Gas Plasma-Oxidized Liquids for Cancer Treatment: Preclinical Relevance, Immuno-Oncology, and Clinical Obstacles

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***Abstract*—Gas plasmas, often referred to as cold physical plasma, are currently being investigated for their potential to serve as anticancer agents. Along similar lines, gas plasma-oxidized liquids as a carrier for reactive oxygen species have found their way into preclinical research. This review focuses on *in vivo* studies that utilized such gas plasma-oxidized liquids for cancer therapies. These preclinical tumor models, treatment modalities, and types of liquids that were used are summarized and critically discussed. Among these studies, significant results were observed, indicating the potential of oxidative liquids to serve as an anticancer treatment. However, several steps have to be taken to enhance the quality and translational capacities of this approach in order to gain clinical acceptance for possible future cancer therapies. The most crucial steps include not only a careful selection of suitable liquids, with respect to their approval as medical products, but also the consideration of orthotopic and immunocompetent animal tumor models. This would increase the relevance of such studies and simultaneously allow studying the contribution of the most potent of all anticancer effectors, the immune system.**

***Index Terms*—PAL, PAM, PCM, physical plasma, plasma-activated liquid, plasma-activated medium, plasma-conditioned media, plasma-treated liquids, PTL.**

I. Introduction

In THE last decade, many gas plasma sources were developed and designed for biomedical applications. The latter include, for instance, the treatment of chronic and infected wounds, cosmetics, and oncology [1]–[9]. Because these ionized gases (gas plasmas) are being generated at physiological temperatures (<40 °C), they are often referred to as cold physical or nonthermal plasmas. In many instances, these gas plasmas are generated via jet devices that allow exposure to nonflat body surfaces, such as skin lesions and tumors. They could, therefore, be suitable for surface treatment in a clinical routine, which is currently the case for the kINPen MED in Europe [10]. Additionally, preliminary results point to an anticancer activity in some patients with skin tumors of the head and neck that received repetitive kINPen plasma treatments [11], [12]. It is important to note that only those patients benefited that underwent several dozen plasma treatment sessions. However, not all tumor entities can benefit from this approach. This is because of: 1) the limited access of the tumor sides for repetitive plasma treatment (repetitive surgeries are usually omitted as they are afflicted with side effects); 2) the rather long treatment times that are needed to reduce the tumor burden; and connected to this 3) the disability to treat a large number of tumor sites. For example, tumors of the peritoneal cavity can be challenging for such a treatment approach as this might require several hours of plasma treatment, which is hard to implement in clinical routine.

Various types of cancers, such as colorectal, pancreatic, ovarian, or gastric carcinoma, as well as melanoma, neuroblastoma, and lymphoma, frequently metastasize into the peritoneal cavity. This body cavity is reached via surgical intervention only. Access to these tumors can also be hampered when being localized between abdominal organs, on the great omentum, or peritoneal pleura. Moreover, macroscopically visible tumors remain an exception, and often the dissemination of small tumor nodules or micrometastasis decreases the efficacy of antitumor therapies, which vice versa increases the mortality [13], [14]. Currently, the administration of chemotherapeutic liquids is one treatment option in such instances, and the extension of such treatment approaches with liquids carrying additional therapeutic agents is conceivable. Thereby, the dosage of chemotherapy needed could be reduced while maintaining the same antitumor activity, effectively reducing drug-related side effects, such as nutritional dysbalance, vomiting, nausea, wound healing disorders, or pain. Other adjuvant therapeutic schemes, such as radiation have similar side effects (e.g., radiodermatitis), or high toxicity toward intestinal organs, and hence often cannot be applied to patients.

Consequently, especially in the field of plasma-oncology, gas plasma-oxidized liquids move into the focus of researchers [15]–[19]. Such plasma-oxidized fluids were already shown to be storable at −20 °C over several weeks, with long-living oxidants found to be stable [20]. Nevertheless, the plethora of reactive oxygen and nitrogen species (ROS/RNS) that are introduced by direct treatment

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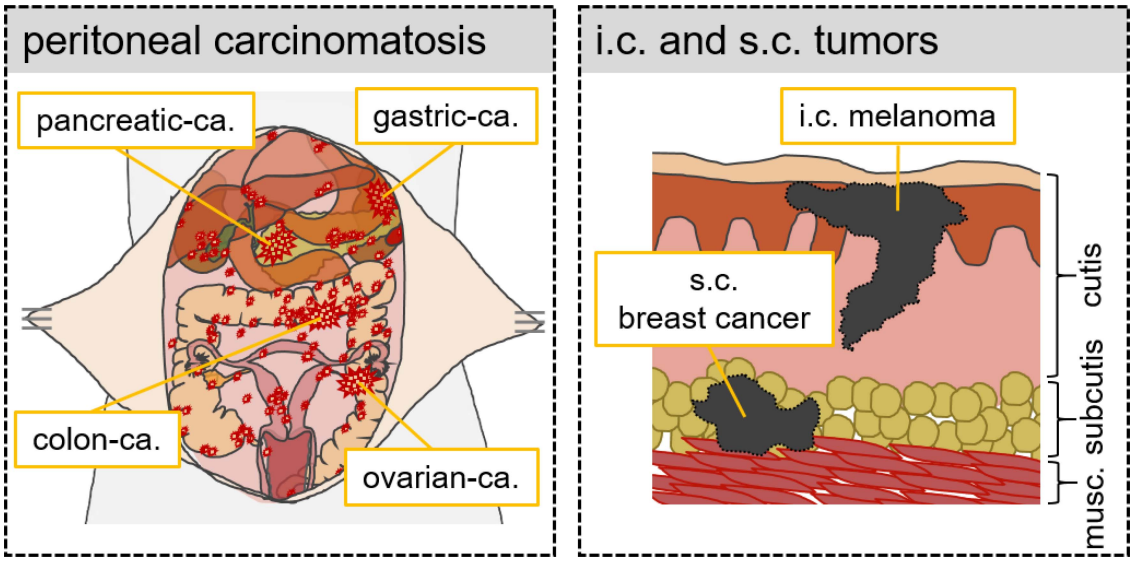

Fig. 1. Schematic overview of cancer cell models utilized in animal experiments of intraperitoneal and cutaneous tumors.

with jet plasmas is reduced to only a few more stable types of oxidants [1], [21]–[23]. Regardless of this, *in vitro* experiments suggested a potential anticancer activity of different plasma-oxidized liquids in a range of different tumor cell lines. This review focuses on the studies that were carried out to validate these effects in animal models (*in vivo*) and to recommend ways to increase the clinical relevance and acceptance of this promising approach.

