



# Oral Neutrophils: Underestimated Players in Oral Cancer

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The composition of the oral milieu reflects oral health. Saliva provides an environment for multiple microorganisms, and contains soluble factors and immune cells. Neutrophils, which rapidly react on the changes in the microenvironment, are a major immune cell population in saliva and thus may serve as a biomarker for oral pathologies. This review focuses on salivary neutrophils in the oral cavity, their phenotype changes in physiological and pathological conditions, as well as on factors regulating oral neutrophil amount, activation and functionality, with special emphasis on oral cancer and its risk factors.

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## INTRODUCTION

Head-and-neck cancer (HNC) of the oral cavity (oral squamous cell carcinoma, OSCC), is one of the leading malignancies worldwide (1). OSCC is located in the area of the tongue, upper and lower gingiva, oral floor, palate and buccal mucosa. It can spread to local lymph nodes in the head and neck, while metastasis to distant organs are rare (2). Despite advances in diagnosis and treatment, the 5-year overall survival rate for OSCC remained below 50% for the last three decades (3). Traditional prognostic factors, such as primary tumor size, regional lymph node metastasis, extracapsular spread, surgical margin involvement and perineural invasion are routinely used to predict OSCC outcome (4). In addition to these, a variety of biomarkers are currently under investigation to predict prognosis, allocate treatment and to follow-up responders or recurrences, including but not limited to circulating DNA, exosomes and automated pathology.

Neutrophils are important players in cancer immunology and their in-depth investigation helps to better understand tumor immune escape mechanisms as well as to establish more suitable biomarkers for cancer diagnostics and therapy. Neutrophils are known to contribute to cancer progression or regression via multiple mechanisms, including the suppression of cytotoxic (5) as well as helper (6) T cell responses and the stimulation of tumor angiogenesis (7, 8). Moreover, neutrophils participate in cancer metastasis via formation of premetastatic niche in target organs (9, 10) or via NET-mediated trapping of circulating cancer cells (11). Clinical studies identified blood neutrophil-to-lymphocyte ratio (12) and the number of tumor-infiltrating neutrophils to be negative prognostic factors in a variety of different cancers, including HNC (5, 13).

Here we provide an up-to-date review on oral neutrophils during the development and progression of oral cancer. We discuss tumor-induced systemic changes in circulating polymorphonuclear leukocytes (cPMN), and further alterations that take place in tumor microenvironment. To evaluate possible prognostic role of oral polymorphonuclear leukocytes

(oPMN) in HNC, we first address their modifications in healthy oral cavity, and then compare it with their activity in conditions known to be HNC risk factors [aging, smoking, chronic periodontitis (CP)] or with neutrophils associated with oral cancer itself. Finally, we discuss the parallels between the activation status of tumor-associated neutrophils (TANs) and oPMNs, and the potential applicability of these cells in diagnostics.

## TUMOR-INDUCED ALTERATION OF CIRCULATING NEUTROPHILS

Most of the current studies on the prognostic role of neutrophils in cancer focus on the blood neutrophils – PMNs, called also circulating PMNs (cPMNs). And so, it is known that in advanced cancer neutrophilia reflects a systemic inflammatory response to cancer progression (14). Neutrophilia and a high ratio of neutrophils-to-lymphocytes are associated with poor prognosis in many different types of cancer (15), including HNC. These changes could be correlated with both tumor burden and spread to lymph nodes (16). Of note, high PMN levels are associated with worse prognosis in HPV<sup>+</sup>, but not HPV<sup>-</sup> oropharyngeal cancer patients (17).

The increased numbers of circulating neutrophils may be the result of tumor-induced emergency myelopoiesis (18), but also of prolonged survival of such cells. PMNs of patients with later stages of HNC were shown to have reduced spontaneous apoptosis in comparison to healthy (19). This was probably due to the increased proportion of immature PMNs in circulation of such patients. To the contrary, in another study including patients with oral cancer, circulating PMNs were shown to have elevated apoptosis due to higher caspase-8 activity and elevated activity of TRAIL-mediated mitochondrial cascade, as compared to healthy (20). The surgical removal of the primary tumor partially decreased the predisposition of such PMNs to apoptosis.

