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ABSTRACT

Plasma medicine is an innovative research field combining plasma physics, life science, and clinical medicine. It is mainly focused on the application cold atmospheric plasma (CAP) in therapeutic settings. Based on its ability to inactivate microorganisms but also to stimulate tissue regeneration, current medical applications are focused on the treatment of wounds and skin diseases. Since CAP is also able to inactivate cancer cells, its use in cancer therapy is expected to be the next field of clinical plasma application. Other promising applications are expected in oral medicine and ophthalmology. It is the current state of knowledge that biological CAP effects are mainly based on the action of reactive oxygen and nitrogen species supported by electrical fields and UV radiation. However, continuing basic research is not only essential to improve, optimize, and enlarge the spectrum of medical CAP applications and their safety, but it is also the basis for identification and definition of a single parameter or set of parameters to monitor and control plasma treatment and its effects. In the field of CAP plasma devices, research and application are currently dominated by two basic types: dielectric barrier discharges and plasma jets. Its individual adaptation to specific medical needs, including its combination with technical units for continuous and real-time monitoring of both plasma performance and the target that is treated, will lead to a new generation of CAP-based therapeutic systems.

I. INTRODUCTION

Plasma medicine refers to the use of physical plasma directly on or in the human (or animal) body for therapeutic purposes. Primarily powered by physicists since the mid-2000s (Stoffels *et al.*, 2004; Fridman *et al.*, 2008; Laroussi 2009; Kong *et al.*, 2009; Weltmann *et al.*, 2010), it developed into an interdisciplinary research field including life sciences and medicine. In its narrow sense, plasma medicine is focused on the use of cold atmospheric-pressure plasmas (CAP), i.e., plasmas that generate temperatures not higher than 40 °C at the target site of treatment. Among the broad spectrum of technologies to generate cold plasma at atmospheric conditions, two basic types of CAP devices are dominating preclinical and clinical research in plasma medicine: dielectric barrier discharges (DBD) and plasma jets (Weltmann *et al.*, 2010; von Woedtke *et al.*, 2013a; Tanaka *et al.*, 2017). DBDs are characterized by plasma ignition in a gap between an isolated high voltage electrode and the target to be treated having a direct contact between plasma and target (volume DBD), or around an individually designed electrode structure (e.g., circular or grid-like), which is isolated from a counter electrode (surface DBD). In the latter case, there is no direct contact of the active plasma with the target to be treated. In DBDs, atmospheric air

usually serves as the working gas for plasma generation (Brandenburg, 2017). In a plasma jet device, the electrode setup for plasma generation is usually located in or around a tube-like arrangement, in most cases inside a pen-like device, where the plasma is ignited using a flowing working gas. Electrode configurations may vary between dielectric-free electrode jets, DBD jets, DBD-like jets, and single electrode jets. The resulting plasma effluent (or afterglow) is carried out along the gas flow and can be brought into direct contact with the target to be treated. Most plasma jet devices are using noble gases (e.g., helium or argon) as working gas, often doped with small amounts of molecular gases (e.g., nitrogen, oxygen) (Winter *et al.*, 2015).

Besides several experimental experiences on the antimicrobial activity of CAP (Laroussi, 1996; Weltmann *et al.*, 2008; Ehlbeck *et al.*, 2011), above all the reports on non-lethal manipulations of mammalian cells in the middle of 2000s (Stoffels *et al.*, 2003) marked the beginning of a decade of intensive *in vitro* research on plasma-cell interactions using microorganisms, mammalian cells including cancer cells, and living tissue models. This is reported in a high and growing body of publications. Starting from this intensive basic research, meanwhile first clinical applications of CAP are becoming reality and the potential of plasma use in medicine is considered to be highly promising (Sorg *et al.*, 2017; Izadjoo *et al.*, 2018; Dubuc *et al.*, 2018; Bernhardt *et al.*, 2019).

II. PRESENT MEDICAL APPLICATION OF CAP DEVICES: WOUND HEALING AND DERMATOLOGY

Research in CAP application in medicine was first focused on treatment of chronic wounds (Kramer *et al.*, 2008; Lloyd *et al.*, 2010). Plasma effects on wound healing are a result of a two-step CAP activity: antisepsis on wound surface in combination with a direct stimulation of tissue regeneration (Fig. 1) (von Woedtke *et al.*, 2018; von Woedtke *et al.*, 2019).

After first positive clinical results of CAP treatment of chronic ulcers using an argon-driven microwave plasma torch (Isbary *et al.*, 2010, 2012), several clinical trials on treatment of chronic ulcers have proven the plasma effect mainly on the reduction of bacterial load on wounds (Shimizu and Ikehara, 2017; Assadian *et al.*, 2019; Bernhardt *et al.*, 2019). Even if the experience in CAP application in clinical settings is predominantly positive, comprehensible and systematic documentation of these practical experience is rare, yet. Therefore, the most important actual challenge in the field of plasma-supported chronic wound healing is the realization of randomized controlled trials (RCT) to consolidate this really promising field of CAP application in the medical practice. However, it has to be kept in mind that controlled trials in the field of wound care are rare, in general, what is mainly caused by the fact that wounds present as part of such a complex presentation that generalization within the scope of a study design is very difficult (Cutting *et al.*, 2017).

Furthermore, beyond studies on healing of chronic and microbiologically infected/contaminated wounds, animal studies as well as clinical trials with healthy volunteers have demonstrated a clear stimulating effect on wound healing independent of antisepsis. It was shown undoubtedly that CAP treatment is useful to accelerate the rate of wound closure at early stages after wounding (Metelmann *et al.*, 2012; Arndt *et al.*, 2013; Vandersee *et al.*, 2014; Schmidt *et al.*, 2017a). In general, the direct combination of wound antisepsis with a stimulating effect on tissue regeneration is the strength and unique feature of CAP in comparison to conventional and established wound care measures, which has to be emphasized both in clinical trials and in clinical practice. Moreover, findings on direct stimulation of tissue regeneration by CAP treatment are the basis to evaluate the applicability of CAP also in the field of acute wound healing. Commonly, it is assumed that acute surgical but also traumatic wounds do not need additional stimulation because they are healing regularly under physiological conditions. However, it has to be analyzed if the initial fastening of wound closure by CAP treatment, possibly together with its antiseptic effect, may have some therapeutic value in prevention of postoperative wound infection and other healing complications. There are first

