS-induced pore formation, which may help surplus extracellular ROS to enter tumour cells.¹⁸⁰ In parallel, lipid peroxidation was predicted to occur, likely via hydroxyl radical formation.¹⁸¹ Albeit several details on gas plasma-induced lipid oxidation remain elusive, it is assumed that lipid head group oxidation takes place prior to tail oxidation.¹⁸² In addition, cholesterol-poor cell membranes, as frequently observed in tumour cells, were predicted to be more sensitive to gas plasma-mediated damage compared to cholesterol-rich counterparts, as suggested by computer simulations.¹⁸³ Recently, a systemic comparison between different tumour cell lines underlined this notion.¹⁴⁴ Another frequently

described class of membrane transporters critical in breast cancer progression is aquaporins.¹⁸⁴ The transmembrane channels are known to transport not only water but also ROS, such as hydrogen peroxide, across cell membranes. Accordingly, a role of aquaporins in gas plasma-induced cancer cell death has been assumed in several works.^{152,185,186} However, a study across 36 cancer cell lines, including several of breast origin, did not support this view.¹⁴⁴

Once gas plasma-derived ROS have entered the cell and/or induced sufficient oxidation of, for example, cell membranes, the tumour cells sense the oxidative stress and respond via specific signalling and altered transcriptional and translational profiles. Several *in vitro* works addressed the mechanisms of gas plasma-induced cytotoxicity in breast cancer cells. One report indicated that gas plasma could inhibit the growth and proliferation of breast cancer cells by reducing STAT3.¹⁶⁴ This was linked to decreased IL-6R expression and Akt phosphorylation, while PTEN was enhanced. STAT3 signalling is associated with breast cancer malignancy by driving progression, proliferation, metastasis and chemoresistance. Therefore, it is investigated as a diagnostic and therapeutic target in breast cancer therapy.¹⁸⁷ Another study found increased phosphorylation of p38 MAPK and decreased ERK phosphorylation following gas plasma exposure in breast cancer cells,¹⁴⁷ suggesting cell death initiation. This was accompanied by elevated p53 and caspase 8 expression levels, and other reports supported this by finding increased caspases 3, 7, 8 and 9 levels in gas plasma-treated breast cancer cells.^{125,163} BAX and PUMA activation, as well as mitochondrial oxidation and cytochrome C release, were also found.^{131,132,153} Apoptosis induction via p53 phosphorylation and increased Bax/Bcl2 ratios were also reported in gas plasma-treated breast cancer tumours *in vivo* in mice.¹²⁸ Elevated apoptotic events increased breast cancer cell expression of ATF3, a molecule implicated in DNA damage responses, which was also mentioned in this study. It is assumed that those findings, along with several studies that reported elevated phosphorylation levels of the DNA-damage indicator histone 2A.X,^{123,127,153} are secondary events and not the action of primary gas plasma-generated ROS reaching the DNA directly.⁷⁴ Gas plasma exposure also increased the expression of HMOX1 in MDA-MB-231 and MCF-7, and glutaredoxin 1 and peroxiredoxins 1 in the latter.¹⁵⁸ These proteins are involved in antioxidant defenses and DNA-damage responses, and the data align with findings of exceptionally high oxidized GSH (GSSG) levels in gas plasma-treated breast cancer cells.¹⁴⁵ This is supported by data on cell death abrogation in gas plasma-treated breast cancer cells by prior addition of the amino acids cysteine and tryptophan,¹⁵⁴ both known to scavenge ROS and support cellular antioxidant defense.

Extracellular matrix broken-down by matrix metalloproteinases after epithelial-mesenchymal transition, together with decreased integrin and cadherin expression, causes cancer cells to enter the bloodstream or lymph vessels, promoting metastasis.¹⁸⁸ Gas plasma treatment of breast cancer patient-derived microspheres in collagen was accompanied by elevated E-cadherin expression.¹²⁰ According, decreased migration was reported for gas plasma-treated breast cancer cells in transwell migration and matrigel invasion as well as 2D time-lapse video microscopy cell motility assays.¹³⁰ This is supported by findings on reduced TRIM31 expression in breast cancer cells following gas plasma exposure,¹⁶⁵ a molecule involved in tumour progression and metastasis. The molecular basis of these changes is not understood yet. A genome-wide epigenetic study on gas plasma-induced changes in CpG methylation sites identified in an IPA analysis genes associated with cell-to-cell signalling as top category,¹⁶⁶ suggesting an involvement of junctional and adhesion kinase signalling processes. As a more basic mechanism underlining gas plasma-derived ROS mechanisms, a recent report suggested oxoguanine (8-oxoG) modifications of mRNA to be critical, hypothesizing that these messengers would be removed and specifically affect S-phase cell cycle progression,¹²⁵ albeit it is unclear how such specificity would be achieved. Recent studies also implied regulation on the micro RNA level, with miR-19a having a particularly tumour-promoting role in breast cancer cells that were attenuated by gas plasma treatment.¹⁸⁹

2.3 | Plasma combination treatment and drug resistance

Several studies combined gas plasma exposure with standard or experimental anti-breast cancer agents *in vitro*. In an extensive comparison study using 14 different breast cancer cell lines with different molecular profiles (luminal, basal A, basal B), gas plasma was combined with radiotherapy.¹²³ Generally, all cell lines were sensitive to both types of treatments, albeit on a different exposure scale. However, what was unexpected was a highly significant correlation between both treatments, that is, cells sensitive to gas plasma were also sensitive to radiotherapy and vice versa. This indicates that radiotherapy—at least *in vitro*—is considerably acting via ROS generation, inducing oxidative stress and cell death. The second intriguing finding of the study was that gas plasma and radiotherapy synergistically combined when exposed together in the seven breast cancer cell lines investigated. The third notion was that gas plasma treatment combined with the PARP-inhibitor olaparib to elevate toxicity, especially in TNBC.

Albeit, underlining data in animal models are missing so far, the many combinations and cell lines employed in this study outline the potential gas plasma treatment might with established breast cancer therapies.¹²³ Another conventional breast cancer chemotherapy is paclitaxel. Gas plasma exposure significantly reversed paclitaxel resistance in MCF-7 cells.¹⁴⁰ Transcriptomic analysis revealed several potential candidates mechanistically linked to this finding, and CEACAM1 and DAGLA were found to regulate sensitivity restoration to the drugs. The two proteins had so far not been ascribed a role in breast cancer drug resistance. Another report investigated the effects of gas plasma-derived ROS in tamoxifen drug-resistant MCF-7 cells.¹⁴¹ The treatment reduced tamoxifen sensitivity in 50% of the cells, and following a gene expression profiling, siRNA-mediated knockdown provided evidence of MX1 and HOXC6 playing a role in drug sensitivity restoration via oxidative stress. The former is known to act in cancer mitosis, while the latter is associated with the mammary gland and described in multidrug-resistant cancer cells. Experimental drugs and approaches have also been combined with gas plasma exposure of breast cancer cells in vitro. For instance, with ascorbate, a substance frequently debated to be beneficial also in breast cancer patients,¹⁹⁰ additive cytotoxicity with gas plasma exposure could not be identified in human breast cancer cells in vitro.¹³⁹ Another study reported a modest combination of gas plasma with gold nanoparticles in two breast cancer cell lines falsely claimed to be synergistic.¹⁹¹ Gas plasma treatment alone and combined with FK866, a nicotinamide phosphoribosyltransferase inhibitor, increased intracellular ROS and cytotoxicity via PARP-1 activation, $\Delta\Psi_m$ disruption and mitochondrial dysfunction and weakened antioxidant defense system (reduction of GSH and NADPH), leading to energy crisis and ATP depletion in MDA-MB-231 breast cancer cells.¹⁵⁷ Gas plasma exposure also potently combined with PU-H71, an HSP90 inhibitor currently in clinical trials, via enhanced degradation of PKD2 and HSP90 β cleavage in breast cancer cells.¹⁴³ However, translational models are lacking to validate any therapeutic implication of the findings, but the mentioned studies nevertheless indicate a role of oxidative stress in combination treatments of breast cancer cells.

2.4 | Plasma-induced ICD in breast cancer

Several physical treatment modalities have already been observed to induce ICD.¹⁹² The idea is to promote anti-tumour immunity by enhancing the quality and quantity of T-cells targeting tumour cells. The concept has

been extended to gas plasma cancer treatment by framing and experimentation (in a syngeneic colon cancer mouse model) a few years ago,^{193,194} with the gas plasma process functioning as a *kick-starter* to set the cancer-immunity-cycle in motion (Figure 4). It should be noted that direct gas plasma-mediated tumour debulking is only a secondary outcome in this process, as there are other methods more suitable to provide such effect. The primary goal is localized ICD induction, sometimes called in situ vaccination.¹⁹⁵ One in vitro report screened the expression of 18 immune-related surface markers after low-toxicity gas plasma treatment and found an increased expression of CD40 and CD112, as well as CD273 and Gal-9 in MDA-MB-231 and CD271 in MCF-7 cells, indicative of oxidative stress translating to differential immuno-suppressive signatures.¹⁴² Of the three studies investigating the anti-cancer efficacy of gas plasma treatment in syngeneic breast cancer mouse models, two deliberately investigated immune effects. In the first study, a helium-driven plasma jet (kinPen) treatment induced hallmarks of ICD in MCF-7 and MDA-MB-231 human breast cancer cells in vitro, such as increased externalization of CRT, HSP70 and HSP90 as well as elevated ATP release.¹³¹ Enhanced CRT externalization was also observed in gas plasma-exposed 3D tumour spheroids. In vivo, live 4T1 cells were injected into both flanks of mice to grow tumours. Only one site was gas plasma-treated, leading to growth reduction on this site. Strikingly, tumour growth also significantly declined on the untreated contra-lateral site, indicating gas plasma inducing an abscopal effect in vivo. TME analysis revealed enhanced apoptosis in both tumours of plasma-treated mice, along with elevated numbers of intratumoural T-cells and DCs.¹³¹ The second study also observed hallmarks of ICD induction in gas plasma-treated 4T1 cells in vitro, such as CRT exposure and enhanced expression of the DC maturation markers CD80 and CD86 in a plasma treatment time-dependent fashion.¹⁹⁶ In vivo, tumours were grown to 500mm² before being surgically removed. The surgical margin was exposed to gas plasma or left untreated, and the tumour relapse growth and animal survival were significantly improved in gas plasma-treated animals. In regrowing tumours, gas plasma exposure significantly increased intratumoural T-cells and their proliferation along with elevated levels of IFN- γ , TNF- α , IL-2, IL-12 and also immuno-suppressive IL-10. In future experiments, it would be interesting to see whether the combination of local gas plasma therapy with systemic checkpoint immunotherapy results in augmented anticancer effects. In syngeneic melanoma models, gas plasma-inactivated melanoma cells in vitro were injected as vaccine in mice, providing partial protection from subsequent live-cell injection-related melanoma growth.^{197,198} Also such gas

plasma-assisted vaccination may be combined with other immunotherapies to test for additive antitumour effects. Last but not least, breast cancer patient-derived samples were recently investigated for their release of inflammatory mediators after gas plasma exposure and incubation *ex vivo*.¹²⁰ Besides several significant but small-scale differences for several cytokines, the largest changes were observed for IL-6 and IL-8 (decrease > 100-fold). Both IL-6 and IL-8 are pleiotropic agents described to perform immunosuppressive functions in breast cancer.¹⁹⁹ Hence, it is possible that gas plasma treatment elevates the immuno-suppressive profiles of breast cancer cells not immediately succumbing to cell death. This has also been observed with other physical modalities for breast cancer treatment before, such as radiotherapy or hyperthermia.²⁰⁰ For those reasons, it is assumed in the field of onco-immunology that physical modalities may help freeing tumour antigen locally to promote antitumour immunity that a later stage should then be re-invigorated using immunotherapy, such as checkpoint inhibitors.

3 | GAS PLASMA TECHNOLOGY FOR ULCERATING BREAST CANCER COMBINATION THERAPY

Several clinical treatment modalities are conceivable using gas plasma technology in breast cancer treatment. First, it should be emphasized that only clinically approved gas plasma devices should be used in patient studies to ensure a safe application and approval based on pre-existing clinical evidence. For plasma jets, devices that have ideal geometries for treating the uneven topology of human body surfaces and surgical wounds, the only approved device for broad applications on the skin or wounds currently is the kINPen MED.⁴⁰

Intuitively, one would assume that plasma could be used to treat breast cancer below the skin. However, this is not feasible as the plasma-generated ROS have only limited penetration depths that depend on the tissue in question. Non-keratinized tissues may show effects between a few dozen to a few hundred micrometers.^{98,201} However, in keratinized skin, plasma effects may not reach much beyond the stratum corneum, as evident in human skin plasma-exposed *ex vivo* and murine skin (nude mice) plasma-exposed *in vivo*.^{97,202} However, deeper tissue effects of the plasma treatment were observed, for instance, in mice skin plasma-treated *ex vivo* using hyperspectral imaging as increased tissue water index and deeper tissue oxygenation were noted in these models.²⁰³ Blood flow elevation, including hemoglobin index, was also found in intact human skin.^{99,100} The mechanisms of action are unclear. Yet, what remains clear is that subdermal breast

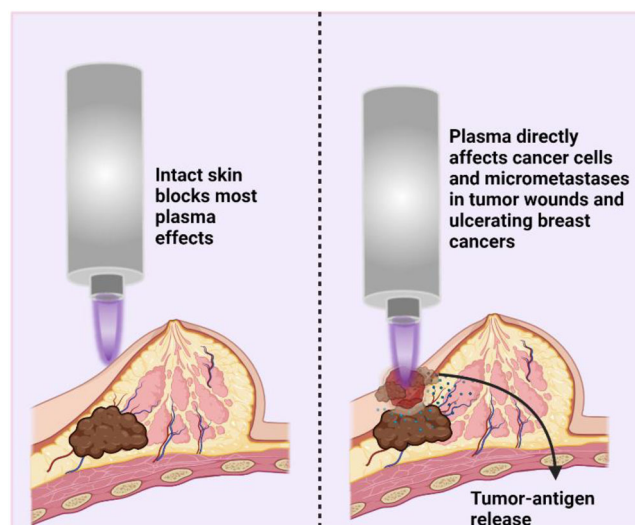


FIGURE 5 Graphic illustrating the advantage of gas plasma treatment of tumour wounds or ulcerations

cancer will likely not be targetable by superficial plasma treatment.

In the case of ulcerating breast cancers, however, plasma therapy may be beneficial (Figure 5). This can be deduced from a case report series of patients suffering from advanced head and neck cancer. The palliative cancer patients had ulcerating tumours in the face or neck region, which were heavily colonized with microorganisms, producing a strong odor that negatively impacted the palliation of these end-stage patients that had failed all standard therapies.¹⁰² With the original aim of reducing the microbial flora, and hence the odor, more than 20 patients received gas plasma therapy regularly (1–3 times per week) over several weeks to months. A dramatic and unexpected decline of tumour mass was observed in some patients after 1–2 dozen treatment sessions. We had recently hypothesized that the result might have been a combined effect of the direct anticancer action of gas plasma and dead microorganisms acting as *in situ* adjuvants to boost antitumour immunity.²⁰⁴ Eventually, all patients relapsed irrespective of the continuation of plasma therapy and deceased. However, these results provide hope that gas plasma technology might be beneficial in ulcerating and infected breast cancer wounds to simultaneously reduce cancer growth and local infection. When breast cancer ulcerates, they cause various symptoms such as pain, itchiness, unpleasant smell, bleeding, leaking or oozing and infection.⁶ Considering the large number of stage III–IV breast cancer patients globally, many patients (about 1% of all cases) are prone to developing fungating or infected superficial breast cancer ulcerations.²⁰⁵

Another concept of utilizing gas plasma technology is its adjuvant application to surgical breast tumour removal.

As emphasized above, recent *in vivo* studies provided compelling evidence that post-operative plasma treatment of the tumour resection margins significantly decreased subsequent tumour relapse, probably by reducing tumour micrometastases in the wound margins and promoting antitumour immunity.¹⁹⁶ Depending on the plasma device to be used, the plasma treatment time might be a reasonable investment for post-surgical breast cancer wound care, even if recurrence rates would be decreased by this method only by a few percent. This is because gas plasma technology is highly advantageous with regard to its superior safety profile and tolerability.⁴⁰ Clinical plasma treatment is straightforward to implement and apply, and single treatment session costs are negligible before the tumour wound is closed.

An additional concept, which has so far not been explored for breast cancer but for melanoma and colorectal cancer in experimental models, is gas plasma-assisted tumour vaccination. Using in the laboratory plasma-inactivated melanoma cells as a vaccine, three independent studies provided evidence of vaccination partially protecting mice from subsequent tumour growth after a second live tumour cell injection.^{194,197,198} In the experiments, plasma treatment did not outperform immunogenic drugs such as the ER-stress inducer mitoxantrone predicted to elicit ICD,¹¹⁸ but possibly, combining plasma treatment with other ICD inducers and adjuvants may improve vaccine-mediated antitumour protection. In the clinical setting, such an approach may re-vitalize the long-standing idea of using autologous tumour material for vaccination purposes by upgrading its immunogenicity using plasma processes. There is no clinical evidence to date to support this concept using gas plasma technology, but autologous tumour lysates and their oxidation using hypochlorous acid were recently shown to induce potent antitumour immunity and improved survival in a cohort of ovarian cancer patients.^{206,207} This promotes the underexplored idea of using ROS as an immune-enhancer and in autologous anticancer vaccination.²⁰⁸

Finally, the idea of post-surgical plasma treatment of breast cancer wounds and the concept of plasma-treating ulcerating and infected breast cancer wounds may be combined with other treatment modalities, such as checkpoint immunotherapies. Other physical, local treatment modalities, such as radiotherapy and PDT, have already been explored similarly.²⁰⁹ Intriguingly, the *in vivo* study using plasma treatment to treat the surgical breast tumour margins convincingly showed elevated antitumour immunity, DC activation, and tumour immune infiltrations in the plasma compared to the control group.¹⁹⁶ Using a syngeneic melanoma model, the same group provided compelling evidence of beneficial combination effects of

local plasma tumour treatment with anti-PD-1 checkpoint antibody immunotherapy.²¹⁰ Hence, it is conceivable that such an *in situ* vaccination using gas plasma technology may be worth testing in the clinical setting. Such as approach, similar to the autologous plasma-upgraded tumour vaccine idea, is especially auspicious since most patients do not die because of the primary tumour but due to metastasis. However, local tumours could be used as biopsy source or *in situ* vaccination target to help promote existing T-cell responses (quantity) or support the generation of novel T-cell clones (quality) adapted to changing mutational landscapes and peptide profiles high-grade tumours often exhibit.²¹¹ As similar effects have been ascribed to radiotherapy via release of DAMPs, inducing ICD, and reasoning radio-immunotherapeutic schemes,^{212,213} it is conceivable that radio- and gas plasma therapy may have synergistic, additive or antagonistic effects in local immuno-stimulation. Our recent combination treatment *in vitro* using melanoma cells at least suggested a combined immuno-stimulatory potential.²¹⁴ However, there seems to be unraised potential in combining both technologies in translationally more relevant models, especially since radiotherapy is already clinically used to treat ulcerating breast cancer.²¹⁵ The ultimate goal remains to enhance the efficacy of immunotherapies by supporting antigen release and inflammatory presentation conditions rather than tumour mass debulking, as recently summarized in the case of adjuvant radiotherapy.²¹⁶ In this frame, gas plasma technology may be an additional future tool in the therapists' toolbox to be added depending on the patient's tumour characteristics to help foster tailored anticancer treatments.

4 | CONCLUSION

Gas plasma-derived ROS are a promising adjuvant tool to support existing breast cancer therapies. Although clinical evidence on plasma breast therapy success is absent, promising proof-of-concept studies are available for other tumour entities in patients and breast cancer animal models. Auspicious applications involve the plasma's capability to increase breast cancer cells' immunogenicity and use the technology for *in situ* or *ex vivo* vaccination approaches. Particularly, patients suffering from ulcerating and infected breasts are expected to benefit from gas plasma therapy.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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ORIGINAL RESEARCH



Gas plasma irradiation of breast cancers promotes immunogenicity, tumor reduction, and an abscopal effect in vivo

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ABSTRACT

While many new and emerging therapeutic concepts have appeared throughout the last decades, cancer still is fatal in many patients. At the same time, the importance of immunology in oncotherapy is increasingly recognized, not only since the advent of checkpoint therapy. Among the many types of tumors, also breast cancer has an immunological dimension that might be exploited best by increasing the immunogenicity of the tumors in the microenvironment. To this end, we tested a novel therapeutic concept, gas plasma irradiation, for its ability to promote the immunogenicity and increase the toxicity of breast cancer cells in vitro and in vivo. Mechanistically, this emerging medical technology is employing a plethora of reactive oxygen species being deposited on the target cells and tissues. Using 2D cultures and 3D tumor spheroids, we found gas plasma-irradiation to drive apoptosis and immunogenic cancer cell death (ICD) in vitro, as evidenced by an increased expression of calreticulin, heat-shock proteins 70 and 90, and MHC-I. In 4T1 breast cancer-bearing mice, the gas plasma irradiation markedly decreased tumor burden and increased survival. Interestingly, non-treated tumors injected in the opposite flank of mice exposed to our novel treatment also exhibited reduced growth, arguing for an abscopal effect. This was concomitant with an increase of apoptosis and tumor-infiltrating CD4⁺ and CD8⁺ T-cells as well as dendritic cells in the tissues. In summary, we found gas plasma-irradiated murine breast cancers to induce toxicity and augmented immunogenicity, leading to reduced tumor growth at a site remote to the treatment area.

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Introduction

Breast cancer is the most frequent type of cancer in women worldwide, with many of the cases being fatal.^{1–3} The development of efficient and new treatment methods is difficult because of the complex nature of that type of cancer.⁴ Most breast cancer-related deaths occur if metastatic cells begin to detach from the primary tumor and start to spread to surrounding tissue or secondary sites. During this process, cancer cells detach from the primary tumor by invading the basement membrane, crossing connective tissue, and penetrating other organs through blood vessels or lymphatic vessels.^{5,6} In general, novel cancer therapies should be effective, safe, and associated with a minimum of side effects.^{7,8}

There is a variety of different traditional and novel treatment options for cancer, such as surgery, chemotherapy, radiotherapy, photodynamic therapy, and immunotherapy.^{9–11} Especially the latter received increased attention during the recent years.¹² Clinical trials using immune checkpoint inhibitors in patients with triple-negative breast cancer, such as atezolizumab targeting PD-L1, showed promising results.^{13,14} This builds the case

for the power of unleashing antitumor immunity in breast cancer patients, which was observed already for other tumor entities.^{15,16} Tumor cells mutate at high frequencies, leading to an elevated mutational burden that correlates to responses to immunotherapy in patients.¹⁷ For breast cancer, on average, somatic mutations are regularly observed,¹⁸ facilitating antitumor T-cells responses for attacking metastatic lesions.

However, for antitumor immunity to form, tumor antigens not only need to be present but also accessible and processable for antigen-presenting cells to promote T-cell mediated immunity. Radiotherapy has been proven to exert immune modulatory effects.¹⁹ Locally applied ionizing radiation can thereby potentiate antitumor immunity, leading to reduced cancer growth also of distant, untreated sites (abscopal effect).²⁰ It is noteworthy that radiotherapy, to some extent, relies on the local generation of reactive oxygen species (ROS).²¹ Further, radiotherapy synergizes with other ROS-inducing treatments such as gas plasma in the induction of cancer cell death with immune stimulatory properties.²² Therapeutic levels of ROS are increasingly appreciated as novel paradigm for cancer treatment.²³

Gas plasma technology is an emerging innovation in oncology.²⁴ It is recognized that the main mode of action of these partially ionized gases operated at body temperature relies on the generation of ROS.²⁵ The unique feature of gas plasma systems is that a plethora of different types of ROS is being produced simultaneously,²⁶ facilitating oxidative damage of tumor cells and, ultimately, leading to their demise. While the concept of ROS-mediated killing using gas plasmas has been explored to some extent,²⁷ the immunological consequences of gas plasma irradiation of breast cancer cells are unknown. To this end, we investigated gas plasma-irradiated breast cancer cells in terms of toxicity and immunogenicity *in vitro* and *in vivo*. Gas plasma irradiation not only effectively controlled tumor growth but also was concomitant with an upregulation of several markers of the immunogenic cancer cell death (ICD), such as calreticulin (CRT) and the damage-associated molecular pattern (DAMP) molecules heat-shock protein (HSP) 70 and HSP90.^{28,29} Finally, we found gas plasma irradiation to mediate an abscopal effect in breast-cancer bearing mice, mounting evidence of a promising role of therapeutic ROS derived from gas plasma systems in promoting anticancer immunity.

Materials and methods

Cell culture and *in vitro* gas plasma irradiation

Human breast cancer cell lines MCF-7 (ATCC: HTB-22) and MDA-MB (ATCC: HTB-26) were cultivated in Dulbecco's Modified Eagles' Medium (DMEM; Corning). The murine breast cancer cell line 4T1 (ATCC: CRL-2539) was cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Corning). All culture media were supplemented with 10% fetal bovine serum, 2% L-glutamine, and 1% penicillin (all Sigma Aldrich), and were kept under standard culture conditions at 37°C, 5% CO₂, and 95% humidity in a cell culture incubator (Binder). Twenty-four hours before experiments, the cells were seeded at 5×10^4 cells per ml of fully supplemented medium in 24-well tc-treated cell culture plates (Eppendorf). Three-dimensional tumor spheroids were formed as described before.³⁰ Briefly, cells were seeded at 4×10^3 cells per 200 μ l fully supplemented medium in 96-well ultra-low attachment plates (Corning). After centrifugation at 1000 $\times$ g for 10 min, the cells were cultured for 72 h before experiments. For the gas plasma irradiation of the cells *in vitro*, a helium-driven plasma jet was utilized. Its technical parameters and physical properties have been described in detail before.²⁶ It was operated with a gas flow four standard liters helium (purity: 99.999%; Air Liquide). The distance of the target to plasma as well as the treatment time was kept constant using a software-controlled xyz-table (CNC Step). Vehicle controls received helium gas only, through the same system.

Reactive species detection

For the detection of reactive species introduced into the cell culture medium following gas plasma irradiation, several different assays were applied. Hydrogen peroxide (H₂O₂) was quantified utilizing the Amplex Ultra Red assay kit (ThermoFisher) according to the manufacturers' instructions.

For the detection of nitrite (NO₂⁻) inside the medium, a deterioration product of nitric oxide (NO), the Griess assay (Cayman Chemical) was carried out. Hypochlorous acid (HOCl) was detected by the colorimetric taurine chloramine assay, as previously described.³¹ The measurements were performed using a multiplate reader (Tecan Infinite F200 PRO) at $\lambda_{\text{ex/em}} = 535 \text{ nm}/590 \text{ nm}$ for fluorescence detection (H₂O₂), or the (Tecan Infinite M200 PRO) at $\lambda = 540 \text{ nm}$ (NO₂⁻) and at $\lambda = 645 \text{ nm}$ (HOCl) for absorbance detection. The pH levels of gas plasma-irradiated cell culture medium were quantified using a pH-meter (Mettler Toledo).