Circulating PMNs in HNC show activated status, with reduced expression of CD62L (L-selectin) in neutrophils, as compared to healthy controls (16). The secretion of various cytokines including IL-1b, VEGF, and IL17 was reported to be increased in blood neutrophils in oral cancer, while the secretion of IL-18 and sTRAIL was reduced (21, 22). The ability to release neutrophil extracellular traps (NETs) by PMNs was reported to be significantly enhanced in early stages of HNC, as compared to healthy (23). Moreover, a statistically significant decrease of ROS production by PMNs from HNC patients was observed (19) and it was associated with poor patient prognosis (24).

Importantly, systemic inflammatory response in different types of cancer, including HNC, results in the activation of cPMNs, increasing their cytotoxic response against tumor cells (25). Moreover, progression of cancer leads to the expansion of immature immunosuppressive PMNs (so called granulocytic myeloid-derived suppressor cells) in low density fraction of blood during HNC, lung cancer, or cancers of bladder and ureter (26). Such cells show upregulated CD11b and CD66b expression, suggesting their enhanced degranulation capacity (26,

27). Expansion of such suppressive neutrophils in human HNC is strongly associated with poor survival of patients (28).

## TUMOR-ASSOCIATED NEUTROPHILS

After transmigration into tumor tissue, blood neutrophils undergo dramatic changes of their phenotype and activity, depending on the cytokines and growth factors available in the tumor microenvironment. Multiple reviews are dedicated to the role of tumor-associated neutrophils (TANs) in tumor progression, therefore here we will only briefly address it to provide a link to the key topic of this review – oral neutrophils in cancer. It is known that TANs contribute to tumor vascularization and metastatic spread via the release of VEGF (Vascular endothelial growth factor) and matrix-degrading enzymes, such as MMP9 (7–9). Moreover, such neutrophils are able to modulate adaptive anti-tumor immune responses. On the one hand, TANs express molecules characteristic for antigen-presenting cells (major histocompatibility complex and co-stimulatory molecules) and release stimulatory cytokines that enhance T cell activity (29). On the other hand, the expression of LOX-1 and arginase by neutrophils was reported to be associated with decreased activity and proliferation of effector T cells in tumor tissue (5). The described complexity of neutrophil functions in cancer has not yet been reflected in the clinical studies concerning oral neutrophils.

The prognostic significance of TANs varies between different types of malignancy, indicating the different role of these cells in tumor. For HNC, increased presence of TANs was shown to be a negative, independent prognostic factor for recurrence, as well as overall survival (19).

In HNC, data concerning TANs are limited, possibly due to the minor size of primary tumors. In other cancer types, such as human lung carcinomas, TANs were revealed to have an activated phenotype with high expression of CD11b, CD66b, ICAM-1 and downregulated CD16, in comparison to blood neutrophils. TANs upregulate chemokine receptors responsible for further homing to lymphoid organs (CCR5, CCR7, CXCR3, CXCR4) and downregulate molecules involved in their migration to the tumor site (CD62L, CXCR1, CXCR2). TANs upregulate Fc receptor CD64, death ligand FasL and co-stimulatory molecules CD86, OX40L, 4-1BBL (29). Moreover, they release high amounts of pro-inflammatory cytokines and chemokines that regulate migration of other immune cells into tumor and so influence tumor growth, angiogenesis, and spread. No significant differences in viability, ROS and phagocytosis between cPMN and TANs were observed (29). Due to their expression of co-stimulatory molecules, TANs can efficiently induce proliferation of T cells and their IFN $\gamma$  production, while blood neutrophils are poor T cell stimulators (29).