II. *In Vivo* Experiments

Besides the vast amount of different *in vitro* studies using gas plasma-oxidized liquids (reviews: [1], [16], [19], [24]–[26], most recent publications: [26]–[56]), there are only a few *in vivo* experiments that were carried out (Table I). These focused not only on growing diffuse peritoneal tumors that were inoculated in mice but also tumors that were inoculated subcutaneously or intracutaneously (Fig. 1). The type of plasma source, the types of oxidized liquids, and the treatment protocols vary significantly among these studies. However, substantial information was retrieved through these studies, bringing new insights for the potential application of gas plasma-oxidized liquids for anticancer therapy. The *in vivo* analysis of tumor responses to gas plasma-oxidized liquids is summarized below.

The first study (that was carried out in 2013) investigated the effect of gas plasma-oxidized Roswell Park Memorial Institute (RPMI) medium in a model of ovarian cancer [57]. The exposure of NOS2 and NOS3 cells to the gas plasma-oxidized RPMI reduced the cells' proliferation rate, and oxidation of the cells and the resulting activation of caspases 3 and 7 were observed *in vitro*. Other NOS cell lines, with resistance to the cytostatic drug paclitaxel (NOS2TR), were more sensitive to the treatment. The two different cell cells were then inoculated subcutaneously into both flanks of Balb/c immunocompromised nude mice and received treatment with 200 μl of gas plasma-oxidized RPMI or with nontreated RPMI. The procedure was carried out starting 24-h post cell implantation and was administered three times a week for overall 29 days, a total of 12 injections. The tumor growth was monitored via caliper measurements and was substantial in the control group. At the same time, the treatment with gas plasma-oxidized RPMI diminished the growth and finally reduced the tumor mass for both cell lines, NOS2, and paclitaxel-resistant NOS2. While this is the first *in vivo* study that demonstrated an antitumor effect of gas plasma-oxidized liquids being highly innovative at its time, the ovarian cancer model was nonorthotopic (i.e., not at the location where the tumor naturally occurs) and focused on human ovarian cancer cells in nude mice. These animals lack functional T-lymphocytes and the model therefore neglects a potential contribution of the immune system to amplify or even dampen such therapeutic effects.

The first study that did not utilize cell culture medium but a more relevant liquid as a carrier liquid for gas plasma-derived oxidation was published in 2016 [58]. The advantage of *Ringer's* lactate in terms of clinical acceptability is that it is already a clinically approved solution and well known for its utilization for infusion or for rinsing wounds. The *in vivo* study was based on findings the authors made with differently composited liquids. The key event was the identification of lactate in *Ringer's* lactate to be responsible for the main antitumor effect. It was found that gas plasma-oxidized L-sodium lactate contains higher amounts of acetyl and pyruvic acid-like groups, which could be plasma-modified mediators of its effects on cancer cells. For animal experiments, human SiHa cells isolated from a squamous cell carcinoma of the cervix were injected in both flanks of immunodeficient Balb/c nude mice. The treatment with *Ringer's* lactate or gas plasma-oxidized *Ringer's* lactate was started 24-h post cell injection. It was continued three times a week for six weeks, a total of 18 injections. During those experiments, the control tumors had a significant gain in volume, while the treatment with 150-μl gas plasma-oxidized *Ringer's* lactate suppressed

the growth of the subcutaneous cervix tumors. The tumor weight was also diminished while the mice's weight remained unaffected. Again, the limitation of this study is that it was nonorthotopic and nonimmunocompetent.

In 2017, the effect of argon gas plasma-oxidized RPMI on gastric cancer, growing in the peritoneal cavity, was investigated [59]. The cells were inoculated into the peritoneal cavity of the mice to induce a diffuse growing peritoneal carcinomatosis, which is causing the growth of tumor nodules in the abdomen. Before the *in vivo* experiments, the group validated an antiproliferative effect of their argon gas plasma-oxidized RPMI on SC2-NU and AGS cells *in vitro*. This effect was dependent on exposure times and the distance of the target to the plasma effluent. The migration of these cells was also reduced, and more interestingly, the adhesion of SC2-NU, AGS, and GCIY cells was diminished post-exposure to gas plasma-oxidized *RPMI in vitro*. For animal studies, the GCIY cells were injected intraperitoneally in Balb/c nu/nu mice. The treatment regimen consisted of the daily application of gas plasma-oxidized RPMI or nontreated RPMI at day 1 to 4 and 8 to 11 post cell injection, a total of only 4 injections. Throughout the experiment, the tumor growth was monitored via luminescence imaging, showing tumor formation in controls and complete growth inhibition in the group that received gas plasma-oxidized RPMI. This model was orthotopic but immunodeficient.

Based on their first results from 2013, Nakamura *et al.* [60] continued their use of gas plasma-oxidized RPMI, this time in an animal model of orthotopic ovarian cancer, growing in the peritoneal cavity of Balb/c nude mice. The *in vitro* analysis showed the reduction of proliferation and migration in the co-culture of ES2 and SKOV ovarian carcinoma cells together with mesothelial cells. In addition, the expression of cell growth-related proteins was investigated, and it was found that gas plasma-oxidized RPMI reduced the amount of MMP-9 *in vitro*, while markers upstream of the MAPK pathway (e.g., p38 and JNK1/2) were downregulated. For the *in vivo* studies, the authors followed this logic of gas plasma-oxidation inhibiting cell attachment and migration, and injected gas plasma-oxidized RPMI 15 min after intraperitoneal inoculation of the cells. The treatment was repeated at days 1 and 2 post-inoculation, which led to a significantly prolonged animal survival and lower tumor burden at the mesentery and the pancreas with its omentum. Despite being orthotopic, the caveat of this experiment is not only the immunodeficient nature of the model, which, however, is unavoidable when testing human cancer cell lines, but also the almost simultaneous injection of tumor cells and the treated liquid only 15 min later. Attacking established tumors embedded in the tumor microenvironment (TME) is more difficult than treating the cells while still being in suspension in the abdominal cavity of the mice. Hence, the translational relevance of such a model is low.