Metabolic activity and cytotoxicity

Resazurin (Alfa Aesar), a non-fluorescent dye becoming fluorescent after reduction via cell-derived NADPH, was added at 22 h or 46 h after gas plasma irradiation at a final concentration of 100 μ M and incubated for 2 h. Fluorescence was detected by a multimode plate reader (Tecan Infinite F200 PRO) at λ_{ex} 535 nm and λ_{em} 590 nm. For assessing cytotoxicity via microscopy, 4,6-Diamidin-2-phenylindol (DAPI, final concentration 1 μ M; BioLegend) was added to each well. Subsequently, the cells were imaged using a high-content imaging analysis system (Operetta CLS; PerkinElmer) with a 20x (NA = 0.4) air objective (Zeiss) equipped with laser-based autofocus (785 nm). Imaging was performed using the brightfield channel (with a 740 nm transmission light source), the digital phase contrast (DPC) channel (generating a single image from differential contrasts that reflect the cytosolic region of a cell), and the DAPI channel (λ_{ex} 365 nm and λ_{em} 465 $\pm$ 35 nm). The data were analyzed using quantitative imaging and segmentation software (Harmony 4.9; PerkinElmer). For analysis of tumor spheroids, they were stained with either MitoTracker Red (MTR) CMXRos (ThermoFisher), sytox green (ThermoFisher), or anti-calreticulin (CRT) Alexa Fluor (AF) 647 (Novus) to assess mitochondrial activity, cell death, and immunogenic cell death, respectively. Imaging was done by using the high content imaging device with a 5x air (NA = 0.16) objective and four z-stacks per spheroid in non-confocal mode. These z-stacks of each spheroid were merged using maximum intensity projections. The spheroids area was segmented and evaluated using Harmony software for the extent of cell death (sytox green intensity per spheroid), the oxidation (MTR intensity per spheroid), and CRT expression (AF647 intensity per spheroid).

Flow cytometry

To investigate the extent of apoptosis following gas plasma irradiation, the cells were incubated with caspase 3/7 detection reagent (ThermoFisher) and DAPI. Cells were detached using accutase (BioLegend) and analyzed using flow cytometry (CytoFLEX S and LX; Beckman-Coulter). For analysis of immunogenic cell death (ICD) markers, the cells were incubated with monoclonal antibodies targeted against CRT and labeled with AF647 (Novus), heat-shock protein (HSP) 70 AF488 (BioLegend), HSP90 PE (Enzo), MHC-I PE-Cy7 (BioLegend), CD274 BV650 (BioLegend), as well as with iFluor 860 (AAT Bioquest). For the detection of intracellular antigens, the cells were fixed and permeabilized (fixation and permeabilization wash buffer; BioLegend) and stained with

antibodies targeted against LC3 AF488 (Cell Signaling), or phospho-eIF2 α labeled with AF488+ (Invitrogen; conjugate ThermoFisher), phospho-ATM labeled with PE (BioLegend), and ERp75 labeled with AF647+ (Santa Cruz; conjugate ThermoFisher). The cells were acquired using flow cytometry, and data analysis was performed using Kaluza 2.1.1 software (Beckman-Coulter).

Supernatant analysis

Cellular supernatants were collected 1 h and 48 h after in vitro treatment procedures. Residual cells were removed via centrifugation. ATP was detected utilizing the Luminescent ATP Detection Kit (Abcam), and luminescence was measured using a multimode plate reader (Tecan). Enzyme-linked immunosorbent assays were utilized according to the manufacturers' procedures for the quantification of HSP70 (ThermoFisher), IFN2 α , IFN γ , and IL-6 (all BioLegend). The absorbance was measured using the same multiplate reader (Tecan).

In vivo tumor model

The in vivo experiments were approved by the Shahid Beheshti University of Medical Science Animal Ethics Committee (approval number IR.SBMU.RETECH.REC.1397.1212) and were conducted at the Shahid Beheshti University. The animals were housed under standard laboratory conditions (room temperature = 23°C), with an ad libitum supply of nutrition and water, and circadian environmental control (12 h light shifts). All interventions at the animals were performed under anesthesia through the injection of ketamine and xylazine. Subcutaneous breast cancers were induced by subcutaneous injection of 5×10^5 4T1 cells (ATCC CRL-2539) in each flank of 6–7 weeks old female Balb/CfC3H mice (Royan Institute). Daily gas plasma irradiations were started after the tumor diameters reached 5 mm at day 12 and were performed for 21 days in total. A helium gas-driven plasma multi-jet was utilized that was operated at 20 kHz frequency with a total input power of 1 W. The gas plasma irradiation time was 300 s per tumor and was only applied to the left tumors in the irradiation group. In order to investigate tumor growth and immune cell infiltration, a portion of the animals was sacrificed at day 36 for further downstream analysis (untreated tumor: n = 16; irradiation left and right: n = 8 each). The survival of untreated and gas plasma-irradiated animals was investigated until day 72 (untreated: n = 10; irradiation: n = 10).

Tumor growth monitoring in vivo

The tumors' greatest longitudinal and transverse diameter were measured every three days. The tumor volume was calculated using a modified ellipsoidal formula ($V_{\text{tumor}} = (d_{\text{longitudinal}} \times d_{\text{transverse}}^2) \times \pi / 6$). The tumor weight at day 36 was measured after tumor explantation via a precision balance. The tumor density was calculated to be 1.056 g/ml ($m = \rho \times V$). For all tumor growth evaluations, the calculated tumor volume was normalized to that of the corresponding initial tumor volume at day 12. Moreover, sonography

(ultrasound) was performed to measure the greatest tumor length, width, and height, as well as to identify hypo or hyperdense structures within the tissue. Representative animals were additionally analyzed via computer tomography (CT) at the Faculty of Veterinary Medicine of the University of Tehran. The calculation of three-dimensional animal reconstructions, surface rendering, and tumor volume quantification was performed with the Horos 3.3.6 medical image viewer (Horos Project).

Tissue sectioning and staining

Tissue sections of 5 μ m thickness were cut and immersed in xylene and different percentages of ethanol to remove the paraffin and perform rehydration. For antigen retrieval, tissue slides were placed in citrate buffer (10 mM, pH 6.0) and were washed three times with PBS containing 1% Tween (PBS-T). Peroxidase blockade was done by adding a H₂O₂ and methanol mixture in PBS-T Tween for 30 min at room temperature, followed by a similar procedure using 3% Bovine Serum Albumin (BSA) in PBS-T. After the blocking buffer was removed, 70 μ l of primary antibodies solution was added. These contained anti-mouse antibodies targeted against active caspase 3, CRT, CD4, CD8, or CD11 (all cell signaling technology). After overnight incubated at 4°C, and followed by incubation with an AF555 secondary antibody for 1 h at room temperature, Antifade Mounting Medium (Vectashield) with DAPI (LSBio) was used for nuclei staining and mounting. Slide imaging was done using the high content imaging system (Operetta CLS; PerkinElmer) in 10 z- stacks with 5 μ m thickness with 20x air objective to capture DAPI and AF555 emission (λ_{ex} 505 nm and λ_{em} 553 $\pm$ 23 nm). Quantitative image analysis was performed using Harmony 4.9 Software (PerkinElmer).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) technology labels DNA fragmentation in the last phase of apoptosis (In Situ Cell Death Detection Kit, Fluorescein, Sigma-Aldrich). Paraffin-embedded tumor tissue sections were deparaffinized and rehydrated by immersing in xylene and different percentages of ethanol. Then, the tissue sections were pretreated with proteinase k working solution (15 μ g/ml in 10 mM Tris/HCl) as a permeabilization solution for 15 min at 37°C. Slides were rinsed three times with PBS and pretreated with 50 μ l of TUNEL reaction mixture (50 μ l enzyme solution mixed with 450 μ l label solution) in a 37°C humidified atmosphere in the dark for 60 min. The label solution also was used as a negative control for accurate target detection. In the following, the slides were incubated with 50 μ l DAPI (20 μ M) for 15 min and were covered by a drop of mounting medium and cover slide. TUNEL-stained and negative control tissues were imaged and quantified, as described above. For the detection of intracellular FOXP3 and IL-17 in CD4⁺ T-cells, the tissue slides were processed as described above and later fixed and permeabilized with TBS-T, as well as Triton X-100 and FOXP3 staining buffer (BioLegend). Afterward, the

nuclei were counterstained with DAPI, and the slides were incubated with antibodies targeting CD4, FOXP3, IL-17A, and IL17F (all BioLegend). The outer tissue border region was quantified by the Harmony software by counting double-positive cells.

Results

Gas plasma irradiation generated ROS that impaired breast cancer cell growth

A helium (He) gas plasma jet was used for the treatment of human breast cancer cells (MCF-7 and MDA-MB) in the *in vitro* experiments (Figure 1a) that expels reactive oxygen and nitrogen species. In gas plasma-irradiated liquids, these species deteriorate then further to oxidants that can be

quantified more conveniently with redox chemical assays. Accordingly, a gas plasma irradiation time-dependent increase of hydrogen peroxide (H_2O_2) as a product of OH (Figure 1b), nitrite (NO_2^-) as a product of nitric oxide (Figure 1c), and hypochlorous acid (HOCl) as a product of O (Figure 1d) was observed in the gas plasma-irradiated cell culture medium. Concomitantly, a modest increase in the pH of the gas plasma-irradiated liquid was observed (Figure 1e). Next, gas plasma irradiation was applied to breast cancer cells *in vitro* (Figure 1f). The treatment led to a significant increase in terminally dead MCF-7 cells (Figure 1g), and a subsequent significant decrease in the metabolic activity of the cells was observed at 24 h and 48 h post-treatment measured in the entire well containing the cells (Figure 1h). In MDA-MB breast cancer cells, gas plasma-induced terminal cell death was more pronounced as compared to MCF-7 cells (Figure 1i), while the

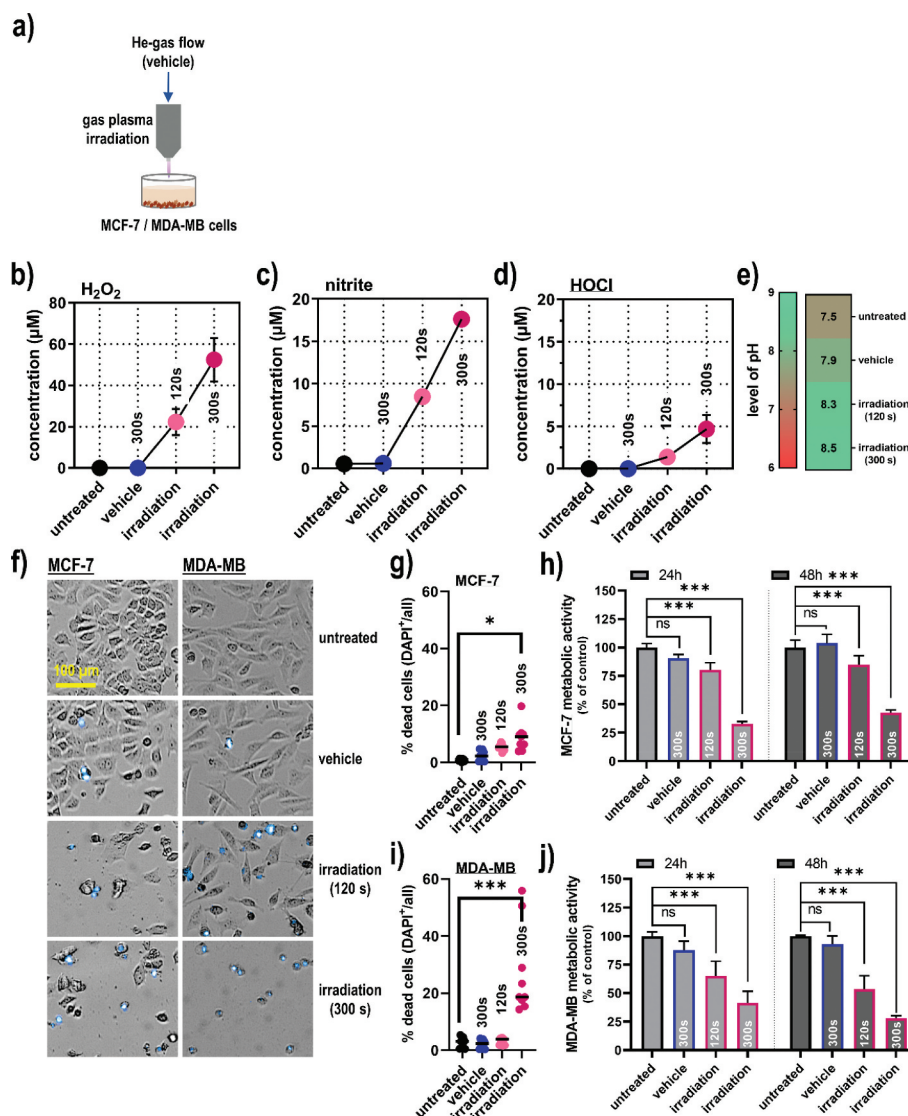


Figure 1. Gas plasma irradiation-generated redox chemistry and metabolic activity in breast cancer cells. (a) scheme of the helium (He) gas plasma irradiation of cells *in vitro*; (b-e) quantification of hydrogen peroxide (H_2O_2 , b), nitrite (NO_2^- , c), hypochlorous acid (HOCl, d), and pH (e) in gas plasma-irradiated cell culture medium; (f) representative brightfield and DAPI (terminally dead cells, blue) images of MCF-7 and MDA-MB breast cancer cells; (g) quantitative image analysis of dead cells in MCF-7 cultures; (h) metabolic activity in MCF-7 cells at 24 h and 48 h post gas plasma irradiation measured in the entire well containing the cells; (i) quantitative image analysis of dead cells in MDA-MB cultures; (j) metabolic activity in MDA-MB cells at 24 h and 48 h post gas plasma irradiation measured in the entire well containing the cells. Cell data are from three independent experiments. Data are presented as mean ($\pm$ SD). Statistical analysis was performed using one-way analysis of variances with $p < .05$ (*) and $p < .001$ (***); ns = non-significant; vehicle = helium gas treatment alone (plasma ignition off); scale bar is 100 μm .

extent of reduction of metabolic activity at 24 h and 48 h post-irradiation measured in the entire well containing the cells was comparable (Figure 1j). Altogether, gas plasma irradiation generated reactive species in the gas phase and treated liquids, which subsequently reduced breast cancer cell viability and metabolic activity significantly.

Gas plasma irradiation promoted toxicity and immunogenicity in 2D and 3D cell models

To identify the mode and immunogenic consequences of gas plasma irradiation in breast cancer cells, flow cytometry was subsequently employed. In MCF-7 cells (Figure 2a), gas plasma irradiation led to a significant decline in the percentage of cells

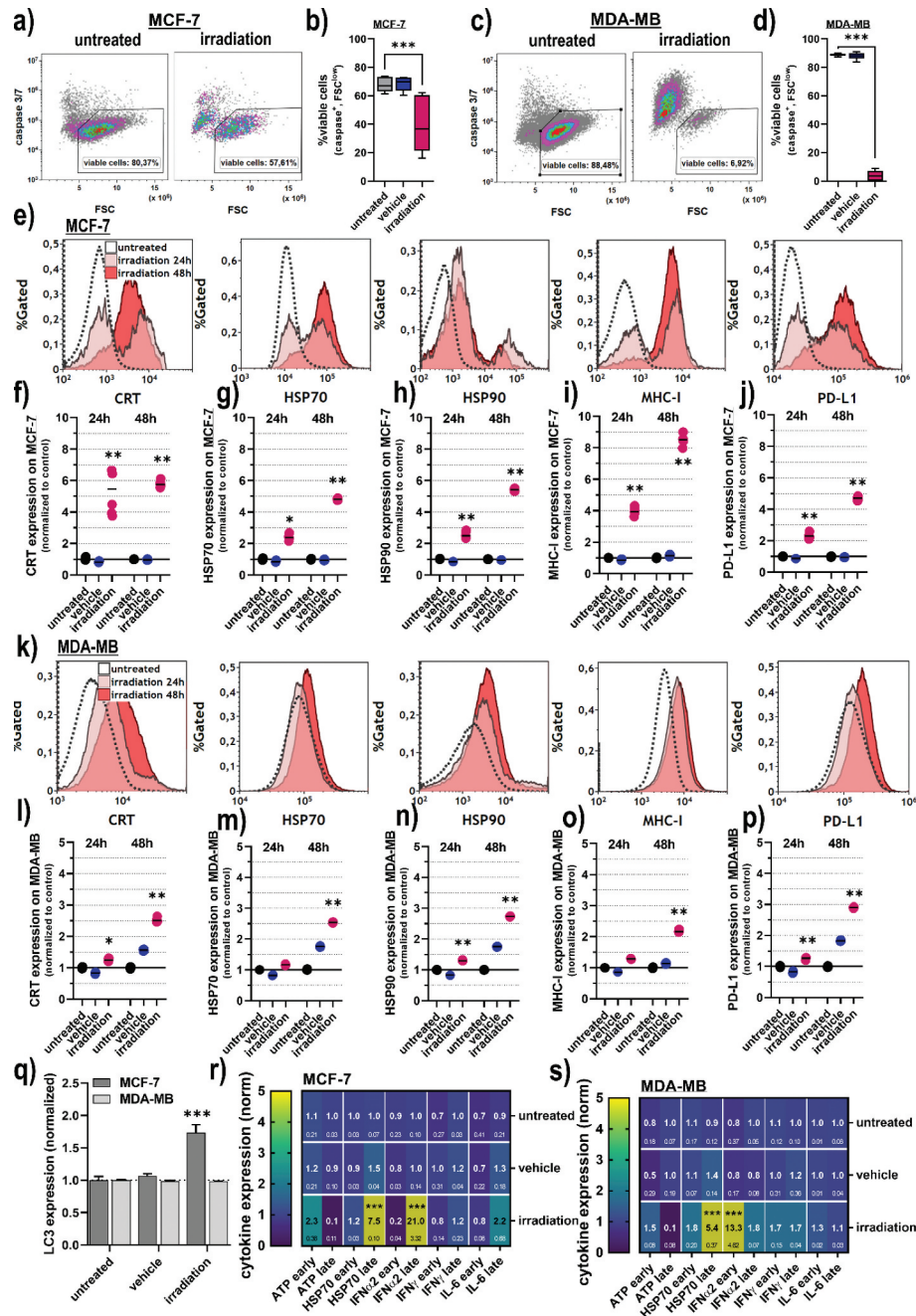


Figure 2. Cell viability and markers of immunogenic cancer cell death in breast cancer cells. (a) representative flow cytometry forward scatter (FSC) and caspase 3/7 dot-plots of MCF-7 cells; (b) quantification of viability data from flow cytometry of MCF-7 cells; (c) representative flow cytometry forward scatter (FSC) and caspase 3/7 dot-plots of MDA-MB cells; (d) quantification of viability data from flow cytometry of MDA-MB cells; (e) overlay histogram from flow cytometry of calreticulin (CRT), heat-shock protein (HSP) 70, HSP 90, major histocompatibility complex (MHC)-I, and programmed death-ligand 1 (PD-L1) in MCF-7 cells; (f-j) quantification of CRT (f), HSP70 (g), HSP90 (h), MHC-I (i), and PD-L1 (j) in viable (caspase 3/7⁻) MCF-7 cells; (k) overlay histogram from flow cytometry of CRT, HSP70, HSP90, MHC-I, and PD-L1 in MDA-MB cells; (l-p) quantification of CRT (l), HSP70 (m), HSP90 (n), MHC-I (o), and PD-L1 (p) in viable (caspase 3/7⁻) MDA-MB cells; (q) quantification of LC3 expression 6 h post treatment in MCF-7 and MDA-MB cells; (r-s) early (1 h) and late (48 h) cytokine expression of adenosine triphosphate (ATP), HSP70, interferon (IFN) α 2, IFN γ , and interleukin (IL)-6 in MCF-7 (r), and MDA-MB cells (s). Data are representative of at least three independent experiments and presented as mean (min-max boxplot; b,d), individual values (e-j, l-p), mean + SD (q), or mean + SEM (r-s). Statistical analysis was performed using one-way analysis of variances with $p < .05$ (*) and $p < .001$ (***).

negative for active caspases 3 and 7 (Figure 2b) being a marker of apoptosis. In MDA-MB cells (Figure 2c), a much more pronounced induction of apoptosis was observed (Figure 2d) as compared to the MCF-7 cells. The latter were subsequently analyzed for the expression of CRT, a molecule known to be increasingly expressed in cells succumbing to immunogenic cancer cell death (ICD), heat-shock-proteins (HSP) 70 and HSP90, major histocompatibility complex (MHC)-1, as well as death-ligand 1 (PD-L1) (Figure 2e). All molecules are reported to be associated with ICD and anticancer immunity,^{32–36} and a significant elevation was observed in gas plasma-irradiated but viable (caspase 3/7[−]) cells across all markers investigated (Figure 2f–j). When analyzing these markers in MDA-MB cells (Figure 2k), a significant increase of CRT was observed (Figure 2l). Also for HSP70 (Figure 2m), HSP90 (Figure 2n), MHC-I (Figure 2o), and PD-L1 (Figure 2p), significantly elevated levels were observed in gas plasma-irradiated tumor cells. In increase in the autophagy-related marker LC3, however, was observed only in MCF-7 but not MDA-MB cells (Figure 2q). As additional markers for cell death signaling, also the endoplasmic reticulum protein 57 (ERp57), the phosphorylated ATM kinase (pATM), as well as the phosphorylated eukaryotic initiation factor 2a (peIF2a) were significantly upregulated in MCF-7, but not MDA-MB cells post-treatment Supplementary (Figure 1a–c). Besides the surface-upregulation of the ICD marker, the secretion of several inflammation and DAMP-factors cytokines was investigated in the cells' supernatants (Figure 2r–s). The levels differed when analyzed at early (1 h) versus late (48 h) collection time points. Both cell lines showed a significant increase of HSP70 as well as interferon (IFN) α 2 in the cell culture supernatant (Figure 2r–s). ATP was increased early (1 h) but not late (48 h) after gas plasma irradiation. IFN γ was only found to be increased after the treatment of MDA-MB cells but not MCF-7 (Figure 2s). In contrast, interleukin (IL)-6,

known as a general pro-inflammatory signal molecule, was elevated 48 h after exposure of MCF-7 cells to gas plasma irradiation (Figure 2r).

To confirm terminal cell death and ICD in a more realistic in vitro model, 3D tumor spheroids were generated and exposed to gas plasma irradiation (Figure 3a). In MCF-7 cells, the effect on terminal cell death (Figure 3b) and cellular oxidation (Figure 3c) was modest, while CRT expression was significantly elevated with the treatment (Figure 3d). For MDA-MB cells, a significantly increased amount of terminally dead cells was observed (Figure 3e), which was concomitant with a significant increase in intracellular oxidation (Figure 3f) and CRT (Figure 3g). The differences between both cell lines in terms of cytotoxic effects might be related to the MCF-7 cells having a more epithelial-like phenotype at the spheroids' vicinity, leading to denser 3D tumors with confined borders. The data analysis was based on the fluorescence signals from the segmented spheroids, not taking into account the viability of individual cells due to the technical limitation of unambiguously identifying all individual cells within the tumor spheroids. In summary, gas plasma irradiation induced apoptosis in human breast cancer cell lines in 2D monolayer and 3D tumor spheroid cultures, and led to a significant elevation of the molecules CRT, HSP70, HSP90, MHC-I, as well as the secretion of ATP and interferons known to be involved in ICD.

Gas plasma irradiation reduced tumor growth in vivo at the treated and remote side

The next question was whether gas plasma irradiation conferred cytotoxic and pro-immunogenic effects not only in vitro but also in vivo. For this, 4T1 syngeneic tumor cells were injected in both flanks of Balb/c mice (Figure 4a). In the control group, both tumors of each

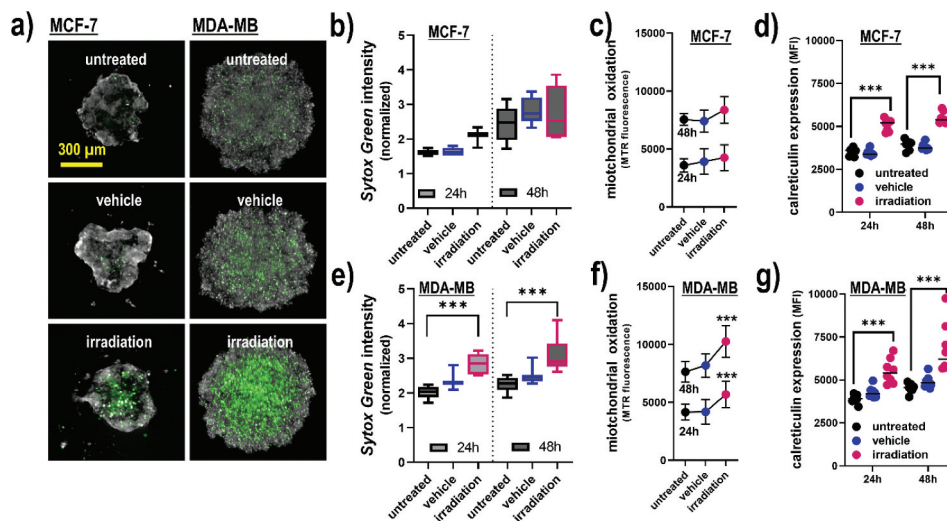


Figure 3. Viability, oxidation, and CRT expression in 3D breast cancer spheroids. (a) representative maximum intensity projection images of MCF-7 and MDA-MB 3D multicellular breast cancer spheroids generated from an overlay digital phase contrast and a sytox green image; (b–d) quantitative image analysis of the dead cell marker sytox green (b), oxidation marker mitotracker red (c), and CRT (d) of the segmented spheroid image area of MCF-7 cells at 24 h and 48 h post gas plasma irradiation exposure; (e–g) quantitative image analysis of sytox green (e), mitochondrial oxidation (f), and CRT (g) of the segmented spheroid image area of MDA-MB cells at 24 h and 48 h post gas plasma irradiation exposure. The data analysis was based on the fluorescence signals from the segmented spheroids, not taking into account the individual viability of the cells (except for sytox green). Data are from three to eight spheroids and presented as mean (min-max boxplot; $\pm$ SD). Statistical analysis was performed using one-way analysis of variances with $p < .001$ (***); scale bar is 300 μ m.