PMNs and TANs are already extensively described in HNC context. However, nothing is known about other type of neutrophils that populate environment localized closely to growing oral HNC – oral neutrophils. These cells represent a unique population of neutrophils with phenotypic and functional properties that are distinct from cPMN or neutrophils in other

biological compartments, such as mucosa or tumor tissue, due to the specific anatomy and physiology of the oral cavity.

## THE COMPLEX ENVIRONMENT OF ORAL CAVITY

Oral cavity represents the first barrier where the external pathogens enter the body and interact with immune defense mechanisms, with saliva as an environment for these interactions. Saliva, secreted by minor and major salivary glands (800–1,500 ml per day, declining during the nighttime) (30–32), is a complex biofluid, containing components from the mucosa, the gingiva crevices, tooth surfaces, nasal secrets and plasma (33–35). Soluble molecules dissolved in saliva are nucleic acids, proteins, mucins/glycoproteins, immunoglobulins, metabolites, drugs and their metabolites (36). The most abundant proteins in saliva are plasma albumin, digestive enzymes and microbicidal proteins, but also variety of hormones, cytokines and chemokines, as well as other molecules with regulatory functions (33). Besides this, saliva contains components with still unknown biological functions (37) that are secreted by exocytosis of the granules of acinar cells in the salivary glands (38). Moreover, saliva contains high amounts of extracellular vesicles, mainly secreted by epithelial cells and salivary glands (39), but also originating from the circulation. Cellular components of saliva include epithelial and immune cells, mostly neutrophils (40).

The mucosal barrier in the oral cavity is considered to be one of the main ecological habitats of the human body (41). Saliva contains bacteria (the most common are *Firmicutes*, *Bacillus*, *Proteobacteria*, *Streptococci*, *Staphylococci*, *Lactobacilli* and *Actinomyces* species) (42–44), fungi (e.g., *Candida* species) (45), viruses (e.g., Herpes-, Papilloma- and Coxsackieviruses) (46, 47), and other exogenous substances that colonize the mouth and can therefore potentially provide an insight into the relationship of the host with the environment (34). Oral bacterial communities are the second most complex in the body, after the communities of the colon (48). Interestingly, a study of healthy volunteers from 12 locations worldwide have found no significant geographical differences between their salivary microbiota (49). This suggests that the diet and the environment do not significantly influence the composition of the oral microbiome and that the host species is the primary determinant (50). Commensal microbiota play an important role in maintaining oral and systemic health (51), as its presence inhibits colonization by pathogens (“colonization resistance”) (52, 53).

## ORAL NEUTROPHILS IN STEADY STATE

The contact of the oral cavity with the external environment, the constant presence of normal or invading microbiota as well as secretion of chemokines by activated epithelial cells attract neutrophils. More than  $10^{11}$  neutrophils are produced daily in the bone marrow (54) and are released into circulation to transmigrate into tissues. The trafficking of neutrophils into oral cavity is a constant process, displaying a circadian rhythm, with

an increase during the day and a decrease at night (55). Around  $10^6$  leukocytes can be isolated after rinsing of the oral cavity. Polymorphonuclear neutrophils represent ca. 83% of cells in such oral rinse, mononuclear cells 17%, and basophils/eosinophils 0.4% (56). Of note, immature forms of neutrophils can also be observed in oral rinse, suggesting direct trafficking from the bone marrow (40). Thus, a redistribution of leukocyte subpopulations in comparison to blood, namely, increased neutrophil and monocyte percentage accompanied by decreased amount of lymphocytes can be observed in oral cavity (57).

The gingival crevices are suggested to be the main point of entry for oPMN into the oral cavity, while only a small proportion of cells originate from salivary glands (55, 58). In agreement, the amount of oPMN in the oral cavity was shown to be significantly decreased in patients without teeth (59, 60). Importantly, there is no correlation between the amount of cPMN and oPMN in healthy individuals (61), which can be explained by a specific local microenvironment, attracting neutrophils to the oral cavity with no impact on the remaining immune system. At the same time, in systemic conditions such as neutropenia or bone marrow transplantation, a shift in numbers of oPMN correlates with cPMN numbers (56, 62).

