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Plasma medical oncology: Immunological interpretation in head and neck squamous cell carcinoma

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List of Abbreviations

CAF	Cancer-associated fibroblasts
CAP	Cold atmospheric plasma
° C	Centigrade
cm^2	Square centimetre
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
EBM	Evidence-based medicine
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papillomavirus
INF-γ	Interferon-gamma
MAMPs	Microbe-associated molecular patterns
min	Minute
mm	Millimetre
MDSCs	Myeloid-derived-suppressor cells
NK-cells	Natural killer cells
PD-L1	Programmed cell death ligand 1
PD-1	Programmed cell death protein-1
slm	Standard litre per minute
TME	Tumour microenvironment
TNF-α	Tumour necrosis factor-alpha
UICC	Union for International Cancer Control

1. Introduction

Over the past several years, various important articles focusing on cancer therapy approaches in head and neck squamous cell carcinomas (HNSCCs) using cold atmospheric plasma (CAP) have been published (SEMMLER et al. 2020 [53], METELMANN et al. 2018 [44], KEIDAR et al. 2011 [33]). This doctoral thesis presents selected results from a prospective observational clinical study in CAP therapy of palliative HNSCC patients, carried out at the Department of Oral and Maxillofacial Surgery/Plastic Surgery of the Greifswald University Medicine. For oral and maxillofacial surgeons, ulcerated surfaces of locally advanced head and neck squamous cell carcinomas (UICC IV) offer a challenging treatment assignment with microbial contamination and tumour progression. The clinical attempt appears to eradicate microbial contamination and to initiate tumour regression. This doctoral thesis will describe the processes of human tumour biology and tumour immunology in HNSCCs and the extent of present knowledge concerning plasma medical oncology as an anticancer modality. In the introduction of the doctoral thesis clinical results of plasma therapy in locally advanced HNSCCs (UICC IV) are set out. This mainly includes the investigation of a therapeutic concept, the treatment phases, the tumour size development and the morphological changes of the infected tumour surface following cold atmospheric plasma therapy. In the main part, a detailed immunological interpretation is proposed on the basis of present preclinical and clinical immunological knowledge. Finally, unexplored questions in plasma medical oncology are highlighted. This is highly significant for future plasma research and clinical anticancer therapy.

1.1 Head and Neck Squamous Cell Carcinoma

Head and neck squamous cell carcinomas (HNSCCs) originate in the epithelial cells of the mucosal linings of the oral cavity, nasal cavity, pharynx, larynx and paranasal cavities (LEEMANS et al. 2018 [37]) (Fig. 1). It is the most common malignant histological variant (90%) of the head and neck region. Approximately 600 000 cases are newly diagnosed annually worldwide (FERLAY 2015 [21]). Despite innovative advances in treatment, the morbidity and mortality in advanced-stage HNSCC (stage IV, UICC) remains high. The overall 5-year survival rate of HNSSC is 65.9% (PULTE and BRENNER 2010 [47]), and for stage IV it is only 4-25 % (LEFEBVRE 2005 [38]). The prognosis in advanced-stage HNSCC just under palliative treatment remains poor with a median overall survival time of 5.1 months (Ferris et al. 2016 [22]).

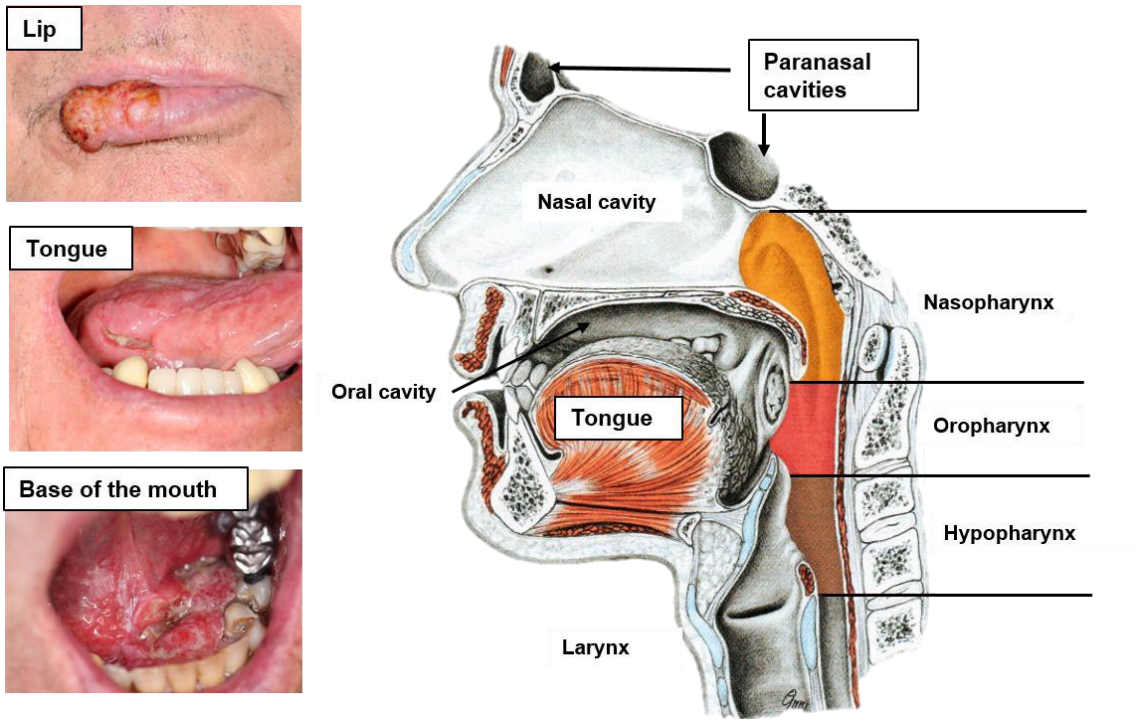

Fig. 1: Anatomical sites of head and neck squamous cell carcinoma on clinical macrophotographs by the Department of Oral and Maxillofacial Surgery (Greifswald University Medicine, Chairman: Prof. Dr. Dr. Hans-Robert Metelmann (anatomic chart by TILLMANN 2005 [54]).

Incidences vary significantly depending on their geographic localisation. Specific etiological agents and a variety of molecular changes cause the transformation of healthy epithelial cells into squamous cancer cells. Here, the classical risk factors are excessive tobacco (smoking, chewing habits), alcohol abuse and an infection with high-risk human papillomavirus (HPV). Based on the etiological risk factor HNSCC could be classified (Fig. 2) as **HPV-negative** (80%, induced by alcohol and tobacco) and **HPV-positive** (20%) (LEEMANS et al. 2011 [36]).

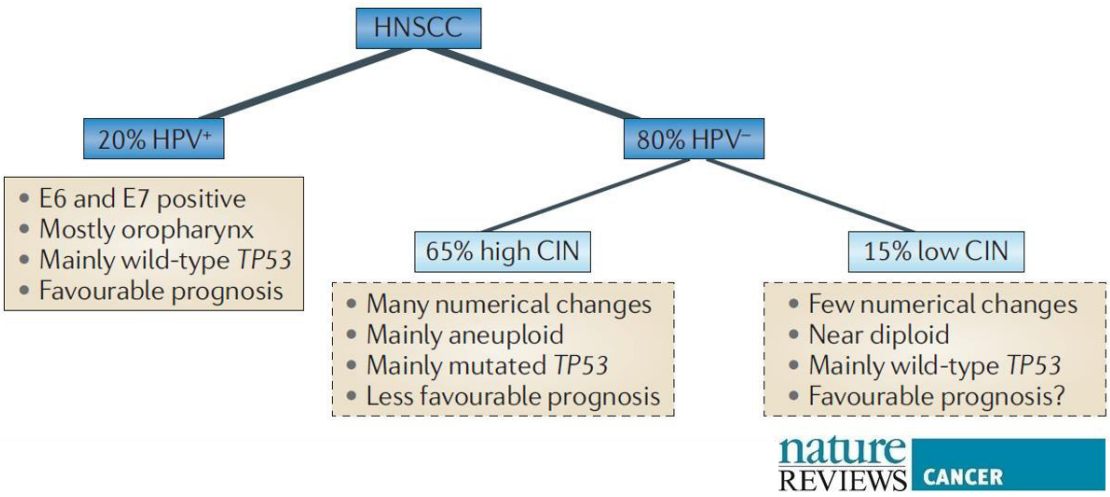

Fig. 2: Etiological classification of HNSCC in HPV$^+$ and HPV$^-$ tumours (LEEMANS et al. 2011 [36]).