In 2017, we used for the first time an immunocompetent animal tumor model to study the effect of gas plasma-oxidized medium [61]. For the experiments, Dulbeccos-modified Eagle's medium (DMEM) was oxidized via kINPen argon plasma and showed dose-dependent toxicity toward PDA6606 murine pancreatic cancer cells but not to nonmalignant fibroblasts *in vitro*. For *in vivo* experiments, the cells were injected into the peritoneum of C57BL/6 mice to induce peritoneal carcinomatosis of pancreatic cancer origin. The cells are syngeneic to C57BL/6 mice, meaning that no shut down of the immune system is needed for the tumor cells to grow and establish in the animals. Starting only at day 7 post tumor cell injection, daily treatment with gas plasma-oxidized DMEM or nonoxidized DMEM was performed. Analysis of magnetic resonance imaging (MRI) as well as a quantification of the tumor weight showed a significant reduction in the size and weight of the tumor nodules through gas plasma-oxidized DMEM. In tissue analysis, the penetration depth of the tumor toxic liquid into the tumor tissue was calculated by counting the dead ($TUNEL^+$) cells in tissue slides extracted from the tumor nodules and was, on average, 250 μm. A decrease of proliferation (lower percentage of Ki-67^+ cells) inside the tumors was found as well. Moreover, additional experiments were carried out using the same treatment regimen that defined the tumor-related death of the animals as endpoint up to 70 days post tumor cell injection. Gas plasma-oxidized DMEM was superior to nonoxidized DMEM during the whole time-course and prolonged the survival of animals with PD6606-induced pancreatic peritoneal carcinomatosis. In contrast to the blood parameters of the animals, they did not show any significant alteration through the treatment, which could be one indicator for the compatibility of this novel cancer treatment approach. In follow-up experiments, an influx of immune cells in the tumor tissue was investigated [62]. In detail, macrophages were found, and the proportion of $CD206^+$ M2 macrophages was decreased, while the amount of proinflammatory $iNOS^+$ M1 macrophages was not changed by the treatment with gas plasma-oxidized DMEM. However, together with findings of elevated numbers of neutrophils and $CD3^+$ T-lymphocytes, this possibly indicated an antitumor immune response that was modulated through the treatment with gas plasma-oxidized DMEM. This hypothesis was supported by significantly elevated levels of the proimmunogenic damage-associated molecular pattern (DAMP) calreticulin (CRT) in tumor tissue that received the gas plasma-oxidized DMEM. For the field of plasma-liquid *in vivo* experiments, this was groundbreaking as, for the first time, the proimmunogenic potential of plasma-related oxidation via liquids was taken into account. The benefits of this model were that the metastatic tumor nodules were located at the site where they appear in the patient (orthotopic) and that immunocompetent and not immunodeficient mice were used to consider effects of the adaptive immune system. Also, the model is realistic as established tumors at day 7 were targeted, and not cell clots apparent at days 1 or 2 after inoculation. The caveats of this study were that a large number of injections (a total of 21 in the first model and even 35 in the second model) was needed to achieve an acceptable but not necessarily full-blown antitumor effect.

The work with gas plasma-oxidized DMEM was extended by Fariba Saadati and her colleagues in 2018 [63]. They utilized a subcutaneous orthotopic model of B16F10 melanoma in immunocompetent C57BL/6 mice. First experiments with B16F10 cell cultures showed a reduction of

TABLE I
CHRONOLOGICAL PRESENTATION OF *in vivo* STUDIES USING GAS PLASMA-OXIDIZED LIQUIDS FOR CANCER THERAPY

authors (first, *correspond.)	cancer model	immuno-competent / orthotopic	plasma-oxidized liquid / # of injections	main results *in vivo* and (*in vitro*)
*Utsumi, *Kajiyama et al. [57]* 12/2013	subcutaneous ovarian cancer / no	no / no	*RPMI* culture medium / 12	growth reduction of NOS2 and paclitaxel NOS2 tumors through argon gas plasma-oxidized RPMI; (decrease of proliferation, induction of caspase 3/7)
**Tanaka/ *Nakamura, Hori et al. [58]* *11/2016*	subcutaneous cervix carcinoma	no / no	*Ringer's* lactate / 18	Growth reduction of SiHa tumors through argon plasma-oxidized *Ringer's* lactate; (plasma-oxidized *Ringer's* lactate is superior to *Ringer's* saline in introducing oxidation and apoptosis induction; plasma treatment creates acetyl and pyruvic acid-like groups)
*Takeda, *Yameda et al. [59]* 01/2017	gastric peritoneal carcinomatosis	no / yes	*RPMI* culture medium / 4	growth inhibition of GCIY tumors through argon gas plasma-oxidized RPMI; (decrease of proliferation and migration of AGS and SC-2-NU cells)
*Nakamura/Peng, *Kajiyama et al. [60]* 07/2017	ovarian peritoneal carcinomatosis	no / yes	*RPMI* culture medium / 3	reduction of ES2 tumors through gas plasma-oxidized (experimental plasma source) RPMI; increased animal survival; animal weight was not affected; (reduced viability of SKOV and ES2 cell; impaired migration and invasion with mesothel- and ES2 cells, downreg. MMP9, JNK, p38)
*Liedtke and Bekeschus, *Partecke et al.; Liedtke, *Bekeschus et al. [61, 62]* 08/2017	pancreatic peritoneal carcinomatosis	yes / yes	*DMEM* culture medium / 21 and 35	reduction of PDA6606 tumor mass through *kINPen*-oxidized DMEM; increased animal survival; tumor apoptosis; influx of neutrophils; influx of immune cells; expression of ICD markers; unaffected blood parameters; (reduced proliferation)
*Saadati, *Abdollahifar/ *Shokri et al.* [63] *05/2018*	subcutaneous melanoma	yes / yes	*DMEM* culture medium / 25	reduction of B6F10 tumors through helium gas plasma-oxidized DMEM; lower effectiveness as direct plasma-treatment; increased effectiveness in combination with cyclophosphamide; increased animal survival; tumor apoptosis; increased p53 and Bax/Bcl-2 ratio; (reduced metabolism)
*Xiang, *Dai, et al.* [38] 08/2018	subcutaneous breast cancer	no / no	*DMEM* (F12) culture medium / 20	reduction of MDAMB but not MCF7 tumor growth through treatment with medium oxidized via an experimental helium gas-plasma jet; (toxicity and growth reduction in DMAMB but nor MCF7 cells; decreases JNK activity)
*Sato, *Yamada et al. [64]* 11/2018	pancreatic peritoneal carcinomatosis	no / yes	*Ringer's* lactate, (*RPMI* culture medium) / 6	reduction of AsPC-1/CMV-luc tumors over time through treatment with argon gas plasma-oxidized *Ringer's* lactate; (oxidation and decrease of proliferation of Capan, MiaPaca, BxPC-3 cells; RNS alone also decrease proliferation)
*Freund, *Bekeschus et al. [65]* 01/2019	colorectal peritoneal carcinomatosis	yes / yes	Sodium chloride (NaCl) / 5	reduction of CT26 tumor mass through *kINPen*-oxidized NaCl; infiltration and activation of immune cells; 3-week storability of the oxidized liquids validated (activation of ICD markers; cell cycle arrest; phenotypical changes)
*Adhikari, *Kaushik/*Choi et al.; Adhikari/Kaushik, *Lee/Kaushik/Choi et al. [66, 67]* 05 and 10/2019	intradermal melanoma	no / yes	*RPMI* culture medium / 3 and 1	reduction of G-361 tumors through μ-DBD-oxidized RPMI medium; synergistic effect with silymarin; LDH and L-DOPA decrease in serum and tissue; induction of AKT, BAX, PARP, caspase 8, p53; downregulation of BCL