## THE LIFE-CYCLE OF ORAL NEUTROPHILS

After their release from the bone marrow, neutrophils circulate for 5–9 h and then migrate into tissues where they survive 8–16 h before they die (63). Recently, a prolonged (up to 5 days) survival of activated neutrophils in tissues has been described (64, 65), which ensures the presence of functional neutrophils at the site of inflammation (66). In healthy volunteers the proportion of viable oPMN is significantly lower compared with cPMN, and their maturation more advanced with elevated apoptosis/necrosis (56, 67). Availability of bacteria-derived endotoxin in the oral microenvironment shifts neutrophil fate from apoptosis to necrosis (68, 69). At the same time, oPMN become more resistant to the additional apoptotic stimuli. Unlike cPMN, exudated oPMN are not sensitive to rhTNF- $\alpha$ /cycloheximide-induced apoptosis (69). In an elegant study, Hotta et al. demonstrated the lack of sensitivity of oPMN to TNF $\alpha$ -stimulated apoptosis, with lower caspase 3 activity, as compared to cPMN. While in cPMN activation of nuclear factor kB (NF-kB) was induced by TNF- $\alpha$ , in oPMN NF-kB was already activated in steady state, and no further activation was observed by TNF- $\alpha$  treatment. Moreover, no significant effect of NF-kB inhibitor in oPMN was observed (70). This shows that neutrophils become more resistant to apoptotic stimuli during their migration from blood to oral cavity and that this resistance depends on the NF-kB pathway.

## THE PHENOTYPES OF ORAL NEUTROPHILS

During transmigration from the circulation to the oral cavity, neutrophils are exposed to the new environment, resulting in an

activation and dramatic changes of their phenotype and function (Figure 1). Significant modulation of their transcriptome has been shown, with 469 genes downregulated and 119 genes upregulated (71). Majority of these genes are involved in cytokine-cytokine receptor interactions, chemokine signaling pathways, hematopoietic cell lineage development and T cell activation (71).

oPMN express typical neutrophil makers, such as CD11b, CD16, and CD66b. These markers were demonstrated to be constantly expressed on neutrophils, independent of the cell location, level of activation or disease state (72). However, the level of their expression can vary in different conditions (see below). As compared to cPMN, oPMN upregulate molecules reflecting their activation in the oral cavity, including CD63 (a marker associated with degranulation of azurophilic granules), CD66a (adhesion), CD10 (marker of neutrophil differentiation), CD64 (Fc-gamma receptor 1), CD55 (complement regulator) and CD11b (adhesion). Other upregulated molecules in oPMN include inhibitory molecules CD85a, CD305 and CD312 (responsible for interaction with immune cells) (72), while CD16 (Fc-gamma receptor 3) is reduced on oPMN (73). Other molecules responsible for intracellular signaling (CD50, CD114, CD132, CD182) as well as molecules responsible for adhesion (CD31), complement regulation (CD46) or regulation of adaptive immunity (CD43, CD44) are reported to be downregulated on oPMNs (72).

Importantly, two distinct subpopulations of oPMN in healthy donors were described, based on the size and granularity of these cells: para-inflammatory 1 neutrophils with size and granularity comparable to cPMN, and para-inflammatory 2 neutrophils, which are smaller and less granular. These populations exhibit also differences in their function and phenotype, with elevated expression of CD55, CD63 and reduced expression of CD16 and CD170 on para-inflammatory 2 neutrophils, as compared to para-inflammatory 1 (see below) (73).