1.2 Current state of tumour biology and tumour immunology in HNSCC

The transformation of healthy epithelial cells into squamous cancer cells is initiated through the aberrant activation of oncogenic pathways and the downregulation of tumour suppressor genes, combined with aberrant immune responses and altered homeostasis in the TME, caused by specific etiological agents (e.g. tobacco, alcohol, HPV) and genetic factors (JOYCE and POLLARD 2009 [30], QUAIL and JOYCE 2013 [48]). As schematically shown in Figure 3, tumour cells are embedded into the tumour microenvironment (TME) which consists of a network of stromal cells, own blood and lymphatic vessels, infiltrating immune cells, cancer-associated fibroblasts (CAFs), cytokines and chemokines (FRIDMAN et al. 2012 [26]).

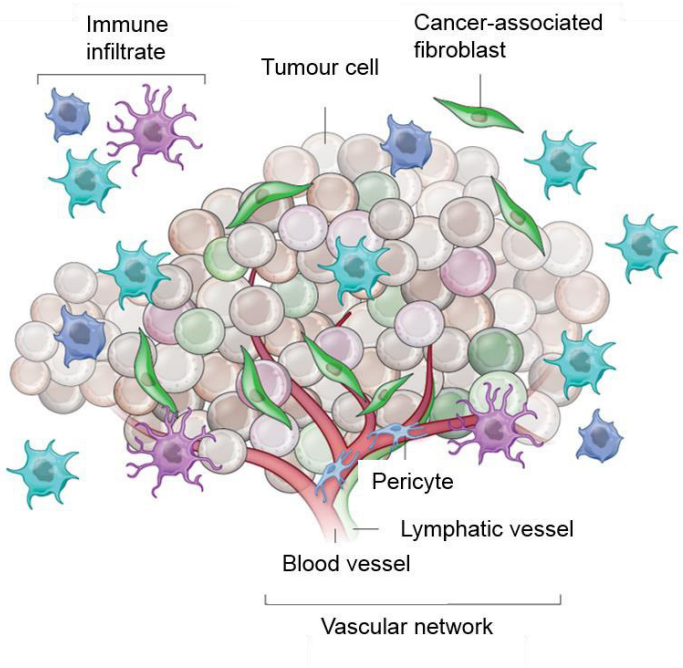

Fig. 3: Schematic depiction of the tumour core embedded in a network of blood- and lymphatic vessels as well as infiltrating immune cells (JUNTTILA und DE SAUVAGE 2013 [31]).

Previous research has shown that the bidirectional communication between cells in the TME is a key factor in normal tissue homeostasis as well as tumourigenesis (QUAIL and JOYCE 2013 [48]). In general, the composition of the TME and especially the immune system play an important role in the cellular biology of cancer (SCHREIBER et al. 2011 [52]). Paul Ehrlich was one of the first researchers who postulated that the cells of the immune system are able to eradicate cancer cells and prevent primary tumour growth (BLAIR and COOK 2008 [15]) under physiological conditions.

Taken together, the current state of **immune-associated eradication of tumour cells** can be summarized as follows (QUAIL and JOYCE 2013 [48], FRIDMAN et al. 2012 [26], FRIDMAN et al. 2017 [27], DUNN et al. 2002 [18]):

• **Natural killer cells** (NK-cells) are able to detect and damage cancerous cells.
• **Dendritic cells** activate cytotoxic T-cells.
• **Cytotoxic T-cells** and **NK-cells** release two separate cytotoxic proteins: (i) perforin that binds to the cellular membrane to form pores for (ii) granzymes to enter tumour cells causing apoptosis.
• **T helper cells** (TH1) release interleukin (IL)-2 and interferon gamma (INF-γ) to recruit and activate more NK-cells to eradicate cancer cells.
• Tumour-suppressive **macrophages** of the extracellular matrix inhibit the formation of a primary tumour. In general, macrophages can alter their polarization state and be classified as either **M1** (pro-inflammatory, anti-tumourigenic) or **M2** (anti-inflammatory, pro-tumourigenic). Cytokines such as interferon gamma (INF-γ) and tumour necrosis factor alpha (TNF-α) secreted by surrounding immune cells contribute to anti-tumourigenic M1-polarization.
• **Fibroblasts** promote haemostasis and inhibit tumour growth.
• **Pericytes** in the vessels act as gatekeepers in terms of primary tumour progression. The secretion of INF-γ initiates a cascade of immune responses whereby the angiostatic chemokines CXCL10 (IPI0), CXCL9 (MIG) und CXCLII (I-TAC) are released and inhibit neovascularisation.

Figure 4 depicts the cellular anti-tumourigenic defence mechanisms within the TME.

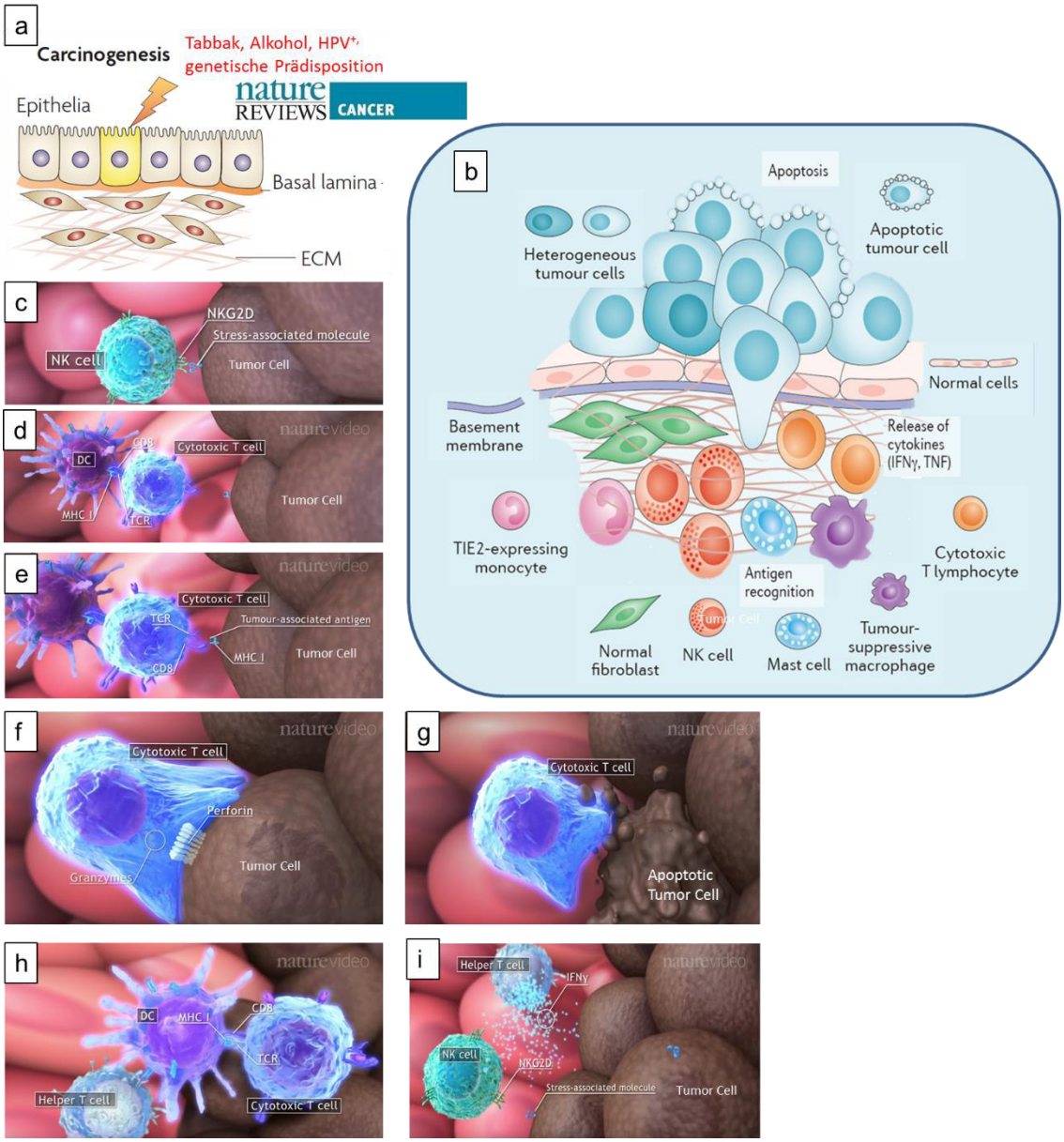

Fig. 4: Model of carcinogenesis on cellular level and cellular anti-tumourigenic defence mechanisms within the TME.
a: Transformation of a healthy epithelial cell into a tumour cell (ALBINI und SPORN 2007 [1]).
b: Schematic model of tumour, stromal, and immune cells within the TME (QUAIL und JOYCE 2013 [48]).
c: Eradication of tumour cells by NK cells (Nature Video) [46].
d: Activation of cytotoxic T-cells by dendritic cells (Nature Video) [46].
e: Binding of the cytotoxic T-cell to the tumour cell (Nature Video) [46].
f: Secretion of granzymes by cytotoxic T-cells and integration of perforin in the tumour cell membrane (Nature Video) [46].
g: Induction of tumour cell apoptosis by cytotoxic T-cells (Nature Video) [46].
h: Activation of cytotoxic T-cells by dendritic cells by T helper cells (Nature Video) [46].
i: Release of INF-γ by T helper cells (Nature Video) [46].