the viability of these cells that was significant but overall lower in comparison to direct treatment with an argon plasma jet. Furthermore, the combination of gas plasma-oxidized DMEM plus the cytostatic drug cyclophosphamide led to an additive effect in tumor-toxicity. This was accompanied by an induction of p53 and an increased ratio of BAX over BCL2 in

the groups with gas plasma-oxidized DMEM, both being enhanced when combined with cyclophosphamide treatment. These results were later validated by western blot analysis of tumor tissue extracted from tumor-bearing mice. The mice received a subcutaneous injection of B16F10 melanoma cells, and after one week (allowing the cells to establish realistic tumors), they were conducted to either 6 min of direct plasma treatment through a jet system or received 400 μl of 6-min oxidized DMEM. The treatment procedure was performed in a daily routine for the following 25 days. The gas plasma-oxidized medium diminished tumor growth when compared to the control group, which was also further accelerated through the combination with the cytostatic drug. While at day 30 of the experiment, all control mice were found dead, 80% of the animals in the combination group were still alive. They had an overall survival of 50 days in the combination group and 40 days in the gas plasma-oxidized DMEM alone group. Interestingly, direct plasma jet treatment more substantially reduced the tumor burden than indirect treatment. This indicates the relevance of short-lived reactive species not being present in the liquid treatment groups that mediate a greater antitumor effect. Validation of the toxic impacts toward the B16F10 melanoma was carried out by analyzing the amount of dead ($TUNEL^+$) cells in tissue slides and by growth monitoring via animal computer tomographic imaging (CT). Unfortunately, an immunological component was not taken into account in this study.

In 2018, MDAMB and MCF7 breast cancer cell lines were compared regarding their response to the gas plasma-oxidized medium [38]. For the oxidation of the DMEM solution, an experimental helium plasma-jet was utilized. However, MCF7 but not MDAMB cells responded with increased apoptosis, necrosis, and lower migration to an *in vitro* treatment with the gas plasma-oxidized medium. This was repeated *in vivo* in Balb/c mice with the subcutaneous injection of the tumor cells. After the tumors reached 5 mm of diameter, daily treatment with gas plasma-oxidized medium or nonoxidized medium was applied until the day of the experiment. The number of injections was not mentioned. The daily caliper measurements showed a significant reduction of MDAMB but not MCF7 tumors. The MCF7 tumors were also not affected in their tumor weight after explanation, which was almost equal to the control group. The study has several weaknesses. Although breast cancer cells can be injected in the breast of mice to make the model orthotopic, the authors chose to inject the tumors into the flank, making the model nonorthotopic. Since human tumor cells were studied, immunodeficient mice had to be used. The number of injections is not specifically mentioned, but from the statement being 'every other day' when tumors reached 5 mm of diameter, it can be extrapolated from the figures that it was 20. Also, the group size of the animal study was small, being only 3 per group. The largest weakness of the study in our hands, however, was that it seemed that one breast cancer cell type was treated with DMEM while the other was treated with DMEM-F12. The latter is known to contain high levels of the antioxidant pyruvate, and the authors showed the H_2O_2 deposition was lower in DMEM-F12. Hence, it is hard to make conclusions from the data presented. *In vivo*, antitumor effects were not observed for MCF7 cells that received the antioxidant-rich DMEM but for MDA-MB, which received the antioxidant-poor regular DMEM. Understanding the contribution of the cell culture medium versus the cell type on the effects observed is hardly possible in this study.

The first study that did not utilize cell culture medium but gas plasma-oxidized *Ringer's* lactate for intraperitoneal administration was carried out in 2018 [64]. The choice of liquid was made after a careful *in vitro* comparison of the effect of gas plasma-oxidized RPMI, gas plasma-oxidized *Ringer's* lactate, and nonoxidized *Ringer's* lactate on four different pancreatic cancer cell lines. The gas plasma-oxidized *Ringer's* lactate introduced oxidation, cell death, and lower adhesion capacities to the pancreatic cancer cells *in vitro*. Their adhesion was also diminished, and these effects could be annulled through the antioxidant n-acetylcysteine (NAC), leaving a residual activity to other oxidants such as nitrite. For the *in vivo* investigations, AsPC-1/CMV-luc cells were inoculated intraperitoneally into Balb/c nu/nu mice. This induced peritoneal carcinomatosis of pancreatic origin in these animals. Notably, during the cell injection, the cells were either diluted already in gas plasma-oxidized *Ringer's* lactate or in nonoxidized *Ringer's* lactate. This was probably done to underline the findings of impaired adhesion *in vitro* in the *in vivo* model but, however, makes this animal tumor model highly artificial due to the reasons outlined above. Repetitive treatments of the mice with 2.5 ml, up to ten times more volume than used in the other studies mentioned above, of gas plasma-oxidized or nonoxidized *Ringer's* lactate were performed at days 2–4 and 8–11 (a total of six injections) before the animals were sacrificed at day 15. During this time course, the tumor growth was monitored via bioluminescence imaging. It was shown that in this treatment regime, the bioluminescence signal of tumor decreased over time in the animals that received gas plasma-oxidized *Ringer's* lactate, while untreated tumors had a significant increase. These results were definitely a step forward to establish the utilization of oxidized liquids in oncology, but necessary steps to gain higher clinical acceptance were not taken: the animal model lacks T-lymphocytes and is therefore not fully immunocompetent. Moreover, the exposure of the cancer cells to the treatment solution during incubation remains a rather nonrealistic scenario for the growth of a tumor. Finally, and with mice weighing only about 20 g, the injection of 2.5 ml of liquid ($>$10% of the body weight, which would be more than 8 l in an average adult human) into the peritoneal cavity lacks not only translational value but also is questionable in terms of side effects of the injection itself. In Germany, the maximum amount of liquid allowed to be injected intraperitoneally into mice ranges between 0.25 and 1.0 ml, as set by ethical guidelines.