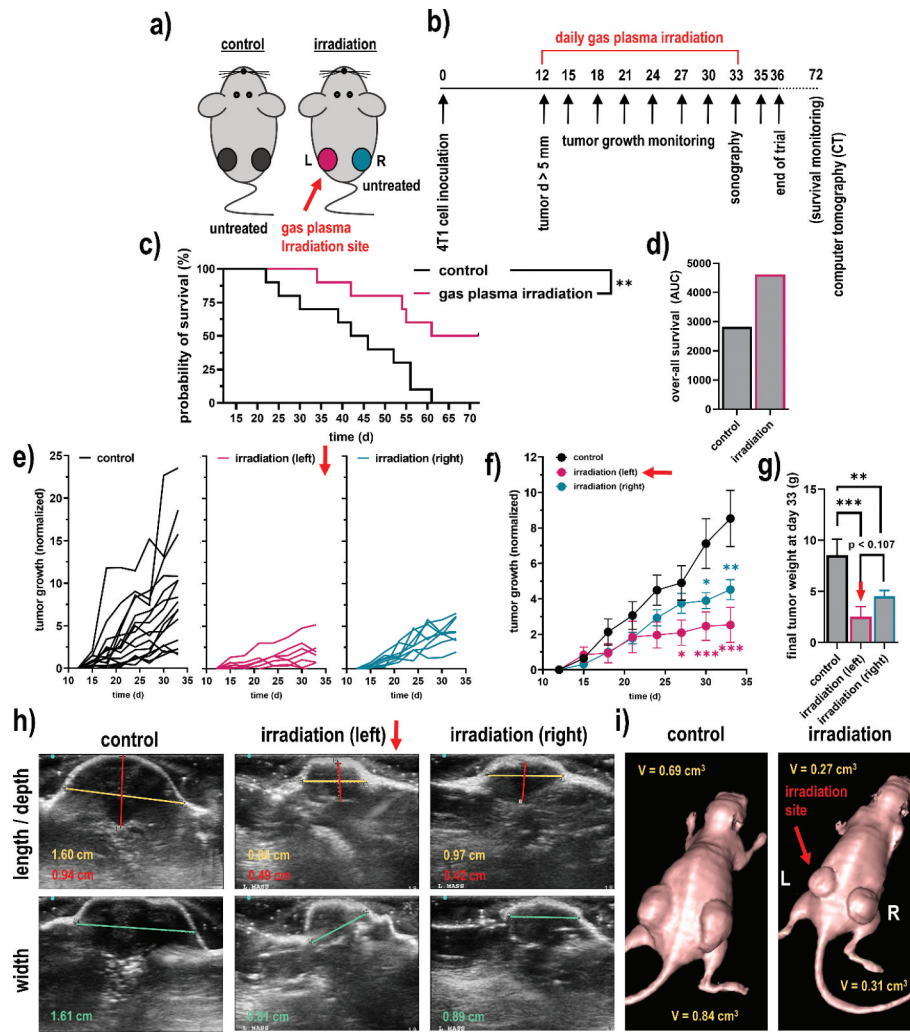


Figure 4. Syngeneic breast cancer animal model and tumor growth. (a-b) scheme of the animal treatment sides (a) and the timeline of the treatment of the breast cancer animal model (b); (c-d) Kaplan-Meier survival curve (c) and area under the curve (d) of the curves; (e) tumor sizes of individual mice of the control group (black) and the gas plasma-irradiated group carrying one tumor on each flank, one being subjected to gas plasma irradiation treatment (magenta) and the tumor on the opposite side remaining untreated (turquoise); (f-g) mean tumor growth (f) and weight (g) of the untreated tumors, gas plasma irradiation-treated tumors as well as untreated opposite flank tumors of the same mice; (h-i) representative images of ultrasound (sonography) measurements of the tumors (h), and of a representative mouse of each group using computer tomography (CT). Data are from eight mice per group. Statistical analysis was performed using one-way analysis of variances with $p < .05$ (*) and $p < .01$ (**).

mouse remained untreated. In the gas plasma irradiation group, only the left tumor in each mouse was treated, while the right tumor of that same animal remained untreated. This way, we aimed at investigating remote, immune-mediated effects of the treatment on distant tumor sites following exposure to the gas plasma irradiation scheme (Figure 4b). The treatment led to a significantly increased survival in tumor-bearing mice (Figure 4c), which was overall prolonged compared to the control group (Figure 4c-d). This was also appreciated when comparing the tumor growth of the individual animals (Figure 4e) and as well as the groups (Figure 4f). Remarkably, the gas plasma irradiation not only decreased the tumor growth and weight of the directly treated breast tumor (left flank) but simultaneously, also the remote breast tumor at the opposite, non-treated side (right flank) showed a significantly impaired growth as compared to tumors of the untreated animals (Figure 4g),

suggesting distant, immune-mediated effects. The principal differences between left and right tumors in the gas plasma-irradiated group, and tumors of the control group, were confirmed using Doppler sonography (Figure 4h) and computer tomography (CT; Figure 4i). All in all, these results implicated irradiation via gas plasma technology not only to decelerate tumor growth, leading to increased survival of breast cancer-bearing mice but also to stimulate possibly immune-mediated effects resulting in impaired tumor growth at distant, untreated sites.

Gas plasma irradiation augmented immune infiltration in the tumor microenvironment

To next understand the composition of the tumor microenvironment that might be related to the antitumor effects observed with gas plasma irradiation, tissue sections were done followed by immunofluorescence staining. The quantification of positively-

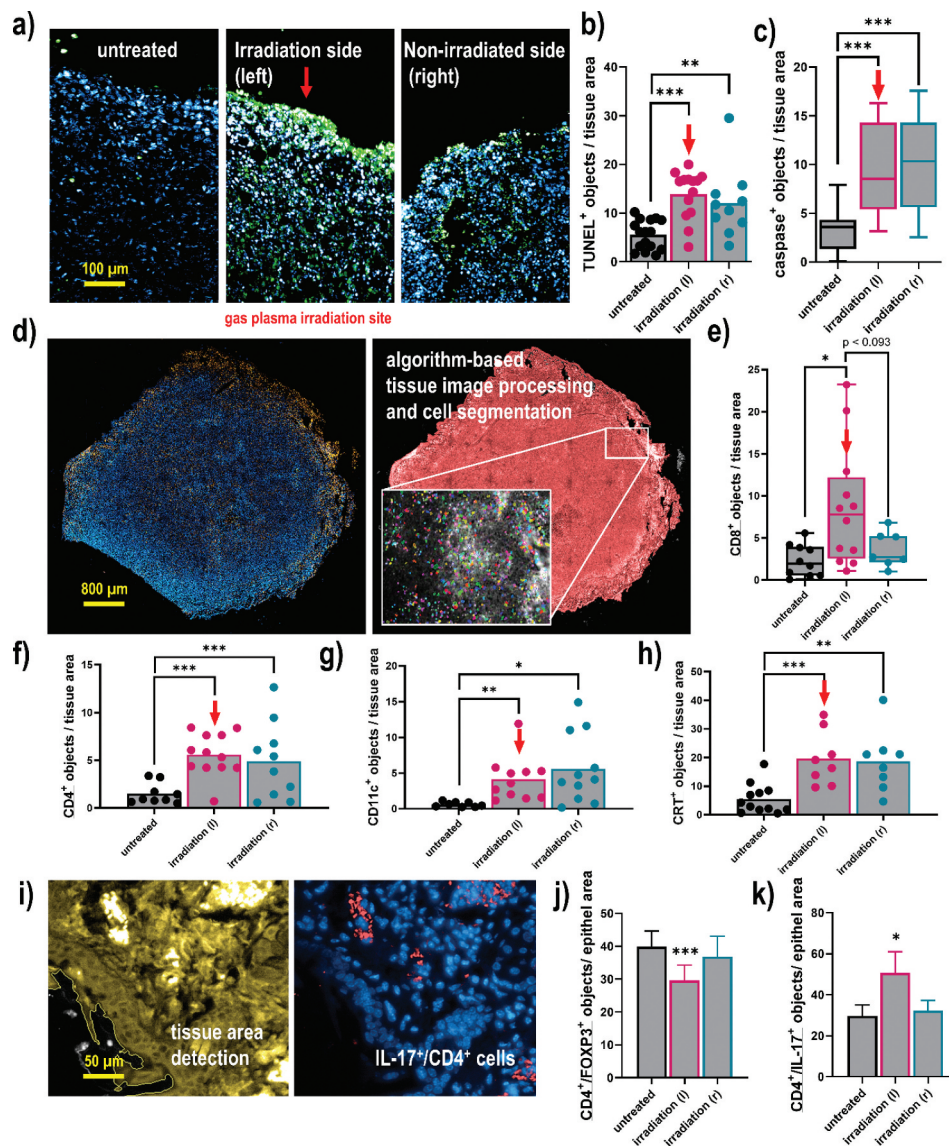


Figure 5. Quantitative imaging analysis of apoptosis, immune cell infiltrates, and CRT in breast cancer tissues. (a) representative images of tumor tissue sections stained with DAPI (nuclei, blue) for all cells and TUNEL (green) for apoptotic cells; (b-c) quantitative image analysis of TUNEL⁺ (b) and caspase 3⁺ (c) cells in tissue sections; (d) representative whole tissue section image that was stained (left) with DAPI and (blue) and anti-CD8 antibodies (orange), and segmented for different image regions (right) based on algorithm-driven image processing for unsupervised detection of cells; (e-h) quantitative image analysis results obtained for objects staining positive for CD8 (e), CD4 (f), CD11c (g), and CRT (h); (i) software detection of tissue border region with the quantification of (j) FOXP3⁺, and (k) IL-17⁺ subpopulations of CD4⁺ cells. The latter analysis was done for all cells staining positive for these markers, not taking into account their viability as technical limitations did not allow for such degree of multiplexing. Data show individual values and mean or min-max boxplots, or as bar graphs with mean +SEM of at least seven tumor sections per group. Statistical analysis was performed using one-way analysis of variances, or t-tests with $p < .05$ (*), $p < .01$ (**), and $p < .001$ (***); scale bars are 100 μm , 800 μm , and 50 μm , respectively.

stained cells was done using algorithm-driven image segmentation. The TUNEL staining is indicative of apoptosis (Figure 5a), and a significant increase of TUNEL⁺ cells was observed not only in gas plasma-irradiated tumors but also in opposite-flank tumors (Figure 5b). Along similar lines, the number of cells staining positive for active caspase 3, an executioner caspase of the apoptotic pathway, was significantly increased in both left and right flank tumors of gas plasma-irradiated mice when compared to untreated control tumors (Figure 5c). Subsequently, the immune infiltrate in tumor tissues was quantified (Figure 5d). For CD8⁺ cytotoxic T-cells, a significant increase was observed in the gas plasma-irradiated tumors (Figure 5e). The change in the untreated tumors of irradiated mice was modest but not significant compared to the control tumors. By contrast, the proportion of CD4⁺

T-helper cells was significantly elevated in both the gas plasma-irradiated and the opposite-flank tumor within the same animal when compared to the numbers identified in control mice (Figure 5f). A similar trend was observed for CD11⁺ cells, indicative of dendritic cells, which were significantly elevated in the gas plasma-treated mice (Figure 5g). Investigating CRT as an indicator ICD, a significantly pronounced staining was observed in both the gas plasma-treated tumors as well as the distant non-treated tumors within the same animal, when compared to those levels found in the tumors of untreated animals. Finally, when staining for subpopulations of CD4⁺ cells and quantifying their numbers in the tissues, a significantly lower amount of FOXP3⁺ CD4⁺ cells was found at the irradiation site of the animals (Figure 5i-j). IL-17 expression showed a pronounced upregulation in CD4⁺ T-cells of

the left irradiated tumor, in contrast to the opposite sided tumor and the untreated group (Figure 5k). The analysis of CD4, CD8, CD11c, CRT, FOXP3, and IL-17 was done for all cells staining positive for these markers, not taking into account their individual viability as technical limitations did not allow for such degree of multiplexing. In sum, analysis of the tumor microenvironment revealed a significant elevation of apoptosis and cell death, which was concomitant with the promotion of immunogenicity and immune cell infiltration following irradiation with gas plasma technology.

Discussion

Despite recent advances in therapy, breast cancer still is fatal in many patients. In thriving for finding novel treatment avenues, we employed an innovative medical gas plasma irradiation approach for tackling breast cancer. Treatment with this technology not only limited tumor growth in vitro and in vivo but also had an immunological dimension. Besides an upregulation of markers of the immunogenic cell death (ICD), we found in vivo untreated tumors showing decelerated growth if present in mice having tumors that had received the treatment, arguing for an abscopal effect (Figure 6).

The term abscopal effect was introduced in 1953,³⁷ describing radiotherapy of one tumor site evoking hampered growth of another, distant, untreated metastatic tumor sites. This phenomenon has occasionally been reported in the medical literature and speculated to be immune-mediated.³⁸ Today, it is well recognized

that the immune system makes a significant contribution to tumor control, as exemplified by the clinical application of check-point immunotherapy.³⁹ This understanding also sheds new light on the abscopal effect observed with predominantly local treatment, such as radiotherapy.⁴⁰ Many studies using experimental tumor models have reported abscopal effects, for instance, in pancreatic cancer,⁴¹ malignant melanoma,⁴² colon cancer,⁴³ lung cancer,⁴⁴ and breast cancer.⁴⁵ The data of our experimental model suggest gas plasma irradiation to mediate an abscopal effect, too. The distant tumor lesion responding to the local therapy usually is characterized not only by retarded growth but also an enhanced immune infiltrate, such as T-cells.⁴⁶ Especially cytotoxic CD8⁺ T-cells were in focus in recent years when it came to harnessing the power of antitumor T-cell responses.⁴⁷ This subtype, however, was not significantly enhanced in our study when investigating the tumor microenvironment (TME). By contrast, we found significantly elevated numbers of CD4⁺ T-cells in the TME, also on the gas plasma irradiated tumor site. This is of note as we previously determined T-cells to be highly susceptible to gas plasma irradiation-induced oxidative stress^{48–50} in vitro, which was predominantly found in antigen-experienced memory T-cells.⁵¹ Hence, gas plasma irradiation-induced T-cell death either is of minor relevance in vivo or not resolved kinetically as new T-cells constantly migrate into the TME from the circulation. In the current studies, the CD4⁺ T-cells expressed pro-inflammatory IL-17, while the immunosuppressive subtype was less pronounced, as FOXP3 expression suggested. CD4⁺ T cells have long been underappreciated in their ability to contribute

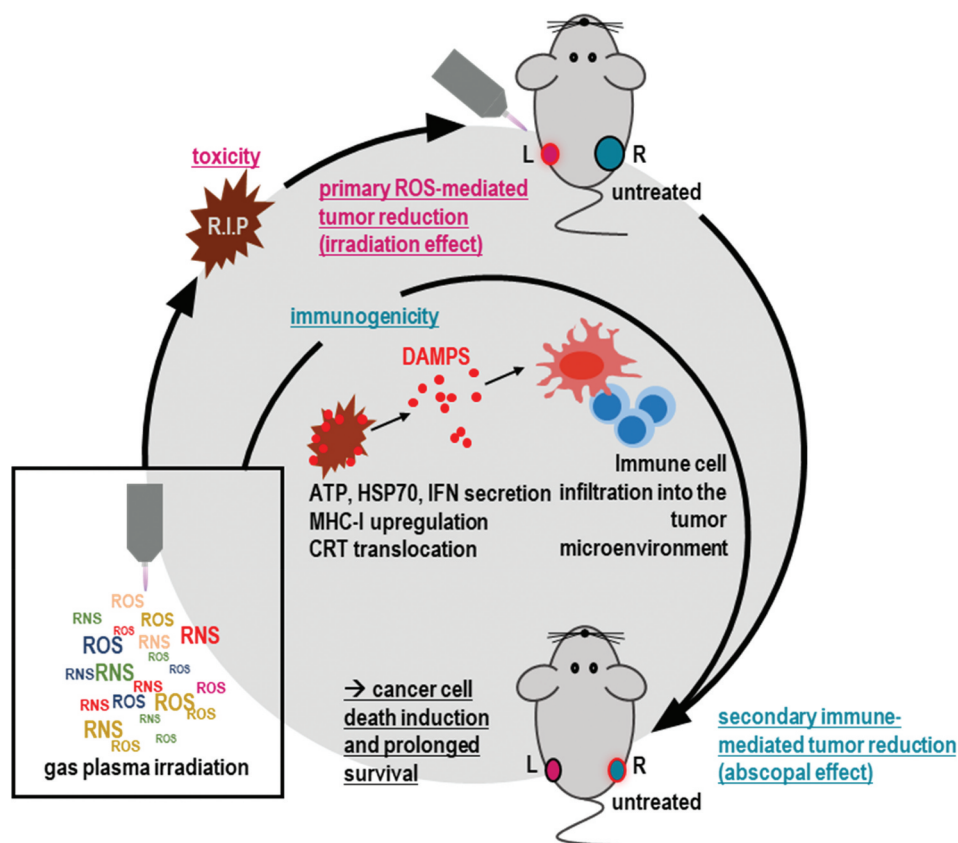


Figure 6. Scheme of gas plasma irradiation in breast cancer treatment. Gas plasma irradiation generates multiple types of ROS and RNS simultaneously, which subsequently induce cytotoxicity in breast cancer cells in vitro and in vivo. Concurrently, cell death occurs in an immunogenic fashion, leading to immune-mediated tumor reduction at a distant site not exposed to gas plasma irradiation (abscopal effect) and improved survival.

antitumor effects in the TME,⁵² albeit their positive prognostic value in cancers of, for instance, the head and neck,^{53,54} the lung,⁵⁵ and the breast.⁵⁶ The value of CD4⁺ T-helper cells lies in their supportive interplay with other types of immune cells, such as cytotoxic CD8⁺ T-cells that subsequently exert the antitumor effects in experimental models.^{57,58} The polyfunctional role of CD4⁺ T-cells is even extended to direct cytotoxic action as premature evidence points into mechanism related to MHCII-expression on tumor cells.^{59,60} CD4⁺ T-cells, moreover, promote the antitumor efficacy of dendritic cells (DCs),⁶¹ a cell type we also found to be increased in the gas plasma-irradiated breast cancers, as compared to controls.

DCs are professional antigen-presenting cells that respond to damage-associated molecular patterns (DAMPs).⁶² DAMPs are intimately linked to the immunogenic cancer cell death (ICD),³⁵ a pro-inflammatory cell death program that overrides the predominantly immunosuppressive nature of many types of regulated cell death.⁶³ In this regard, we found the DAMPs and ICD-associated molecules HSP70, HSP90, calreticulin (CRT), and MHC-I, as well as ATP secretion, IFN α 2, IFN γ , and the interleukin 6 (IL-6) to be upregulated following gas plasma irradiation. Also, the programmed death-ligand 1 (PD-L1) which contributes to a muted immune response was found to be upregulated as it is also known for radiation therapy.^{64,65} However, this might be outbalanced by the pro-inflammatory molecules that were increasingly present as well, such as CRT. CRT levels depend on the type of chemotherapeutic agent used and are associated with elevated rejection rates of tumors in vivo.²⁸ Intriguingly, also localized therapies such as ionizing radiation and photodynamic therapy (PDT) led to increased CRT levels in tumor cells and subsequently hampered tumor growth in vivo that was associated with increased immune infiltrates into the TME.^{66–69} The effects of both PDT and radiotherapy are known to be mediated significantly via reactive oxygen species (ROS).^{70–72} ROS, in turn, have significant implications in the release and appearance of DAMPs and ICD in terms of not only oxidative damage but also redox regulation.^{73–76}

Medical gas plasma systems are a novel technology for oncotherapy. Gas plasmas expel a multitude of different ROS and RNS onto the irradiated target in a localized fashion.²⁵ In contrast to radiotherapy and PDT, these reactive species are not generated inside the target cells but are being deposited from the extracellular gas plasma phase. Our results showing gas plasma irradiation-mediated cytotoxicity in breast cancer cells are in line with a previous study reporting increased apoptosis and cell damage related to antioxidant profiles and degradation of PKD2.^{77–79} Using a microcapillary plasma jet, a limitation of breast cancer growth in vivo and an increase in the number of TUNEL⁺ cells was also noted before.⁸⁰ Interestingly, gas plasma irradiation was shown to induce hallmarks of immunogenic cell death before in malignant melanoma cells,^{81,82} lung cancer,⁸³ and colorectal cancer.^{84,85} Some reports also observed ICD-induction in tumor cells exposed to cell culture medium⁸⁶ or phosphate-buffered saline⁸⁷ previously irradiated with gas plasma, implicating that some of the long-lived ROS may contribute to effects observed.⁸⁸ These

treatments were also associated with higher tumor immuno-infiltrates in vivo when applied as intraperitoneal lavage to target peritoneal carcinomatosis of colorectal or pancreatic cancer origin.^{87,89} In vivo, direct gas plasma-induced ICD was not only found to correlate with anticancer efficacy and immune infiltration^{85,90,91} but also showed protection of subsequent re-challenge using the syngeneic vaccination model with gas plasma-inactivated tumor cells.^{31,81} Along with our findings, these results suggest gas plasma irradiation to be a novel and potent tool for promoting antitumor immunity by eliciting ICD in tumor cells. An open question, however, remains, which single or composite of reactive species in terms of quantity and quality contribute to gas plasma irradiation-induced ICD. Among the species being generated, especially in our system, is, for instance, hydroxyl radicals, superoxide anion, nitrite, singlet delta oxygen, hydrogen peroxide, nitrate, atomic oxygen, nitric oxide, ozone, peroxyxynitrite, and several types of metastables, each having distinct spatio-temporal distributions and reaction kinetics with other molecules.^{92–96} While it is known that the multi-ROS nature of gas plasmas exceeds the extent of ICD when compared to individual species,⁸¹ it is also clear the individual distribution within this ROS cocktail affects ICD-related outcomes as well.³¹ The proof-of-concept of ROS-induced ICD remains exciting as a recent study had suggested increased tumor cell immunogenicity and more potent therapeutic effects of autologous DCs in patients suffering from metastatic ovarian cancer.^{97,98}

Novel cancer therapies not only need to be effective but also safe. While exact technical parameters and the type of feed gas differ between gas plasma systems, their unifying theme is the generation of ROS together with other plasma components, such as electrons and ions, electric fields, and UV-emission. A large body of evidence, however, points to ROS and RNS to be the primary mode of action.²⁵ For some gas plasma jets, extensive safety testing has been performed before.⁹⁹ This includes the absence of mutagenic effects using the OECD-accredited assays for HRPT¹⁰⁰ and micronucleus in vitro¹⁰¹ and in vivo.¹⁰² Moreover, a one-year follow-up study in mice¹⁰³ and human volunteers¹⁰⁴ did not report on any tumorigenic effects of repeated gas plasma irradiation. Moreover, gas plasma irradiation seems to target malignant over nonmalignant cells selectively.^{24,105} It is suggested that this is due to faster proliferation,¹⁰⁶ the different lipid composition of the cell plasma membrane,¹⁰⁷ lower concentration of cholesterol,¹⁰⁸ lower levels of antioxidants,¹⁰⁹ and/or higher ROS concentration in cancer cells.^{110,111} For breast cancer cells, it was speculated that their accelerated genome mutation rate, as well as hyper-activated MAPK/JNK and NF- κ B pathways, promote their vulnerability toward gas plasma irradiation when compared to nonmalignant cells.¹¹²

A limitation of our study was the lack of the gold standard prophylactic vaccination. The non-orthotopic model of breast

cancer is only partially realistic in terms of microenvironment and cellular differentiation. The blockage of effector cell subtypes and its effect on tumor growth was not investigated but cellular subtypes and tumor immuno-infiltration was analyzed.

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Originality disclosure

The data presented in this manuscript are original and have not been published elsewhere.

Declaration of interest statement

The authors report no conflict of interest.

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Article

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 μ L 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 μ m cell strainer, and the eluent was filtered using a 40 μ m cell strainer. The eluent containing single breast cancer cells and debris was discarded while the material on the filter, being 40–100 μ m 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 $-20\text{ }^{\circ}\text{C}$ 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.

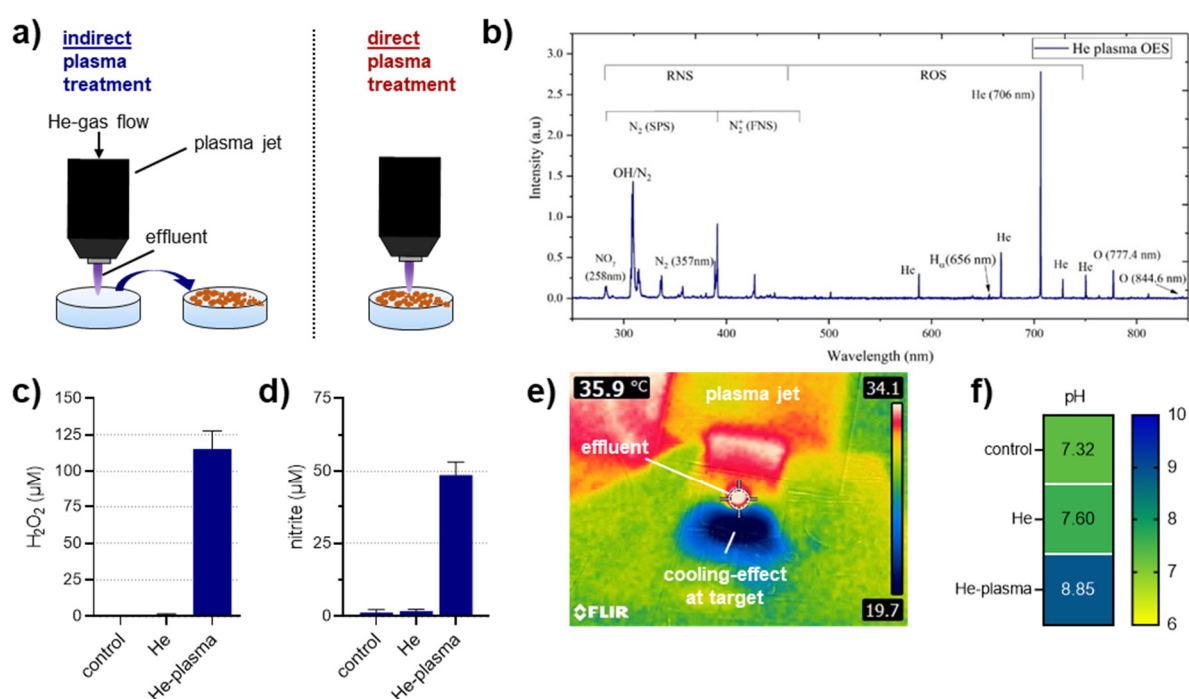


Figure 1. Characteristics of the gas plasma jet used in this study. (a) scheme of the indirect vs. the direct treatment regimens; (b) optical emission spectroscopy of the plasma jet; (c) hydrogen peroxide (H_2O_2) production of the gas plasma jet; (d) nitrite production of the gas plasma jet; (e) thermal 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).

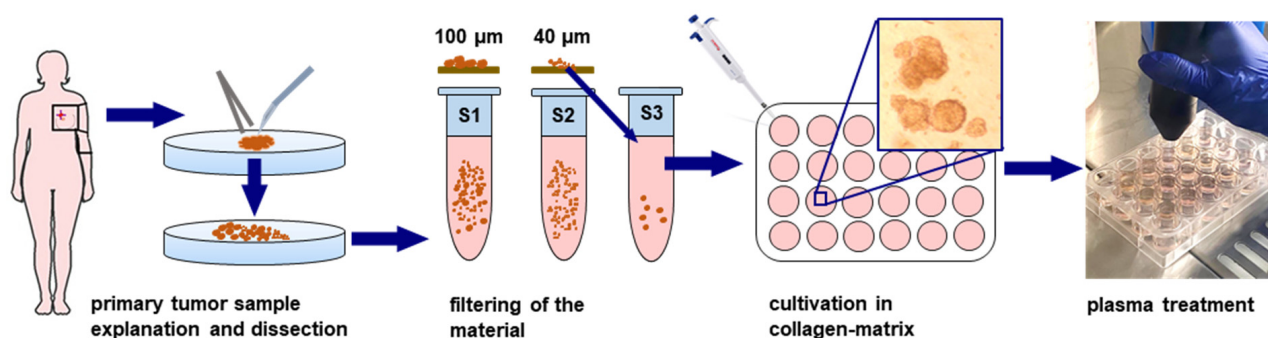


Figure 2. Scheme of sample preparation and treatment. Human patient-derived breast cancer tissue was surgically removed and dissected ex vivo. Subsequently, the material was filtered several times to achieve microtissues with a size $< 100\ \mu\text{m}$ and $> 40\ \mu\text{m}$, prior to mixing with collagen. 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.

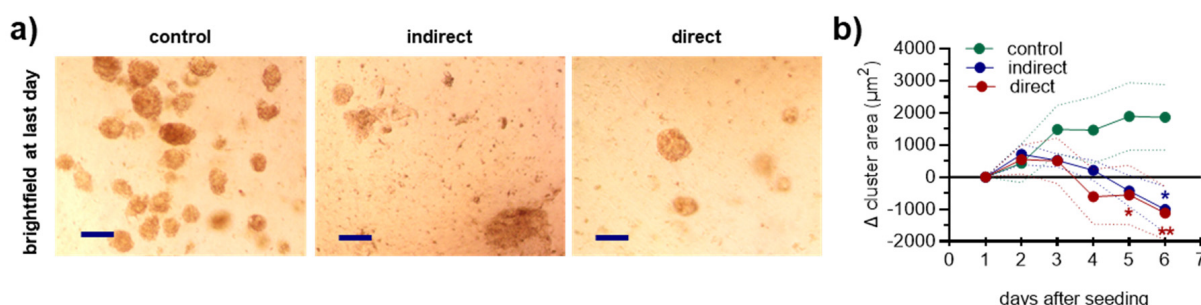


Figure 3. Gas plasma-treated human breast cancer tissue. (a) representative images of human patient-derived breast cancer tissue colonies in collagen 120 h after seeding and being either untreated or exposed to direct or indirect plasma treatment; (b) quantification of colonies with plasma treatment 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 $250\ \mu\text{m}$.