The literature shows, that immune cells (such as macrophages) and non-immune cells in the TME (such as fibroblasts) have a high degree of plasticity and functional diversity. At both extremes, they can accommodate either anti-tumourigenic or pro-tumourigenic effects (QUAIL and JOYCE 2013 [48]). Figure 5 depicts the cell populations in the TME that can promote either anti-tumourigenic or pro-tumourigenic effects during tumourigenesis and tumour development.

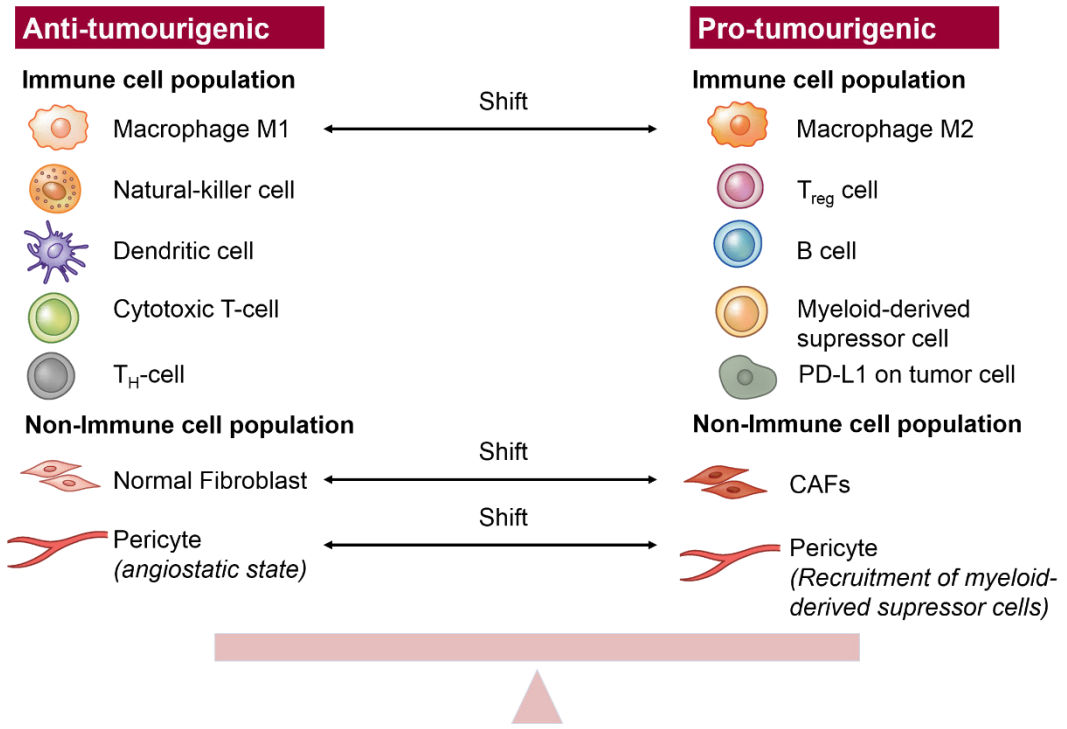

Fig. 5: Cell populations in the TME with distinct functions during tumourigenesis and tumour development (Images of the cells adopted from QUAIL and JOYCE 2013 [48]).

The current **concept of tumourigenesis** is explained by the model of cancer immunoediting, based on the immunosurveillance hypothesis by Burnet and Thomas and confirmed by investigations in vivo and clinical observations (DUNN et al. 2002 [18], QUAIL & JOYCE 2013 [48]).

According to this concept, the immune system plays a dual role in cancer (SCHREIBER et al. 2011 [52]). On the one hand, the immune system is capable of eradicating tumour cells; on the other hand, it can establish conditions within the TME that promote tumour escape from immune destruction (DUNN et al. 2002 [18]).

Here, solid tumours establish an immunosuppressive microenvironment (Fig. 6), partially via reprogramming of host cells such as monocytes into M2 macrophages (pro-tumourigenic) and fibroblasts into cancer-associated fibroblasts (CAFs, pro-tumourigenic) (FRANKLIN et al. 2014 [23], LIAO et al. 2018 [39]). Unfortunately, clinical studies of

HNSCC revealed that mortality positively correlated with the number of CAFs in the TME (BELLO et al. 2011 [13], VERED et al. 2010 [57]). Another mechanism which involves the escape of tumour cells from adaptive immune responses by high surface expression levels of programmed cell death-ligand 1 (PD-L1) has been described. PD-L1 binds to programmed cell death protein-1 (PD-1) on cytotoxic T cells, whereby the cytotoxic T- cell receives an inhibitory signal. Here, a remarkable decrease in T-cell-mediated tumour cell lysis is reported. For more details on this topic, see ESCORS et al. 2018 [20]. Other approaches of immunosuppression include the recruitment of myeloid-derived-suppressor cells (MDSCs) by pericytes, leading to immunosuppression of anti-tumour effector cells via (a) the promotion of angiogenesis, (b) the malfunctioning of antigen presentation by dendritic cells, (c) the inhibition of NK cells, (d) the inhibition of the T-cell activation, and (e) the reduction of M1 macrophages (QUAIL & YOYCE 2013 [48]).

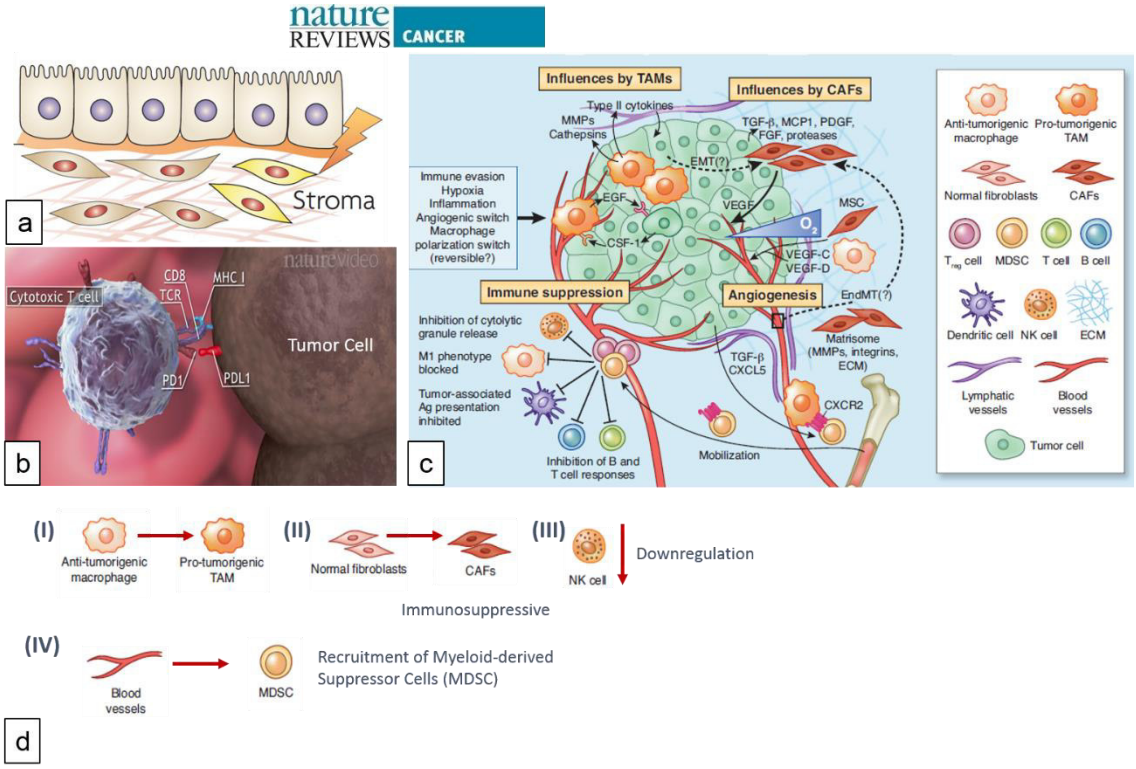

Fig. 6: Mechanisms of tumour growth.
a: Modification of stromal cells in the TME (ALBINI und SPORN 2007 [1]).
b: Model of pro-tumourigenic mechanisms in the TME (QUAIL und JOYCE 2013 [48]).
c: Inhibition of cytotoxic T-cells by PD1-PDL1 blocking (Nature Video) [46]).
d: Specific modifications in the TME promoting tumourigenesis (Images of the cells adopted from QUAIL and JOYCE 2013 [48]).