The above-mentioned caveats led us to design a study with high clinical relevance, which was published in 2019 [65]. Our model of colorectal CT26 cancer was syngeneic in fully immunocompetent Balb/c mice. It was orthotopic as colon cancer frequently initiates peritoneal carcinomatosis, which we modeled by injecting the cells into the peritoneum of the animals. It was relevant from the clinical point of view, as

we used 0.9% sodium chloride for the *in vivo* experiments, a medical product frequently used in clinical routine for different purposes. An additional innovative step was taken: the gas plasma-oxidized NaCl was stored at −20 °C for up to three weeks and used successfully thereafter. This, for the first time, suggested an additive translational value of using premanufactured liquids aiding to a potential storability being one primary requirement for easy and fast usage during, e.g., surgery. The *in vivo* study was precluded by extensive *in vitro* experiments, which, however, used the phosphate-buffered saline (PBS) and not NaCl for the treatment of the cells. Here, the metabolic activity and viability of the CT26 colorectal tumor cells were reduced by gas plasma-oxidized PBS, which was superior to a hydrogen peroxide control in 3-D tumor spheroids, but not in 2-D cell cultures. This effect was accompanied by phosphatidylserine translocation and caspase 3/7 activation. Besides this tumor-toxicity, it was found that the gas plasma-oxidized PBS changed the morphology of the cells to an elongated phenotype with rearrangements in the actin cytoskeleton independent of activation of markers of the epithelial-mesenchymal transmission pathways. However, gas plasma-oxidized PBS decreased the cells' motility and led to the expression of proimmunogenic molecules. The key findings were validated for MC38 colorectal and PDA6606 pancreatic cancer and were absent or of lower magnitude in nonmalignant HaCaT keratinocytes. The keratinocytes showed, in contrast to the cancer cells, no proinflammatory marker profile after exposure to gas plasma-oxidized PBS. For the investigation of the *in vivo* effect of this gas plasma oxidized liquids and to assess the treatment of colorectal peritoneal carcinomatosis, the murine cells were inoculated into the peritoneal cavity of Balb/c mice. Gas plasma-oxidized NaCl was injected beginning at day 2 for every second day. In total, five injections were performed, which reduced the tumor mass by two-thirds (>70%) of the tumor mass seen in the control group that received nonoxidized NaCl. Moreover, the importance of the immune system for this tumor therapies was pointed to, finding an increased fraction of macrophages, and a higher activation status of T-lymphocytes that were restimulated with dead tumor cells *ex vivo*. The caveat of our study was the relatively early onset of the treatment (day 2) and, similar to all other studies published in the field on this topic, the lack of a large-scale plasma generator for taking the next step toward a clinical application. We had used the kINPen to treat 50 ml of liquid for 60 min. This was sufficient for 200 doses with 250 μl in mice, but for humans, several liters per day would be required to fulfill the clinical need during, e.g., HIPEC therapy of several patients [68].

The spectrum of investigations on the gas plasma-oxidized medium was extended to the treatment of skin cancers in a murine model in Korea. In the first study in 2019, directly exposed G-361 melanoma cells (air μ-DBD plasma device) were investigated *in vitro* in combination with a silymarin nanoemulsion to analyze a potential additive anticancer effect [66]. *In vivo*, human melanoma cells were inoculated intracutaneously in the flanks of CAnN.CG-Foxn1 nude mice. The freshly prepared gas plasma-oxidized RPMI medium was injected into the tumors for three consecutive days, while the animal received silymarin one day ahead. In the combination regimen, the tumor volume and weight were reduced significantly. Unfortunately, no information about the tumor reduction through the gas plasma-oxidized RPMI or silymarin alone was available from this study. The only information that is available for these treatment groups is the increase of the mice's body weight over time, while in the untreated control group, a severe weight loss was observed, indicating that these animals are suffering from the tumor growth and its metabolic side effects. In the same year, the authors continued their investigations of gas plasma-oxidized RPMI in another study that utilizes the same melanoma and animal model [67]. The mice received 200 μl of the freshly gas plasma-oxidized RPMI subcutaneously into the flanks (tumor sites) at day 10 post tumor cell injection and were observed for another 12 days. The treatment significantly reduced the tumor burden, and for further experiments, the tumor tissue was explanted. The tumors that were exposed to the gas plasma-oxidized RPMI had elevated levels of carbonylated proteins, as well as markers for lipid peroxidation (MDA). Furthermore, negative prognostic markers, such as LDH and L-DOPA were also found to be reduced in the serum of mice that received gas plasma-oxidized RPMI. Another step of this *ex vivo* analysis was the quantification of the ratio BAX over BCL2, and the apoptosis-inducing p53, that were elevated here as observed before [63]. Also, caspase 8 and PARP were elevated while the growth-promoting AKT (protein kinase b) was decreased in the tumor tissue. While only a few injections were shown to have large effects, the caveats of the studies were the usage of the cell culture medium over medically relevant liquids and the immunodeficient nature of the animal models.

Although the analysis of the *in vivo* antitumor effect of gas plasma-oxidized liquids is at their very beginning, it is important to note that the studies described above already offer a good compilation of different tumor models and oxidized liquids (all with their advantages and disadvantages). Several tumor models were under investigation and they all have shown overall good response to gas plasma-oxidized liquids. However, the number of injections varied strikingly between those studies. Some used very early injections of oxidizing liquids although at those time points tumor formation might not have already been successful. Also, the time that the animals were investigated during tumor growth varied. Furthermore, as in most studies from plasma medicine, a lot of different plasma devices were utilized to generate the oxidation and ROS. Here, the field still lacks standardization and also the treatment regimen of the liquids was different. Most importantly, research should focus on liquids that are suitable for clinical application. Some studies did not even quantify the reactive species that mediated the antitumor activity. However, the first steps are done to pave the way for further research in this field. A summary of the advancement through the experiments is outlined in Box 1. In the following sections, it will be explored how to gain more clinical acceptance of plasma-treated liquids in oncology, and how to standardize the preclinical *in vivo* research to break existing obstacles when it comes to novel routes in tumor therapy.

- gas plasma-oxidized liquids are able to diminish the tumor growth in a broad spectrum of tumor models, but the effect size can vary between different cell lines tested
- the anti-cancer activity is also observed in immunodeficient animals, but altered infiltration rates of immune cells in syngeneic models suggest a vital role of the immune system in supporting this activity
- different liquids as carrier solutions for oxidative species showed comparable effects, but the clinical relevance is elevated through the use of medically approved solutions such as sodium chloride (NaCl) and *Ringer's* lactate
- experiments that used a combination of gas plasma-oxidized liquids and cytostatic drugs (such as cyclophosphamide or silymarin) were promising and should be further promoted, albeit the usage of drugs should be oriented to standard-of-care compounds in the specific oncological disease intended to be targeted
- across all studies, no side effects of the treatment with gas plasma-oxidized liquids were reported, and beyond, blood parameters and animal weight were mostly unaffected

Box 1. Findings of studies that utilized gas plasma-oxidized liquids for cancer treatment *in vivo*.

III. Mechabisms of Action and Selection of Suitable Liquids

Most studies on gas plasma-oxidized liquids utilize cell culture medium (such as RPMI or DMEM) as a carrier for the reactive species. Although it might appear logical to apply the plasma to cancer cells while they are growing in their respective culture medium at optimal growth conditions, this experimental setting has severe weaknesses (Table II).