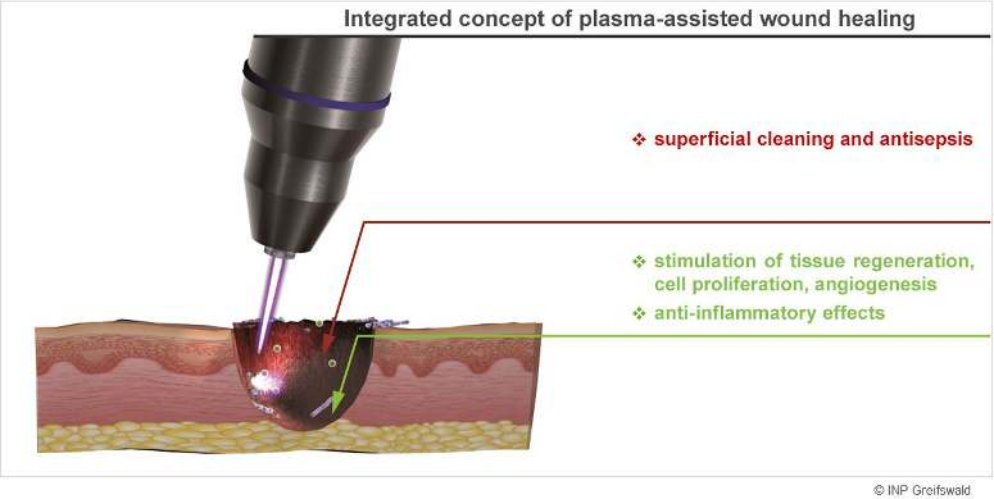

FIG. 1. Concept of the two-step effectivity of CAP-supported wound healing. Reprinted by permission from Podmelle *et al.*, *Comprehensive Clinical Plasma Medicine. Cold Physical Plasma for Medical Application*, edited by H.-R. Metelmann, T. von Woedtke, and K.-D. Weltmann (Springer Nature Customer Service Center GmbH: Springer Nature, 2018).

approaches to use CAP to support healing of acute surgical wounds in cases where because of the patient's health status the risk of retarded healing is enhanced (Hartwig *et al.*, 2017). In this context, also the impact of plasma-supported acceleration of acute wound healing on scar formation should be clarified. Together with possible preventive effects on wound infection, CAP treatment is a promising option to support several interventions in the field of plastic surgery and esthetic medicine (Podmelle *et al.*, 2018). Moreover, preventive and therapeutic use of CAP in wound treatment is also under discussion for field applications in emergency and military medicine (Fridman *et al.*, 2008; Izadjoo *et al.*, 2018).

Beyond wound healing as the most investigated CAP application in the clinical context, plasma devices are also used in treatment of infective and inflammatory skin diseases like herpes zoster, atopic eczema, acne, athlete's foot, and others (Heinlin *et al.*, 2010, 2011; Isbary *et al.*, 2013a; Emmert *et al.*, 2013a; Tiede *et al.*, 2014; Bernhardt *et al.*, 2019). It is known that medical doctors who are engaged in plasma medicine also use CAP for such kind of indications. However, experience in these dermatological applications is rarely documented or reported in larger case series. It is an ongoing task in clinical research to clarify and systematize the indications of CAP application in wound healing and dermatology.

Generally, it can be stated that the application of CAP devices for therapeutic purposes is on its way to clinical routine. This was favored not least by the fact that any enhanced risk of genotoxic and mutagenic effects of CAP treatments could be excluded by several meaningful and well-established *in vitro* tests as well as by a long-term animal trial (Boxhammer *et al.*, 2013; Wende *et al.*, 2016; Kluge *et al.*, 2016; Maisch *et al.*, 2017; Schmidt *et al.*, 2017b; Bekeschus *et al.*, 2018a; Boehm and Bourke, 2019).

III. PREDICTABLE FIELDS OF CAP APPLICATION IN MEDICINE

Aside from wound healing and dermatology, several other potential CAP applications in medicine are under research. In recent years, the most important field of investigation is plasma application in cancer treatment. First reports on clinical application of CAP treatment for actinic keratoses, i.e., *in situ* squamous cell carcinomas of the skin, achieved good results in patient studies (Friedman *et al.*, 2017; Wirtz *et al.*, 2018). In patients with advanced squamous cell carcinoma of head and neck, CAP application is used successfully in palliative care to treat infected tumor ulcerations to reduce microbial load and the resulting typical fetid odor. In some particular cases, also transient tumor remission occurred (Metelmann *et al.*, 2015; Schuster *et al.*, 2016; Metelmann *et al.*, 2018).

Currently, the focus in CAP research lies in specific plasma applications for cancer eradication or at least selective reduction of cancer cells by enhancing immunogenic cell death (ICD). This is based on several studies on effective inactivation of different cancer cell lines *in vitro* by induction of programmed cell death with tentative experimental evidence of at least partial selectivity of CAP toward cancer cells in comparison to non-cancerous cells (Schlegel *et al.*, 2013; Ratovitski *et al.*, 2014; Hirst *et al.*, 2016; Dubuc *et al.*, 2018; Semmler *et al.*, 2020). First animal studies on transcutaneous plasma treatment of subcutaneously induced solid tumors (Vandamme *et al.*, 2010) could prove the general concept of plasma-supported tumor treatment leading to the very optimistic prognosis of a "paradigm shift in cancer therapy" in 2011 already (Keidar *et al.*, 2011). However, CAP application in cancer treatment is in the state of preclinical and clinical research and not yet part of applicable therapy concepts. For the time being, two approaches seem to be most promising for CAP application in cancer treatment. On the one hand, based on the experimental proof of effective inactivation of single layers of cancer cells by local plasma treatment (Partecke *et al.*, 2012), supportive plasma application in combination with surgical tumor resections in cases, where large-scale tumor removal is impossible, seems to be realistic. Here, CAP could be used for post-treatment of the operation field after surgery to inactivate possibly remaining cancer cells (von Woedtke and Metelmann, 2014; Yoon *et al.*, 2018). On the other hand, CAP application in melanoma treatment is a promising field of application because melanomas typically occur in the skin, are highly susceptible for immunogenic therapies, and are, therefore, good locatable and amenable for direct plasma treatment (Fridman *et al.*, 2008; Daeschlein *et al.*, 2013; Chernets *et al.*, 2015; Bekeschus *et al.*, 2017a; Pasqual-Melo *et al.*, 2018; Sagwal *et al.*, 2018; Gandhirajan *et al.*, 2018). Due to the potentially induced immunogenic cell death by plasma, the melanoma treatment efficacy may be enhanced by combining CAP together with established immunotherapies for metastasized melanoma treatment.

In general, it can be predicted that CAP will not preferentially appear as a single therapeutic option in cancer treatment but as part of combined therapeutic strategies. Besides its combination with surgical tumor resection or immunotherapies as mentioned above, other options under investigation are combinations with radiotherapy, pulsed electric fields, and chemotherapy (Brullé *et al.*, 2012; Köritzer *et al.*, 2013; Masur *et al.*, 2015; Pasqual-Melo *et al.*, 2018; Wolff *et al.*, 2019; Pasqual-Melo *et al.*, 2020; Chung *et al.*, 2020).

Another promising field of CAP application in cancer treatment is the use of plasma-treated liquids as it is mentioned in Sec. VI.