1.3 Plasma Medical Oncology

1.3.1 Therapeutic concept of plasma medical oncology in Greifswald

Cold atmospheric plasma is supplied by the plasma jet device kINPen® MED (neoplas tools GmbH, Germany, Greifswald, Fig. 7). The kIN Pen® Med is a portable plasma jet device. It consists of a hand-held unit, a direct current power unit and an Argon gas reservoir that discharges cold plasma under atmospheric conditions. The maximum temperature of the plasma jet is 38 °C. The working gas is delivered with a flow rate of 3 to 5 slm. For further details on the physics and the plasma chemistry of the kINPen® MED, see Reuter et al. (2018) [49].

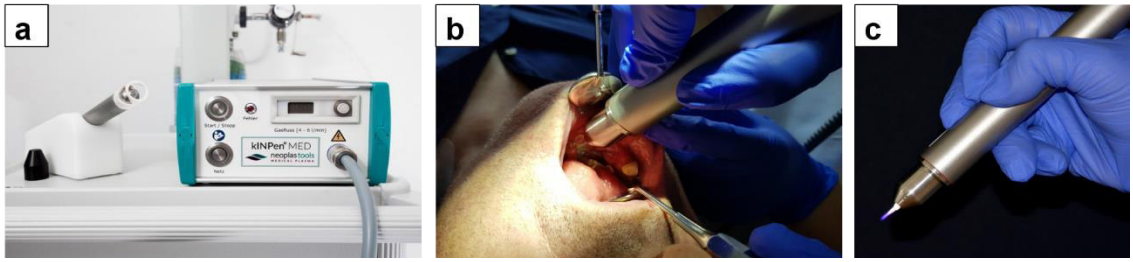

Fig. 7: Plasma jet kIN Pen® Med. (a) Portable cold atmospheric pressure plasma jet device with a hand-held unit, power unit and an Argon gas reservoir (kIN Pen®, neoplas tools GmbH, Greifswald, Germany). (b) CAP-treatment of a patient with a wound healing deficit at the base of the mouth after HNSCC resection. (c) The effluent plasma extends approximately 10 mm from the ceramic capillary.

The plasma treatment parameters are listed in Table 1.

Tab. 1: Plasma treatment parameters (from METELMANN et al. 2018 [44]).

Duration of one therapy cycle	1 week including three single plasma applications
Duration without plasma therapy	1 week
Treatment time	Approximately 1 min/cm^2
Vertical distance jet nozzle-tumour tissue	8 mm
Palliative care (including the use of wound dressings)	Continued

1.3.2 Clinical long-term results of plasma medical oncology in HNSCC

The prospective observational study by METELMANN and colleagues [44] aimed to assess the therapeutic effect of plasma treatment in locally advanced HNSCC (UICC IV) patients who suffered from contaminated tumour ulcerations with no lasting remission following curative tumour treatment. The study focused on palliative care was conducted in accordance with the ethical standards of the institutional and national research committee as well as the Declaration of Helsinki from 1964 and its later amendments. Between January 2015 and March 2017, recruitment, treatment and follow-up of all patients were performed at the department of oral and maxillofacial surgery of the Greifswald University Medicine. Patients underwent a staging for oropharyngeal cancer according to the standard guidelines of the Union for International Cancer Control (UICC). A total of six patients, diagnosed with a malignant locally advanced squamous cell carcinoma of the oropharynx (H2-H6) and the naso-oropharynx (H1) enrolled in the clinical trial and were treated with cold atmospheric plasma supplied by the plasma jet device kINPen® MED.

The mean age of all patients was 62.3±11.4 years (mean±s.d.). The demographical data and the clinical outcome of the six patients (H1-H6) in the study are listed in Table 2.

Tab. 2: Demographical and tumour-specific data for head and neck cancer patients (modified from METELMANN et al. 2018 [44]).

ID#	Age (years)	Gender	Main etiological factor	TNM-classification/ stage	Effect on tumour growth	Effect on contamination	Side effects	Effect on pain medication
H1	76	f			NR	ROO	E, P,	No
H2	55	f			PD	ROO	E, S, DM, (B)	LD
H3	78	m			PD	NR	EON, (B), DM	No
H4	56	f		pT4	PD	ROO	No	LD
H5	53	m	tobacco		PR	ROO		LD
H6	56	m			PR	ROO	No	LD

n=six patients; HPV, human papillomavirus; py, pack years; Tumour stage of HNSCC according to the Tumour-Node-Metastasis (TNM) staging system of the Union for International Cancer Control (UICC), refers to TNM stage at the time of study inclusion; Effect on contamination, abbreviations after CAP treatment: NR, No response; PD, Progressive disease; PR, Partial remission; ROO, Reduction of odour; NR, No reduction; CAP Side effects abbreviations: E, Exhaustion; P, Pain; S, Sialorrhea; DM, Dry mouth; B, Bleeding, EON, Oedema of the Neck, Effect on Pain Medication following CAP treatment abbreviation: LD, Less demand;

1.3.2.1 CAP-induced morphological changes of the tumour surface

Figure 8 gives an example of an ulcerated tumour surface in a CAP-responder patient with a large lymph node metastasis of a well to moderately differentiated HNSCC (stage IVb, pT2 pN2c cM0 pL1 pV1 pR2 G2). The ulceration is localized in the carotid triangle on the left side of the neck. The wound is characterized by a defect in the epidermis and the underlying connective tissue. Bacteria, cell detritus and a thick fibrin layer are abundant in the wound. Additionally, a painful erythema around the ulceration is obvious. Interestingly, a significant reduction of the wound area was clearly detectable after CAP application. The wound bed regenerated up to 80% of its original size and an excessive fibrin layer production was reduced. New blood vessels populate the wound area and the migration of epithelial cells was observed (Fig. 8b and c).

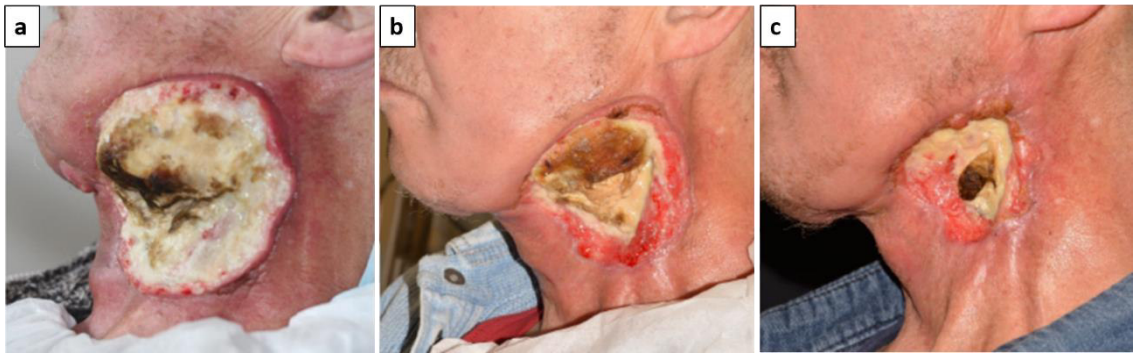

Fig. 8: Macrophotographs of the tumour surface development by CAP treatment in a responder-patient with a large lymph node metastasis of a well to moderately differentiated HNSCC. (a) Tumour without previous CAP treatment. (b) 3-month follow-up of CAP response to the tumour surface. (c) 5-month follow-up. (from METELMANN et al. 2018 [44]).

1.3.2.2 Tumour surface size following CAP therapy

Of the six patients who entered the observation study, one third showed a partial tumour remission under CAP treatment regime. All patients could be confidently identified as either a responder or non-responder to CAP treatment within the first two weeks after intervention.

To illustrate the effectiveness of CAP therapy, six graphical curves of tumour surface size development as a function of time are displayed in Figure 9, showing a difference between CAP-responders and non-responders at the baseline.