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)- $\alpha 2$, interleukin (IL)-17A, IL18, and IL33. Despite the significant increases, the absolute changes 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.

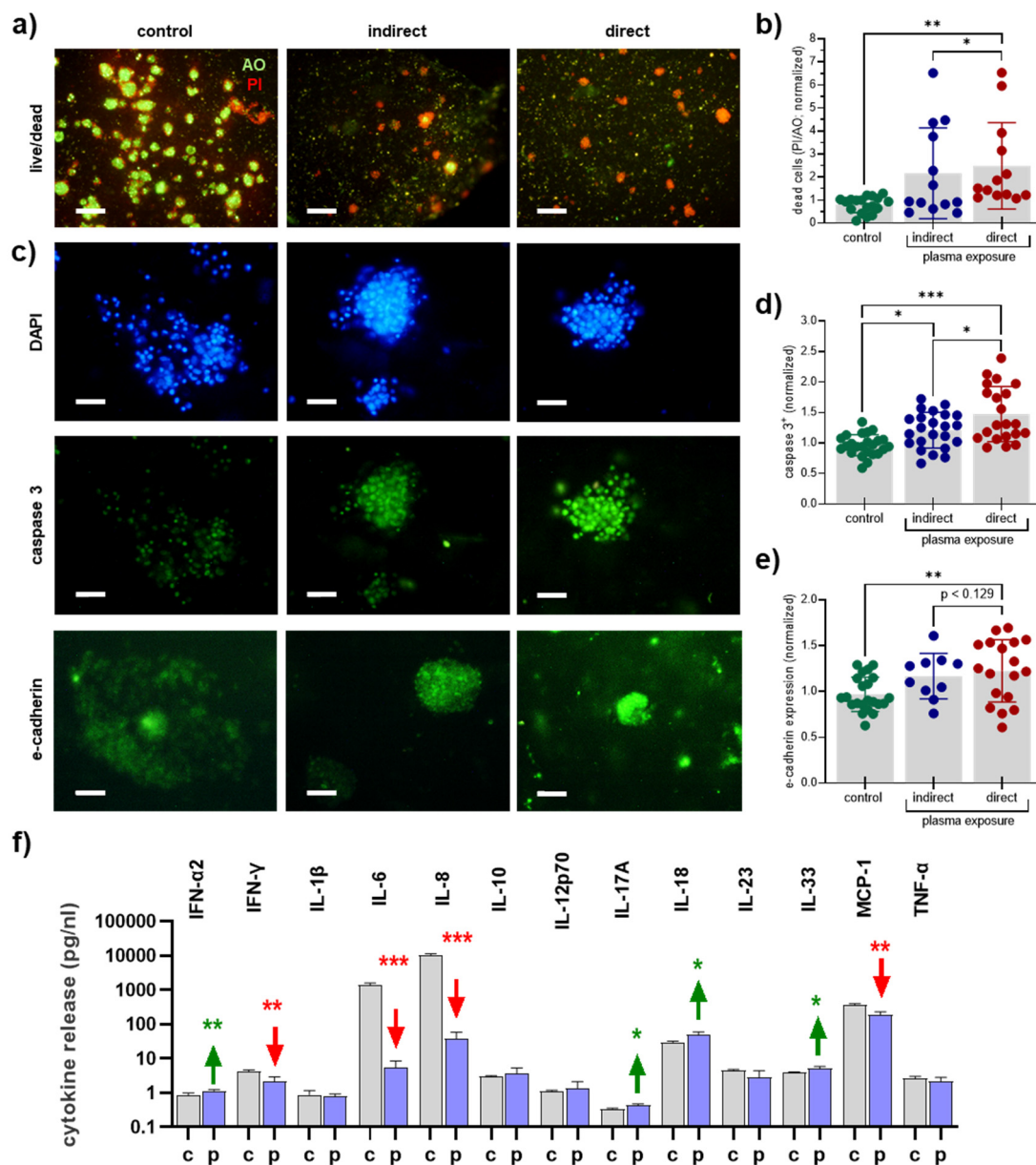


Figure 4. Microscopy and supernatant analysis. (a) representative fluorescence microscopy images of human patient-derived breast cancer tissue 24h after the second plasma treatment followed by staining with acridine orange (AO) and propidium iodide (PI); (b) quantification of dead cells; (c) representative 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 μ m (AO/PI and E-cadherin) and 100 μ m (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 to 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 difficult 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.

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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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Article

Optimized High-Content Imaging Screening Quantifying Micronuclei Formation in Polymer-Treated HaCaT Keratinocytes

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Abstract: Research on nano- and micro-plastic particles (NMPPs) suggests their potential threat to human health. Some studies have even suggested genotoxic effects of NMPP exposure, such as micronuclei (MN) formation, while others found the opposite. To clarify the ability of NMPP to induce MN formation, we used non-malignant HaCaT keratinocytes and exposed these to a variety of polystyrene (PS) and poly methyl methacrylate (PMMA) particle types at different concentrations and three different sizes. Investigations were performed following acute (one day) and chronic exposure (five weeks) against cytotoxic (amino-modified NMPPs) and genotoxic (methyl methane-sulfonate, MMS) positive controls. An optimized high-content imaging workflow was established strictly according to OECD guidelines for analysis. Algorithm-based object segmentation and MN identification led to computer-driven, unsupervised quantitative image analysis results on MN frequencies among the different conditions and thousands of cells per condition. This could only be realized using accutase, allowing for partial cell detachment for optimal identification of bi-nucleated cells. Cytotoxic amino-modified particles were not genotoxic; MMS was both. During acute and long-term studies, PS and PMMA particles were neither toxic nor increased MN formation, except for 1000 nm PS particles at the highest concentration of unphysiological 100 µg/mL. Interestingly, ROS formation was significantly decreased in this condition. Hence, most non-charged polymer particles were neither toxic nor genotoxic, while aminated particles were toxic but not genotoxic. Altogether, we present an optimized quantitative imaging workflow applied to a timely research question in environmental toxicity.

Keywords: algorithm; micro-plastic; nano-plastic; cytotoxicity; Operetta CLS



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1. Introduction

Nano- and micro-plastic particles (NMPPs) are becoming a major environmental concern due to their small size, lack of biodegradability, and significant bio-permeability [1,2]. There is a continuous increase in the use of plastic in a variety of industries. A large proportion of plastic-containing products end up as waste in the environment due to the lack of efficient collection and improper management. In addition, many plastics do not enter the recycling chain [3]. These plastics are continuously degraded due to environmental stressors, such as salty sea water and UV radiation, and ultimately break down into smaller fragments to form NMPPs [4]. There are many ways through which humans can be exposed to NMPPs due to environmental contamination [5,6]. The skin is one of the primary human barriers against environmental pollution. Because it is the largest organ in the body, it can be a significant entry point for NMPPs into the human body. Skin and hair cosmetic products, such as scrubs,

lotions, gels, and exfoliating products, are in contact with the various parts of the human body. A large-scale study of over seven thousand cosmetics and personal care products found 87% of them to contain micro-plastics [7]. Daily skin contact with micro-plastics can have destructive effects over time.

To date, only a few studies investigated whether plastic particles cause toxicity or genotoxicity in human skin cells. To reduce this shortcoming, the present study used human HaCaT keratinocytes to investigate genotoxic effects. The cell line is non-tumorigenic and capable of differentiation and regular keratinization as well as three-dimensional skin equivalent formation [8–10]. Accordingly, several types of toxicity analysis, e.g., silver nanoparticles [11] and cadmium [12], have been performed with this cell line, particularly on protection from DNA damage-inducing agents such as UV-radiation and oxidative stress [13–15]. Therefore, it seems plausible to have a well-established, non-tumorigenic, stress-sensitive skin cell line as a model system to study the biomedical consequences of plastic particle exposure, not only because the skin route is heavily understudied (despite the ample presence of NMPPs in cosmetic products and air pollution daily exposing the skin as the largest organ of the body to environmental particles) but also because of the suitability of the model system itself. At the same time, we aimed to provide a robust and donor-independent model system based on a commonly used cell line, which is more economical and reproducible in technical terms.

The literature on the genotoxic properties of NMPPs particles is not only still infrequent but also contradictory. Studies have shown both an increase in DNA damage when exposed to NMPPs and the absence of genotoxic damage [16–18]. The technical restrictions imposed on them can often lead to contradictory results, especially regarding the manual analysis of microscopy images. Therefore, we aimed to develop a new approach for evaluating genotoxicity based on the OECD-micronucleus protocol *in vitro* using high-content imaging and algorithm-driven quantitative image analysis. Micronuclei formation was chosen as the endpoint for genotoxicity because it detects an already-transmitted mutation [19]. Micronuclei are chromatin-containing bodies that appear due to DNA damage and may reflect a chromosome aberration. The *in vitro* micronucleus assay is a genotoxicity test system used to detect micronuclei in the cytoplasm of interphase cells. The micronuclei originate from chromosomes that cannot migrate to the poles during cell division [20]. It should be mentioned that the results of the *in vitro* studies published have investigated only short-time exposures to micro-plastic particles and, in most cases, high concentrations of micro-plastic particles [21].

Accordingly, this study aimed to investigate the toxic and genotoxic effects of several types (polystyrene and poly methyl methacrylate; PS and PMMA) and concentrations of polymer particles in human HaCaT keratinocytes *in vitro*. Besides acute effects, chronic effects were also investigated by analysing long-term polymer-HaCaT cultures for up to five weeks. We used a high-content imaging system and algorithm-driven quantitative image analysis to demonstrate these particles' short- and long-term impact on HaCaT keratinocytes. Methyl methanesulfonate (MMS) was used as a positive control test chemical for micronuclei induction and method optimization, and amino-modified (NH₂) PS particles with a diameter of 50 nm were used as positive particle control to monitor particle-induced toxicity. Accordingly, we comprehensively screened plastic particle exposure across different polymer types, particle sizes, and incubation times in HaCaT keratinocytes and *in vitro* for micronuclei formation using optimized quantitative high-content imaging and unsupervised algorithm-driven data analysis to infer the particles' toxic and genotoxic effects.

2. Experimental Section

2.1. Cell Culture and Short-Term Polymer Treatment

HaCaT keratinocytes (Cell Line Service, CLS300493CP; passage number 40) were cultured in Roswell Park Memorial Institute (RPMI) medium 1640 containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. The cells were incubated at 37 °C and 5% CO₂ in a humidified atmosphere and were passaged in a 1:3 ratio once

per week when they reached approximately 80% confluency. Experiments were performed in 96-well plates containing a final volume of 100 μL of complete medium per well. For experiments, cells were seeded at an initial density of 1×10^3 HaCaT keratinocytes per well. After incubation overnight to allow attachment, positive control dose-response analysis was performed by treating cells with various concentrations of PS-NH₂ and MMS (methyl methanesulfonate) to find appropriate concentrations for toxicity and micronuclei induction. To achieve the optimum dose to score maximum bi-nucleated cells (BNCs) with less toxicity, cells were treated with cytochalasin B concentrations ranging from 0–9 $\mu\text{g}/\text{mL}$ for 48 h. In the present study, 70–1100 nm PMMA and 50–1000 nm polystyrene particles were used. Particles were suspended in the PBS to set up the main stock solution. Following identifying dose-response data, the cells were treated with the desired dose of positive controls, 1 or 100 $\mu\text{g}/\text{mL}$ concentrations of PS (50 nm, 200 nm, 1000 nm, and mix of three sizes) or PMMA (70 nm, 400 nm, 1100 nm, and mix of three sizes) particles as a single treatment for 6 h. An exceedingly detailed analysis of the particles used in this study by dynamic light scattering for size, zeta-potential, and polydispersity, along with information on the manufacturer solvents, has been provided in a previous study [22]. The lower concentration of 1 $\mu\text{g}/\text{mL}$ is a medium concentration that was claimed to be found in human blood of probands [23], although the methods of identifying this concentration are currently questioned in the community, and the results and concentration need to be verified in future studies. As complement, one higher (100 $\mu\text{g}/\text{mL}$) concentration was used. The cells were then treated with cytochalasin B at a final concentration of 2 $\mu\text{g}/\text{mL}$ to block cytokinesis and allow the identification of MN in the cells that have completed one cell division and created BNCs.

2.2. Long-Term Polymer Treatment of HaCaT Keratinocytes

To begin this process, 5×10^4 HaCaT keratinocytes were suspended in complete cell culture media and seeded in T-25 flasks. Immediately after, the cell suspension was treated with 1 $\mu\text{g}/\text{mL}$ NMPPs and incubated under optimum cell growth conditions. The culture medium containing NMPPs was replaced with culture medium containing NMPPs every two days to ensure continuous exposure. The cells were split every week. The experiments lasted approximately 5 weeks. This was to mimic constant exposure to plastic particles spanning approximately more than 30 cell division cycles to allow micronuclei to accumulate during each cellular (and nuclear) division. Considering a mean cell cycle time of 13 days in the epidermis [24], our long-term culture mimics more than 1 year of keratinocyte proliferation *in vivo*. Then, the cells of each group were detached from the flask and seeded separately in a 96-well plate. The next day, two different concentrations of NMPPs were added to the plate, similar to the acute treatment regime. Cell supernatants were replaced by fresh media containing 2 $\mu\text{g}/\text{mL}$ cytochalasin B after 6 h (Figure 1a). The experiments were continued for two more days before imaging and MN assessment.

2.3. Staining and Imaging

The 96-well wells containing the cells were washed once with PBS and treated with 50 μL accutase for 10 min at room temperature. Accutase was deactivated by adding 50 μL of complete cell culture media, and the well plate was transferred to the incubator for 1 h. This additional incubation time helped a few residual cells that had become floating due to the accutase treatment to settle down to the well bottom again. The supernatant was then removed, and the cells were then fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature, followed by washing twice with PBS. Cells were permeabilized by adding 0.1% Triton X-100 for 10 min. Non-specific binding was blocked by incubating cells with 2% bovine serum albumin in PBS for 30 min at room temperature. Alexa Fluor (AF) 594 phalloidin (BioLegend, Amsterdam, The Netherlands) working solution was prepared fresh by diluting 10 μL stock solution in 1 mL of PBS. Cellular actin was then stained using 50 μL of this working solution per well. Nuclei were then counterstained using a 2 μM working solution of DAPI at room temperature, followed by twice washing with PBS. Images were

acquired using a high-content imaging system (Operetta CLS; PerkinElmer, Hamburg, Germany) in a single plane with a 20x air objective (NA = 0.4) to capture DAPI and AF594 fluorescence emission light. The non-confocal mode was used to acquire the images, and the binning was adjusted to 2. DAPI fluorescence was acquired with a λ_{em} 430–500 nm filter (λ_{ex} 365 nm), while a λ_{em} 570–650 nm filter (λ_{ex} 550 nm) was used to capture AF594 fluorescence. Per well and technical replicate and biological replicate, 61 fields of view were acquired. More than 12,000 individual fluorescence microscopy images were analyzed in this study. Algorithm-based image analysis was performed using *Harmony 4.9* software (PerkinElmer).

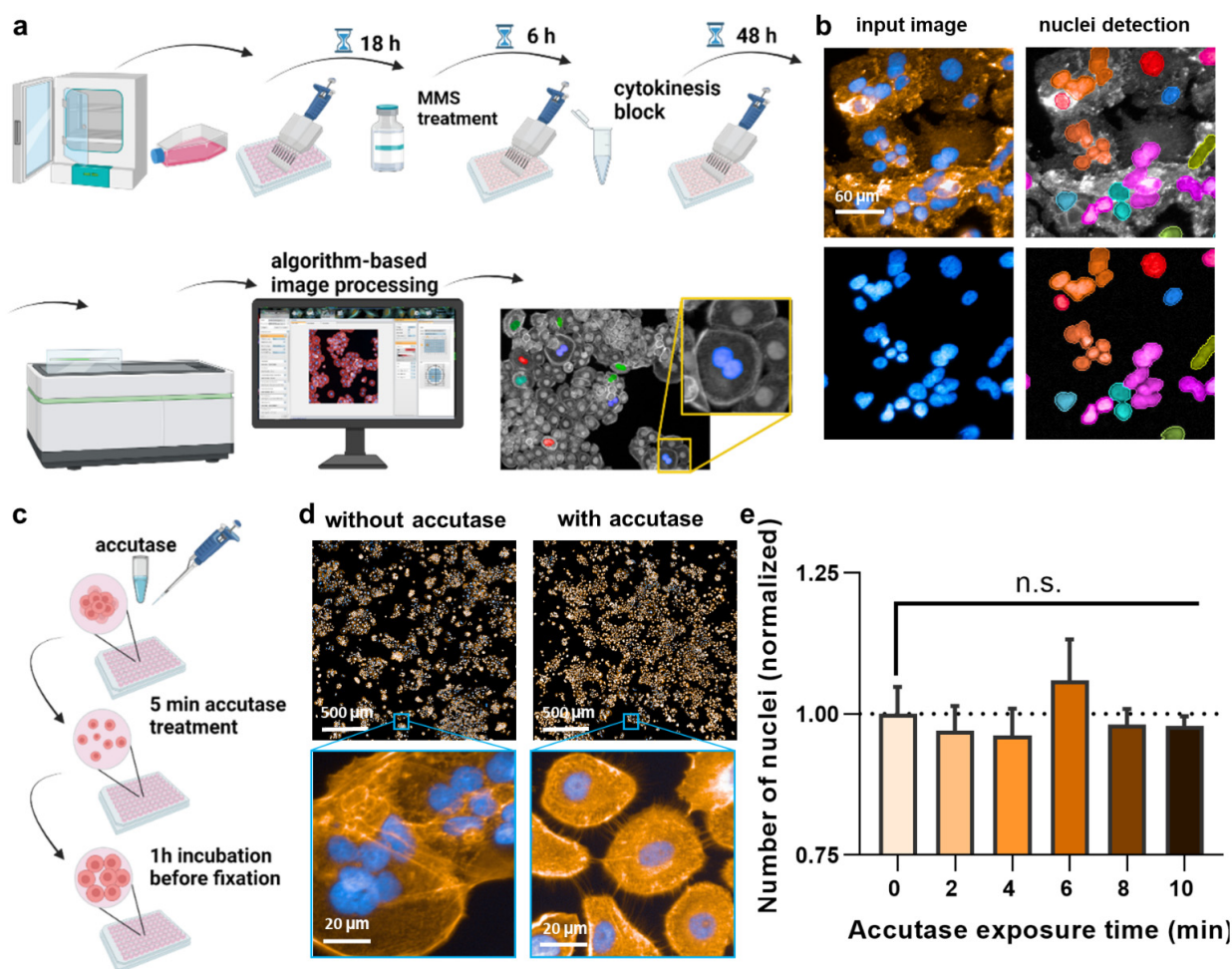


Figure 1. Study design and cell detachment. (a) Study design; (b) failure of original nuclear segmentation by software due to HaCaT keratinocytes' aggregated cell growth; (c) scheme using a 5 min accutase treatment before fixation to lift and separate the cells without detachment; (d) representative image of the effect of accutase treatment on the separation of cells; (e) number of nuclei after 2–10 min of accutase exposure detachment, followed by addition of cell culture medium and 1 h incubation, and data normalization to cells not exposed to accutase (0), indicating that the initial accutase treatment does not lead to complete cellular detachment with subsequently less number of cells being identified by the software. Data are representative of mean + S.E.M. from at least three independent experiments. Statistical analysis was performed using one-way analysis of variances with Dunnett post hoc testing for multiple comparisons (e). n.s. = not significant.

2.4. Thiol Content

To begin this process, 1×10^5 HaCaT keratinocytes were seeded in 24-well plates for 24 h, followed by exposure to either MMS (positive control) or NMPPs (at the previously described concentrations) for 6 h at 37 °C. Then, cells were washed with PBS afterward to

remove excess NMPPs, and fresh culture medium containing cytochalasin B at 2 µg/mL was added for 48 h. Next, HaCaT intracellular thiol content was performed using a glutathione detection reagent (ThiolTracker Violet; Thermo Fisher Scientific, Bremen, Germany), and Zombie NIR (BioLegend) staining was used to mark dead cells. Cells were harvested and stained for 20 min at 37 °C. Single-cell acquisition was performed using flow cytometry (CytoFLEX LX; Beckman–Coulter, Krefeld, Germany). Flow cytometry data files were analyzed utilizing *Kaluza* 2.1.3 software (Beckman–Coulter).

2.5. Intracellular ROS

HaCaT keratinocytes (1×10^5) were seeded in 96-well plates (Nunc; Thermo Fisher Scientific) at 37 °C for 24 h. Afterward, cells were exposed to MMS to either MMS (positive control) or NMPPs (at previously described concentrations) at 37 °C for 6 h. Cells were washed with PBS and stained with CM-H₂DCF-DA (2 µM; Thermo Fisher Scientific) at 37 °C for 1 h. Images were acquired using a 20x objective and the Operetta CLS device described above. Excitation and emission settings were λ_{ex} 505nm and λ_{em} 510–570 nm for H₂DCF-DA and digital phase contrast (DPC) was used for cells. Algorithm-based image quantification was performed using *Harmony* 4.9 imaging and analysis software (PerkinElmer). After flat field and bright field corrections, cells were detected by segmenting their cytoplasm using DPC signals. Then, the fluorescence intensities of cells were quantified, and the results were normalized to the total cell area analyzed. The final results were expressed as mean fluorescence intensity (MFI) per cell area and were normalized against vehicle controls.

2.6. Statistical Data Analysis

Statistical analysis was performed using one-way analysis of variances with Dunnett's post hoc test to correct for multiple testing or *t*-test. Levels of significance were indicated as follows: $\alpha = 0.05$ (*), $\alpha = 0.01$ (**), $\alpha = 0.001$ (***). Graphing and statistical analysis were performed using *prism* software 9.4.1 (GraphPad; San Diego, CA, USA).

3. Results

3.1. Method Optimization of MN Formation Analysis in HaCaT Keratinocytes

To date, a reliable method to analyze micronuclei (MN) formation with an algorithm-based method is not available for HaCaT keratinocytes. This is because of the clustered growth of the cells, impeding image-segmentation-based detection of individual cells and nuclei. This, again, is important because, according to the OECD protocol No. 487, the MN must only be counted in bi-nucleated cells (BNCs) and ultimately related to the absolute numbers of the latter. Quantitative fluorescence analysis has the advantage of identifying hundreds to thousands BNCs relatively quickly, out-competing manual counting attempts in terms of number, accuracy, and unbiasedness. Accordingly, a cytokinesis-block micronucleus assay was set up (Figure 1a). In regularly fixed and stained cells (blue: nuclei; orange: actin/cytosol), neither unambiguous identifications of BNCs nor their cytosolic region could be identified (Figure 1b). Strikingly, the clustered growth can be alleviated by incubating the cells with accutase (Figure 1c) long enough to become single cells in the 96-well plate (Figure 1d) but too short for the cells to detach completely. Quantifying the absolute number of nuclei (and normalization to control-wells corresponding to “0 min” that had not received accutase) showed no difference for incubation times up to 10 min with accutase (Figure 1e), indicating the accutase effect only separated but not completely detached the cells. Next, cytochalasin B was used, an agent shown to prevent metastasis [25] and cytotoxic at higher concentrations (Figure 2a). It is added to cells to inhibit actin polymerization, thereby inhibiting cytokinesis and allowing the identification of MN in cells that had undergone one cell and nuclear division but not separation. Cells were sampled after 48 h, a time point equivalent to about 1.5–2.0 normal cell cycle lengths. The cytokinesis-block proliferation index (Figure 2b) and the percentage of BNCs (Figure 2c) were significantly increased for low to modest cytochalasin B concentrations used. The ideal dosage of cytochalasin B was determined to be 2 µg/mL since it was not

highly toxic to HaCaT keratinocytes and allowed us to obtain 50% bi-nucleated cells at 48 h reliably. Next, the incubation time post-accutase treatment was optimized (Figure 2d), and a manual quantification of MN revealed 1 h incubation to be significantly superior to 0 h (Figure 2e). Next, the dose–response of methyl methanesulfonate (MMS) treatment, a genotoxic compound and positive control in this study [26], was analyzed (Figure 2f). The number of manually quantified MN reached a maximum at about 400 μ M (Figure 2g), but as this concentration already was toxic in 50% of cells, 300 μ M was used for further analysis. The final algorithm-driven image analysis could now be set up. Representative images of the object segmentation workflow are provided (Figure 2h), showing the benefit of accutase treatment to identify nuclei, and algorithm-based sub-grouping from the total population into bi-nucleated cells (BNCs), multinucleated cells, and mono-nucleated cells. From there, bi-nucleated cells (BNCs) were identified from the DAPI-stained nuclear over the identification of the cytosolic area via the phalloidin-stained actin (orange), the removal of cross-contaminating nuclei in these image regions, and the automatic identification of micronuclei (MN) related to bi-nucleated objects (Figure 2i) as highlighted (Figure 2j).

3.2. Acute PS and PMMA Polymer Particle Toxicity and Genotoxicity Were Negligible

We next applied the newly established method to analyze the acute toxicity and genotoxicity of two types of polymer particles (PS and PMMA) across three sizes each (50 nm and 70 nm, 200 nm and 400 nm, 1000 nm and 1100 nm, respectively) as well as one size mix for two different concentrations (1 μ g/mL and 100 μ g/mL) in HaCaT keratinocytes *in vitro*. The cells were incubated with particles for 6 h, followed by performing the CBMN assay as described above (Figure 3a). As positive control, aminated (NH_2) particles (size: 50 nm) were used, and their IC_{50} was determined (Figure 3b); 10 μ g/mL were used for subsequent experiments. We then calculated the CBPI of cells. The results indicate lower proliferation in cells treated with MMS or aminated particles, as both substances are cytotoxic (Figure 3c). By contrast, none of the particle exposure conditions affected the calculated CBPI values of HaCaT cells significantly. This, however, was not the case for the number of mononucleated (Figure 3d) and bi-nucleated cells identified (Figure 3e) as well as the number of micronuclei among BNCs (Figure 3f). Specifically, the larger particle sizes at the high concentrations elevated MNC and BNC counts. At the same time, only 1 μ m PS at 100 μ g/mL led to a modest but significant increase in the MN frequency. It has been suggested that increased MN frequencies are associated with oxidative stress. To this end, we tested intracellular ROS production in HaCaT keratinocytes exposed to polymer particles (Figure 4a). ROS production was elevated for positive controls. In contrast, intracellular ROS levels were significantly decreased for 1000 nm PS and 1100 nm PMMA particles at the high 100 μ g/mL concentration (Figure 4b). As this was surprising, we analyzed whether this ROS decrease affected the cells' antioxidative defense capacity. By quantifying intracellular free thiols, we identified profound changes with the positive control treatment of HaCaT keratinocytes, while none of the polymer particle conditions led to significant alterations in the antioxidant defense capacity (Figure 4c).