However, before CAP application in cancer treatment may become realistic, some questions with special regard to its safety have to be answered. On the one hand, it has to be proven if there is a real selectivity of CAP against cancer cells (Keidar, 2018) or rather to what extent and with which consequences neighboring healthy tissue might suffer from cancer cell-inactivating plasma impact. On the other hand, much more important is the question if (accidental) sub-efficient CAP treatment intensity, e.g., in the edge zone of plasma impact, may induce an acceleration of cancer cell growth and proliferation comparable to the CAP effect in tissue regeneration, with the final consequence of metastasis. First *in vitro* studies to investigate the latter problem give some evidence that this danger does not exist but needs much more confirmation (Bekeschus *et al.*, 2019). However, some *in vivo* studies could demonstrate that CAP treatment can enhance tissue oxygenation and modulate blood flow, which could support metastasis on the one side but could conversely enhance effects of radiotherapy and chemotherapy in combination treatments on the other (Collet *et al.*, 2014; Kisch *et al.*, 2016; Daeschlein *et al.*, 2018). Consequently, much more research is needed to estimate the pros and cons of CAP applications for cancer treatment under different therapeutic settings.

Finally, the question of accessibility of bulk tumors by CAP treatment is an open question. On the one hand, in an experimental *semi-in vivo* bulk tumor model, inactivation of cell layers up to a depth of 40 μm by plasma was demonstrated (Partecke *et al.*, 2012). On the other hand, in animal trials, a reduction of the bulk volume of

subcutaneous tumor xenografts was demonstrated by transcutaneous plasma treatment (Keidar *et al.*, 2011; Vandamme *et al.*, 2012), i.e., under conditions where a direct plasma contact to the tumor bulk *in vivo* can be excluded. Here, some very urgent questions on possible plasma-induced stimulation of immunogenic cell death (ICD) have to be discussed. The ICD concept is based on the assumption that plasma-treated cancer cells are able to enhance their visibility to body's own immune cells by the presentation of so-called damage-associated molecular patterns (DAMPs). Thus, the protective effects of the patient's own immune system are exploited to specifically target the cancerous cells. It is well-known that some chemotherapeutics and also physical therapies like photodynamic therapy or radiation therapy can be potent ICD inducers. There is some experimental evidence that this is also true for CAP treatment. This would be a highly promising therapeutic approach for cancer treatment in cases where direct plasma contact cannot be realized (Miller *et al.*, 2016; Mizuno *et al.*, 2017; Bekeschus *et al.*, 2018b; Bekeschus *et al.*, 2018c; Lin *et al.*, 2019; Khalili *et al.*, 2019). Therefore, CAP-induced ICD is one of the most interesting research fields in plasma-supported cancer treatment. Additionally, these questions are not only important with regard to plasma cancer treatment but also, in general, with regard to potential systemic effects of local CAP applications.

Another field investigated from the beginning of research in plasma medicine is the use of CAP for blood coagulation and haemostasis (Fridman *et al.*, 2008). Blood coagulation using plasma is an established part of electro surgery, e.g., as argon plasma coagulation (Raiser and Zenker, 2006; Manner *et al.*, 2008). With these techniques, haemostasis is achieved by heat-based closure of bleeding areas via "sealing" and shrinking the tissue (Shimizu and Ikehara, 2017). In recent years, several experimental studies *ex vivo* and *in vivo* have demonstrated that CAP is also useful to induce blood coagulation without thermal tissue damage. There are different hypotheses on potential mechanisms of direct interaction of CAP with specific components of the physiologic blood coagulation cascade (Kalghatgi *et al.*, 2007; Chen *et al.*, 2009; Kuo *et al.*, 2010; Ikehara *et al.*, 2013; Ikehara *et al.*, 2015; Miyamoto *et al.*, 2016; Nomura *et al.*, 2017; Bekeschus *et al.*, 2018d). By CAP application, blood coagulation can be realized in a much localized manner without detrimental changes of tissue by necrosis or shrinking. Therefore, it could become a valuable supporting technique in surgery, above all in specific applications like laparoscopy or minimal invasive surgery.

Another large and long-time studied field of investigation on CAP application is oral medicine. CAP applications are possible here for both preventive and therapeutic purposes. The most prevalent oral diseases are caries and periodontitis. These diseases are initiated by dysbiotic biofilms and their progression is caused by them. Consequently, therapeutic interventions supported by plasma aim primarily at influencing, reducing, or eliminating biofilms on tooth substances, surrounding tissues, or prosthetic/implantological surfaces. There is a broad spectrum of possible oral and dental CAP applications under research ranging from treatment of infections and wounds of oral mucosa, inactivation and removal of biofilm on teeth, dentures and on dental implants, disinfection of tooth root canals, plasma-assisted cleaning and optimization of tooth and implant surfaces to improve bone integration up to improvement of bonding of dental fillings and prostheses, decontamination and coating of dental prosthesis, or tooth whitening (Rupf *et al.*, 2011; Idlibi *et al.*, 2013; Kim *et al.*, 2013; Cha and Park, 2014; Gherardi *et al.*, 2018). However, despite a growing number of promising experimental results over a time of far more than 10 years, there are nearly no applications of CAP in clinical settings in dentistry. The reason for that is not really clear. One reason could be that in most cases dental problems or diseases are not that wearing or life-threatening as non-healing chronic wounds or cancers are. Therefore, the risk-benefit-balance may be assessed in dentistry with different emphasis compared to other medical fields. However, this kind of reservation should be removed at large because it was specifically proven for oral mucosa, too, that no long-term side effects caused by CAP treatment exist (Jablonowski *et al.*, 2019). Moreover, for most of the problems in oral medicine more or less satisfactory treatment options are existing. Therefore, any CAP application has to demonstrate a significant improvement compared to established therapies to convince dental surgeons to accept such innovative technology. Consequently, a main current task of application-oriented research in plasma medicine is to evaluate the realistic potential of CAP application in oral medicine also from an economic point of view and, where appropriate, to foster its implementation into clinical settings.

Finally, ophthalmology is a field of potential clinical application of CAP which has been surprisingly subject of little research yet although the results are really promising. Here, possible CAP applications are focused on treatment of infections as well as ulcerations of the cornea. It was repeatedly demonstrated *in vitro* and *in vivo* that CAP impact on the surface of the eye may inactivate microorganisms without any destructive effects on the cornea (Martines *et al.*, 2013; Alhabshan *et al.*, 2013; Alekseev *et al.*, 2014; Nikmaram *et al.*, 2018; Nejat *et al.*, 2019). Moreover, in a first-in-human trial on patients with therapy-resistant corneal ulcers the clinical potential of CAP was demonstrated (Reitberger *et al.*, 2018). Therefore, CAP application in ophthalmology as a mild and effective local anti-infective, anti-inflammatory, and wound healing therapy should be kept in mind in the next time.

Other medical applications of CAP should be possible or were suggested casually, e.g., application to treat neurological diseases (Xiong, 2018).

However, whether plasma medicine continues to move into clinical practice will depend on several factors. Current applications of CAP above all in wound healing have to be consolidated in clinical practice and by randomized controlled trials (RCT) to finally prove its practical value and cost effectiveness in therapy. Based on such positive experience, further clinical needs that possibly can be solved using plasma technology will be defined by medical doctors. This has to be supported by ongoing basic research efforts to deepen the understanding of mechanisms of biological plasma effects that can be translated into specific medical applications. Finally, plasma physics and technology is needed to improve and optimize CAP devices to meet specific needs of medical applications, e.g., larger electrode arrangements to treat larger wounds or skin surfaces at once or the combination of plasma devices with suitable wound dressings. Last but not least, therapeutic CAP applications have to become part of guidelines for medical care to embed plasma medicine into rational therapeutic concepts. In Germany, a guideline project on "Rational therapeutic use of cold physical plasma" has been started in 2018 (AWMF, 2020).