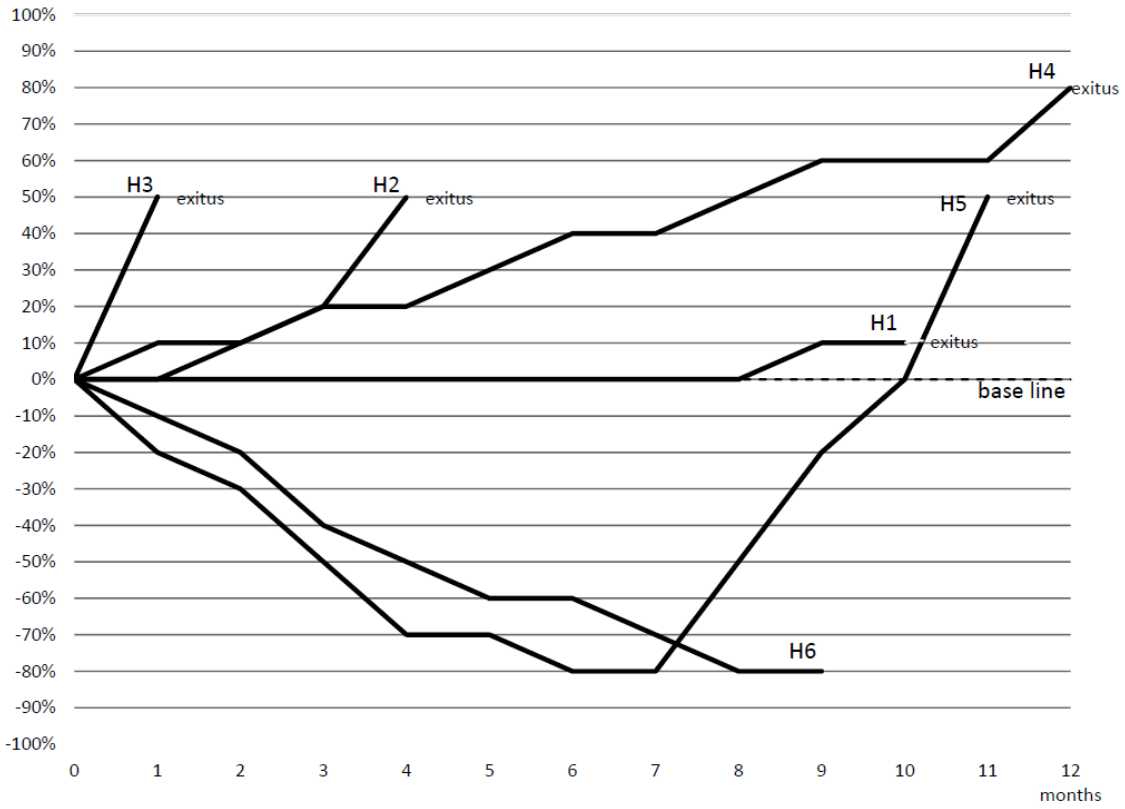

Fig. 9: Tumour surface size development following CAP-therapy as a function of time (from METELMANN et al. 2018 [44]).

In all four patients (H1-H4) that were identified as non-responders, the tumour surface size significantly increased in the course of the treatment. In one patient (H1), there was no evidence of tumour growth at the 7-month follow-up. The patient considered this as an encouraging development. However, in the 8th month, the tumour started to continue growing. The patient gave up and went on to die from HNSCC. In patient H2, the tumour surface growth rate became slower shortly after one month of CAP therapy, but went on to increase in months three and four. In the patient (H3) with an oro-pharynx HNSCC and a tumour surface growth rate of 50% per month, CAP treatment was initiated, but the patient passed away after just one cycle of treatment shortly after inclusion. The tumour surface of patient H4 increased 80% compared to its original size.

Both patients (H5, H6) who were identified as CAP-responders showed an identical rate of decrease relating to the tumour surface, i.e. approximately 10-15% per month compared to the baseline. In the group of responder patients, the ulcerated tumour surface decreased by 80% in size compared to the baseline within seven months (*regressive phase*). Then, however, it must be noted that the effectiveness of the plasma treatment

expired following a minimum *plateau phase*[2] of one month and the tumour returned to the *progressive phase* (METELMANN et al. 2018 [44], WITZKE et al. 2020 [59]).

1.3.2.3 CAP-induced treatment phases

Based on the development of tumour surface size compared to the baseline, the CAP treatment phases in palliative patients with locally advanced HNSCC could be classified as **regressive phase, plateau phase and progressive phase** (Fig. 10) (METELMANN et al. 2018 [44], WITZKE et al. 2018 [58], WITZKE et al. 2020 [59]). Regressive phase means a significant reduction of tumour surface size in the course of CAP treatment. During the plateau phase there is no change of tumour surface size. Progressive phase means an increase of tumour surface area.

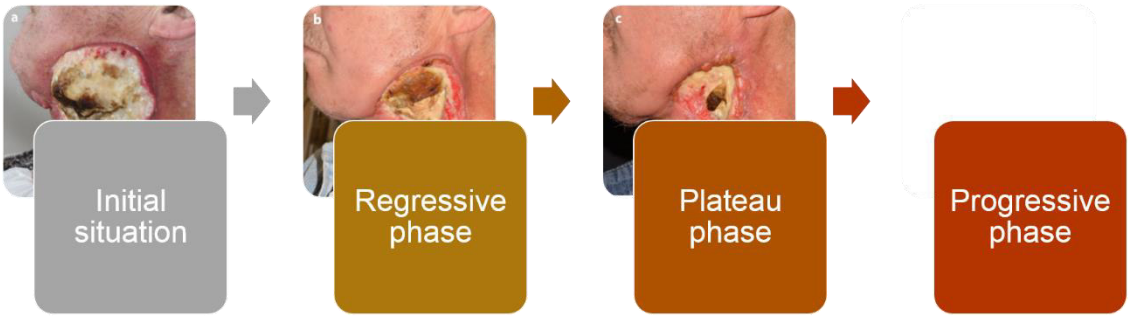

Fig 10: Characteristic treatment phases in HNSCCs in CAP responder-patients (macrophotographs by METELMANN et al. 2018 [44]).

1.3.2.4 Survival time of HNSCC patients under CAP treatment

The prospective study by METELMANN et al. (2018) [44] had a median survival time of 7.4 months (H1-H5) between the date of study inclusion and the date of exitus letalis. A total of five patients died. One of the patients (H6) from the pilot study was still alive at the study end date. Figure 11 shows the survival time of HNSCC patients under CAP-therapy.

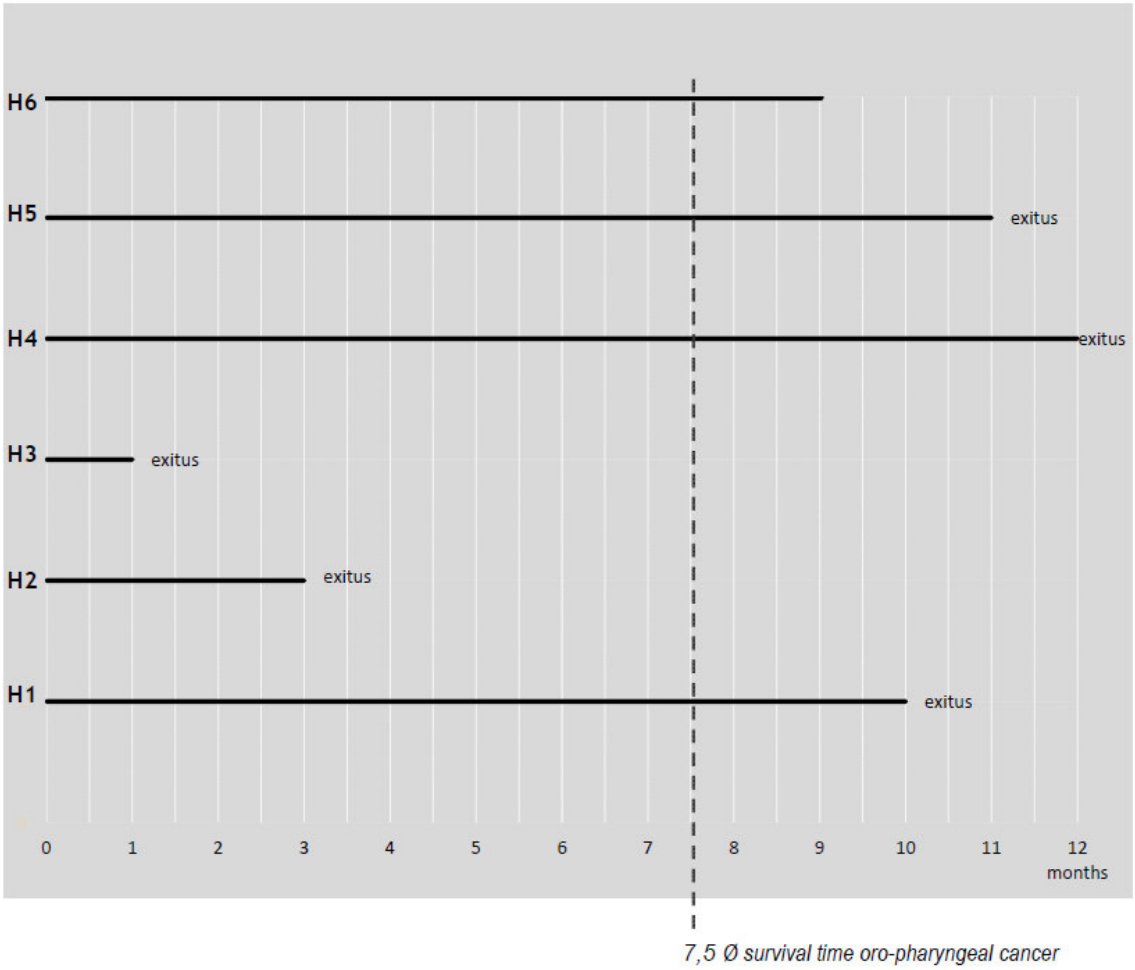

Fig. 11: Survival time of HNSCC patients under CAP treatment (from METELMANN et al. 2018 [44]).