In a biological system, such as a living animal or a human patient, it is unrealistic that the cancer cells are exposed to oxidative liquids for an extended period of time, as the cells that are growing in a cell culture medium presented to physical plasma would do *in vitro*. Added *in vivo*, these liquids are rather quickly absorbed by blood and lymph vessels and lose their local activity within minutes [69], [70], at least in the peritoneal cavity. The constant exposure of the cells to that oxidized-medium could instead lead to cancer cell growth retardation and senescence because of metabolic disturbances rather than actual tumor-toxicity [45]. Short exposure times of cancer cells to gas plasma-oxidized liquids, such as sodium chloride (NaCl), *Ringer's* lactate, or PBS, which after a limited exposure time to cells are subsequently replaced with fresh cell culture medium, could help to enhance the applicability of such *in vitro* experiments. It was already shown that the inherent toxicity of such liquids remains low at such short incubation times [20], [51].

Moreover, compared to NaCl, *Ringer's* lactate, and PBS, cell culture medium has a much more complex and inconsistent composition [20], [71], which can lead to a redox-chemistry that is more difficult to control and to analyze. For example, the type of culture medium, the amount of FCS, and other ingredients can play a significant role in the long term plasma effect [28], [71]–[73]. By contrast, using either the simple composition of NaCl or additive anticancer effect through lactate in *Ringer's* lactate [58], [65] helps to improve the standardization of such oxidative solutions. In 2019, several different clinically approved and nonapproved liquids were compared for their anticancer effect after their exposure to the effluent of the *kINPen* argon plasma jet [20]. It was found that also for nonbuffered solutions, the decrease in the level of pH was negligible (except for HES), which was also shown in other studies [51], [74], [75]. The glucose solution G-5 and *Ringer's* lactate had the highest levels of superoxide production or deposition during plasma treatment, while RPMI had the lowest. Also, the amount of hydrogen peroxide (H_2O_2) that was introduced by the plasma-treatment varied in the different liquids. More importantly, all liquids had different capacities of H_2O_2 storability. Two weeks after the plasma-treatment and storage of the liquids at -20 °C, almost no H_2O_2 was left in RPMI (containing FCS; also supported by other studies that utilized EMEM [76]), while NaCl, *Ringer's* lactate, and PBS had the highest levels. Other studies also reported opposite results showing similar anticancer activity and concentrations of H_2O_2 (but not nitrite/nitrate) in gas plasma-oxidized DMEM that was stored for up to 6 months [42].

Another critical point is the nomenclature of these oxidized liquids being plasma-*activated* medium or PAM. The wording is likely to develop significant importance in terms of clinical acceptance of gas plasma-oxidized liquids. Medical products and reagents are always subject to restrictions, need to be easy to understand, and contain information about their supplements (e.g., G-5 is a 5% glucose solution; HES stands for hydroxyethyl starch; E153 contains electrolytes at 153 mval/l. By contrast, a description of a product as being *activated* not only is inaccurate but also has an esoteric touch, and therefore is rather less acceptable for science or modern medicine, at least in terms of clinical utilization. A more accurate description could be *gas plasma-oxidized*. It is essential to include the information that it is a medical gas plasma-oxidized type of liquid (not: blood plasma) and that the kind of conditioning was oxidation. It goes without saying that all other plasma components (e.g., UV-radiation and electric fields) will not be "stored" in such a solution. Moreover, from a clinician point of view, it is unrealistic to support cell culture medium as a potential anticancer agent. Other liquids are much better characterized, already have their clinical approval, and physicians gained experiences with their application in different medical settings [77]–[79]. It is, therefore, paramount to reconsider the usage of cell culture medium for future *in vivo* experiments because the clinical translation of research in the field of plasma medicine should be of prime importance.

TABLE II
OVERVIEW OF ADVANTAGES AND DISADVANTAGES FOR LIQUIDS TO BE UTILIZED AS GAS PLASMA-OXIDIZED LIQUIDS IN CANCER THERAPY IN PRECLINICAL ANIMAL MODELS AS WELL AS FUTURE APPLICATIONS IN PATIENTS

plasma-oxidized liquid	advantages	disadvantages
cell culture medium	"Mimics" the *in vitro* conditions	not clinically approved, weakest anti-cancer efficacy
Ringer's lactate	clinically approved, good storability of oxidants, additional anti-cancer effect of lactate indicated, high anti-cancer efficacy, highest *in vitro* superoxide production	low buffer capacity
sodium chloride (NaCl)	clinically approved, good storability of oxidants, high anti-cancer efficacy	low buffer capacity
phosphate-buffered saline (PBS)	good storability of oxidants	not clinically approved
double-distilled water (H_2O)	clinically approved	low buffer capacity

Having a rather simple and well-characterized solution as the carrier for the plasma-derived ROS/RNS also has some other advantages. It is known that the plethora of reactive species that are induced by the gas plasma are condensed to only a few stable ones in the liquid phase [1], [16], [21]–[23], [80]. However, the reactions and productions of intermediate species in these liquids remain complex but could produce very effective mediators of an anticancer effect [19], [24]. Identifying the main mechanisms of action that mediate the antitumor immune response and affiliate them with oxidants that can be found in the gas plasma-oxidized liquids could lead to a more manageable production of oxidizing anticancer agents. These could be matched to concentrations that are introduced by gas plasmas or set-in supraphysiological concentrations if they show promising results. *In vitro* investigations already mimicked the effect of gas plasma-oxidized liquids by utilizing concentration matched controls of H_2O_2 alone [33], [65] or with the addition of nitrite [51], [81], [82]. Interestingly, this was observed in 2-D cell cultures, but in experiments with 3-D tumor spheroids, gas plasma-oxidized PBS reached higher levels of tumor-toxicity than H_2O_2 alone [43], [65]. One explanation could be that the H_2O_2 concentration-matched controls could have different permeabilization capabilities to also reach deeper cell layers in 3-D tumor spheroids. This thesis is supported by a study that showed differences in the gas plasma-induced and H_2O_2-induced growth arrest of human colorectal cancer spheroids (HCT116) [43]. The gas plasma-oxidized PBS was significantly more efficient, while it also developed an additive effect in combination with pulsed electric fields (PEFs), while the H_2O_2 control could not. Identifying the major mediators that differentiate the gas plasma-oxidized liquids from other oxidizing agents should, therefore, continue to be a target of future research to, in the end, optimize the process of their generation or the development of surrogates.