IV. ACTUAL CHALLENGES FOR BASIC RESEARCH IN PLASMA MEDICINE

According to the actual state of knowledge, biological plasma effects are mainly based on the action of reactive oxygen and nitrogen species (ROS, RNS) which are generated and transferred into the (liquid) cell and tissue environment resulting from CAP impact (Graves, 2012, 2014; Wende *et al.*, 2019; Privat-Maldonado *et al.*, 2019). The reactive species are generated inside the plasma or as a result of plasma interactions with media that come into contact with the plasma, like surrounding air, liquids or surfaces. In these complex interactions, plasma-generated electrical fields as well as (V)UV radiation have a supporting function. All CAP sources for biomedical applications are working under atmospheric air conditions or use ambient air as working gas. Consequently, generation of ROS and RNS from air-based oxygen and nitrogen is a corresponding feature of all these plasma sources. Merely composition and quantity of plasma-generated ROS and RNS are dependent on specific physical and technical plasma source and device parameters as working gas composition, power input, or temperature (Lu *et al.*, 2016). It was demonstrated that medically relevant plasma effects like stimulation of tissue regeneration or inactivation of cancer cells are based on stimulation or manipulation of redox-controlled cellular processes (Bauer and Graves, 2016; Yan *et al.*, 2017; Schmidt *et al.*, 2019; Semmler *et al.*, 2020). Therefore, plasma medicine can be considered as a field of applied redox biology (von Woedtke *et al.*, 2019).

Detailed understanding of mechanisms of biological plasma effects is not only necessary for elucidating the basic scientific principles of plasma medicine but also for control and monitoring of therapeutic CAP applications and eventually for any further development and optimization of CAP devices for medical application. It is still not finally clarified whether, and if so, to what extent single plasma-generated ROS and RNS are specifically responsible for distinct biological effects. Answering this question could be a precondition for further optimization of CAP treatment by "tunable" plasma devices. By variation of parameters like feed gas composition or input power, a modification of reactive species composition could allow different effects during the course of a CAP-supported therapy (Weltmann and von Woedtke, 2017). In chronic wound healing, for example, it could be an increased antimicrobial effect in the beginning followed by an intensified stimulation of tissue regeneration in the further course of therapy. In an animal wound healing study using a torch-like plasma device called plasma needle, coagulation and subsequent stimulation of wound closure was realized by consecutive treatment by argon and helium plasma (García-Alcantara *et al.*, 2013). Admixtures of molecular gases (N_2, O_2) and variation of humidity of the working gas argon of a HF-driven plasma jet showed different influence on the metabolic activity of mammalian cells but did not change the mutagenic potential of CAP *in vitro* (Bekeschus *et al.*, 2018a). In another study, different effects on plasma-induced platelet activation for blood coagulation were estimated (Bekeschus *et al.*, 2018d). With a radio frequency driven atmospheric plasma jet, pro-apoptotic anticancer effects could be modified by different mixtures of the working gas helium with O_2 or changing gas humidity, respectively (Bekeschus *et al.*, 2017b). By variation of N_2/O_2 admixture to an argon plasma jet by a specifically designed gas shielding device, reactive species composition was modulated resulting in different effects on viability of microorganisms and mammalian cells (Reuter *et al.*, 2012; Jablonowski *et al.*, 2015). A more intensive combination of such *in vitro* cell treatment results with both more detailed investigations in plasma diagnostics and molecular mechanisms of cell effects should allow adaptation of plasma treatment parameters to special needs of therapy in future.

In connection with research on modification of plasma composition with special regard to ROS and RNS, another problem has to be taken into consideration more strongly, namely, possible feedback effects of the treated target on the plasma composition. Conductivity of the target has influence on the plasma properties (Darny *et al.*, 2017; Judée and Dufour, 2019). This is not that surprising because of the conductive characteristics of the plasma. Moreover, there is some evidence that also other characteristics of the microenvironment of living targets like its humidity, but also its chemical composition may influence the plasma (Riès *et al.*, 2014; Du *et al.*, 2017; Pouvesle *et al.*, 2018; Zhao and Nie, 2019). Current research efforts have to be focused much more on these aspects of plasma-target interactions to find out if and to what extent this may influence therapeutic effects because living tissue is a target whose characteristics can change depending on several factors of influence. Therefore, it has to be an aim of basic research in plasma medicine to define a target that is representative as best as possible for a living organism which can be used to "calibrate" plasma devices and compare its performance characteristics (Judée and Dufour, 2019; Stancampiano *et al.*, 2019).

Another very important research issue is the depth effectiveness of plasma beyond barrier layers like the epidermis as protective skin layer as well as in deeper tissue layers. Using cultivated whole-skin biopsies *ex vivo*, plasma-induced stimulation of skin cell proliferation could be detected in the *Stratum basale*, the deepest cell layer of the outer skin (epidermis) that has a mean thickness of 50–200 μm and is protected on the surface by the less permeable *Stratum corneum* (Hasse *et al.*, 2016). As it was mentioned earlier, in a tumor bulk model, the inactivation of cancer cells up to a depth of 40 μm was demonstrated experimentally (Partecke *et al.*, 2012) and a reduction of the volume of subcutaneous tumor xenografts was demonstrated by transcutaneous plasma treatment of mice (Keidar *et al.*, 2011; Vandamme *et al.*, 2012). Since diffusion characteristics of a plasma are similar to that of a gas, a direct penetration of plasma itself or its effluent to deeper cell layers to realize a direct plasma-cell interaction should be improbable. On the one hand, any depth effectiveness could be based on diffusion of ROS and RNS through several cell layers which can be accelerated by physical plasma components like electrical field. Because of their reactivity and the availability of several organic reaction partners in living tissue, very complex reaction chemistry including generation of secondary and tertiary ROS and RNS has to be taken into consideration. So far, a lot of research was focused on plasma-liquid interaction as a first and simple approach to living tissue taking into consideration that cells in the tissue environment are surrounded by an aqueous extracellular environment (Jablonowski and von Woedtke, 2015; Bruggeman *et al.*, 2016; Wende *et al.*, 2019; Lu *et al.*, 2019; Khlyustova *et al.*, 2019). Recently, more detailed research on penetration and diffusion of plasma-generated reactive species has been started based on cell membrane models, gel-based models, and models using living tissue *in vitro* (Szili *et al.*, 2018; Lu *et al.*, 2019]. It should also be taken into consideration that less reactive (and therefore more stable) species may diffuse over longer distances and may generate more reactive and short living species in some specific pH microenvironments (Tarabová *et al.*, 2019). Another possibility to explain

plasma effects in deeper tissue layers is the cell-to-cell communication via signaling molecules released by plasma-treated cells from upper cell layers initiating a relayed response chain in the tissue environment to transmit specific biological signals (Graves, 2012, 2014; Lu *et al.*, 2019).