2 Material and Methods

This doctoral thesis summarizes the current state of clinical research on plasma medical oncology in head and neck cancer, with special focus on modern scientific knowledge of immunity aspects in the tumour microenvironment. Within the scope of this, the clinical plasma therapy results of the department of oral and maxillofacial surgery of the Greifswald University Medicine were summarized. The amount of clinical results of plasma treatment as anticancer modality in palliative medicine requires a detailed immunological interpretation. This was done using the current literature concerning tumour biology and tumour immunology.

3. Results

The immune system plays a key role in the CAP treatment of cancer patients. There are studies supporting the notion that the plasma treatment affects both immune cells and the immunogenicity of tumour cells. Table 3 and Table 4 summarize the plasma effects on immune cell populations in vitro (WITZKE et al. 2020 [59]).

Tab 3: Plasma effects on the myeloid lineages in vitro (modified from WITZKE et al. 2020 [59]).

Cell type	Function	Plasma effect	References
Myeloid lineages			
Macrophages	M1 anti-tumourigenic, secrete T_H1 cytokines M2 pro-tumourigenic	Upregulation of M1 Macrophages, downregulation of M2 macrophages	LEE et al. 2018 [35], CRESTALE et al. 2018 [17], KAUSHIK et al. 2019 [32]
Dendritic cells	Antigen-presenting cells from the bone marrow, initiate innate and adaptive immune responses for tumour regression; activate cytotoxic T-cells	Recruitment of antigen-presenting cells into tumours No change in intratumoural dendritic cells DC maturation with plasma - treated tumour cells	LIN et al. 2018 [42] LIEDTKE et al. 2018 [40] VAN LOENHOUT et al. 2019 [55]
Neutrophils	Phenotypically plastic, opposing functions in tumour progression	Induction of neutrophil extracellular trap formation with resulting increase of IL-8 release	BEKESCHUS et al. 2016a [5]
Mast cells	Recruited to the tumour to promote tumour angiogenesis	No published data available	-
Myeloid-derived suppressor cells	Suppression of the host immune system	No published data available	-

Tab 4: Plasma effects on the lymphoid lineages in vitro modified from WITZKE et al. 2020 [59]).

Cell type	Function	Plasma effect	References
Lymphoid lineages			
Natural-killer cells (NK)	Anti-tumourigenic; detect and eradicate tumour cells	Non-stimulated cells are sensitive; mitogen-activated cells are robust	BEKESCHUS et al. 2013a [2]
Cytotoxic T-cells	Bind to the MHC-I receptor of cells; release of granzymes and initiate (tumour) cell apoptosis	Non-stimulated cells are sensitive; mitogen-activated cells are robust Enhancement of cytotoxic T-cell infiltration into tumours	BEKESCHUS et al. 2013a [2] MIZUNO et al. 2018 [45]
T helper cells	Subgroups T_H1 (anti-tumorigenic) and T_H2, (pro-tumourigenic), ratio of both lineages correlates with tumour stage and grade	nNn-stimulated cells are sensitive; mitogen-activated cells are robust T_{emra} are most robust to plasma treatment Proliferation of activated cells is not selectively inhibited by plasma; plasma treatment does not lead to proliferation Changes in the redox balance after plasma treatment Increased influx in tumour tissue exposed to plasma	BEKESCHUS et al. 2013a [2] BEKESCHUS et al. 2016c [7] BEKESCHUS et al. 2013b [3] BEKESCHUS et al. 2013c [4] FREUND et al. 2019b [25]
Regulatory T-cells	Primarily pro-tumourigenic by suppressing mechanisms of immunosurveillance; divergent role	No published data available	-
B-cells	Humoral immunity, promote tumour progression by releasing pro-tumourigenic cytokines and altering T_H1 (anti-tumourigenic) and T_H2, (pro-tumourigenic) ratio	Non-stimulated cells are sensitive; mitogen-activated cells are robust	BEKESCHUS et al. 2013a [2]

Evidently, plasma treatment not only affects the viability of different leukocyte subpopulations differentially (BEKESCHUS et al. 2013a [2]), but it also induces molecular and phenotypic alterations in lymphocyte (BEKESCHUS et al. 2016c [7], BUNDSCHERER et al. 2013 [16]) as well as myeloid subsets (FREUND et al. 2019a [24], BEKESCHUS et al. 2016a [5], b [6], 2017a [8], 2018a [10], b [11], SAGWAL et al. 2018 [50]). There is also evidence for the induction of immunogenic cancer cell death in vitro (BEKESCHUS et al. 2018c [12], LIN et al. 2017 [41], BEKESCHUS et al. 2017 b

[9]) and in vivo (LIN et al. 2018 [42], FREUND et al. 2019 b [25], LIN et al. 2019 [43]) by plasma treatment. This type of cell death emerges as an important aspect in the formation of anti-tumour immunity (GALUZZI et al. 2017 [28]). The concept of plasma assistance in anticancer immunity has been postulated recently by KHALILI et al. (2019) [34]. Moreover, METELMANN et al. (2018) [44] have also found evidence of alterations in immune cell infiltrates in plasma-treated tumour tissue of HNSCC patients. Biopsies from the CAP treated HNSCC-tumour site revealed a minor presence of myeloid cells CD11b$^+$ compared to high levels in non-CAP treated patients.

Recent work by LEE et al. (2018) [35] showed that CAP has the ability to alter the polarization state of macrophages. As shown in Figure 12, CAP promotes an upregulation of anti-tumourigenic M1 macrophages and a downregulation of pro-tumourigenic M2 macrophages. Another study by LIN et al. (2018) [42] revealed that plasma treatment leads to a recruitment of dendritic cells into the tumour. In addition, the infiltration of cytotoxic T-cells into tumours is enhanced (MIZUNO et al. 2018 [45]).

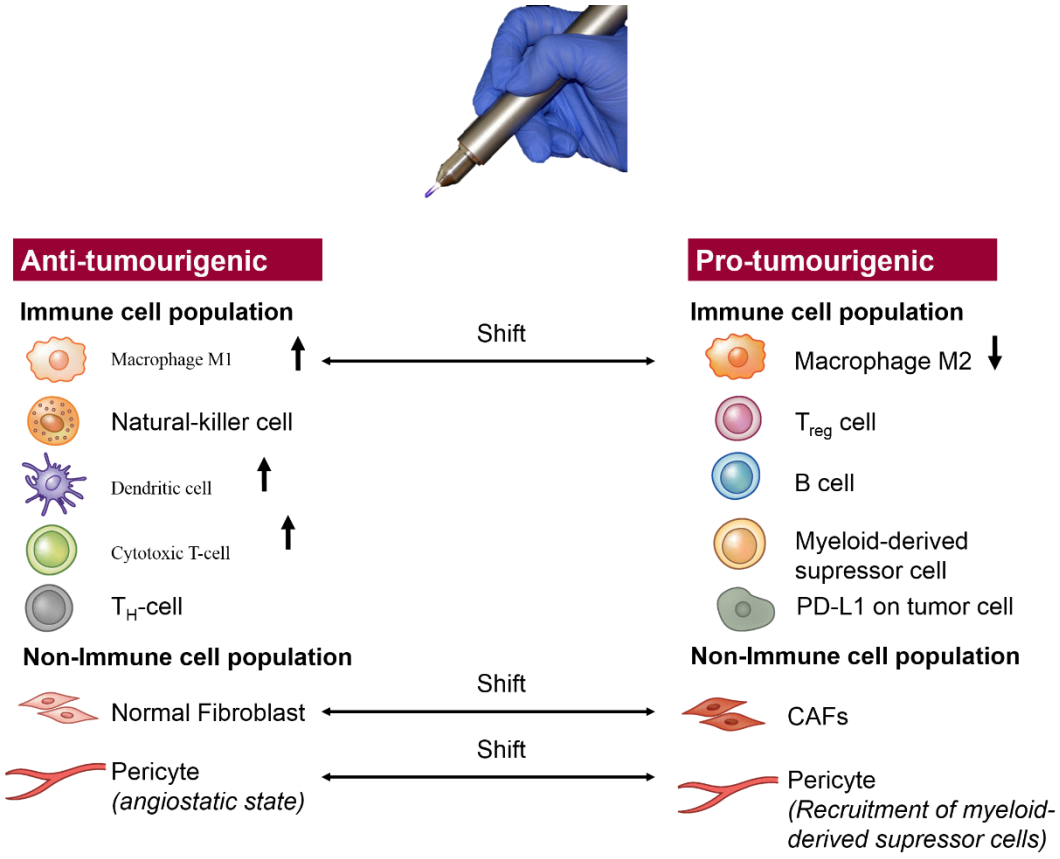

Fig. 12: Effect of plasma gas treatment on the cell populations in the tumour microenvironment (Images of the cells adopted from QUAIL and JOYCE 2013 [48]).