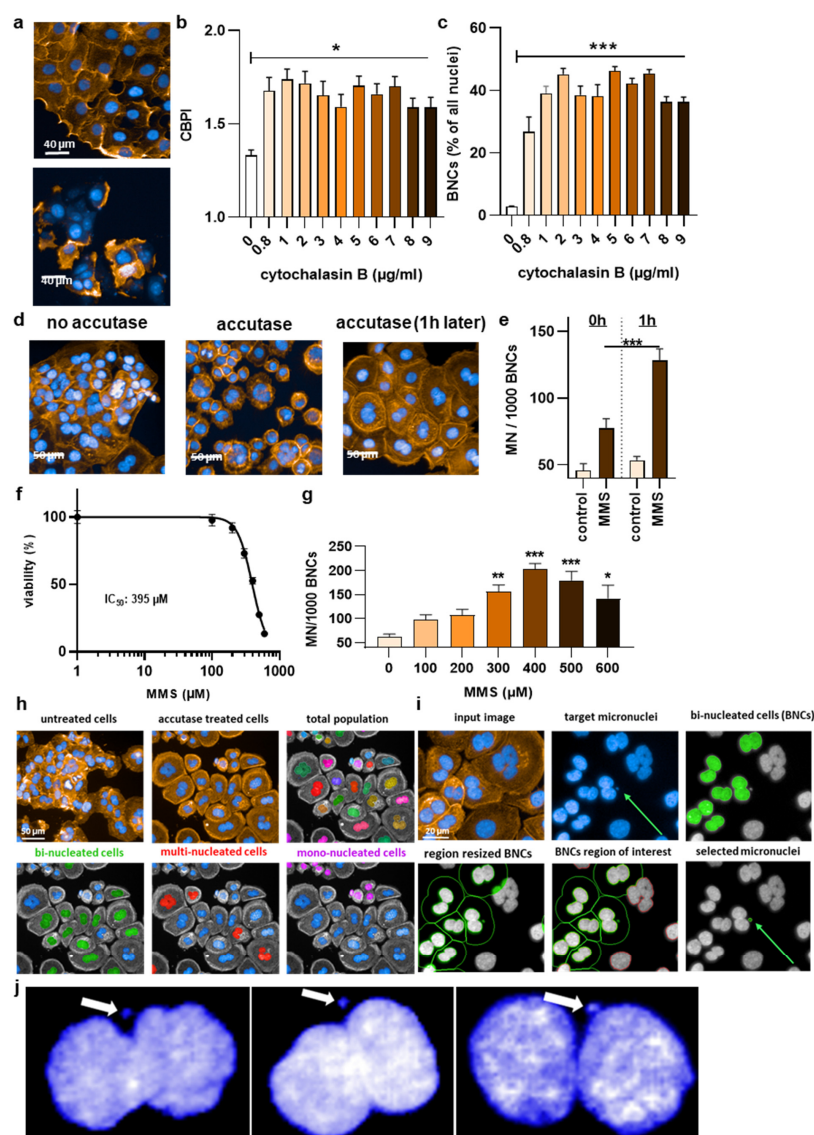


Figure 2. High-content imaging protocol optimization. (a) Representative images for the effects of low (upper image) and high (lower image) cytochalasin B concentrations; (b) cytokinesis block proliferation index (CBPI) in relation to different cytochalasin concentrations; and (c) bi-nucleation rate in relation to different cytochalasin concentrations; (d) representative images of the effect of accutase on nuclear segmentation and single cells; (e) micronucleation rate at 0 h and 1 h post accutase treatment; (f) MMS dose–response curve; (g) MMS concentrations in relation to micronucleation rate among bi-nucleated cells (BNCs); (h) representative images showing the benefit of accutase treatment to identify nuclei, and algorithm-based sub-grouping from the total population into bi-nucleated cells (BNCs), multinucleated cells, and mono-nucleated cells; (i) representative images from the algorithm-driven object segmentation workflow starting with the input image and the identification of bi-nucleated cells from the DAPI-stained nuclear over the identification of the cytosolic area via the phalloidin-stained actin (orange), the removal of cross-contaminating nuclei in these image regions, and the automatic identification of micronuclei (MN) related to bi-nucleated objects; (j) representative magnified fluorescence microscopy images of DAPI staining in bi-nucleated cells with micronuclei. Data are representative or mean $\pm$ S.E.M. from at least three independent experiments. Statistical analysis was performed using one-way analysis of variances with Dunnett post hoc testing for multiple comparisons (b,c,g) or *t*-test (e) with * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$.

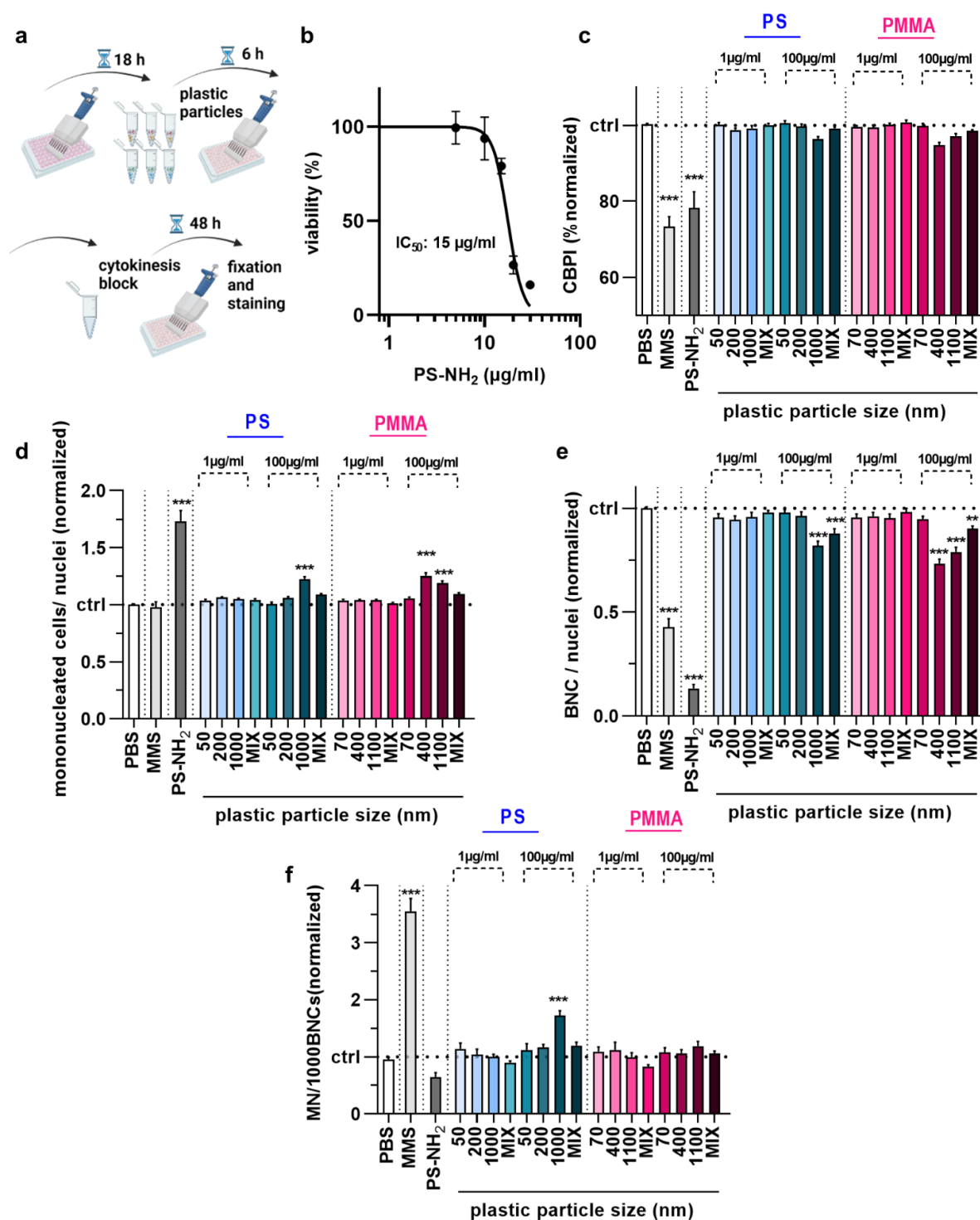


Figure 3. Acute toxicity and genotoxicity testing in HaCaT keratinocytes upon polymer particle exposure. (a) Acute particles exposure scheme; (b) aminated (PS-NH₂) polystyrene (PS) particle dose–response curve 54 h post-exposure; (c–f) CBPI (c), number of MNCs (d), number of BNCs (e), and MN frequency per 1000 BNCs (f) in HaCaT keratinocyte samples exposed to vehicle (PBS), MMS (positive control for genotoxicity), PS-NH₂ particles (positive control for polymer toxicity), and PS and PMMA polymer particles of different sizes (50–1100 nm) and concentrations (1 μg/mL and 100 μg/mL). Data are representative or mean + SEM from at least six independent experiments. Statistical analysis was performed using one-way analysis of variances with Dunnett post hoc testing for multiple comparisons (c,d,e,f) with * = $p < 0.01$ and *** = $p < 0.001$.

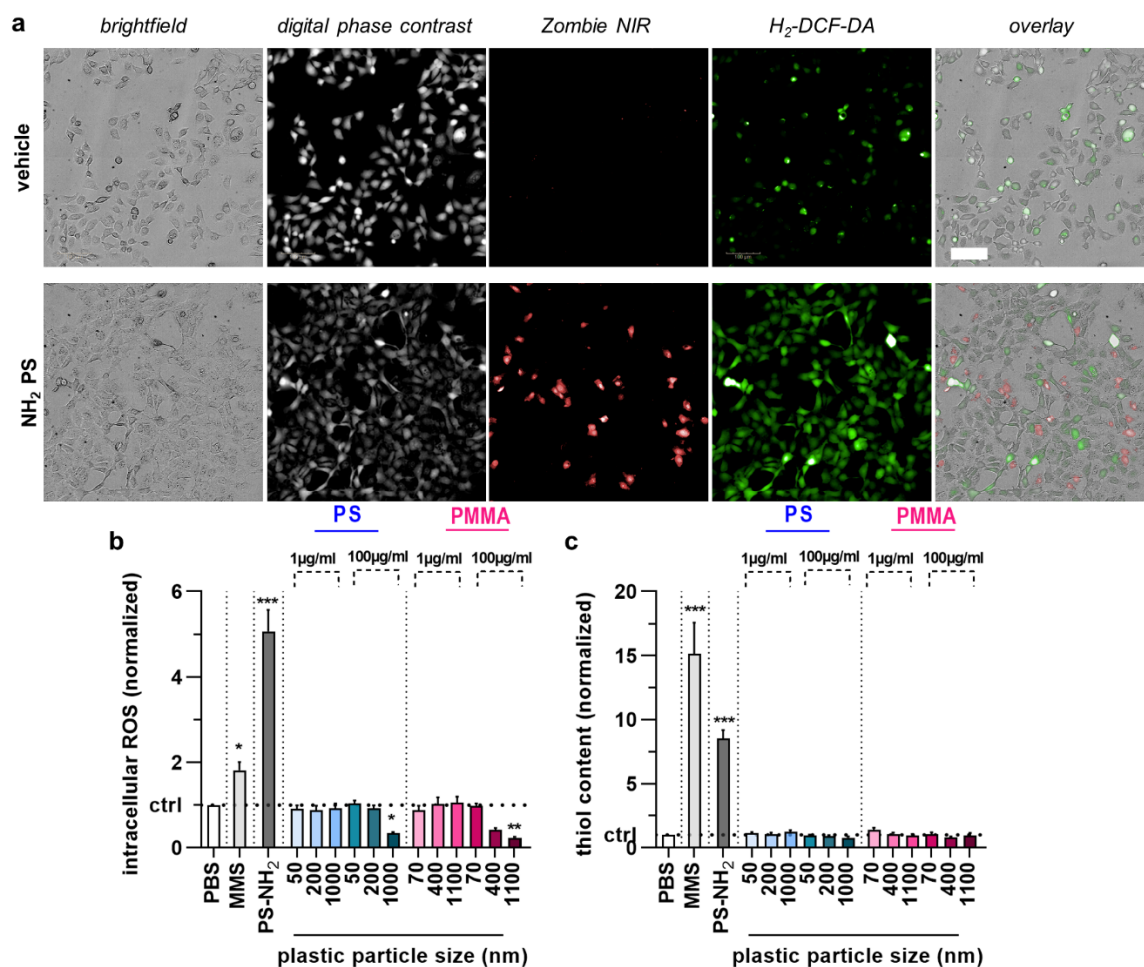


Figure 4. Cell cycle arrest, intracellular ROS, and antioxidant defense in HaCaT keratinocytes upon acute polymer particle exposure. (a) Representative brightfield, digital phase contrast, Zombie NIR, $\text{H}_2\text{-DCF-DA}$, and overlay images of vehicle and NH_2 particle treated HaCaT keratinocytes; (b,c) intracellular ROS (normalized DCF mean fluorescence intensity) (b), and antioxidant defense (normalized thioltracker violet mean fluorescence intensity) (c) in HaCaT keratinocyte samples exposed to vehicle (PBS), MMS (positive control for genotoxicity), PS-NH₂ particles (positive control for polymer toxicity), and PS and PMMA polymer particles of different sizes (50–1100 nm) and concentrations (1 µg/mL and 100 µg/mL). Data are representative or mean + SEM from at least four independent experiments. Statistical analysis was performed using one-way analysis of variances with Dunnett post hoc testing for multiple comparisons with * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$.

3.3. Chronic PS and PMMA Polymer Particle Toxicity and Genotoxicity Were Similar to Acute Toxicity In Vitro

The majority of studies investigating the effects of plastic exposure in vitro focus on short-term investigations reflecting acute consequences. By contrast, we also wanted to elucidate the long-term effects of plastic exposure by setting up a 5-week permanent plastic particle culture model (Figure 5a). Before conducting the MN analysis of the cells permanently cultured in the presence of NMPPs (chronic exposure) at a final concentration of 1 µg/mL, the cells were exposed to the CBMN assay protocol exactly as described for the short-term (acute) exposure. Overall, the results obtained for mononucleated (Figure 5b) and bi-nucleated (Figure 5c) HaCaT keratinocytes were similar to those of acute exposure. Specifically, larger particles at higher concentrations of 100 µg/mL led to elevated MNC and decreased BNC numbers in HaCaT keratinocytes. Additionally, the CBPI (Figure 5d) was, in contrast to short-term exposure, significantly decreased for the cells incubated in

the presence of particles of larger sizes and at higher concentrations. Regarding the MN frequency, the same 1 μm PS culture conditions at 100 $\mu\text{g}/\text{mL}$ gave a low but significant increase in MN frequency (Figure 5e), similar to findings with acute exposure (Figure 3f). All other plastic particle culture conditions did not induce changes in HaCaT keratinocytes. Collectively, our data suggest polymer particles of larger sizes (>1000 nm) to have greater effects on cell cultures in vitro.

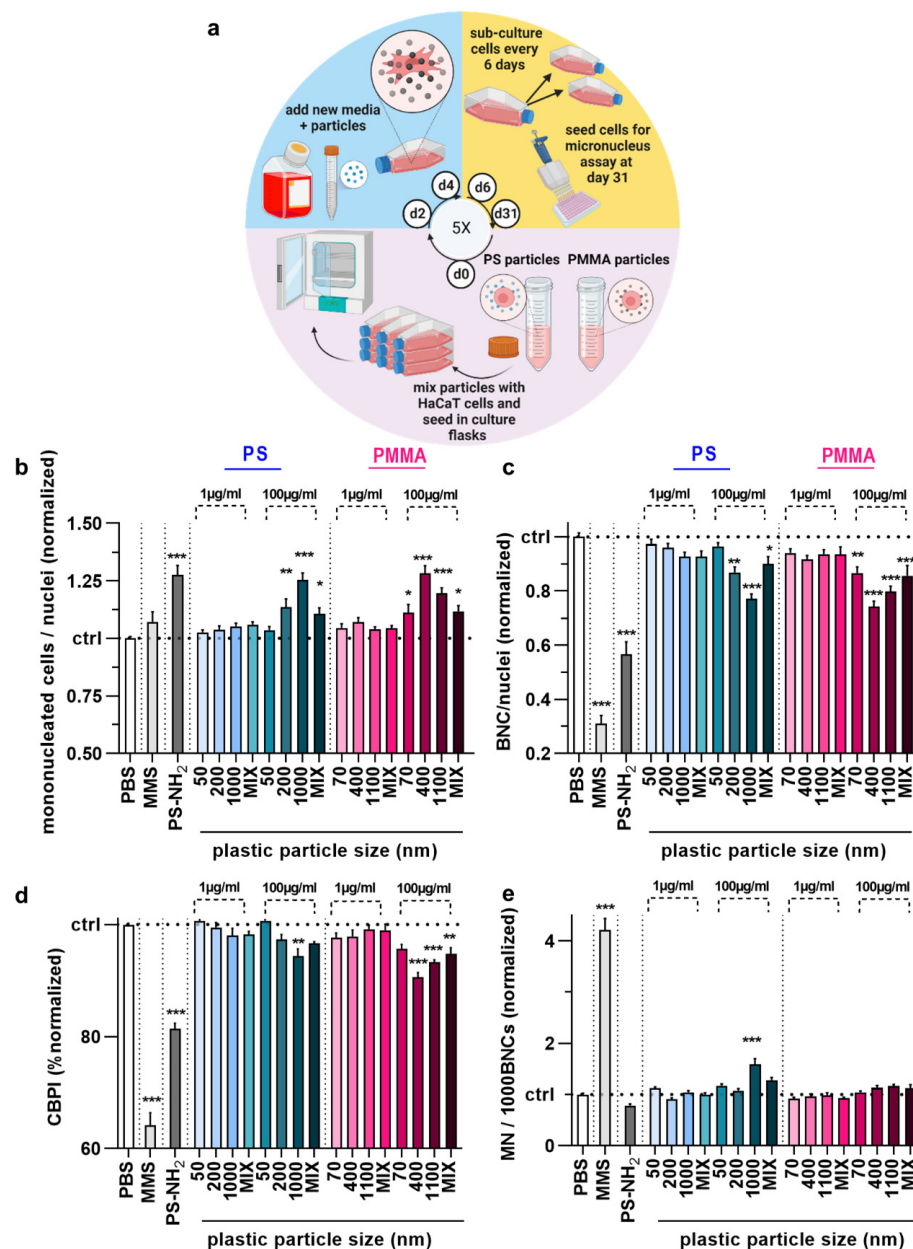


Figure 5. Long-term (5-week) genotoxicity testing in HaCaT keratinocytes upon polymer particle exposure. (a) study scheme; (b–e) mononucleated (b), bi-nucleated (c), CBPI (d), and micro-nucleated (MN) ϵ cells per 1000 BNCs of HaCaT keratinocytes exposed to vehicle (PBS), MMS (positive control for genotoxicity), PS-NH₂ particles (positive control for polymer toxicity), and PS and PMMA polymer particles of different sizes (50–1100 nm) and concentrations (1 $\mu\text{g}/\text{mL}$ and 100 $\mu\text{g}/\text{mL}$) for 6 h post chronic exposure to the respective particles for 5 weeks at 1 $\mu\text{g}/\text{mL}$. Data are representative or mean $\pm$ SEM from at least six independent experiments. Statistical analysis was performed using one-way analysis of variances with Dunnett post hoc testing for multiple comparisons (b–e) with * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$.

4. Discussion

The exponential growth of plastic waste has been observed worldwide, which causes environmental contamination [27]. However, the reports on the effects of nano- and micro-plastic particles (NMPPs) on human health are conflicting, ranging from little to more pronounced effects. Yet, even though several studies demonstrate that NMPPs may accumulate in cells without producing acute toxicity, the fact that certain NMPPs have been found to impact cells is a reason for concern [28–31]. This confirms that their interactions with biological systems should be investigated for the potential to cause toxicity and genotoxicity. We attempted to investigate this thoroughly across several iterations on polymer type, size, concentration, acute or chronic exposure, and by using algorithm-driven quantitative imaging.

NMPPs can enter cells and interact with them using different pathways. The uptake of NMPPs was evaluated and proved in our previous study in three different cell lines [22]. Here, we aimed to explore the genotoxic effects of PS and PMMA particles in human epidermal keratinocytes. The high-content imaging-based method and software were established to identify and quantify micronucleus formation in an unprecedented fashion. The most crucial factor while performing an MN assay based on OECD guidelines is verifying that the cells being scored have completed mitosis throughout the treatment or post-treatment period [32]. Cytochalasin B is a substance that has been frequently used to block cytokinesis. It blocks actin assembly during cytokinesis and prevents daughter cell separation after mitosis, resulting in bi-nucleated cell formation [33–35]. Humans can be exposed to NMPPs for a long period at low concentrations. Notwithstanding, OECD guidelines on the micronucleus assay suggest that the treatment period with toxicants should be short in cytogenetic assays [36]. Accordingly, higher concentrations of toxicants should be preferred for exposure to detect subsequent genetic damage. In this study, we tested low (1 µg/mL) and high (100 µg/mL) concentrations of NMPPs in *in vitro* conditions. NMPPs at high concentrations may produce biologically irrelevant positive results. However, based on the results obtained from relatively higher concentrations of NMPPs, influential information can be deduced from evidence obtained for lower concentrations for a long period of exposure [37,38]. In our previous study, we monitored the uptake and intracellular localization of NMPPs and found that 6 h was enough for particles to enter the cells despite varying uptake kinetics for various cell lines [22].

The NMPPs' potential ability to induce genotoxicity can be divided into direct and indirect damage. As the direct mechanism of genotoxicity, the damage can occur through the direct physical interaction of NMPPs with the DNA of a cell, and the effect of indirect genotoxicity can be induced through oxidative stress causing damage to proteins involved in the replication of DNA and the division of cells [29,39]. Hence, investigating ROS could be a fundamental point in determining the underlying mechanism involved in the toxicity and genotoxicity of NMPPs. Therefore, we evaluated ROS production induced by NMPPs exposure and analyzed and quantified cellular thiol content. PS-NH₂ particles have been used here as a positive control to illustrate particles' toxic, especially ROS-provoking effect, given their impact on cell proliferation [40]. Surprisingly, despite the toxic effect, the number of MN/1000 BNCs was lower in PS-NH₂-treated cells compared to untreated cells. Additionally, mononucleated cell numbers were significantly higher in the cells treated with these particles. Comparing the number of mononucleated cells and BNCs in PS-NH₂ treated cells explained how these particles might potentially interfere with DNA replication and cell division [41,42]. Hence, the data suggest that the treatment with PS-NH₂ interfered with the cell cycle progression and inhibited DNA synthesis in HaCaT keratinocytes [42]. In A549 lung cancer cells, Kim and colleagues reported that PS-NH₂ induced cell death and interrupted cell cycle progression, and cell cycle arrest occurred gradually at two points along the cell cycle [41]. Lunov and colleagues previously investigated the effect of PS-NH₂ on macrophages differentiated from human monocyte and reported that these particles potentially led to cell death and inflammation [43]. However, additional experiments are needed to confirm this point and further elucidate cell cycle arrest mechanisms.

Interestingly, PS particles similar to the PS-NH₂ but without surface modification in three different sizes do not show any cytotoxic effect over two concentrations in acute treatment. However, the rate of BNCs per nuclei suggests that a high dose of NMPPs can induce cell cycle arrest, depending on the exposure dose. The number of mononucleated cells confirms these results, which are also in line with other studies [44,45]. Genotoxicity was another crucial toxicological endpoint that was evaluated. Our study did not find any relevant genotoxic effect in the cells exposed to NMPPs for 6 h, except a small but significant increase for one 1 µm PS particle condition at 100 µg/mL. For all other conditions, we observed that the treatment of the NMPP's low concentration did not change the frequencies of MN as a genomic instability in the HaCaT keratinocytes compared to the background. Although in some studies, higher levels of DNA damage have been recorded in cells treated with NMPPs, others have found no correlation between NMPP exposure and genotoxic damage [17,39,46], confirming our findings. The same is true for the absence of major cytotoxic effects for particles of different types, origins, sizes, and concentrations [47–49]. Yet, cell membrane alterations could result from the internalization of the NMPP and direct physical contact between the membrane and NMPPs. Bigger particles have more contact with the cells' surface. It has been suggested that, especially during prolonged exposure of cells to NMPPs *in vitro*, the particles interact continuously with the cells and elicit various changes in the cell membrane [50–53]. As a result, cells may be more vulnerable to high particle dosage, potentially leading to cell cycle arrest and cell death after prolonged exposure. This explains why statistically significant results were not found at the lower concentrations, while significant changes were observed at 100 µg/mL concentrations after prolonged exposure in our study. Similar to acute treatment, the BNCs rate showed that NMPPs treatment caused cell cycle arrest, and cells could not pass the cell division process. As a result, there are more mononucleated cells and fewer BNCs [50,54–57]. Regarding NMPP size, we investigated not only individual sizes but also the effects of NMPP mixtures of different sizes. Interestingly, the results of this “mix” treatment in HaCaT cultures permanently incubated in the presence of plastic particles resembled the effects seen with the smaller than larger particles (Figure 5). The authors were not fully prepared for the result of exposing the cells to a mixture of plastic particles of various sizes. Hence, there is no acceptable explanation for these findings.

Several studies reported that, following NMPPs uptake by cells, they aggregate in lysosomes, causing lysosomal swelling and membrane damage, which releases proteolytic enzymes into the cytosol and triggers cell death [50,58]. Along those lines, it has also been suggested that NMPPs exposure is associated with increased oxidative stress. However, since some studies have reported direct toxicity without ROS generation, the assumption that ROS generation is necessary for NMPPs-induced toxicity and genotoxicity may be incorrect. The results of our study corroborate these findings. However, to predict NMPP-induced toxicity, ROS generation in cells exposed to NMPPs must be characterized to determine whether ROS generation plays a role in NMPP-induced harmful effects [30,59,60]. In addition, various potential molecular mechanisms might be responsible for creating MN in response to the presence of NMPPs. Micronuclei may be clastogens, chromosome fragments created when DNA is broken, or aneugens, complete chromosomes created when the mitotic apparatus is disrupted. NMPPs seem to be somewhat in between, suggesting they are both clastogenic and aneugenic. However, more details on the mechanisms will be investigated in future studies on this subject.

The limitation of this study was that the results on NMPP genotoxicity are only applicable to *in vitro* HaCaT keratinocytes cultures, and we do not extrapolate our findings to draw conclusions on the limited genotoxicity of NMPPs in human skin. From a dermatological point of view, testing primary keratinocytes and using 3D skin models would be desirable to obtain results in model systems that more closely mimic *in vivo* conditions. In addition, the chronic exposure cultures could have been performed for longer, and extending the assay toward other cell lines is desirable. Moreover, we only tested homogenous,

commercially available plastic particles, and it would be interesting to test environmental nano- and micro-plastic samples in future studies.

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Communication

Cold Physical Plasma Toxicity in Breast and Oral Squamous Carcinoma In Vitro and in Patient-Derived Cancer Tissue Ex Vivo

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Abstract: Breast cancer (BC) and oral squamous cell carcinoma (OSCC) are among the most common types of cancer, but current clinical outcomes remain unsatisfactory. Available therapies have limitations in terms of efficacy and may also cause severe side effects. Cold physical plasma is a promising approach for selectively eliminating cancer cells while avoiding genotoxic effects on non-malignant cells. In this study, we investigated the potential of cold physical plasma as a therapeutic intervention for BC and OSCC through in vitro and ex vivo studies on toxicity. For the in vitro study, T-47 BC cells and SCC-4 and SCC-9 OSCC cell lines were used, and we found cold plasma to be toxic in a treatment time-dependent manner. Moreover, we investigated the safety of physical plasma therapy and found no genotoxic potential in plasma-treated human keratinocytes in vitro. Finally, for the first time, 20 BC and OSCC patient-derived tumor tissues were punch biopsied and ex vivo-exposed to cold physical plasmas to study responses in the tumor microenvironment (TME). Cold physical plasma caused significant apoptosis in patient-derived BC and OSCC tumor tissues, and decreased the number of CD163⁺ cells (e.g., tumor-associated macrophages, TAM) in BC tissue plasma-treated ex vivo. Collectively, our findings motivate the investigation of cold physical plasma as a potential adjuvant treatment in oncology.