However, the main challenge to get a deeper insight into these complex interactions is ROS and RNS because their reactivity and resulting short lifespan are difficult to detect both in liquid phases and in tissue layers. Therefore, research on both plasma-liquid interaction and tissue spreading of CAP and its reactive compounds has to be based not only on chemical analytics, but also significantly on modeling of plasmas and its interaction with liquid and tissue (Chen *et al.*, 2014; Babaeva *et al.*, 2014; Babaeva and Naidis, 2018; Verlackt *et al.*, 2018; Semenov *et al.*, 2019).

V. CAP DEVICES FOR MEDICAL APPLICATION: CURRENT STATE AND CHALLENGES

Medical plasma devices for clinical application are the argon-driven HF plasma jet kINPen® MED (neoplas tools GmbH, Greifswald, Germany), the argon-driven microwave plasma torch SteriPlas (ADTEC, Hunslow, UK), and the DBD-based devices, PlasmaDerm® (CINOGY GmbH, Duderstadt, Germany) and plasma care® (terraplasma medical GmbH, Garching, Germany), the latter two using atmospheric air as working gas. Their specific purpose is the treatment of chronic wounds as well as pathogen-associated skin diseases. Besides their European Conformity (CE) certification as medical devices class IIa according to the European Council Directive 93/42/EEC, all these devices are distinguished by comprehensive physical and biological characterization of the respective plasma source accompanied by detailed preclinical and clinical investigations (von Woedtke *et al.*, 2013b; Isbary *et al.*, 2013b; Tanaka *et al.*, 2017). This is worth to be pointed out because several other devices are on the market that are offered to be useful for "plasma medicine" but have no or very inadequate physical, technical, biological or clinical references that could prove this.

Here, it is necessary during the next years to establish a much better specification and systematization of plasma devices that are offered for applications in the medical field. Above all, a better differentiation of CAP devices from other plasma-based medical devices is needed. The following categories might be useful to get some more order:

- Cold atmospheric-pressure plasma (CAP) devices that generate plasma in direct contact or in close vicinity to the target to be treated (wound, skin, etc.) with a temperature below 40 °C at the site of treatment. This is true for the CAP devices as mentioned above.
- Devices using plasma to generate gases or gas mixtures for therapeutic use, e.g., nitric oxide (Vasilets *et al.*, 2015; Shekhter *et al.*, 2019) or ozone (Martínez-Sánchez *et al.*, 2005; Gupta and Deepa, 2016; Tiwari *et al.*, 2017).
- Electrosurgical plasma devices for blood coagulation, cauterization, tissue ablation, and cutting, respectively, the effects of which are mainly based on thermal impact (Raiser and Zenker, 2006; Canady *et al.*, 2006; Keller *et al.*, 2013; Weiss *et al.*, 2018).

One of the most important technical disadvantage with regard to plasma device application is the current unavailability of a parameter or set of parameters to control and monitor plasma performance that is applicable as a kind of "dose" as it is used in phototherapy, radiation treatment, or laser therapy. According to the actual state of knowledge, biological CAP effects are results of complex interactions of plasma components with structures and components of the living tissue with a dominating role of reactive oxygen and nitrogen species (ROS, RNS), supported by (V)UV irradiation and electrical fields (von Woedtke *et al.*, 2019). Single components or plasma parameters that can clearly be correlated with specific biological effects or therapeutic outcomes, respectively, could not be identified so far. Consequently, plasma impact on biological experiments as well as on therapeutic applications is usually controlled via treatment time and/or energy supply to the device. Because of the different technical setups of CAP devices under investigation or in medical application, this has to be defined for every device specifically and a generalization is not possible. Identification and definition of such a parameter or set of parameters for device-independent control of biological plasma effectivity is possibly the biggest challenge in preclinical research in plasma medicine (Adamovich *et al.*, 2017).

In the meantime, a preliminary but feasible comparability of different CAP devices might be based on a test panel for basic characterization of CAP performance. With the German DIN SPEC 91315 "General requirements for plasma sources in medicine," a first proposal of such a test panel is given (DIN SPEC 91315:2014–6, 2014; Mann *et al.*, 2016). As physical evaluation criteria, plasma/gas temperature, thermal output, optical emission spectra and irradiance measurements in the range between 200 and 900 nm, current flows (patient leakage current), and gas emission are proposed. Biological evaluation criteria comprise the *in vitro* determination of inactivation of specified microorganisms as well as viability tests of eukaryotic cell cultures *in vitro*. The detection of chemical species generated by CAP treatment of aqueous liquid is additionally proposed to roughly evaluate the composition and extent of ROS and RNS generated by the plasma device. This combination of physical, biological, and chemical tests recommended by the DIN SPEC 91315 is a first step on a further road toward standard characterization of plasma sources intended for medical application. This test panel evaluates their effectiveness as well as gives some information with regard to safety for users (investigators, patients, and therapists). A further approach to improve plasma characterization *in vitro* is the identification of a technical target that is representative for the human body to identify plasma-target interactions and its effects on plasma characteristics (Judée and Dufour, 2019; Stancampiano *et al.*, 2019). For more detailed characterization of biochemical reactivity of CAP to estimate biological effectivity, biorelevant tracer substances like cysteine or hemoglobin can be used (Lackmann *et al.*, 2018; Ki *et al.*, 2018). Generally, the aim of all these efforts is to characterize CAP devices as broad as possible but also as effective as possible to estimate their expected biological effects both with regard to therapeutic usability as well as to safety. Eventually, such standardization will also improve the transfer of experimental results into industrial development of medical devices for plasma medicine. Over the next years, a consensus-based international standard has to be developed to make such a basic characterization of plasma devices mandatory (Hahn *et al.*, 2018).

Another ongoing challenge in the field of conception and development of CAP devices is their adaptation to specific needs of particular medical applications. Currently, CAP devices certified for medical applications are oriented toward application on the body surface

(wounds, pathologically altered skin areas). Specifically in wound healing, there is a need to treat larger surface areas. Planar and flat DBD electrode arrangements can be designed to cover such areas completely. A commercially available example is the PlasmaDerm Dress system (CINOGY GmbH, Duderstadt, Germany). Large area treatment is also possible by plasma jet arrays. Spot-like plasma jets have to be moved across the surface to be treated which can be supported by automated systems (Park *et al.*, 2012; von Woedtke *et al.*, 2013a; Setsuhara 2016; Weltmann and von Woedtke, 2017).

With a further spread of plasma technology into medical applications, need of adaptation of plasma devices to more specific application conditions can be expected. In ophthalmology, similar CAP devices to be used on body surfaces may be acceptable, possibly with a more precise control of treatment parameters like distance or temperature. For CAP application in oral medicine, customized plasma devices have to be provided to enable effective and ergonomic plasma treatment in the oral cavity (Weltmann and von Woedtke, 2017). For endoscopic applications as well as minimal invasive surgery, catheter-shaped and miniaturized plasma devices are under development (Kim *et al.*, 2010; Polak *et al.*, 2012; Robert *et al.*, 2013; Mirpour *et al.*, 2016; Chen *et al.*, 2017; Winter *et al.*, 2019). The main challenge for these applications is to guarantee a stable and effective plasma generation in small and long body cavities which are wet and less well aerated. This may be also true for laparoscopic plasma devices (Hirst *et al.*, 2014). Additionally, for all these plasma applications inside the body, an effective navigation of the device has to be realized.