## THE FUNCTION OF ORAL NEUTROPHILS AND ITS MODULATION BY THE SALIVA

Antibacterial functions of neutrophils include adhesion and internalization of bacteria (phagocytosis), production of reactive oxygen species (ROS) that damage membranes and genetical material, the release of neutrophil extracellular traps (NETs) capturing pathogens and the secretion of antibacterial proteins. oPMN isolated from healthy controls demonstrate slightly elevated adhesion to pathogens and significantly higher internalization of bacteria (*A. actinomycetemcomitans*, *P. gingivalis*, *E. coli*) in comparison to cPMN (63). Higher phagocytotic activity in para-inflammatory 2 oral neutrophils was also demonstrated, as compared to para-inflammatory 1 (73). Activation of neutrophils upon phagocytosis (61, 74) led to dramatically increased ROS production by oPMN, but not by cPMN (40). A comparison of both subpopulations of oPMN reveals only slightly increased ROS production by para-inflammatory 2 neutrophils (73). Importantly, the ability

to respond to the stimulation with PMA is preserved in both populations in healthy donors (73).

To immobilize and kill distantly localized microorganisms, neutrophils release NETs (75). Importantly, increased NET formation by oPMN, in comparison to cPMN, was reported (63), with para-inflammatory 2 subpopulation showing significantly higher release (73). This was in line with their elevated phagocytosis. In addition to changes in effector function, migratory capacity of neutrophils decreases significantly once they have transmigrated from the bloodstream to the oral cavity. oPMN exhibit random chemotactic movement with a shorter distance as well as decreased fMLP receptor expression (63).