Keywords: gas plasma technology; genotoxicity; plasma medicine; reactive oxygen species; ROS

1. Introduction

Oral squamous cell carcinoma (OSCC) and breast cancer (BC) are common types of cancers with some similarities in their molecular profiling, such as the expression of genes and proteins involved in cell proliferation, differentiation, and survival. Both cancers also have a relatively high incidence of metastasis, which can lead to poor prognosis and reduced survival rates [1,2]. Despite the implementation of different treatments, the 5-year survival rates for advanced OSCC and BC have remained relatively unchanged over time [3,4]. In recent years, there have been significant advances in the diagnosis and treatment of both OSCC and BC, including chemotherapy, radiotherapy, the development

of targeted therapies, and immunotherapies [5–8]. Additionally, ongoing research aims to improve current treatments' effectiveness and develop new therapies that may offer better outcomes for patients with these types of cancer. It is known that endogenous and exogenous reactive oxygen species (ROS) can have various effects on cancer cells and immune cells within the tumor microenvironment (TME) [9–11]. Cold physical plasma is a partially ionized gas that can generate various types of ROS, which have been shown to induce ROS, toxicity, and immunogenicity in tumor cells, reducing tumor growth and increasing cell death [12–14]. Additionally, physical plasma has been shown to increase the expression of specific chemokines and cytokines within the TME, which can attract immune cells such as T cells and natural killer cells to the tumor site, such as melanoma *in vivo* and in patient-derived melanoma tissues *ex vivo* [15,16]. Such responses can potentially enhance the immune response against cancer cells and improve the overall response to treatment [17]. However, proof-of-concept studies are lacking on whether cold physical plasma is effective against BC and OSCC cancers in models resembling phenotypes found in the clinics.

This study involved conducting *in vitro* investigations to evaluate the treatment time-dependent toxicity of an atmospheric pressure argon plasma jet on T-47D, SCC-4, and SCC-9 cell lines of BC and OSCC, respectively. In addition, an OECD-approved *in vitro* micronucleus test was performed to detect micronuclei formation in the cytoplasm of interphase human keratinocytes after cold physical plasma treatment to determine if the argon plasma had any harmful effects on non-malignant cells. Finally, *ex vivo* punch biopsies of BC and OSCC were generated from patient-derived cancer tissue samples to assess the effects of cold physical plasma therapy on cancer cells in human tumor tissues rather than relying solely on cell lines that may not fully represent the characteristics of actual tumors. These experiments aimed to understand whether cold physical plasma has the potential as a putative adjuvant treatment in future clinical cancer management.

2. Materials and Methods

2.1. Cell Culture

SCC-4 and SCC-9 OSCC cell lines (derived from human tongue) and the T-47D cell line (derived from female human breast) were obtained from ATCC and cultured in 5% CO₂ and 95% air-humidified atmosphere at 37 °C. RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin was used to grow OSCC cell lines, while T-47D cells were cultured in DMEM high glucose supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin. The cells were subcultured at 80% confluency, and experiments were conducted on cultures with the same confluency.

2.2. *In Vitro* Plasma Treatment

For the experiments, 1×10^4 cells were seeded in a 96-well plate in 90 µL of fully supplemented cell culture media. After 18 h of incubation, the medium was replaced, and 200 µL of RPMI was added to the cells. The cells were then incubated for an additional hour and treated with various treatment times of cold physical plasma (0, 20 s, 40 s, 60 s, 90 s, 120 s, 180 s, 240 s, and 300 s) using the atmospheric pressure argon plasma jet kINPen IND (neoplas, Greifswald, Germany) [18] operated with 2.5 and 1.0 standard liters of argon gas per minute for cytotoxicity and genotoxicity analysis, respectively. The lower feed gas flux was necessary for the latter as the higher fluxes were too toxic for the assay to be performed adequately based on our previously optimized OECD protocol with algorithm-driven image quantification analysis [19]. The distance between the plasma jet nozzle and the cell culture liquid covering the adherent cells was 10 mm. To compensate for the water evaporation effect caused by extended cold physical plasma treatment, pre-established volumes of double-distilled (dd) water (H₂O) were added to the treated cells. To rule out effects of water and argon gas alone on cell toxicity, the cells were treated with the

highest volume of ddH₂O, and one group was exposed to the argon gas for 300 s since this represented the longest cold physical plasma treatment time.

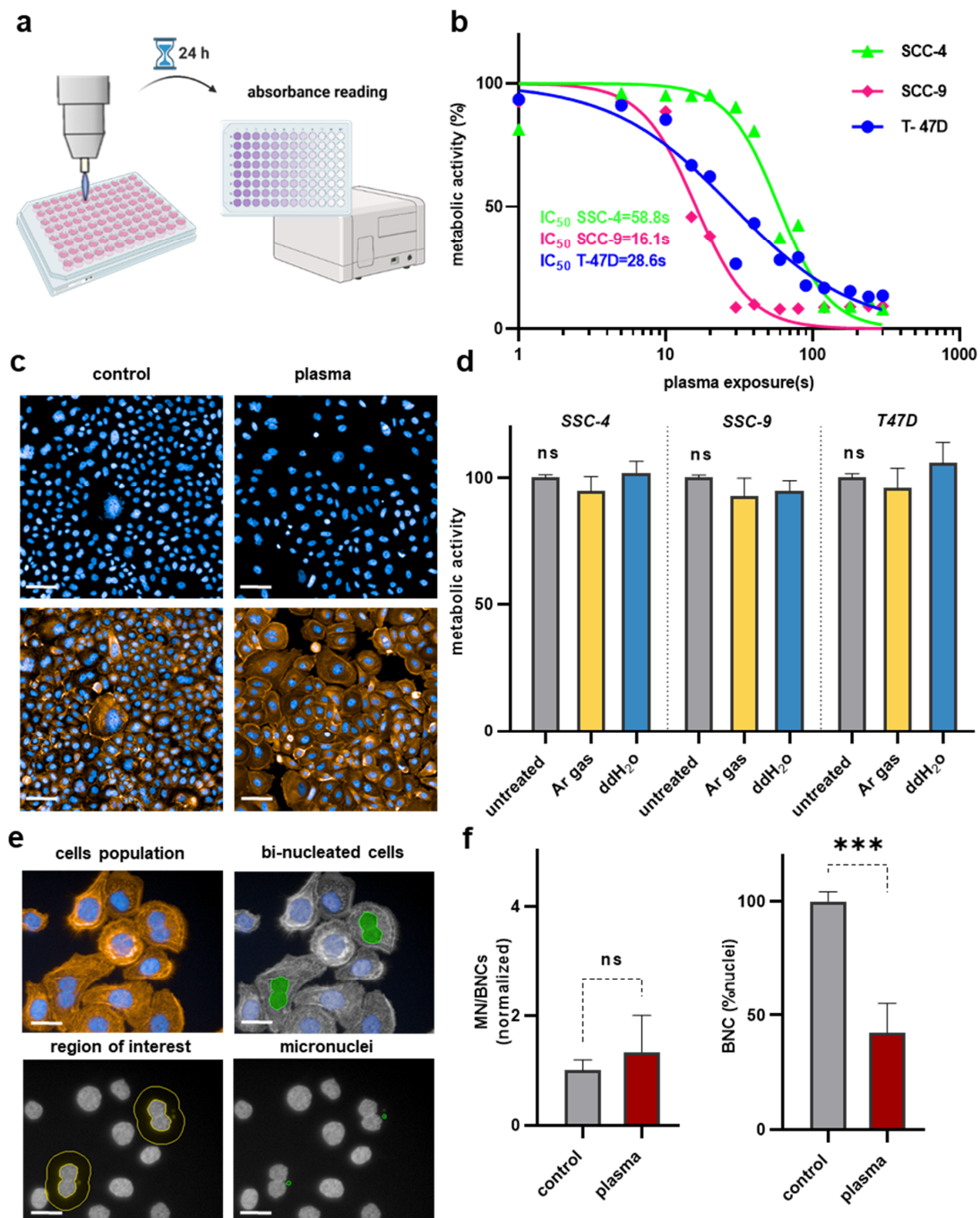


Figure 1. Toxicity and genotoxicity testing upon argon plasma treatment. (a) plasma exposure scheme; (b) argon plasma treatment time response curve 24 h post-exposure in SCC-4, SCC-9, and T-47D cells; (c) representative images showing cold plasma-mediated decrease cell viability in cancer cell lines; (d) confirmation of absence of toxicity of argon gas treatment alone or addition of ddH₂O (negative controls) in SCC-4, SCC-9, and T-47D cells after 24 h of incubation; (e) representative images of the algorithm-based object segmentation process in human HaCaT keratinocytes after argon plasma exposure, from the input image over binucleated cell and micronuclei segmentation through DAPI-stained identification. Statistical analysis was done using one-way analysis of variance with Dunnett post hoc testing for multiple comparisons (d) or *t*-test (f). Levels of significance were indicated as follows: $\alpha = 0.001$ (***). n.s. = not significant.

2.3. Metabolic Activity Assay

After 20 h of incubation following treatment, 10 µL of resazurin (stock solution: 2 mM) diluted in a complete growth medium was added to each well, followed by a 4 h incubation period. Absorbance changes (Figure 1a) were monitored using a microplate reader (M200; Tecan, Männedorf, Switzerland).

2.4. OECD-Based Micronucleus Assay

To investigate if gas plasma treatment showed any potential genotoxic effects, human HaCaT keratinocytes were seeded at an initial density of $1\text{--}10^4$ cells/well in 96-well plates. After incubation overnight to allow attachment, cells were exposed to cold physical plasma for 10 s (1 slm) and incubated for 6 h. To achieve maximum binucleated cells (BNCs) as per OECD guideline [20], cells were treated with cytochalasin B (final concentration: 2 µg/mL) to block cytokinesis and allow the identification of MN in the cells that have completed one cell division and created BNC. The assay was performed as recently described [19].

2.5. Ex Vivo Biopsies and Exposure to Cold Physical Plasma

A total of 20 patients suffering from BC and 20 patients suffering from OSCC (who had not undergone any prior therapy) were recruited for the study. The Ethics Committee of Motamed Cancer Institute approved both studies (approval number: IR.ACECR.IBCRC.REC.1400.007), and each patient gave written informed consent before participating. Tumors were surgically removed from the patients and transported to the laboratory in complete cell culture media. The ex vivo tumor tissue biopsy samples were obtained by punching each sample with a 5 mm diameter punch and divided into groups that were either treated with cold physical plasma or left untreated as controls. The patient-derived punch biopsies were placed onto an impact plate and were exposed to cold physical plasma for 300 s using an atmospheric pressure argon plasma jet operated at a flow rate of five standard liters per minute and at a distance of 10 mm from the nozzle to the tissue. The jet has been described in detail regarding its physico-chemical traits and biological effects in vitro and in vivo before [21], and the optical emission spectrum profile of this argon plasma jet is similar to that found for the kINPen [22]. After plasma treatment, the samples were transferred to a 6-well plate and incubated in fully-supplemented RPMI1640 at 37 °C for 20 h. After incubation, the tissues were fixed, processed, and encased in paraffin wax for preservation.

2.6. Tissue Section Preparation, Immunofluorescence Staining, Imaging, and Data Evaluation

The samples were cut into seven-micrometer-thick FFPE cross sections using a Cryo microtome (Leica Microsystems, Wetzlar, Germany). The sections were placed on Superfrost Plus slides (Thermo Fisher Scientific, Bremen, Germany) and baked overnight at 60 °C. To prepare for the pretreatment steps, the slides were deparaffinized in xylene and dehydrated in EtOH. Sequential sections were chosen for the immunofluorescence staining of samples. The tissue was subjected to heat-induced antigen retrieval in citrate buffer, permeabilized using Triton X-100 (0.25%), and non-specifically blocked by incubating samples in 1% bovine serum albumin. CD45- (Ventana, SP57 Y04591), CD163- (Ventana, SP57 Y04591), and Foxp3- (Ventana, SP57 Y04591) reactive antibodies were added to the samples at previously optimized concentrations, and Alexa Fluor 647-conjugated secondary antibodies were used to detect the primary antibodies. DAPI (4',6-diamidino-2-phenylindole; Sigma-Aldrich, Taufkirchen, Germany) was used to stain the nuclei. Positive and negative controls were added to the evaluation to ensure no technical errors interfered with the outcome. The TUNEL assay (Roche Diagnostics, Rotkreuz, Switzerland) was used according to the manufacturer's instructions to detect dead cells with DNA degradation during the late stages of apoptosis. After staining, the sections were microscopically examined using an Operetta CLS high-content imaging device (PerkinElmer, Hamburg, Germany) and a 10× objective (Zeiss, Jena, Germany). Quantification was performed using algorithm-based image analysis software Harmony 4.9 (PerkinElmer, Hamburg, Germany), which helped calculate the positive staining area relative to the total number of cells.

2.7. Statistical Data Analysis

Statistical analysis was performed using one-way analysis of variances with Dunn's post-hoc test to correct for multiple testing, or *Student's t*-test. Levels of significance were indicated as follows: $\alpha = 0.05$ (*), $\alpha = 0.01$ (**), $\alpha = 0.001$ (***). Graphing and statistical analysis were performed using *prism* 9.5.1 (GraphPad Software; San Diego, CA, USA).

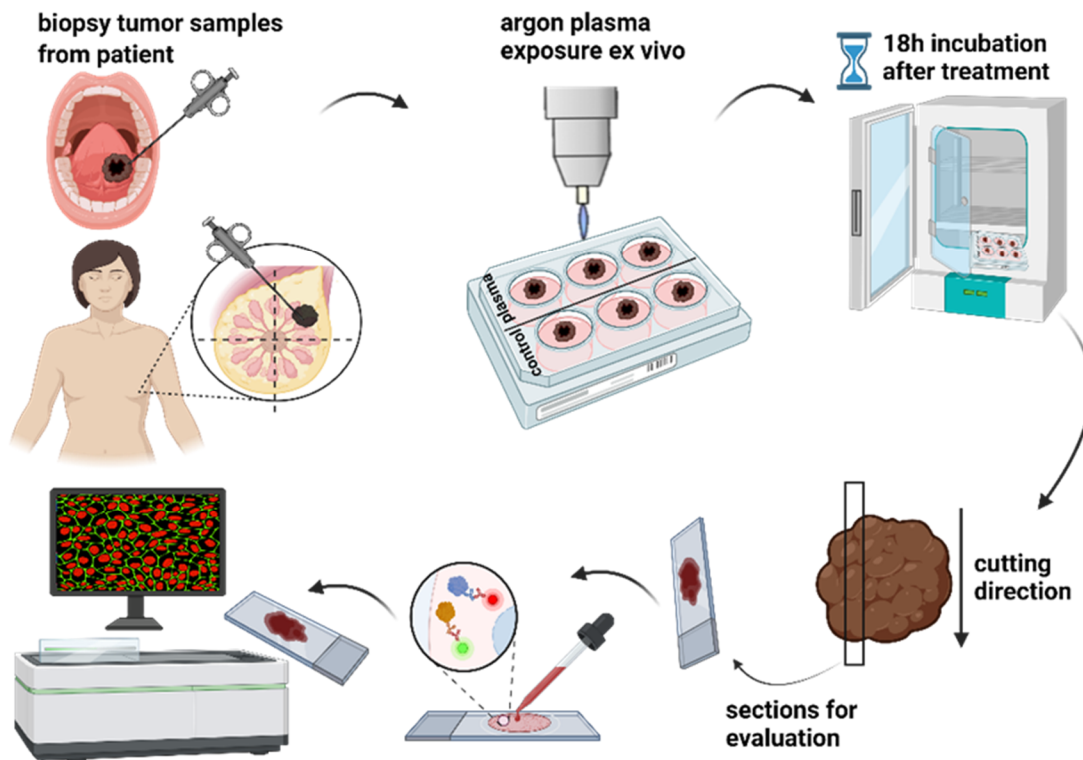


Figure 2. Ex vivo study design.

3. Results

3.1. Argon Plasma Showed Treatment Time-Dependent Toxicity in T-47D, SCC-4, and SCC-9 Cells but Did Not Lead to Genotoxic Effects in Human Keratinocytes

In this study, we investigated the effects of physical plasma on cancer cell viability by conducting cold physical plasma treatments on two cell lines for OSCC and one cell line for BC. There was a cold physical plasma exposure-time-dependent reduction in cell viability in SCC-4, SCC-9, and T-47D cells *in vitro* (Figure 1b). The dose-response curve demonstrated that the IC₅₀ value of physical plasma exposure dose was 60 s for SCC-4 cells, 30 s for T-47D cells, and 16 s for SCC-9 cells, indicating that these doses induced significant cytotoxicity (Figure 1c). Further increasing the physical plasma treatment time decreased the metabolic activity, indicating a clear dose-dependent response. It was observed that all cells were dead after being exposed to physical plasma for 300 s, which was the highest physical plasma exposure dose used in the experiment. To compensate for water evaporation caused by cold physical plasma exposure, a pre-determined amount of ddH₂O was added to the cells based on the evaporated water volume. Neither this water compensation nor the exposure of cells to argon gas alone for 300 s (plasma = off) showed any negative effects on the tumor cells' metabolic activity (Figure 1d). To evaluate whether cold physical plasma conferred any genotoxic effects, human HaCaT keratinocytes were subjected to argon plasma treatment. The micronuclei rate in binucleated cells was measured as a measure of genotoxicity (Figure 1e). The findings revealed that cold physical plasma treatment did not induce significant micronuclei in the HaCaT cells, indicating the absence of genotoxic effects of physical plasma on non-malignant cells (Figure 1f).

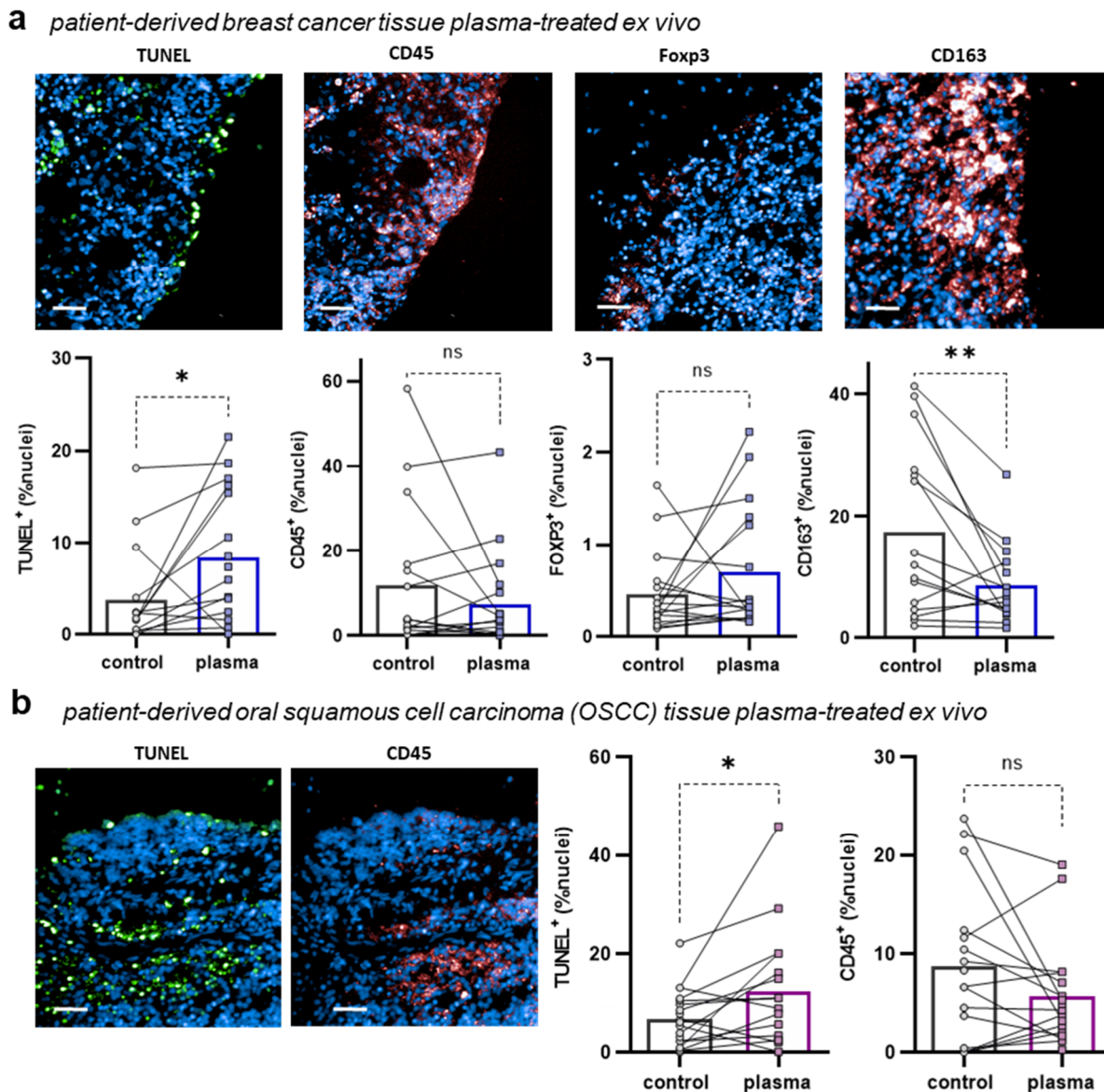


Figure 3. Immunofluorescence analysis. (a) representative images and quantitative imaging data of ex vivo generated patient-derived tumor tissue samples for stained with DAPI to indicate nuclei, TUNEL to indicate apoptotic cells, CD45 to label leukocytes, CD163 to label M2 macrophages, and FoxP3 for labeling regulatory T-cells in gas plasma-treated and untreated samples; (b) DAPI, TUNEL, and CD45 staining in ex vivo gas plasma-treated and untreated OSCC patient-derived tissues. Data are representative or mean from 20 BC and OSCC patient samples, respectively. Statistical analysis was performed using paired *t*-test. Levels of significance were indicated as follows: $\alpha = 0.05$ (*) and $\alpha = 0.01$ (**). ns = not significant.

3.2. Argon Plasma Induced Apoptosis in Patient-Derived BC and OSCC Cancer Tissues

We next examined the effects of cold physical plasma on ex vivo-generated punch biopsies of BC and OSCC tissue derived from a total of 40 cancer patients. The question of our study in this part was whether plasma treatment induced cytotoxic responses in cancer tissues investigated 24 h after treatment (Figure 2). After the tissue was sectioned, stained, and imaged, apoptotic cells (TUNEL positive, green) were quantified, and the ratio of green nuclei (apoptotic cells) over the total number of nuclei (DAPI⁺ objects) was calculated. Our quantitative analysis revealed a significant increase in apoptosis in the cold physical plasma-treated BC (Figure 3a) and OSCC (Figure 3b) samples, particularly at the borders of the tissues where the cold physical plasma treatment was applied. The results showed that

physical plasma treatment led to an on-average three-fold increase in apoptotic cells in BC tissue samples and a two-fold increase in OSCC samples compared to untreated samples. Apart from apoptosis, we also investigated the impact of cold physical plasma treatment on the number of stainable immune cells (CD45-staining), regulatory T cells (Foxp3-staining), and tumor-associated macrophages (TAM, CD163⁺-staining). Using immunofluorescence staining and analysis on tissue slides (Figure 3), we counted the number of these immune cells in both cold physical plasma-treated and untreated samples. The results indicated a significant decrease in the number of TAM in plasma-treated BC tissue samples. However, cold plasma exposure did not cause any significant effects on total leukocyte counts and regulatory T cells in BC tumor samples. Taken together, our findings suggest that cold physical plasma treatment induces apoptosis in cancer cells in vitro and in patient-derived tumor tissue ex vivo. At the same time, genotoxic effects were not observed in vitro. These results may have important implications for the potential use of cold physical plasma as a novel adjuvant treatment approach for cancer.

4. Discussion

The use of cold physical plasma to selectively target cancer cells has been a topic of interest in recent years [23–25]. Our study provides further evidence of its potential effectiveness by investigating physical plasma's effects in tongue cancer and breast cancer cell lines and found a clear dose-dependent reduction in cell viability in all cell lines tested. The IC₅₀ values for physical plasma exposure indicate that even relatively short exposure times can induce significant cytotoxicity in cancer cells. Several studies have investigated physical plasma effects on various cancer cell types, including brain, lung, blood, cervical, melanoma, breast, colorectal, head and neck, hepatocellular, prostate, ovarian, osteosarcoma, pancreatic, bladder, gastric, thyroid, uterine, and epidermal cancer cells [26]. These studies have shown promising results in inducing cancer cell death which is in line with our results. Liu et al. investigated the effect of physical plasma treatment on triple-negative breast cancer cells, which are known to be particularly aggressive and difficult to treat. They found that physical plasma treatment was able to induce cell death in these cancer cells through ROS-dependent cellular pathways [27]. Xiang et al. investigated the effect of physical plasma treatment on triple-negative breast cancer cells. The researchers found that physical plasma treatment was able to induce cell death in these cancer cells, as well as inhibit their migration and invasion abilities both in vitro and in vivo [28]. Ramireddy et al. investigated the effect of physical plasma treatment on Oral squamous cell carcinoma and found that physical plasma treatment was able to inhibit cell growth in OSCC cells in a manner that is dependent on the dosage and triggers apoptosis by generating reactive oxygen and nitrogen species. The induced apoptosis is linked to an increase in Bax expression and a decrease in Bcl-2 expression. Additionally, plasma treatment of cancer cells was found to cause cell cycle arrest through the ATM/P53/P21 signaling pathway, and cellular senescence [29].

Novel treatment options need to be not only effective but also safe. One aspect of assessing safety is studying genotoxic effects [30]. We have previously established a high-throughput, high-content imaging micronucleus assay incorporating the requirements of OECD guidelines on the CBMN assay and utilizing the power of algorithm-driven object segmentation for unsupervised analysis and quantification of micronuclei in binucleated cells [19]. Several studies have demonstrated that cold physical plasma-induced reactive oxygen species (ROS) are responsible for plasma-induced toxicity, have been associated with some hallmarks of cancer and are thought to spur malignancy and DNA damage when applied chronically at higher concentrations [31]. However, we could not find any genotoxic effects of gas plasma exposure in human HaCaT keratinocytes. These findings align with recent studies indicating that physical plasma did not cause DNA damage or abnormalities like micronuclei [32,33]. The absence of genotoxic effects in physical plasma-treated cells underline the favorable safety profile for human use. Our study also evaluated the potential toxicity of argon gas and ddH₂O as negative controls and found no reduction

in metabolic activity after 24 h. This suggests that the observed toxicity in the physical plasma experiments is attributed to ROS rather than gas flow.