At the same time, it needs to be appreciated that bacteria accumulate and proliferate on tumour surfaces, where they can provide a plethora of stimuli to the immune system via microbe-associated molecular patterns (MAMPs) as reported by DUONG et al. (2019) [19].

In summary, the initial tumour regression in palliative HNSCC stage IV patients under plasma therapy, followed by a steady-state growth and progressive disease could be explained via several different mechanisms that affect tumour cells, the immune system and the tumour microenvironment. There is evidence (Fig.13) for a decrease of tumour cell apoptosis and a low tumour proliferation in HNSCC patients after CAP treatment beginning (METELMANN et al. 2018 [44]), suggesting that these mechanisms predominate in the regressive phase.

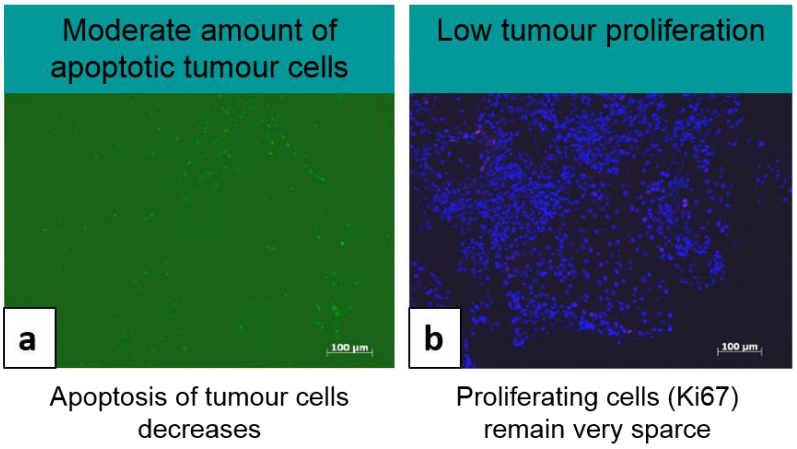

Fig. 13: CAP-specific modification of the tumour microenvironment (a and b) in locally advanced oropharyngeal carcinomas with contaminated ulcerations during regression phase (modified from METELMANN et al. 2018 [44]).

We propose three different mechanisms (Fig. 14) inducing CAP-resistance and disease relapse (WITZKE et al. 2020 [59]):

(I) A first effector mechanism of plasma resistance may be **tumour adaption**. Tumour adaption is supposed to be the principle limiting factor to achieving therapeutic cures in patients suffering from cancer (VASAN et al. 2019 [56]). The CAP, as tumour-eradicating agent, targets the tumour cells within the tumour microenvironment. In general, tumour-eradicating agents put a microevolutionary pressure on cancer cells and promote the growth of cancer cells that are inherently less sensitive to the treatment and withstand the anti-tumour treatment.

(II) A second possible effector mechanism of CAP-resistance is based on **"microbial assistance"**. Bacteria have the capability to initiate anti-tumour immune responses like an activation of macrophages and dendritic cells, which can contribute to an efficient anti-

tumour microenvironment (DUONG et al. 2019 [19]). In terms of plasma medical oncology, several publications have appeared in recent years documenting that plasma treatment has the ability to decrease the microbial burden on tumours (METELMANN et al. 2018 [44], ISBARY et al. 2010 [29]). Once the microbial colonisation is eradicated, an immunostimulant may be lost and pro-tumourigenic immunosuppressive conditions may predominate within the tumour microenvironment.

(III) A third possible mechanism is based on **desmoplastic reaction and wound healing** processes. Under repeated plasma treatments, the relatively soft and infected tissue changes into a comparatively hard and noninfected tumour tissue. Hereby an analogy to fibrotic and wound healing reactions becomes apparent (METELMANN et al. 2018 [44]). In general, both reactions are anti-inflammatory at final stages. In general, the growth of fibrous or connective tissue is a barrier against anti-tumourigenic immune cells and drugs by decreasing angiogenesis and blood flow (BHAW-LUXIMON and JHURRY 2015 [14]).

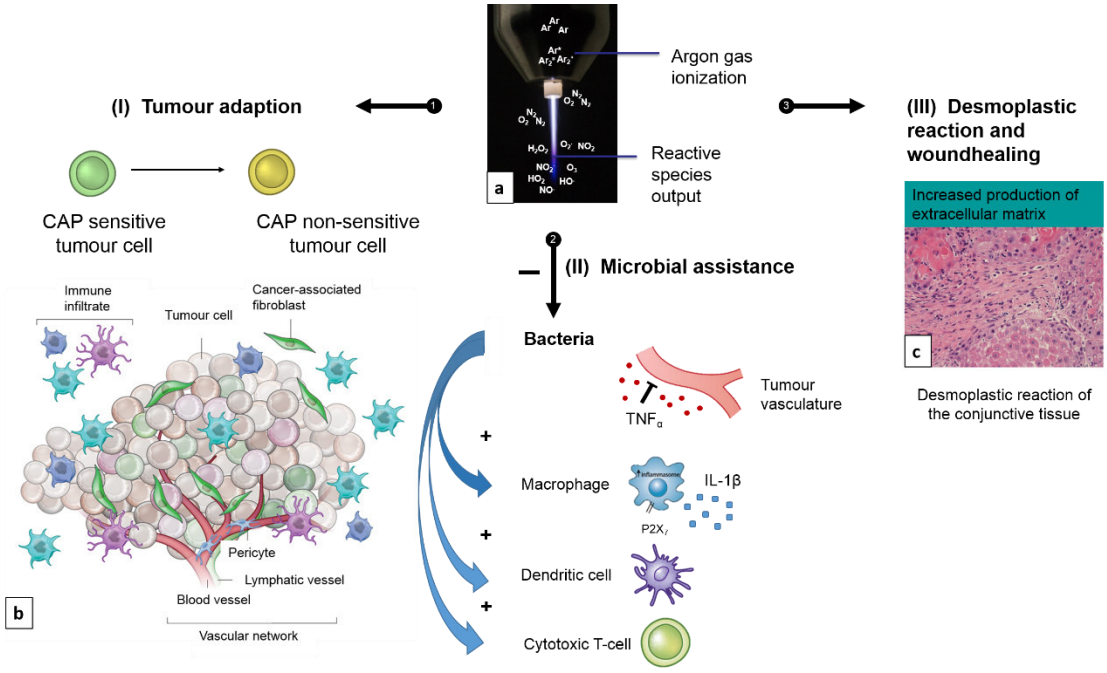

Fig. 14: Possible principles of CAP-resistance in cancer.
a: The effluent plasma and its composition (from WITZKE et al. 2020 [59]).
b: Tumour adaption, depicting the transformation of a CAP-sensitive tumour cell to a CAP non-sensitive tumour cell (figure of the schematic tumour core taken from JUNTTILA und DE SAUVAGE (2013) [31]).
c: Desmoplastic reaction and wound-healing showing an increased production of extracellular matrix following CAP-therapy in a HNSCC (figure taken from METELMANN et al. 2018 [44].

4 Discussion and Outlook

4.1 Clinical results

The 1-year survival time of palliative patients receiving CAP treatment at University Medicine Greifswald was 7.4 months (H1-H5), whereby one patient (H6) was still alive at the study end date, suggesting that the median survival time is even higher. Comparable survival times in palliative HNSCC patients (UICC IV) with CAP therapy do not exist in the literature. FERRIS et al. (2016) [22] studied the median survival in patients with HNSCC (UICC IV) under nivolumab therapy and under standard palliative therapy and found a median survival of 7.5 months for nivolumab therapy and 5.1 months for standard palliative therapy. The results of CAP therapy are generally consistent with the studies in the literature regarding the prognosis in advanced-stage HNSCC just under palliative treatment and under anti–programmed death 1 (PD-1) monoclonal antibody therapy. Admittedly, the literature is very limited regarding survival rates in patients with HNSCC.