The complexity of both plasma generation, monitoring, and control on the one hand and specific application conditions and demands in medicine on the other have to be kept in mind from the beginning when plasma devices for specific medical applications are designed and developed. The fact that most of the critical features of CAP devices have to be identified and optimized at the best before first *in vivo* testing makes CAP device research and development a unique and highly important field in plasma medicine (Fig. 2).

In general, for clinical investigations plasma devices have to be approved as medical devices according to the medical device regulations of the respective country. In the case of plasma devices that are under development, all product requirements on technical and biosafety testing and preclinical evaluation as well as specific occupational safety and accident prevention measures to ensure the protection of subjects according to the respective medical device regulations have to be fulfilled (DIN EN ISO 14155, 2018).

VI. SPECIAL FIELD: PLASMA-TREATED LIQUIDS

More or less as a result of basic research on plasma effects on living systems and the resulting insight into the role of the liquid cellular environment as a kind of "transfer phase" of plasma-generated species, a side-branch of plasma medicine arose: the use of plasma-treated liquids, which are very often called plasma-"activated" liquids, e.g., plasma-activated water (PAW) or plasma-activated medium (PAM). However, even if the term "activation" sounds slightly "magic" and should be avoided if possible, there is no doubt that plasma treatment of liquids usually results in changing of their biological characteristics. This phenomenon was demonstrated at first with simple aqueous

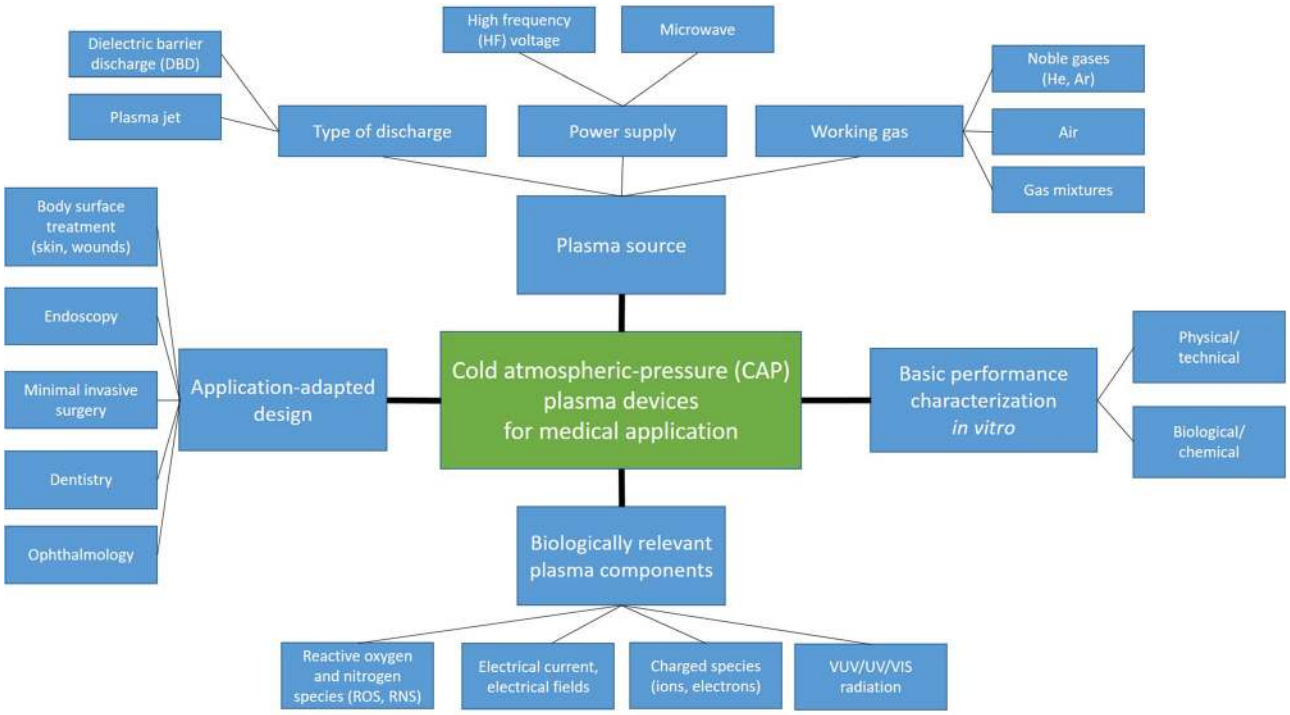

FIG. 2. Most important aspects that have to be taken into consideration before *in vivo* application when CAP devices for medical application are developed and optimized for specific applications.

liquids like water or physiological saline that became antimicrobial effective as a result of CAP treatment (Ikawa *et al.*, 2010; Naïtali *et al.*, 2010; Oehmigen *et al.*, 2011; Julák *et al.*, 2012). Furthermore, plasma treatment of more complex liquids like cell culture media could prove its effectivity also on different characteristics of mammalian cells including its ability to inactivate cancer cells (Partecke *et al.*, 2012; Hoentsch *et al.*, 2014; Utsumi *et al.*, 2013). Sometimes, this kind of application of plasma treated liquids is called "indirect" plasma application because the biological targets (microorganisms, cells, tissue) are not directly present during plasma treatment but are exposed to the plasma-treated liquid. Meanwhile, a huge number of experiments have proven that the effects of plasma-treated liquids are mainly based on the activity of relatively long-lived ROS and RNS (Kaushik *et al.*, 2019; Khlyustova *et al.*, 2019). The most important difference of the use of plasma-treated liquid compared to direct plasma application is that any direct effect of UV radiation or electric fields on the biological target can be avoided. Meanwhile, biological effectivity of such plasma-treated liquids is well-proven with a clear focus on its ability to kill microorganisms and cancer cells. Despite these experimental findings and the resulting estimation of several biological and medical applications (Kaushik *et al.*, 2019), their real practical benefit has to be clarified yet. One option is its use as disinfectants or antiseptics (Kramer *et al.*, 2015). Therefore, clear advantages in comparison to conventional liquid disinfectants and antiseptics have to be demonstrated also taking into account economical aspects. However, a very promising application is the use of plasma-treated liquids for abdominal lavage in the case of disseminated tumors as a result of intraperitoneal metastasis of abdominal cancers (Kajiyama *et al.*, 2014). Because of its spreading inside the abdomen, both surgical eradication and radiation treatment is impossible and lavage using chemotherapeutic drugs causes severe side effects. First very promising animal studies confirm this therapeutic approach of using plasma-treated liquid (Utsumi *et al.*, 2013; Liedtke *et al.*, 2017; Freund *et al.*, 2019). However, much more research has to be done before a clinical application will be possible. One of the key questions is the classification of such plasma-treated liquids from a regulatory point of view.