To investigate the effects of physical plasma on the patient-derived tumor samples, punch biopsies of BC and OSCC tissues were generated ex vivo and were investigated by immunofluorescence after cold plasma exposure. The results acquired from TUNEL staining demonstrate that physical plasma treatment significantly impacts cancerous tissue by inducing apoptosis. Similar findings were reported previously in patient-derived cutaneous squamous cell carcinoma, basal cell carcinoma, melanoma, urothelial carcinoma, and glioblastoma samples following cold physical plasma treatment ex vivo [16,34–36]. Moreover, a quantitative evaluation of CD45, CD163, and FoxP3 staining was performed, focusing on the most active areas in the tumor's periphery following physical plasma treatment. The decrease in the number of tumor-associated macrophages (CD163⁺ cells) following physical plasma treatment in BC tissue samples suggests that cold plasma exposure may aid in lifting immunosuppressive actions of, e.g., TAM, which is associated with improved antitumor T-cell responses [37,38]. In our previous study, we found that physical plasma treatment had the potential to induce toxicity and promote an immunogenic cell death (ICD) response in BC cells in vitro and in vivo, resulting in the infiltration of effector T cells into the BC TME [39]. Such an aspect might be beneficial beyond sole cytotoxic actions of cold physical plasma applications in future oncology. Further research is necessary to fully understand the mechanisms underlying the effects of cold physical plasma on cancer cells and to determine the optimal conditions for utilizing this novel technology as a clinical adjuvant anticancer treatment.

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Article

Patient-Derived Human Basal and Cutaneous Squamous Cell Carcinoma Tissues Display Apoptosis and Immunomodulation following Gas Plasma Exposure with a Certified Argon Jet

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Abstract: Reactive oxygen species (ROS) have been subject of increasing interest in the pathophysiology and therapy of cancers in recent years. In skin cancer, ROS are involved in UV-induced tumorigenesis and its targeted treatment via, e.g., photodynamic therapy. Another recent technology for topical ROS generation is cold physical plasma, a partially ionized gas expelling dozens of reactive species onto its treatment target. Gas plasma technology is accredited for its wound-healing abilities in Europe, and current clinical evidence suggests that it may have beneficial effects against actinic keratosis. Since the concept of hormesis dictates that low ROS levels perform signaling functions, while high ROS levels cause damage, we investigated herein the antitumor activity of gas plasma in non-melanoma skin cancer. In vitro, gas plasma exposure diminished the metabolic activity, preferentially in squamous cell carcinoma cell (SCC) lines compared to non-malignant HaCaT cells. In patient-derived basal cell carcinoma (BCC) and SCC samples treated with gas plasma ex vivo, increased apoptosis was found in both cancer types. Moreover, the immunomodulatory actions of gas plasma treatment were found affecting, e.g., the expression of CD86 and the number of regulatory T-cells. The supernatants of these ex vivo cultured tumors were quantitatively screened for cytokines, chemokines, and growth factors, identifying CCL5 and GM-CSF, molecules associated with skin cancer metastasis, to be markedly decreased. These findings suggest gas plasma treatment to be an interesting future technology for non-melanoma skin cancer topical therapy.

Keywords: chemokines; cold physical plasma; cytokines; reactive oxygen species; ROS; skin cancer

1. Introduction

Basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) are highly prevalent skin cancers [1,2]. SCC is a relatively slow-growing skin cancer that can expand to the

surrounding tissue and further into the body (metastasis). On the contrary, BCC rarely metastasizes but has invasive and destructive local growth [3]. Changes and diversity in the tumor microenvironment (TME) and the unknown nature of several other factors partially explain the failure of definitive treatment for these diseases in some cases [4,5]. Based on recent studies, the tumor microenvironment (TME) plays a significant role in tumor progression, response to treatment, growth, and the acquisition of the metastatic pattern by tumor cells [6]. In addition to all the classic strategies of tumors escaping the immune system responses, e.g., antigen expression reduction, resistance to cell lysis by the immune cells, and the expression and secretion of immune suppressor factors, the tumor also escapes the immune system under TME complexity [7,8]. Thus, looking for different treatment methods to target these tumors, especially in cases where resection is impossible, and examining the TME response to treatment is of utmost importance.

In recent decades, cold physical plasma has been used as a powerful tool for inhibiting growth and destroying cancer cells [9,10]. Numerous studies have demonstrated the physical plasma's toxicity on different tumor entities, including glioblastoma [11,12] and skin [13,14], breast [15–17], colorectal [18,19], head and neck [20,21], and lung cancer [22,23]. The anticancer mechanism of physical plasma is complex, but it is assumed that the plethora of reactive oxygen species (ROS) generated by the plasma discharge affects cell functions by disrupting the intracellular redox balance and membrane antioxidant enzymes [24]. ROS are relatively short-lived molecules containing oxygen that are chemically active [25]. At supraphysiological concentrations, ROS damage membranes, DNA, mitochondria, and the endoplasmic reticulum, eventually leading to, e.g., apoptosis, necrosis, senescence, or autophagy [26,27]. Moreover, the mechanism of chemotherapy, photodynamic therapy, and radiotherapy are at least partially based on ROS generation and subsequent apoptosis induction [28,29]. Due to tumor cells' strong reliance on physiological homeostasis with ROS, they are prone to perturbations in redox homeostasis and oxidative distress [30]. Importantly, such stress is also considered a central prerequisite in many types of chemotherapy [31].

Many cancers express a strong network of cytokines and chemokines as well as their receptors. For instance, tumor cells stimulate endothelial cells by chemokine and cytokine expression to loosen their contact inhibition for invasion and metastasis to other tissues [32]. In general, the immune system plays a pivotal and influential role in maintaining the body's homeostasis and destroying tumors, in addition to its fundamental role in dealing with external and infectious agents [33]. Tumor cells can manipulate immune cells by, for instance, adapting their metabolic needs to ultimately enhance instead of halt cancer growth [34]. Hence, investigating the TME is of great importance in oncology.

In this study, the toxicity of a clinically certified atmospheric pressure argon plasma jet on skin cancer cells was investigated *in vitro*, as well as primary patient-derived SCC and BCC tumor samples *ex vivo*. We found that gas plasma treatment can induce apoptosis *in vitro* and *in vivo* and to modulate the inflammatory profiles of the skin tumor biopsies regarding the secretion of chemokines and cytokines as well as the expression of immune-related surface markers.

2. Results

2.1. Toxicity of Gas Plasma Treatment in Skin Cancer Cells *In Vitro*

The toxicity of different doses of plasma irradiation was examined in four cell lines. A resazurin-based assay was used, which is indicative of total metabolic activity (Figure 1a). Two treatment modes were investigated. The first addressed toxicity in cell lines in suspension, while the second was performed in the cell lines that were allowed to adhere before treatment. An exposure time-dependent decline of metabolic activity was found in the former approach (Figure 1b). Based on these data and the calculated inhibitory concentration 25 (IC₂₅), non-malignant HaCaT keratinocytes were less prone to gas plasma-induced metabolic activity reduction than the skin cancer cell lines. However, in the latter approach treating cell lines in adherent states, reduced abrogation in metabolic activity was identified

(Figure 1c). Again, HaCaT cells' resistance to gas plasma treatment was much greater than those of the other three skin cancer cell types. Due to the fact that the plasma treatment of cells in suspension is less relevant for the therapy of human disease, we focused on the stress responses of adherent plasma-treated cells in the following experiments. Thereby, the previous results were re-iterated by assessing the viability of cells based on caspase 3 and 7 activity and terminal cell death (Figure 1d). A comparable viability reduction was observed after gas plasma exposure across all cell types treated in adherent states (Figure 1e). By analyzing the different cell cycle phase in two tumor cell lines (Figure 1f), changes in the quantitative distribution of the different cell cycle phases were observed in both cell lines, which are indicative of cell cycle arrest (Figure 1g), as confirmed by the calculation of G1/G2 ratios (Figure 1h). The flow cytometry analysis of markers for oxidative stress (Figure 1i) subsequently showed overall treatment time-dependent elevated levels in gas plasma-treated skin cancer cells (Figure 1j), especially for phosphorylated (γ) histone 2A.X (γ H2AX), suggesting oxidative stress-induced cell death.

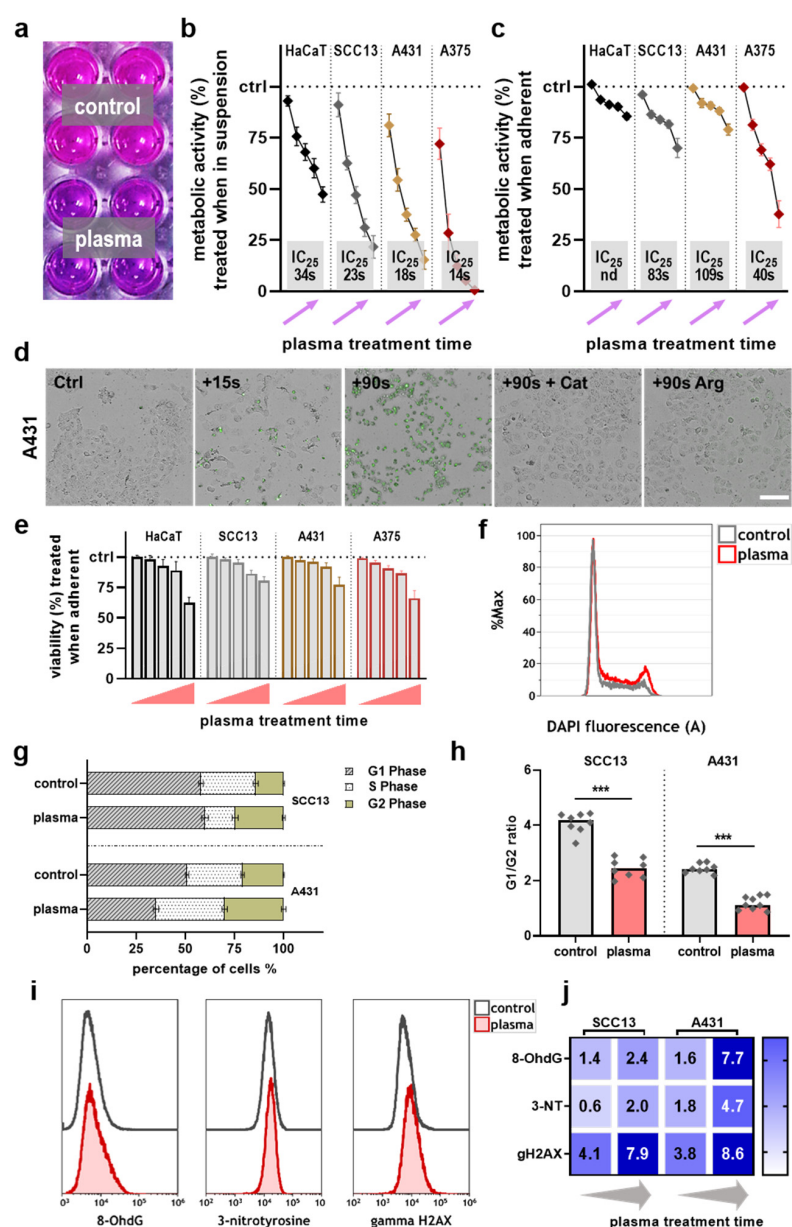


Figure 1. Gas plasma treatment in vitro: (a) image of the metabolic activity assay with higher (control) and lower (bottom) resazurin transformation, respectively; (b,c) quantitative analysis of gas plasma

treatment effects in four cell lines exposed in suspension (b) or adherent (c) states, where grey boxes indicate IC_{25} values; (d) representative brightfield and fluorescence (caspase 3/7, green) overlay images of cells exposed to various conditions, catalase (cat) and argon gas treatment (Arg) served as controls; (e) viability of the four cell lines exposed in adherent state to several plasma treatment times (15 s, 30 s, 45 s, 60 s and 90 s); (f) representative overlay histogram of the DAPI intensity of control and gas plasma-treated SCC13 samples; (g) quantification of cell cycle phases in fixed and permeabilized DAPI-stained SCC13 and A431 cells after acquisition using flow cytometry and analysis of individual cell cycle phases using mathematical modeling according to the Michael H. Fox algorithm employed in the Kaluza analysis software; (h) G1/G2 ratios calculated from cell cycle data; (i,j) representative flow cytometry overlay histograms (i) and normalized quantification of three oxidative stress-related markers (j). Data were normalized to control and are displayed as the mean with SEM of three experiments. Statistical analysis was performed using *t*-test with $p < 0.001$ (**). Scale bar is 100 μ m.

2.2. Toxicity and Immunomodulation of Gas Plasma-Treated Skin Cancer Biopsies

SCC and BCC skin cancer samples were collected from patients to generate biopsies of equal sizes and volumes, followed by gas plasma exposure *ex vivo*, incubation, and analysis of immunofluorescence tissue analysis and screening for soluble inflammatory markers in the tissue culture supernatants (Figure 2a). To verify that the kINPen used in this study generates reactive oxygen and nitrogen species as collectively described before [34], the stable and long-lived products hydrogen peroxide (H_2O_2), nitrite (NO_2^-), and nitrate (NO_3^-) were quantified in 200 μ L of phosphate-buffered saline (PBS) after plasma exposure of different treatment times. Argon gas alone (applied at the maximum exposure time used for plasma treatment) served as the control in addition to untreated liquid (i.e., 0s). For all three types of oxidants investigated, their concentration increased in a plasma exposure time-dependent manner (Figure 2b). While the generation of H_2O_2 was nearly linear to the plasma treatment time, nitrite and nitrate were quickly generated during short plasma exposure but showed a slower rise for longer treatments. Tissues were sectioned and analyzed for apoptosis and the expression of several markers expressed by cells of the TME (Figure 3a). For BCC, gas plasma exposure significantly increased the percentage of apoptotic cells, while the expression of CD206, a marker of tumor-supportive M2 macrophages, remained unchanged (Figure 3b). Interestingly, a significantly lower expression was found for CD86, a molecule associated with professional antigen-presenting cells, and FOXP3, a transcription factor of regulatory T cells (T_{reg}). Elevated levels of apoptosis were also found in *ex vivo* gas plasma-treated patient-derived SCC tissue, while CD206 remained unchanged as in BCC (Figure 3c). Similar to BCC, CD86 was significantly reduced, however, by contrast, the T_{reg} numbers remained unchanged. The immunomodulatory effects of gas plasma treatment on primary skin cancer samples were further investigated by quantifying more chemokines, cytokines, and growth factors released into the culture medium during the incubation period. For BCCs, a significant reduction in CCL5, G-CSF, GM-CSF, and IL-1 β was observed, while granzyme A and IL-17A release was significantly elevated (Figure 4). In SCC samples, three targets were significantly regulated, namely CCL5 and GM-CSF which were upregulated as observed in the BCC samples, and PDGF-aa being reduced (Figure 5). These results suggest gas plasma treatment to confer toxicity in SCC and BCC and to have an immunomodulatory role.

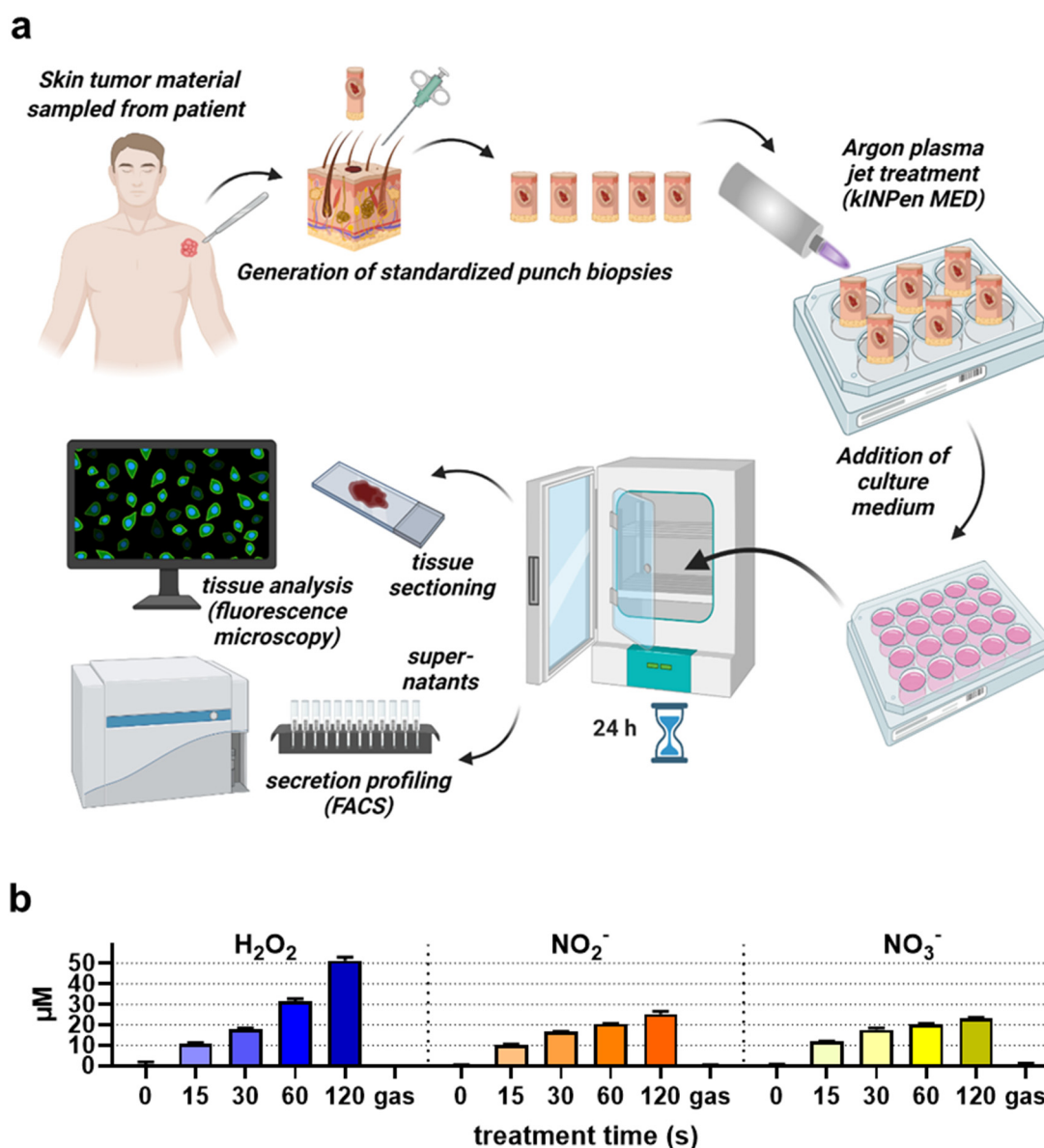


Figure 2. Human tumor tissue sampling and treatment scheme: (a) excised skin tumors were retrieved and used to generate punch biopsies of identical sizes. The punch biopsies were subsequently added to microtiter well plates for standardized gas plasma treatment. Afterward, cell culture medium was added to each well and the samples were incubated for 24 h, before supernatants were collected and tissues were cryo-sectioned and stained. Supernatants were analyzed using multiplex flow cytometry, and cryo-sections were stained with antibodies, followed by quantitative immunofluorescence imaging; (b) quantification of ROS (hydrogen peroxide, H_2O_2) and RNS (nitrite, NO_2^- ; nitrate, NO_3^-) in plasma-treated liquids (200 μL of PBS). Gas refers to 120 s of exposure of the liquid with argon gas only (i.e., plasma = off).

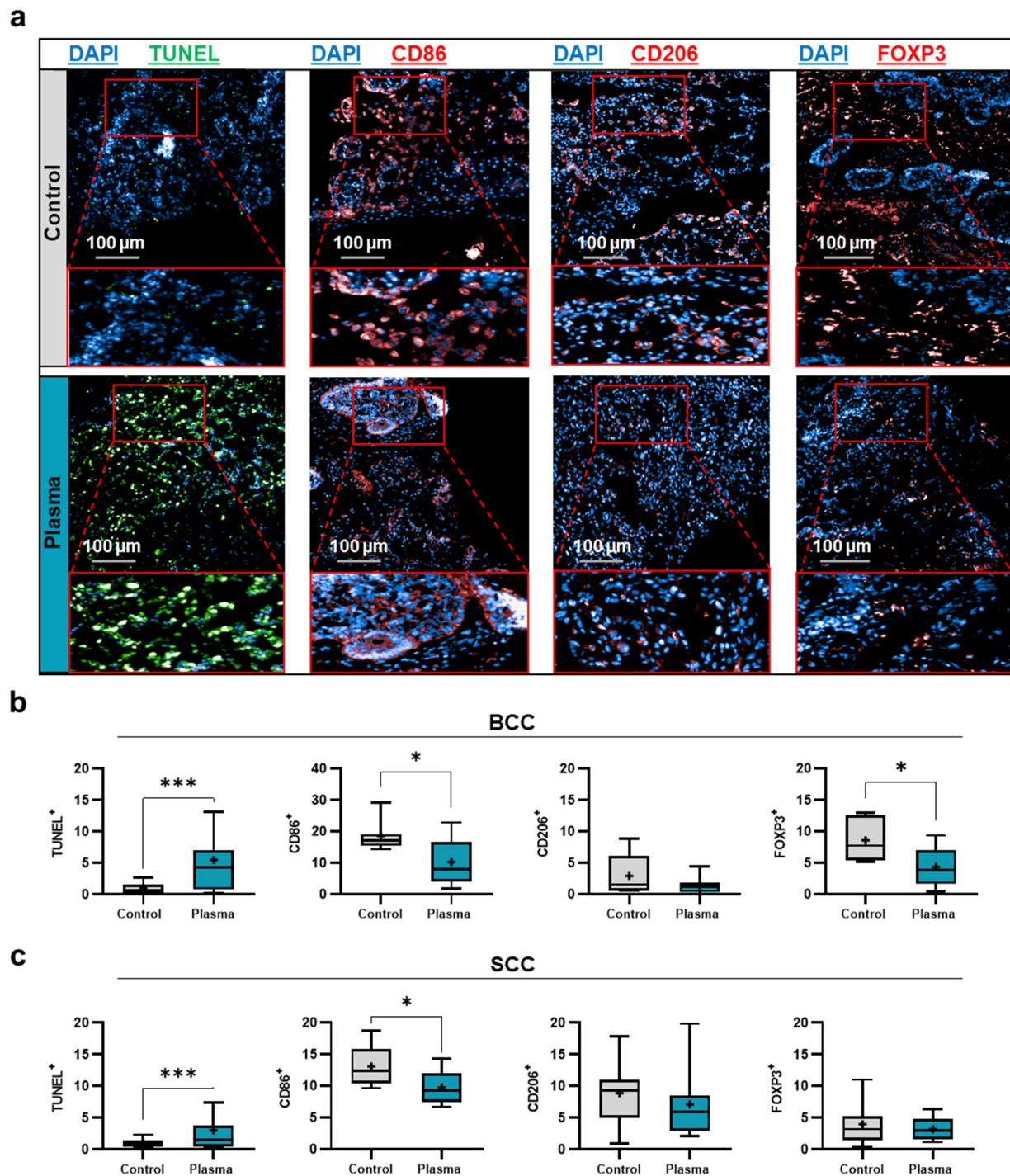


Figure 3. Immunofluorescence analysis: (a) representative immunofluorescence images of DAPI, TUNEL, CD86, CD206, and FOXP3 staining in gas plasma and argon gas (control)-treated BCC tissues; (b) quantitative imaging data for all markers in BCC samples; (c) quantitative imaging data for all markers in SCC samples. Data show boxplots from 6–8 patients, and statistical analysis was performed using *t*-test with $p < 0.05$ (*) and $p < 0.001$ (***). Scale bars are 100 μm .

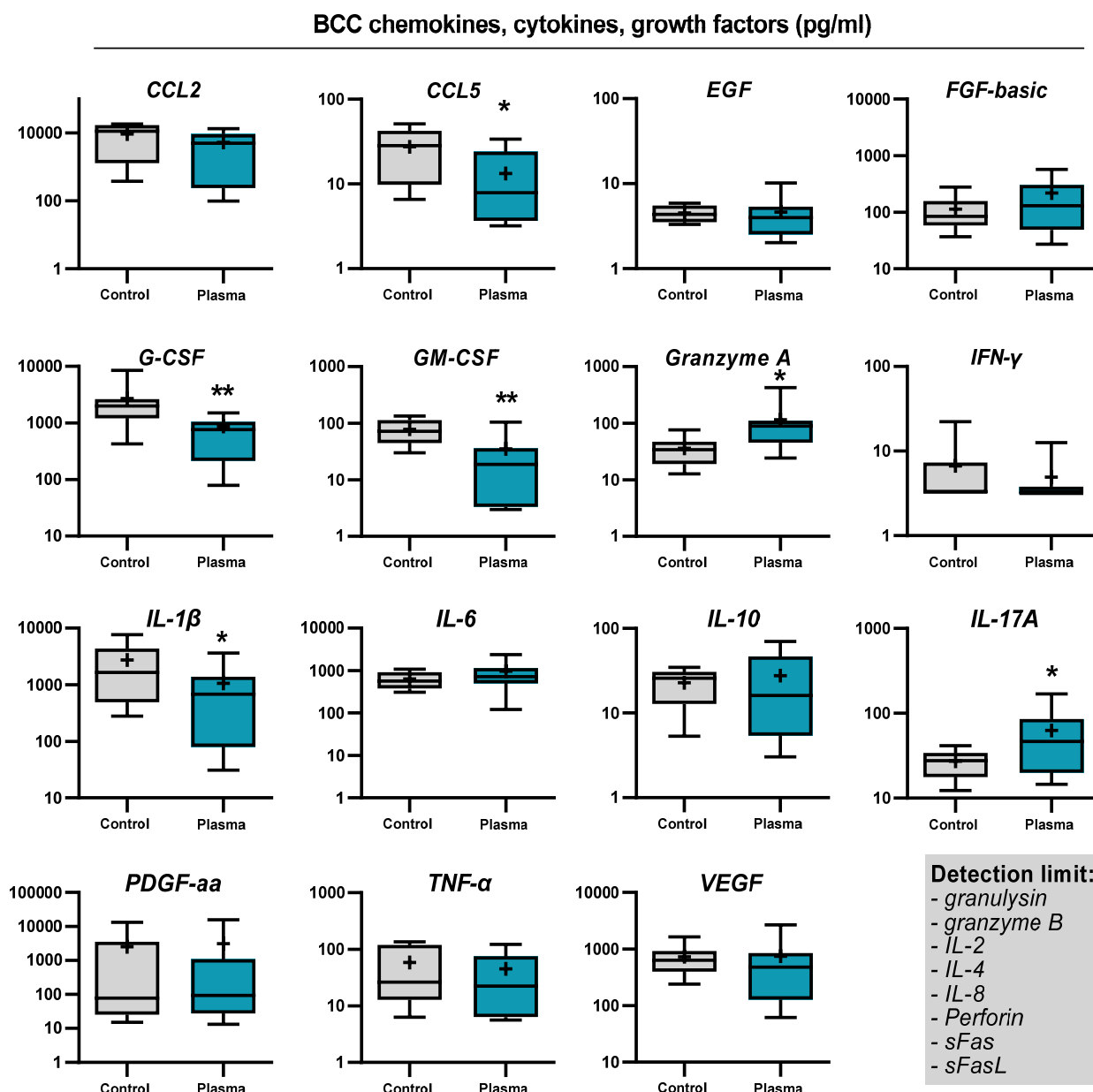


Figure 4. Secretion profile of BCCs: quantification of chemokines, cytokines, and growth factors in pg/mL in BCC tissue culture supernatants 24 h after exposure to gas plasma or argon gas. Data show boxplots from 6–8 patients, and statistical analysis was performed using *t*-test with $p < 0.05$ (*) and $p < 0.01$ (**).