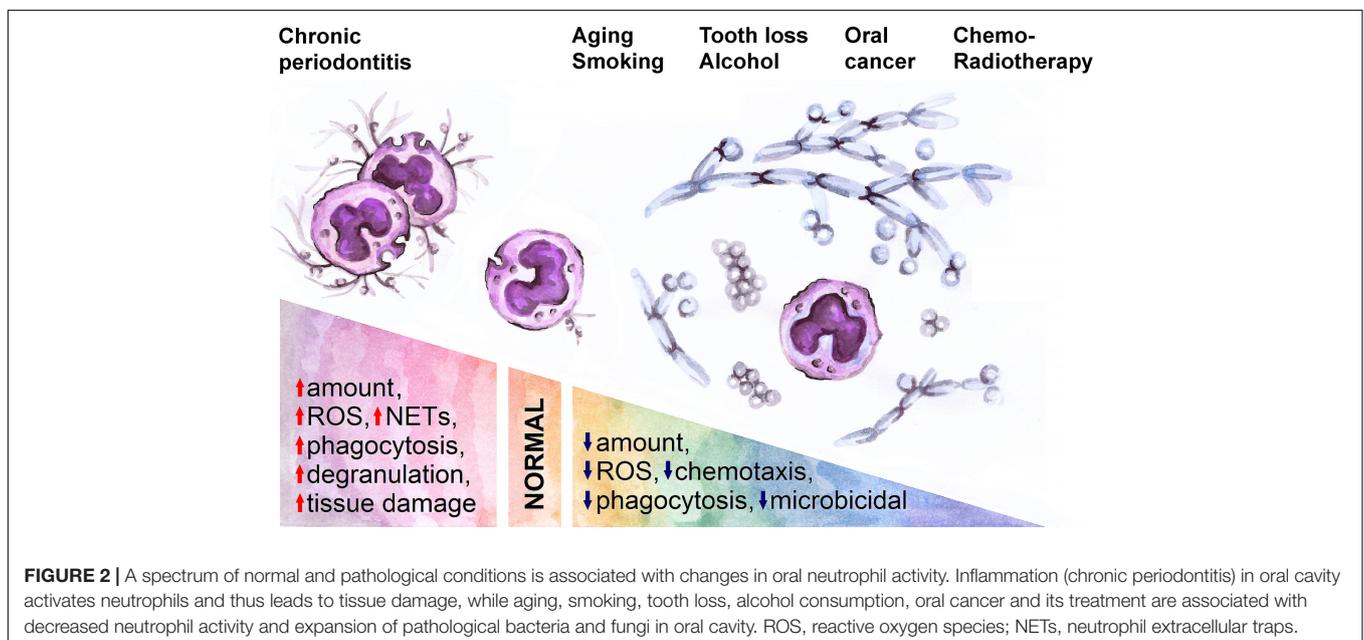
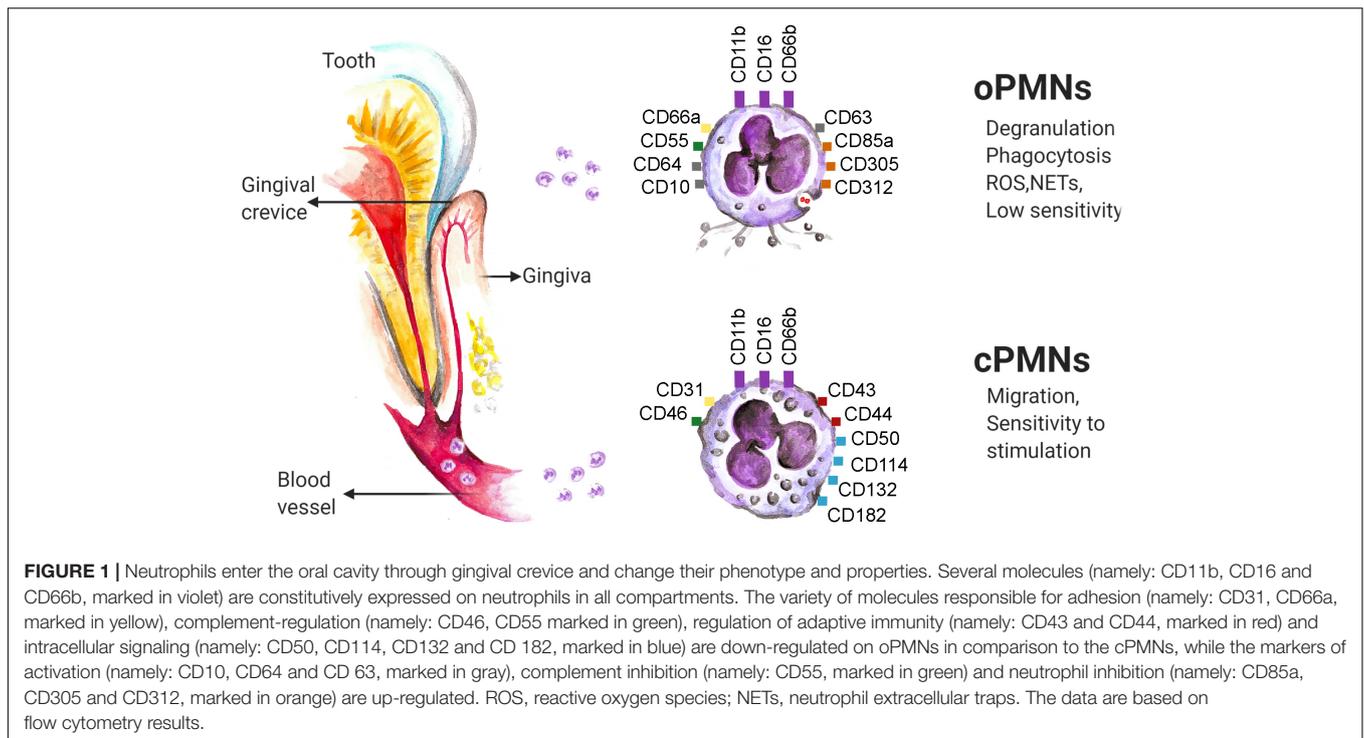
The effect of saliva on neutrophils in the oral cavity is complex and depends on multiple mediators, such as cytokines, chemokines, various proteins or glycoproteins, as well as commensal and pathogenic bacteria and fungi. Several bacterial species are reported to modulate neutrophil functions *in vitro*. The effect of different oral microorganisms, both commensal (*S. oralis*, *S. sanguinis*, *S. salivarius*) and pathogenic (*S. mutans*, *A. actinomycetemcomitans*, *P. gingivalis*), on the activation status of cPMN was described by Oveisi et al. (76). While CD63 and CD11b/CD18 markers were upregulated after exposure to both commensal and pathogenic bacteria, commensal microorganisms in biofilms induced the selective increase of CD66, CD64, CD55, while pathogenic bacteria induced the expression of lipopolysaccharide receptor CD14. Moreover, only commensal bacteria in biofilms stimulated degranulation, phagocytosis, ROS production and NET formation, while pathogenic bacteria showed no effect (76). Coexistence of *F. alocis* with other pathogens induced the secretion of proinflammatory cytokines from epithelial cells and promoted apoptosis of neutrophils (77). This was responsible for increased pathological conditions in oral cavity. Interestingly, *F. alocis* has been shown to be resistant to oxidative stress and to inhibit PMA-induced NET-production. Moreover, this bacterium can survive within neutrophils, repressing their ROS release and maturation of granules. This in turn prolongs neutrophils lifespan and leads to elevated inflammation and tissue damage (77, 78). At the same time, components of bacteria-free saliva (e.g., carbohydrates) limit tissue-damaging neutrophil inflammatory responses (ROS production and release of hydrolytic enzymes) to microbiota (61, 79, 80).