For an effective application, e.g., for intraperitoneal lavage comparable to hyperthermic intraperitoneal chemotherapy (HIPEC) (Valle *et al.*, 2016), several liters of plasma-treated liquid are needed. Previous experiments are mostly based on small volumes of a maximum of several milliliters. Therefore, technical question of effective and economic treatment of larger liquid volumes is a question to be solved. It is also possible to store smaller volumes of plasma-treated liquid by refrigeration and to pool it before application because it was demonstrated that plasma-treated liquid can be stabilized at lower temperatures (Ikawa *et al.*, 2016).

Despite several questions that are unsolved yet, plasma treatment of liquids to generate, modify, or stabilize biologically effective components could open a very interesting field of CAP application which can be called "plasma pharmacy" to differentiate it from plasma medicine which means the direct application of CAP for therapeutic purposes (von Woedtke *et al.*, 2013b; Joslin *et al.*, 2016).

VII. PLASMA APPLICATION IN COSMETICS: TWILIGHT ZONE OR PROMISING APPLICATION FIELD?

The continuing success and visibility of plasma applications in medicine, specifically in dermatology, exerts growing attraction to the field of cosmetics, too. There are a large number of proposals in the World Wide Web that offer promising effects of plasma application for corrective treatments and skin improvement, some of them explicitly referring to plasma medicine (Crofford, 2019). However, up until now, this field appears very unclear, not really regulated and only badly supported by systematic research. Therefore, it should be an important task for the plasma medicine community to monitor this special field of plasma application to see where experiences from plasma medicine can be used for cosmetic applications, but also to define where demarcations are necessary to avoid harmful consequences for plasma medicine.

As in plasma medicine, also in cosmetic applications plasma is used directly on the human body. Moreover, the idea of application of plasma-treated liquids is present in cosmetics, too. The main difference might be the intention of plasma application. According to the U. S. Federal Food, Drug, and Cosmetic Act (FD&C Act), cosmetics are "articles intended to be rubbed, poured, sprinkled, or sprayed on, introduced into, or otherwise applied to the human body…for cleansing, beautifying, promoting attractiveness, or altering the appearance" [FD&C Act, sec. 201(i)] (FDA, 2018a). Furthermore, cosmetic devices are defined by the U. S. Food and Drug Administration as "devices that are used to improve appearance and do not impart any health benefits" (FDA, 2018b).

However, one must be aware that the decision what "health benefits" are is not that easy and clear and that there are smooth transitions between "therapeutic use" as it is intended with plasma medicine devices and "improvement of appearance" as it has to be intended with plasma cosmetic devices. A first approach to simplify this differentiation could be to state that plasma medical applications are targeted at harmed skin and also deeper tissue layers, whereas cosmetic applications are targeted at more or less intact skin and body surface or restricted to the upper dermal layers, respectively (Fig. 3).

However, this definition cannot be that tight because some plasma application on intact skin can have medical indications (e.g., skin disinfection and antisepsis), whereas some applications that are claimed to be cosmetic affect harmed skin (e.g., acne treatment). Additionally, there is no doubt that some cosmetic treatments also have therapeutic impact (e.g., from a psychological point of view) and primary medical therapies may have cosmetic effects. Only these short remarks should demonstrate that a strict separation between plasma medicine and plasma cosmetics is not easy and probably impossible.

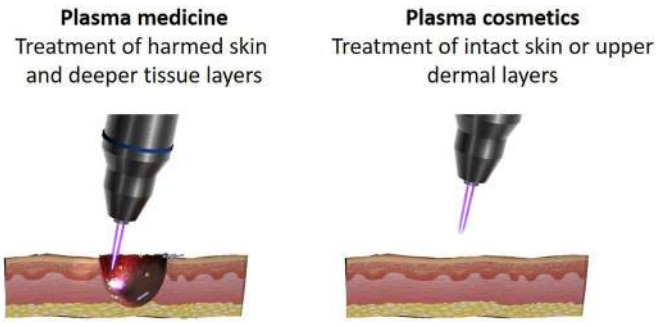

FIG. 3. Preliminary differentiation between plasma medicine and plasma cosmetics by the target to be treated; several overlapping between medical and cosmetic treatments is possible.

Therefore, the best way should be to coordinate both fields of plasma application to make it successful here and there and to avoid conflicts that are eventually disadvantageous for patients and customers. In November 2019, the First International Meeting on Plasma Cosmetic Science (IMPCS-1) was held in Orléans, France, which highlighted the importance of establishing a strong scientific foundation for plasma application in cosmetics (http://www.lestudium-ias.com/event/international-meeting-plasma-cosmetic-science).

A first step for such coordination has to be the systematization of cosmetic plasma devices and their intended use in a similar way as it is necessary in plasma medicine. An important field of cosmetic indications of plasma treatment is focused on skin tightening, wrinkle removal, face and body lifting, skin rejuvenation, blepharoplasty (tightening of eyelids), etc. It seems that these plasma effects are mainly based on thermal impacts. To the best of our knowledge, the most described and investigated plasma device for cosmetic and esthetic applications is the nitrogen plasma-based jet-like Portrait® PSR^3 system (Kilmer *et al.*, 2007; Bogle *et al.*, 2007; Wade Foster *et al.*, 2008; Holcomb *et al.*, 2009; Pourazizi and Abtahi-Naeini, 2017). It is mainly applied for skin regeneration. Its effect is based on rapid energy transfer to the skin surface by plasma application resulting in instantaneous heating in a controlled, uniform manner without explosive effect on tissue or epidermal removal. No tissue vaporization occurs. Nowadays, several plasma cosmetic applications are based on needle-like devices where an electrical arc is generated between the needle-tip and the skin causing a "sublimation" of the fluid in superficial parts of skin avoiding heat transmission to the adjacent tissue regions. An immediate result of this treatment are dark spots on the skin as a result of tissue carbonization that resolve during the next days. Main application fields of these devices are also blepharoplasty and skin tightening (Sotiris *et al.*, 2014; Gloustianou *et al.*, 2016; King, 2017). Even if it is stated that tissue heating is drastically reduced in comparison to laser application for the same indications, it has to be pointed out that any reference to plasma medicine above all with respect to safety is illegitimate because nearly all recent investigations in plasma medicine are based on non-thermal plasma-cell and plasma-tissue interactions. Moreover, it seems really strange that such kind of manipulations on skin and above all in close proximity to the eyes with such arcing devices are allowed to be done by non-medical personnel within pure cosmetic settings if one takes into account what high safety standards are demanded for medical plasma applications. Here, a "twilight zone" of plasma applications on human body seems to exist that should be much more regulated and restricted. Otherwise, with a continuing non-critical application of plasma devices in the field of cosmetics and its direct referring to plasma medicine, there is the real danger that any negative events will shed a bad light on medical plasma applications.

Besides these more stringent investigations and possibly regulations of cosmetic plasma devices whose effectiveness is based on thermal impact, it should be an additional aim of research for the next years to decide if and to what extent CAP is useful for cosmetic applications. There is a single report on a DBD-based CAP device for acne treatment and esthetic skin improvement tested in a clinical setting (Chutsirimongkol *et al.*, 2014). However, as it has been discussed before it has to be decided if acne treatment is a medical or a cosmetic indication.