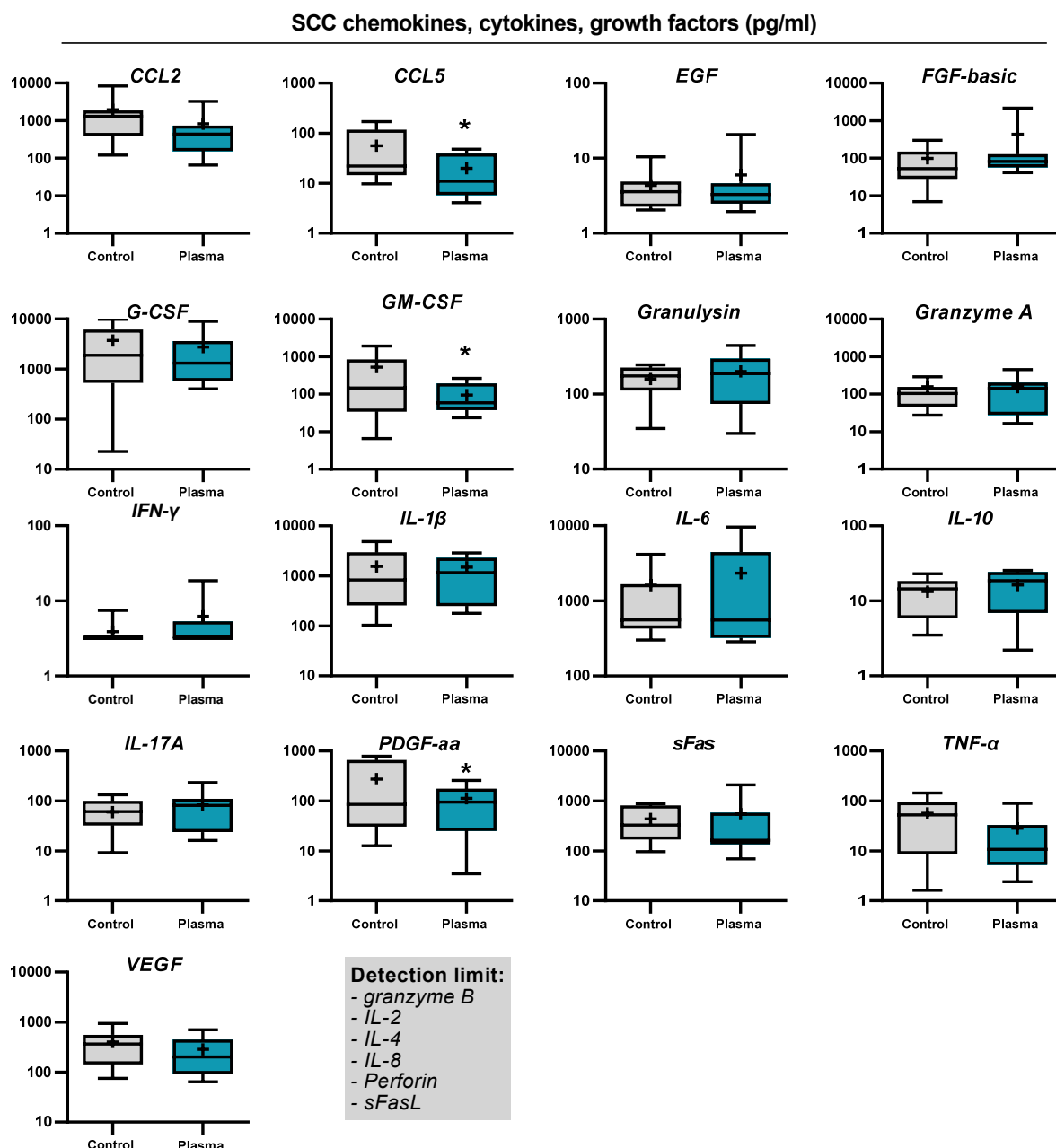


Figure 5. Secretion profile of SCCs: quantification of chemokines, cytokines, and growth factors in pg/mL in BCC tissue culture supernatants 24 h after exposure to gas plasma or argon gas. Data show boxplots from 6–8 patients, and statistical analysis was performed using *t*-test with $p < 0.05$ (*).

3. Discussion

The capacity of cold physical plasma employed in medical gas plasma technology in killing tumor cells was predicted via the direct induction of ROS in tumor cells and the inhibition of tumor cells' defense mechanism and antioxidant mechanisms [35,36]. This study demonstrated gas plasma-induced toxicity in vitro and in patient-derived skin cancer samples ex vivo together with immunomodulatory effects.

In vitro, a gas plasma exposure time-dependent and selective toxicity was observed. In general, the selectivity of gas plasma treatment is a matter of debate and depends on the type of cell line used as comparator [9]. Hence, both selectivity [37,38] and non-selectivity [39,40] have been observed. Our findings of cell cycle arrest underline previous findings as well [41,42]. H2AX phosphorylation following gas plasma treatment has

also been described before [43,44], albeit it should be noted that γ H2AX induction is a consequence of apoptosis rather than directly short-lived ROS-mediated DNA double-strand breaks [45]. Pro-apoptotic effects of gas plasma in three-dimensional tumor tissues have been observed several times using, for instance, in vivo tumors [46,47] and tumor animal models [48,49]. However, regarding patient-derived skin tumor biopsies, we here describe gas plasma-induced apoptosis in cutaneous SCC and BCC. For oral SCC, gas plasma-induced apoptosis using the kINPen plasma jet has been reported before [50].

Gas plasma treatment of skin cancer tissues changed the expression and release of immuno-related molecules, respectively. CD86 is readily expressed by professional antigen-expressing cells to support T cell activation and was found to be decreased in gas plasma-treated SCC and BCC tissue samples. However, CD86 also is readily expressed on tumor cells [51–53], for reasons which to date remain unknown. High CD86 has been linked to unfavorable prognosis in different types of tumors [54,55]. CD206 remained unchanged in our study. The molecule is a M2a macrophage polarization marker (also called tumor-associated macrophage (TAM)) [56] and is associated with disease severity in BCC [57]. FOXP3-expressing T_{regs} are also a poor prognostic marker in BCC as well as SCC [58,59]. Although realistic immunodynamics, including extravasation and the infiltration of new leukocytes, is not reflected in our ex vivo model, our data at least suggest that gas plasma treatment also has an immunological dimension. The findings in tissue-cultured supernatants underlined this. The changes in BCC samples were more pronounced compared to SCC after gas plasma treatment. Both had a marked downregulation of CCL5 (RANTES) in common. There are many sources of CCL5 in skin cancer, for instance, dendritic cells, macrophages, cancer-associated fibroblasts, keratinocytes, mast cells, and tumor cells [60–62]. CCL5 serves as chemoattractant for lymphocytes and promotes tumor-supportive T_{H2} polarization [63]. The molecule is associated with poor prognosis in many types of cancers [64–66]. In BCC, but not SCC samples, gas plasma stimulated elevated levels of IL-17A. This was probably due to the higher baseline expression of IL-17A in BCC that is associated with a favorable prognosis [67]. GM-CSF levels significantly declined in SCC and BCC following gas plasma exposure. GM-CSF has pleiotropic roles in tumor biology, and expression is elevated in SCC [68], while at the same time, it has also been used as a therapeutic agent in non-melanoma skin cancer [69].

It is widely assumed that ROS are the main drivers of gas plasma-mediated tumor cell toxicity, especially in vitro [24]. Regarding the significance of ROS for tissue-mediated effects, less evidence is available. This is because tools that are capable of detecting ROS in tissues are generally scarce. Recently, we showed for the first time the direct oxidation of plasma-derived ROS into human subcutaneous tumors grown in immunodeficient mice using a luminescent probe [70]. In a human head and neck cancer patient, changes in the microcirculation were directly observed after plasma treatment [71], pointing to a role of fast effectors such as ROS and/or electric fields. Regarding the latter, mounting evidence suggests that electric fields modulated in gas plasma sources are in contact with targets [72,73]. Final evidence and models are awaited to distinguish the role of different effectors in the gas plasma treatment of tissues. This includes the types of species dominating biological effects in tissues. While in plasma-treated liquids [74] and in vitro, depending on the feed gas admixture used, the dominant presence of a few long-lived species were described [75], the main types of ROS reaching the target tissue at sufficient depth and quantity as well as the putative secondary redox chemistry remains to be elucidated. The physics and redox chemistry of the kINPen in the plasma gas phase is, by contrast, very well explored [76].

4. Materials and Methods

4.1. Cell Culture

The human skin epidermoid squamous cell carcinoma cell line A431, the human epidermal squamous carcinoma cell line SCC-13, the human melanoma cell line A375, and the human non-malignant HaCaT keratinocyte cell line were grown in fully supplemented

(10% fetal bovine serum, 1% penicillin/streptomycin, and 2% glutamine) Roswell Park Memorial Institute (RPMI) 1640 medium (Pan-Biotech, Aidenbach, Germany). All cell lines were passaged twice per week and maintained under standard culture conditions (37 °C, 5% CO₂, 95% humidity). For experiments, 1×10^5 cells were seeded per well of a 24-well plate (NUNC, Roskilde, Denmark) in 500 µL of fully supplemented cell culture media.

4.2. Primary Skin Tumor Material

Study approval was received from the University of Duisburg-Essen (ethical committee of the University of Duisburg-Essen) under the Institutional Review Board protocol number 12-4961-BO. Patient samples were retrieved upon informed consent to perform the experiments and anonymously publish the data. Primary tumor material from human cutaneous squamous cell carcinoma (SCC) and basal cell carcinoma (BCC) was collected from ten patients each during surgery. Subsequently, 3 mm punch biopsies (without hampering histological diagnosis) were generated for each tissue in the laboratory to generate skin cancer tissue samples for further experiments with a standardized size and volume. The biopsies were added to customized conical receptacles, which were added to 96-well holders to ensure a standardized location and maintain their upright position.

4.3. Gas Plasma Exposure

The atmospheric pressure plasma jet kINPen (neoplas GmbH) was utilized for the in vitro experiments. The ambient air conditions were not monitored as it was recently shown that its role in the kINPen plasma jet's tumor toxicity is minor [14,77]. For in vitro experiments, the argon feed gas flowed through a capillary (inner diameter 1.6 mm) with 1.5 standard liters per minute and plasma was ignited by a radio frequency voltage (1.1 MHz/2–6 kV, peak to peak, non-pulsed). A motorized and computer-controlled xyz-table (CNC, Germany) was used to hover the gas plasma jet over the central position of each well for different dwell times (15 s, 30 s, 45 s, 60 s, and 90 s). The exposure distance from the nozzle to the liquid target (as common in cell culture experiments) was 15 mm. The main reactive species being generated in the kINPen plasma gas phase and treated liquids have been thoroughly described before [76,78,79]. As a control, the samples were exposed to argon gas alone. Liquid evaporation was compensated for by adding predetermined amounts of double-distilled water immediately following the treatment. For the treatment of primary human skin biopsies, the atmospheric pressure argon plasma jet kINPen MED (neoplas MED, Greifswald, Germany) was used. The jet is accredited as medical product class IIa in the European Union for dermatological applications, especially wound healing [80], but it has not yet received licensing for cancer treatment. The gas plasma was generated at a frequency of 1 MHz and using five standard liters per minute of argon gas with an effective power of 1 W. The samples received gas plasma or argon gas treatment for 120 s at a distance of 1 cm between the capillary and the tumor tissue, so that the jet was touching the tissue surface (conductive mode). The jet was operated at body temperature and does not cause thermal damage to treated tissues [80]. Following plasma treatment ex vivo, the tumor samples received fully supplemented cell culture medium. After 24 h of incubation under standard culture conditions, the supernatants were collected and stored at −80 °C until analysis. Then, the tissue samples were embedded in OCT, added to disposable molds, snap-frozen using liquid N₂, and stored at −80 °C.

4.4. Metabolic Activity

Twenty hours after exposure, resazurin (7-Hydroxy-3H-phenoxazin-3-one 10-oxide) was added to the cells (final concentration: 100 µM). Metabolically active cells transformed the non-fluorescent resazurin into fluorescent resorufin. After 4 h of incubation in the cell culture incubator, the well plate was transferred to a multimode plate reader (F200; Tecan, Mennedorf, Switzerland), and fluorescence was determined at λ_{ex} 535 nm and λ_{em} 590 nm. The data were background-subtracted using wells containing fully supplemented cell culture medium without cells and resazurin and normalized against the argon gas-treated

control, in which the plasma of the jet has been switched off. Pilot experiments confirmed no effect of the argon gas treatment compared to untreated cells.

4.5. Apoptosis Detection

Twenty-four hours after exposure, CellEvent caspase 3/7 detection reagent (ThermoFisher, Bremen, Germany) was added, and the cells were cultured for another 30 min. The dye quickly entered cells and contained a fluorescent moiety with high affinity and fluorescence once bound to DNA but only after it has been released by enzymatic activity performed by the caspases 3 and 7. Afterwards, the plate was imaged using fluorescence microscopy (Operetta CLS; PerkinElmer, Hamburg, Germany) by the acquisition of bright-field and fluorescence (λ_{ex} 475 nm and λ_{em} 525 nm) images, and quantitative image analysis (Harmony 4.9 software; PerkinElmer, Germany) was performed.

4.6. Flow Cytometry

Twenty-four hours after exposure, cells were detached using accutase and subsequently fixed and permeabilized. For cell cycle analysis, DAPI (4',6-Diamidino-2-phenylindol) was added (final concentration: 10 μM) and incubated at 4 °C for 1 h. After washing, cells were resuspended in FACS buffer and acquired using flow cytometry (CytoFLEX LX; Beckman-Coulter, Krefeld, Germany). Alternatively, fixed cells were stained using fluorescently labeled monoclonal antibodies targeting 8-OHdG (8-Hydroxy-2'-deoxyguanosine), 3-NT (3-nitrotyrosine), and γH2AX (phosphorylated histone 2A family member X). After 1 h of incubation at 4 °C, cells were washed and acquired using flow cytometry. Data analysis was performed utilizing Kaluza 2.1 software (Beckman-Coulter, Germany). The quantification of cells in the G1, S, and G2 phases of the cell cycle was done using mathematical modeling based on the Michael H. Fox algorithm [81].

4.7. Supernatant Analysis

To detect inflammatory mediators, multiplex analysis was performed using bead-based assay kits (BioLegend, Amsterdam, The Netherlands) to simultaneously assess several cytokines, chemokines, and growth factors according to the manufacturing company's instructions. To achieve this, the supernatant collected from the tumor tissue cultures was incubated with antibody-coated beads. After washing, the addition of streptavidin beads, and another washing step, the analytes' mean fluorescent intensity was determined using a flow cytometry device (CytoFLEX S; Beckman-Coulter, Germany) and the LegendPlex Software (BioLegend, The Netherlands). For absolute quantification, 5-log standard curves were generated for each of the targets.

4.8. Tissue Immunofluorescence Analysis

Tissue material was sectioned using a cryotome to retrieve 7 μm -thick sections placed on the microscopy slides (SuperFrost Plus Adhesion slides; ThermoFisher, Germany). The tissues were fixed using 4% paraformaldehyde for 10 min at room temperature. To retrieve the antigens of interest and increase permeability, the tissues were placed into sodium citrate and Triton X-100 solution for 20 min. Washing with PBS was performed three times, each time lasting 5 min, between different staining stages. Then, the tissues were blocked in 3% BSA solution in PBST for 30 min to prevent the non-specific binding of antibodies which were added afterwards. For staining, anti-CD86 (Abcam: Ab53004), anti-CD206 (Novus: NBP1-90020), and anti-FOXP3 (Novus: NB100-39002) antibodies were used and incubated in PBST solution containing 1% BSA overnight at 4 °C. After washing, appropriate Alexa Flour 647-conjugated secondary antibodies (Thermo Fisher, Germany) were added. Then, TUNEL staining was performed according to the manufacturer's instructions (Sigma-Aldrich, Taufkirchen, Germany) for frozen tissues to identify apoptotic cells. Finally, nuclei staining was performed by using DAPI. The slides were imaged using the fluorescence microscope's 20x air objective ($\text{NA} = 0.4$) (Operetta CLS). The bandpass centers for fluorescence capturing were λ_{ex} 365 nm and λ_{em} 450 nm for detecting DAPI,

λ_{ex} 475 nm and λ_{em} 525 nm for detecting TUNEL-stained apoptotic cells and λ_{ex} 630 nm and λ_{em} 700 nm for detecting the antibody-labeled cells. Image acquisition, analysis, and quantification were performed by using Harmony 4.9 software.

4.9. Reactive Species Analysis

To quantify the amounts of the long-lived oxidants H_2O_2 , NO_2^- , and NO_3^- , known products of short-lived ROS/RNS generated in the plasma gas phase [34], 200 μL of PBS were exposed to the kINPen as described for the treatment of cell cultures. After evaporated liquid was compensated for, analysis was performed as previously described in detail [78].

4.10. Statistical Analysis

Data graphing and statistical analysis were conducted using Prism 9.2 (GraphPad Software). The *T*-test was used to compare the difference between two groups statistically. The determination of IC_{25} values was done using nonlinear regression analysis against log-2 transformed exposure times. The confidence interval was 95% for all tests, and statistically significant differences were marked with * ($p < 0.05$), ** ($p < 0.01$), or *** ($p < 0.001$).

5. Conclusions

Our proof-of-concept study shows cytotoxic and immunomodulatory effects of gas plasma jet treatment in patient-derived BCC and SCC samples, calling for future clinical exploration of this technology in non-melanoma skin cancer treatment. At the same time, substantial efforts are needed to decipher the gas plasma-derived reactive species chemistry necessary to induce the tumor toxic effects and to elucidate potential optimization routes of this technology to serve as an adjuvant oncological therapeutic option in the long term.

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

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Institutional Review Board Statement: Study approval was received from the University of Duisburg-Essen (ethical committee of the University of Duisburg-Essen) under the Institutional Review Board protocol number 12-4961-BO.

Informed Consent Statement: Patient samples were retrieved upon informed consent.

Data Availability Statement: The underlying data are available from the corresponding author upon reasonable request.

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8. Supplements

8.1) Own contribution to the publications

I. The potential of gas plasma technology for targeting breast cancer

Authors: Bekeschus, S. and **Saadati, F.**; Emmert, S.

Journal: *Clinical and Translational Medicine*

doi: 10.1002/ctm2.1022

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My contribution: I summarized the in vitro and in vivo study tables, designed most of the figures, and contributed to the manuscript writing and preparation.

II. Gas plasma irradiation of breast cancers promotes immunogenicity, tumor reduction, and an abscopal effect in vivo

Authors: Mahdikia, H.; **Saadati, F.** and Freund, E.; Gaipf, U.S.; Majidzadeh-a, K.; Shokri, B.; Bekeschus, S.

Journal: *Oncoimmunology*

doi: 10.1080/2162402X.2020.1859731

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My contribution: I performed the in vivo and part of the in vitro experiments, analyzed the in vivo data, and contributed to figure design and manuscript preparation.

III. Antitumor effects in gas plasma-treated patient-derived microtissues. Adjuvant therapy for ulcerating breast cancer?

Authors: Akbari, Z. and **Saadati, F.**; Mahdikia, H.; Freund, E.; Abbasvandi, F.; Shokri, B.; Zali, H.; Bekeschus, S.

Journal: *Applied Sciences*

doi: 10.3390/app11104527

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My contribution: I was involved in collecting most of the clinical samples and performed sample processing and plasma treatment as well as sample analysis in the laboratory, created the first draft of figures, and contributed to writing the manuscript.

IV. Optimized high-content imaging screening quantifying micronuclei formation in polymer-treated HaCaT keratinocytes

Authors: Saadati, F.; da Silva Brito, W.A.; Emmert, S.; Bekeschus, S.

Journal: *Nanomaterials*

doi: 10.3390/nano12244463

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My contribution: I performed the in vitro experiments, analyzed the data, designed the figures, and prepared the manuscript draft.

V. Cold Physical Plasma Toxicity in Breast and Oral Squamous Carcinoma In Vitro and in Patient-Derived Cancer Tissue Ex Vivo

Authors: Saadati, F.; Jahanbakhshi, F.; Mahdikia, H.; Abbasvandi, F.; Ghomi H.; Yazdani, N.; Aghazadeh, K.; Emmert, S.; Bekeschus, S.

Journal: *Applied Sciences*

doi: 10.3390/app13116472

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My contribution: I performed the tissue sample plasma treatment procedures, performed the tissue section staining experiments, analyzed the data, designed the figures, and prepared the manuscript draft.

VI. Patient-derived human basal and cutaneous squamous cell carcinoma tissues display apoptosis and immunomodulation following gas plasma exposure with a certified argon jet

Authors: Saadati, F.; Moritz, J.; Berner, J.; Freund, E.; Miebach, L.; Helfrich, I.; Stoffels, I.; Emmert, S.; Bekeschus, S.

Journal: *International Journal of Molecular Sciences*

doi: 10.3390/ijms222111446

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Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

My contribution: I performed the in vitro experiments, analyzed the data, designed the figures, and prepared the manuscript draft.

8.2) Curriculum vitae

PERSONAL INFORMATION

Name	Fariba Saadati
Place of birth	Ahar, Iran
Residence	Germany

WORK EXPERIENCES

2020-2024	Researcher at Leibniz Institute for Plasma Science and Technology (INP), Greifswald, Germany
2017-2020	Researcher at the School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Science, Tehran, Iran
2016-2017	Researcher (master thesis) at the Department of Anatomy and Biology, Shahid Beheshti University of Medical Science, Tehran, Iran

ACADEMIC BACKGROUND

2021-2024	PhD student in the field of Experimental Dermatology at the Clinic and Polyclinic for Dermatology and Venereology, University Medical Center Rostock Supervisor: Prof. Dr. Steffen Emmert, Thesis title: Cold plasma-mediated toxicity in cutaneous and ulcerating cancer tissues
2014-2017	Master of Science in Physics (Biophysics focus) Shahid Beheshti University, Shahid Beheshti University of medical science, Tehran, Iran Thesis title: Application of cold atmospheric pressure plasma in cancer therapy in-vivo and in-vitro
2009-2013	Bachelor of Science in Physics University of Urmia, Urmia, Iran

INTERNATIONAL CONFERENCE

- The 2022 Microplastics conference at the Congressi Stefano Franscini, Monte Verità, in Ascona, Switzerland
- XVIth International Congress of Toxicology in Maastricht, the Netherlands Under review papers

Publications

a) Published peer-reviewed international papers

1. Bekeschus, S+., **Saadati, F.**, Emmert, S., 2022. The potential of gas plasma technology for targeting breast cancer. *Clinical and Translational Medicine*, 12(8), p.e1022.
2. **Saadati, F.**, Mahdikia, H., Abbaszadeh, H.A., Abdollahifar, M.A., Khoramgah, M.S. and Shokri, B., 2018. Comparison of Direct and Indirect cold atmospheric-pressure plasma methods in the B16F10 melanoma cancer cells treatment. *Scientific reports*, 8(1), pp.1-15.
3. **Saadati, F.**, Moritz, J., Berner, J., Freund, E., Miebach, L., Helfrich, I., Stoffels, I., Emmert, S. and Bekeschus, S., 2021. Patient-derived human basal and cutaneous squamous cell carcinoma tissues display apoptosis and immunomodulation following gas plasma exposure with a certified argon jet. *International Journal of Molecular Sciences*, 22(21), p.11446.
4. **Saadati, F.**, Jahanbakhshi, F., Mahdikia, H., Abbasvandi, F Ghomi, H., Yazdani, N., Aghazadeh, K., Emmert, S., and Bekeschus, S., 2023. Cold Physical Plasma Toxicity in Breast and Oral Squamous Carcinoma In Vitro and in Patient-Derived Cancer Tissue Ex Vivo *Applied Sciences*, 13(11):6472
5. Akbari, Z., **Saadati, F.**, Mahdikia, H., Freund, E., Abbasvandi, F., Shokri, B., Zali, H. and Bekeschus, S., 2021. Antitumor effects in gas plasma-treated patient-derived microtissues. Adjuvant therapy for ulcerating breast cancer? *Applied Sciences*, 11(10), p.4527.
6. Mahdikia, H., **Saadati, F.**, Freund, E., Gaipf, U.S., Majidzadeh-a, K., Shokri, B. and Bekeschus, S., 2021. Gas plasma irradiation of breast cancers promotes immunogenicity, tumor reduction, and an abscopal effect in vivo. *Oncoimmunology*, 10(1), p.1859731.
7. **F Saadati**, WADS Brito, S Emmert, S Bekeschus. 2022. Optimized high-content imaging screening quantifying micronuclei formation in polymer-treated HaCaT keratinocytes. *Nanomaterials*. 12(24):4463.
8. Mahdikia, H., **Saadati, F.**, Alizadeh, A. M., Khalighfard, S., Bekeschus, S., & Shokri, B. (2023). Low-frequency magnetic fields potentiate plasma-modified magneto-electric nanoparticle drug loading for anticancer activity in vitro and in vivo. *Scientific Reports*, 13(1), 17536.
9. Jeibouei, S., Hojat, A., Mostafavi, E., Aref, A.R., Kalbasi, A., Niazi, V., Ajoudanian, M., Mohammadi, F., **Saadati, F.**, Javadi, S.M. and Shams, F., 2022. Radiobiological effects of wound fluid on breast cancer cell lines and human-derived tumor spheroids in 2D and microfluidic culture. *Scientific reports*, 12(1), pp.1-21.
10. Gelbrich, N., Miebach, L., Berner, J., Freund, E., **Saadati, F.**, Schmidt, A., Stope, M., Zimmermann, U., Burchardt, M. and Bekeschus, S., 2022. Medical gas plasma augments bladder cancer cell toxicity in preclinical models and patient-derived tumor tissues—*Journal of Advanced Research*.
11. Bekeschus, S., Ispirjan, M., Freund, E., Kinnen, F., Moritz, J., **Saadati, F.**, Eckroth, J., Singer, D., Stope, M.B., Wende, K. and Ritter, C.A., 2022. Gas Plasma Exposure of Glioblastoma Is Cytotoxic and Immunomodulatory in Patient-Derived GBM Tissue. *Cancers*, 14(3), p.813.
12. da Silva Brito, W.A., Singer, D., Miebach, L., **Saadati, F.**, Wende, K., Schmidt, A. and Bekeschus, S., 2023. Comprehensive in vitro polymer type, concentration, and size correlation analysis to microplastic toxicity and inflammation. *Science of The Total Environment*, 854, p.158731.

13. A Schmidt, WADS Brito, D Singer, M Mühl, J Berner, **F Saadati**, C Wolff, L Miebach, K Wende, S Bekeschus., 2023. Short- and long-term polystyrene nano- and microplastic exposure promotes oxidative stress and divergently affects skin cell architecture and Wnt/beta-catenin signaling. *Particle and Fibre Toxicology*.
14. Dorsch, A., Förschner, F., Ravandeh, M., da Silva Brito, W.A., **Saadati, F.**, Delcea, M., Wende, K. and Bekeschus, S., 2024. Nanoplastic size and surface chemistry dictate decoration by human saliva proteins. *ACS Applied Materials & Interfaces*, 16(20), pp.25977-25993.
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b) Published conference abstracts

1. **F Saadati**, WA Da Silva Brito, A Schmidt, K Wende, S Bekeschus., 2022. LP-31 Polystyrene and PMMA particle toxicity in chicken embryos and particle genotoxicity using a novel high-content imaging workflow. *Toxicology Letters* 368, S294-S295.
2. WA Da Silva Brito, D Singer, B Honnorat, **F Saadati**, K Wende, S Bekeschus., 2022. LP-41 Laser-ablated polypropylene microplastics and their biological responses in human cell lines. *Toxicology Letters* 368, S298.

8.3) Danksagung

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8.4) Eidesstattliche Versicherung

Unter Eid und mit voller Verantwortung erkläre ich hiermit, dass die vorliegende Arbeit ausschließlich von meiner eigenen Hand verfasst wurde und dass ich lediglich die angegebenen Quellen als Hilfsmittel genutzt habe. Jegliche Stellen, die direkt oder indirekt aus bereits veröffentlichten Quellen entnommen wurden, sind sorgfältig gekennzeichnet und zitiert.

Rostock, 2024

Fariba Saadati