Also, the specification of pathways of action could help to identify the most crucial oxidative species. The variations in effects observed with different gas plasma-derived oxidants are considerable [1], [19], [83], [84]. The clearest effects are made by the activation of caspases, or the involvement of p53 and increased BAX over BCL-2, as described above [33], [57], [63], [64], [66], [67], [85]. Further *in vitro* experiments that utilized gas plasma-oxidized liquids for cancer treatment made further suggestions for plasma effects playing an important role in the formation of cell death dependent on autophagy [50], [52], the downregulation of survival proteins that are involved in the AKT pathway [86], [87], targeting cell organelles, such as mitochondria and the endoplasmic reticulum [88]–[90], and redox-regulated changes in cell pathway activation and gene expression [91]–[94]. Moreover, alteration of the metastatic capacities of tumor cells [60], [95] and their morphology, oxidation of the membrane [75], [96], [97], senescence (through intracellular zinc) [36], [45], and disturbances in the cells' metabolic profile [47] are examples of promising research.

IV. IMMUNOGENICITY: OBSTACLE OR CHANCE?

Out of several *in vivo* experiments, there are only three that were performed in syngeneic cancer animal models. The other studies used human cell lines that were implanted in immunodeficient mice known to be (partially) void of adaptive immune responses. Deciding which model is appropriate for the specific research goals is always a double-edged sword. On the one hand, the usage of the human cell lines seems to increase translational relevance. On the other hand, this practice hinders adequate immunological research [98]. We know that the immune system has a significant impact, not only in tumor development but also in its metastatic spread [99], [100]. Not only since the 2018's *Nobel Prize for Physiology and Medicine* for the development of checkpoint inhibitors [101], it is well established that a deliberated antitumor T-cell response significantly reduces tumor growth and shows good clinical results among different oncological disciplines [102]–[104].

Among the different *in vitro* studies, two ways of increasing an antitumor immune response are investigated: 1) changing the tumor cells' expression of proimmunogenic molecules and 2) stimulating immune cells to differentiate and initiate an immune response [1], [105]. Several studies have shown that treatment with directly applied gas plasma [106]–[108] or dielectric barrier discharge (DBD) [109]–[111] can increase

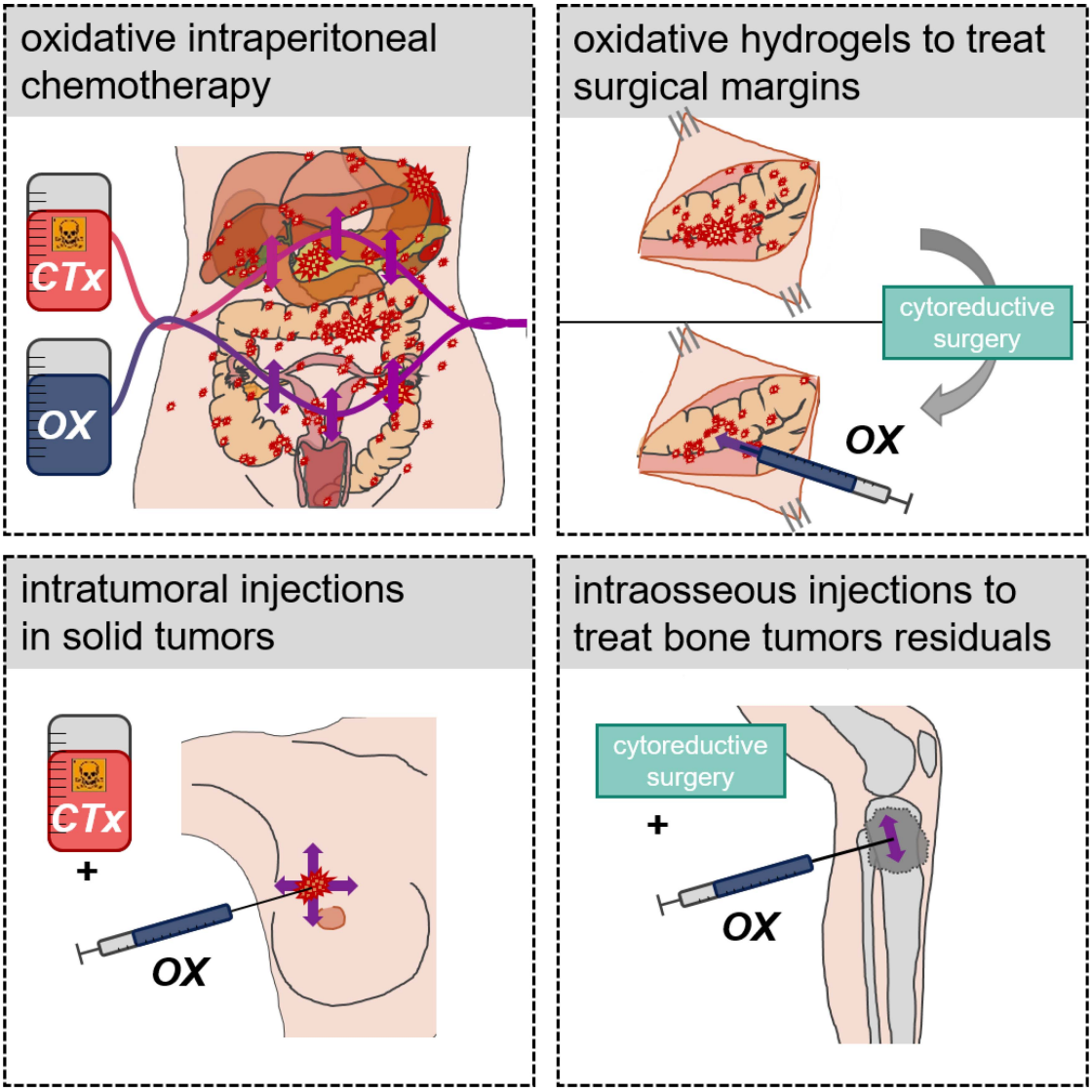

Fig. 2. Future applications of gas plasma-oxidized liquids and hydrogels for cancer therapy.

the immunogenicity of tumor cells. In particular, the ER-chaperon calreticulin (CRT) is proposed to have a significant impact on the tumor cells' immunogenicity [112]. The gold-standard assay for determining the immunogenicity of treatments is *in vivo* vaccination experiments [98]. Here, immunocompetent animals are stimulated with material from tumors that were killed through different treatment regimens. Later, the animals are rechallenged with viable tumor cells. The number of animals developing tumors tells the efficacy of a treatment, and gas plasma treatment (both kINPen plasma jet and DBD) was already shown to initiate tumor-suppressive immune effect [113], [114]. Both of these treatment regimens also observed an increased amount of CRT on the tumor cell surface, and with plasma jet treatment, we were also able to demonstrate increased activation of adaptive immune cells (T-cells) [113]. Similar experiments reported the *in vitro* differentiation of macrophages [115], [116], as well as their altered migration [117]. The gas plasma-derived reactive species were also shown to have an impact on the differentiation profile of other cell types, as changes in the activation status of mononuclear blood cells were observed [118], [119]. Additionally, it is reported that gas plasma is able to support the formation of neutrophil extracellular traps [120].