Another effect that is sometimes mentioned within complex cosmetic treatment procedures including CAP application is its potential ability to enhance skin permeability and to enhance the penetration of substances via the very strong barrier layer of the *Stratum corneum* into deeper skin layers (Lademann *et al.*, 2011a, 2011b; Kalghatgi *et al.*, 2015; Shimizu *et al.*, 2015; Gelker *et al.*, 2018; Kristof *et al.*, 2019). Even if these experimental data may open a promising field of CAP application also in cosmetics, its usability *in vivo* has to be proven yet because only a few data on this effect on living skin are available (Choi *et al.*, 2014). Above all, some more research is needed to identify exact mechanisms of skin permeabilization by CAP, its dependence on specific plasma parameters as well as its limitations with regard to other characteristics of molecules or particles like size or hydrophilicity/hydrophobicity.

VIII. SUMMARY, CONCLUSIONS AND OUTLOOK

In April 2012, a workshop on "Clinical Concepts in Plasma Medicine" was held in Greifswald, Germany, where all German research groups that were active in the field of plasma medicine at that time participated. In a resulting paper of consent, it was stated that due to the then-status quo in clinical research, plasma treatments in dermatology as well as plastic and esthetic surgery have the best prospect to succeed, whereas the use of antimicrobial plasma effects, plasma supported stimulation of tissue regeneration, and inflammation modulating plasma effects will be in the focus of therapeutic indications. Consequently, as first promising application areas, the support of the healing process with focus on the treatment of chronic wounds, the treatment of infected skin diseases, and the treatment of dermatitis were identified (Emmert *et al.*, 2013b). However, looking back today, this prediction was more or less fulfilled. Cold atmospheric plasma is on its way to clinical routine. First CAP devices have received CE certification as medical devices class IIa for treatment of chronic wounds as well as pathogen-based skin diseases. Based on this success story, additional medical CAP applications are under research, whereas plasma application in cancer treatment is the most promising but also the most challenging field. To establish plasma medicine as part of clinical practice, close cooperation between clinical research and laboratory basic research is essential. A continuing biological research to better understand and eventually control and optimize medically useful plasma applications must go hand-in-hand with constant improvement in plasma device technology.

A critical task in this connection is an international harmonization of methods and criteria to characterize plasma devices with regard to both their physical and technical parameters as well as their biological performance characteristics to allow a better comparability. An international standardization is considered to be an important stimulus to trigger the interest of industry and subsequently to allow a larger breakthrough of plasma devices in medicine.

Nowadays, we have more or less "static" CAP devices working with fixed settings of working parameters like input power and working gas. Medical treatments are mainly controlled via treatment time. There are no feedback mechanisms taken into consideration, yet, neither with respect to changes in plasma parameters caused by variations of the target nor to biological effects as a result of the plasma treatment.

Therefore, the next important step should be to upgrade CAP treatment devices to treatment systems including not only a CAP generating device but also technical units for continuous and real-time

monitoring of both plasma performance and the target that is treated (Fig. 4).

In such an integrated therapy system, the plasma treatment unit should be controllable according to the actual demands of treatment. Any changes of plasma parameters are adjusted based on a continuous monitoring of both electrical parameters of the device and the plasma itself. Moreover, these signals might also be used to get information about changes of the target resulting from or occurring during the plasma treatment. For the different plasma-generating technologies (DBD, plasma jet), variable and specifically adapted solutions have to be developed to realize such feedback regulation.

In addition to this device-orientated control, an innovative target-orientated control will be helpful to optimize CAP-based medical treatments. Currently, CAP treatments take place without any possibility to register if there was sufficient plasma-tissue interaction to realize the intended medical effect. Currently, first efforts have been made to visualize immediate and short-term physiological effects, specifically hemodynamic parameters resulting from complex plasma-tissue interactions by hyperspectral imaging techniques (Rutkowski *et al.*, 2017; Daeschlein *et al.*, 2018; Kulcke *et al.*, 2018). The next step will be the use of more sophisticated spectroscopic methods to visualize biochemical effects on a molecular tissue level which will be based on the dominating role of redox processes in plasma- tissue interaction (Meyer *et al.*, 2019).

The realization of such integrated CAP therapy systems demands the development of compact and miniaturized tools for device, plasma, and target monitoring to equip clinically applicable CAP devices. Bringing together all these different signals in a data processing and control unit, a direct feedback regulation should be the result. Here, innovative methods of machine learning and artificial intelligence have to be used to realize such complex data analysis, data processing, and feedback regulation (Mesbah and Graves, 2019; Meyer *et al.*, 2019). Finally, as a most advanced version of such a CAP therapy system, it will be part of a robotic system for precise data-based CAP positioning and treatment control (McKinney *et al.*, 2019).

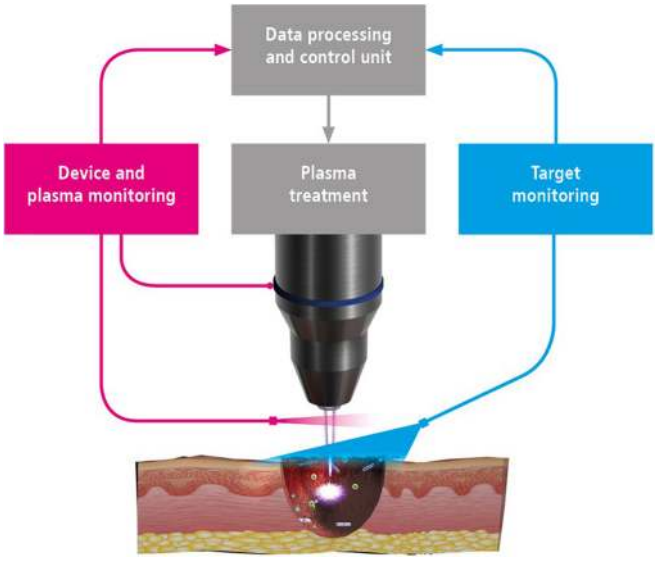

FIG. 4. Schematic picture of an integrated CAP-based therapy system including plasma treatment device, plasma monitoring and target monitoring unit as well as unit for feedback control of plasma treatment.

Currently, improved knowledge about molecular processes of wound healing is also a result of the strong scientifically based progression of plasma medicine from the beginning. Consequently, plasma medicine was able to create benefits also beyond its actual field of research. This kind of interdisciplinary knowledge gain will be accelerated with a closer interconnection of plasma medicine with redox biology (von Woedtke *et al.*, 2019). Moreover, such mutual profiling in different fields by interconnections can also be expected if plasma medicine is able to use innovative techniques of monitoring, data processing, machine learning, and robotics.

In the 20th century, laser was a physical technology that was integrated very successfully into medicine, creating its own medical field of expertise called laser medicine. Plasma has the chance to repeat this successful integration of a physical technology into medicine to be in the fore of scientific and technological development in medicine of the 21st century.

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DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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