4.2 Immunological interpretation

The current state of preclinical and clinical research on cancer therapy includes two different therapeutic approaches. The biochemical-molecular approach using immune checkpoint-inhibitor molecules like Nivolumab (immunotherapy) and the physical approach using CAP (plasma therapy). Recently, plasma application in cancer is moving up the pyramid of evidence-based medicine (EBM) and has reached EBM-level III in head and neck squamous cell carcinoma (METELMANN et al. 2018 [44]). Our experience in clinical observational studies using CAP in HNSCC patients require a detailed immunological interpretation. To the author’s best knowledge, very few publications are available in the literature that address the issue of immunological investigations in HNSCC patients under plasma therapy. TME is composed of both, anti-tumourigenic and pro-tumourigenic immune cell populations and conditions. Multiple cells within the TME are functionally plastic and can alter their polarization state to accommodate either anti-tumourigenic or pro-tumourigenic conditions, suggesting that during the regressive phase following plasma therapy, where the tumour surface size decreases, anti-tumourigenic cell populations and conditions predominate. During the steady state phase, we assume a balance between pro-and anti-tumourigenic cell populations and conditions, and when the tumour returns to progressive disease, we suggest a predomination of pro-tumourigenic conditions. It is hoped that future research

will shed light on the ratio of pro- and anti-tumourigenic immune cell populations and conditions during the regressive, steady state and progressive phase in HNSCC patients in detail. And also, making one-person trials happen - which is a key component in the direction of precision medicine (SCHORK 2015 [51]).

4.3 Outlook

The present developments in plasma medical oncology look particularly promising. To further validate the cellular and molecular modifications taking place during the course of plasma treatment, biopsies of responder patients are needed at the regressive, steady state and progressive phase. In addition, we need to investigate the correlation of microbial contamination and the efficacy of plasma therapy over a period of time. “Likewise, tissue sampling and tumor cell DNA sequencing (or RNA sequencing) would allow following either genetic or transcriptional adaptions of tumour cells over the course of plasma treatment. Tissue sectioning and enumeration of tumor-infiltrating lymphocytes and macrophages would identify whether the anti-tumour efficacy of plasma is directly linked with intratumoral leukocytes. If so, and if high levels of immunosuppressive molecules are detected in the tumor (e.g., PD-L1, cytotoxic T-lymphocyte-associated protein 4 [CTLA4]), checkpoint therapy may be an option to combine with gas plasma treatment. To prevent desmoplastic reactions, novel agents such as PEGPH20 (a Phase II trial on this drug, NCT01839487, has been completed in 2018 Stage IV pancreatic cancer) may be used in combination with plasma treatment. Synchronizing the tumor characterization (e.g., contamination, desmoplastic reaction, and immune infiltrate) with the appropriate medication may also further tailor oncological therapy toward precision medicine. Furthermore, clarifying the molecular and TME differences between gas plasma responders and non-responders in HNSCC patients may generate patient selection criteria for gas plasma therapy in the future. Another topic of future research is whether HNSCC cells can become resistant to gas plasma treatment or whether immunological aspects can predominate, or both” (WITZKE et al. 2020 [59]).

The next few years will reveal the therapeutic potential of plasma therapy. Although all available research results seem to support the idea that CAP has the ability to decrease tumour progression, some important questions remain unanswered. Which plasma treatment parameters will provide optimal results? Do we need combinations with different small molecules to improve the plasma effect? How do we reach cancer patients?

Appropriate answers to these questions have the potential to improve the quality of life and to save the lives of more than 600 000 HNSCC patients per year.

5 Summary

This cumulative doctoral thesis focuses on summarising recent clinical long-term results in the field of plasma medical oncology. It covers a broad spectrum of tumour biology and immunology in HNSCC including a detailed immunological interpretation of medical plasma treatment. METELMANN and colleagues (2018) [44] evaluated long-term survival outcomes of plasma-treated stage IV locally advanced HNSCC patients who failed to respond to curative tumour treatment. Taken together, the tumour went into remission under CAP therapy in approximately one third of the patients (responders) and the tumour surface decreased by 80% compared to the baseline. However, during the course of the treatment the tumour went into progression after a steady state growth. On the basis of current preclinical and clinical immunological knowledge, we propose the following three possible effector mechanisms as explanation of CAP-resistance: (I) tumour adaption, (II) microbial assistance and (III) desmoplastic reaction and wound healing. This is highly significant for future plasma research and paves the way for the development of improved anticancer therapeutic strategies, giving hope of reducing the morbidity and mortality of HNSCCs. With this discussion, we hope to provide a starting point for future studies, whose results might help to overcome CAP-resistance.

List of Publications

5.1 Articles

1. **Witzke K.**, Seebauer C., Metelmann H.-R. *(2018):* Plasma Medical Oncology: Long-term results with focus on immunity research, Clin Plasma Med 9 (2): 48

2. Semmler M.L., Bekeschus S., Schäfer M., Bernhardt T., Fischer T., **Witzke K.**, Seebauer C., Rebl H., Grambow E., Vollmar B., Nebe J.B., Metelmann H.-R., Woedtke T., Emmert S., Boeckmann L.: Molecular Mechanisms of the Efficacy of Cold Atmospheric Pressure Plasma (CAP) in Cancer Treatment. Cancers 2020, 12, 269.

   (Impact factor 6,162)

3. **Witzke K.**, Seebauer C., Jesse K., Kwiatek E., Boeckmann L., Weltmann K.-D., Metelmann H.-R., Bekeschus S.: Plasma medical oncology: immunological interpretation in head and neck squamous cell carcinoma, in Plasma Processes & Polymers (**Impact factor 3.173**)

5.2 Book chapter

1. Seebauer C, Metelmann HR, **Witzke K**, Pouvesle JM: Palliative Treatment of Head and Neck Cancer. In: Metelmann HR, Woedtke T, Weltmann KD (ed), Comprehensive Clinical Plasma Medicine. Cold Physical Plasma for Medical Application, Cham, 185-195, 2018, Springer Verlag

5.3 Conference contributions

1. **Witzke K.**, Seebauer C., Metelmann H.-R. *(2018):* Plasma Medical Oncology: Long-term results with focus on immunity research (Poster, 5th International Workshop on Plasma for Cancer Treatment, 20.th – 21.th March of 2018 in Greifswald, Deutschland)

2. Kwiatek E., **Witzke K.**, Suhm C., Metelmann H.-R.: TP5b: Ethische Aspekte bei der Entwicklung innovativer Tumorbehandlungsverfahren (Onkother-H Statusseminar 11.02.2020)

3. Kwiatek E, Suhm C., Bethke B., Metelmann H.-R., **Witzke K.**: Family centering in clinical studies with cold atmospheric pressure plasma for the palliative treatment of patients with squamous cell carcinoma or melanoma, 7th International Workshop on Plasma for Cancer Treatment (**Accepted,** IWPCT 2020, 30.th September-02.October 2020, Raleigh, NC, USA)

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EXPERT OPINION

PLASMA PROCESSES AND POLYMERS

Plasma medical oncology: Immunological interpretation of head and neck squamous cell carcinoma

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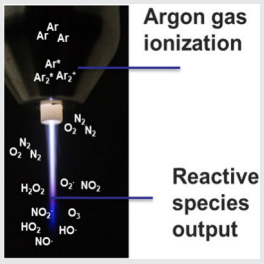
Abstract

The prognosis of patients suffering from advanced-stage head and neck squamous cell carcinoma (HNSCC) remains poor. Medical gas plasma therapy receives growing attention as a novel anticancer modality. Our recent prospective observational study on HNSCC patients suffering from contaminated tumor ulcerations without lasting remission after first-line anticancer therapy showed remarkable efficacy of gas plasma treatment, with the ulcerated tumor surface decreasing by up to 80%. However, tumor growth relapsed, and this biphasic response may be a consequence of immunological and molecular changes in the tumor microenvironment that could be caused by (a) immunosuppression, (b) tumor cell adaption, (c) loss of microbe-induced immunostimulation, and/or (d) stromal cell adaption. These considerations may be vital for the design of clinical plasma trials in the future.



KEYWORDS
cold physical plasma, HNSCC, kINPen, plasma medicine, tumor microenvironment

Abbreviations: CCND1, cyclin D1; CDKN2A, cyclin-dependent kinase inhibitor 2A; CTLA4, cytotoxic T-lymphocyte-associated protein 4; DNA, deoxyribonucleic acid; EBM, evidence-based medicine; EGFR, epidermal growth factor receptor; FAT1, protocadherin; HNSCC, head and neck squamous cell carcinoma; HPV, human papillomavirus; IFN-γ, interferon gamma; IL, interleukin; MAMP, microbe-associated molecular pattern; MDSC, myeloid-derived suppressor cells; MUL1, mitochondrial E3 ubiquitin protein ligase 1; NK-cell, natural killer cell; p16, tumor suppressor protein p16; p53, tumor suppressor protein p53; PD-1, programmed cell death protein-1; PD-L1, programmed cell death-ligand 1; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase; PTEN, phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase; R1, microscopic tumor-positive resection margins; R2, macroscopic tumor-positive resection margins; RNA, ribonucleic acid; RNS, reactive nitrogen species; ROS, reactive oxygen species; TCGA, The Cancer Genome Atlas; TP53, tumor suppressor p53; UICC, Union for International Cancer Control.