Both syngeneic tumor models that studied the effect of gas plasma-oxidized liquids *in vivo* found increased infiltrating immune cells in the tumors [62], [65]. Especially T-cells played a role and showed higher activation or presence in the TME. This indicates that gas plasma-oxidized liquids have the potential to spur antitumor immunity. We hypothesize this to be one primary working principle of gas plasma treatment in *in vivo* systems. Nevertheless, future studies are needed to block T-cell activity *in vivo* to identify their contribution to the effects observed. *In vitro*, promising studies with gas plasma-oxidized liquids for immunomodulation have been published. An altered differentiation profile of bone marrow-derived macrophages was observed [121]. Changes in the tumor cells' phenotype, accompanied by upregulation of CRT, as well as heat shock proteins and HMGB1, were identified,

- Sodium chloride and *Ringer's* lactate are state-of-the-art liquids for *in vivo* experiments with gas plasma-oxidized solutions. They are already in clinical use for other applications, were validated to be effective, and have high capabilities to store oxidants. Oxidized cell culture medium should not be considered for *in vivo* studies.
- The relevance of the immune response in mediating an anti-tumor response is high. Hence, immune activation needs to be considered in experiments with gas plasma-oxidized liquids by employing syngeneic murine tumor models rather than xenografts
- To gain clinical acceptance, the wording of liquids that were exposed to physical plasma should be specified to *gas plasma-oxidized*. The phrase "*activated*" is inaccurate, has an esoteric touch, and is therefore not appropriate for a future medical product.
- A goal of the *in vivo* pre-clinical research should be to combine gas plasma-oxidized liquids with standard chemotherapeutic drugs.

Box 2. Summary of suggested steps to enhance the clinical acceptance of research regarding gas plasma-oxidized liquids for cancer treatment.

while immunosuppressive markers, such as PD-L1 (crucial for T-cell function) on tumor cells were found to be downregulated [65], [67]. Moreover, induction of the immunogenic cell death after application of gas plasma-oxidized PBS as well as higher activation of dendritic cells and tumor cell phagocytosis was identified [122]. Immunosuppressive stellate cells (associated with pancreatic cancer cells) were eliminated to a greater extent with that treatment [122]. All these findings motivate the utilization of immunocompetent animal models for future preclinical research with gas plasma-oxidized liquids to unravel putative immune stimulation and finding their way into future cancer therapies.

V. Future Perspectives

Before novel cancer therapies can find their way into clinical routine, they are always tested and compared side by side with standard therapies. To enhance the transferability of preclinical animal models, gas plasma-oxidized liquids should, therefore, be compared to or combined with standard cytostatics. However, across all *in vivo* experiments, only two studies combined gas plasma-oxidized liquids with drugs [63], [67], with both studies using compounds not being standard drugs for systemic melanoma treatment [123]. This is not necessarily a weakness of these studies, because through their combination with the oxidizing solutions, old or new substances can move into the focus of research (if an additive effect is observed). In our recent *in vitro* studies, we screened 80 kinase inhibitors for their additive effect with low dose H_2O_2 and found rapamycin and GF109203X to be highly cytotoxic in colorectal cancer cells in combination treatment [124]. Our other gas plasma-liquid and drug (cisplatin and gemcitabine) combination study in pancreatic cancer cells was highly promising as both drugs used are part of clinical routine [74]. The same is true for a study on hepatocellular carcinoma cells in combination with doxorubicin [30]. The animal models of peritoneal carcinomatosis that were investigated seem to be ideal for the combination treatment with drugs. In the clinical situation of metastasized peritoneal cancer, cytoreductive surgery is followed by the intraperitoneal administration of cytostatics during hyperthermic intraperitoneal chemotherapies (HIPEC) that often is accompanied by severe side effects [13], [125], [126]. A reduction of the drug doses, when combined with gas plasma-oxidized liquids, would be conceivable to reduce the potential side effects while inducing the same tumor reduction (Fig. 2).

Also, intratumoral injections, as in skin or breast tumors, could have potential in the future [127], [128] (Fig. 2). Intratumoral injections of HOCl, for instance, showed auspicious results [129]. However, the injections in solid tumors are not a standard in cancer treatment. A higher need is seen for the treatment of resection margins of tumors that were subjected to surgery. Here, tumor microresidues are macroscopically invisible, while resection margins are kept small in order to save critical tissue (Fig. 2), leading to cancer recurrence. Such resection margins, in the pancreas, the colorectum, or in tumors of the central nervous system, would benefit from the application of a gas plasma-oxidized liquid or even a hydrogel. Such hydrogels are suggested to function as a carrier of the reactive species, with some other characteristics compared to gas plasma-oxidized liquids, such as an increased viscosity. This makes a topical application of the gas plasma-derived species possible, while also having the advantage of the lack of extended treatment times as evident for direct gas plasma applications. At the same time, early *in vitro* studies suggested alginate hydrogels to have a higher capacity of storing reactive species as compared to *Ringer's* saline [130]. The therapy of bone tumors could benefit from gas plasma-oxidized liquids or hydrogels that are injected into the resection cave of the tumors (Fig. 2). *In vitro* studies already reported cytotoxic activity of gas plasma-oxidized medium to osteosarcoma cells, while the regenerative stem cells were left unaffected [131].

For all these forms of application, it is crucial that the oxidative liquids are easy to store, fast to produce, and to have a reliable chemical composition based on already approved liquids in the sense of a pharmacological agent. Therefore, it is a need for future research on gas plasma-oxidized liquids to develop plasma sources that can oxidize large volumes of liquids. As a proof-of-concept, we have shown with conventional plasma jet systems (kINPen) that sufficiently larger liquid volumes (50-ml NaCl treated for 1h) can be treated [65]. Now

is the time to upscale such systems. For instance, the wIN-Plas discharge is capable of treating 500 ml at once [132], but ideally, 5000-ml reactors are needed that produce plasma-oxidized liquids in a pharmaceutical quality that can be subsequently stored and shipped for future adjuvant cancer therapies in the clinics.

VI. Conclusion

Until today, the *in vivo* research on the effect of gas plasma-oxidized liquids in murine systems was revealing and brought the field of plasma medicine new insights into potential possible plasma applications. The oxidizing liquids showed tumor-toxic effects in a broad spectrum of tumor animal models, focusing on peritoneal carcinomatosis and intracutaneous or subcutaneous tumors. Most interestingly, the effective combination with cytostatic drugs, intraperitoneal administration, and immune effects, make gas plasma-oxidized liquids a promising candidate for future anticancer therapies. However, most *in vivo* studies so far lack immunocompetent animal models and utilized the cell culture medium as a carrier solution for the reactive species, and therefore compromised clinical transferability of the results. Before future research can gain clinical relevance and acceptance under physicians, several steps have to be taken (Box 2).

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