## RISK FACTORS FOR ORAL CANCER AND THEIR INFLUENCE ON THE ACTIVITY OF oPMN

Main risk factors for oral cancer include age (81), tobacco and alcohol consumption (82), and chronic inflammation (e.g., periodontitis) (83, 84). All these factors have also the potential to influence the phenotype, activation and functions of oPMN (Figure 2).

### Aging

Aging is associated with a significant impairment of neutrophil responses in the oral cavity. Elderly individuals (60–85 years



old) in comparison to younger persons (20–50 years old) show a reduction of oPMN numbers, which is in line with overall decreasing innate immune responses in elderly (85). Moreover, elevated granulocyte-macrophage colony-stimulating factor (86) in the saliva of aged individuals was reported, which could be responsible for the sensitivity of oPMN to bacteria-induced apoptosis (86). Also, cPMN from the elderly group express lower amounts of CXCR1, CD11b/CD18 integrin and CD62L (L-selectin), which could be responsible for the decreased migration

of such cells to the oral cavity (86). Aging is associated with diminished salivary flow rates and reduced production of antimicrobial factors (transferrin and lactoferrin) by oPMN (87). Neutrophil antimicrobial functions, such as phagocytosis, are impaired with age in both, cPMN and oPMN (86). Moreover, aged oPMN show decreased generation of ROS and reduced killing activity (87). All these changes in oPMN functionality could be responsible for the elevated risk for the development of oral neoplasia in elderly.

## Smoking and Alcohol Consumption

The influence of smoking on oral cells is mediated by nicotine as well as a wide range of other accompanying hazardous substances that are included in tobacco smoke. Besides the direct influence, tobacco also affects the pH of saliva (88) and can thus potentially influence the functions of neutrophils. *In vitro* experiments on the effect of nicotine on cPMN demonstrate a disturbed balance between antibacterial and tissue-damaging properties of neutrophils: on the one hand, a dose-dependent suppression of chemotaxis, phagocytosis (89) and diminished ROS-dependent killing is observed (90), on the other hand, such cells show enhanced degranulation and elastase release (89), but their viability is not influenced (89, 90). The data obtained from *in vivo* studies of the effect of smoking on the oPMN functions remain controversial. While some studies show an increased phagocytic activity of oPMN directly after smoking (91), suggesting a direct effect of tobacco smoke on these cells, others demonstrate reduced phagocytic activity and viability of oPMN in smokers, as compared to non-smokers (92, 93). The observed differences may be explained by the different time point of analysis, directly after exposure or chronic changes, or the different impact of various components of the smoke (94), and should still be investigated.

While no data concerning the effect of alcohol consumption on oPMNs are available, data about cPMNs report the correlation of blood level of gamma-glutamyl transpeptidase (as a measure of alcohol consumption) with compromised neutrophil bacterial killing (95).

## Inflammation in the Oral Cavity

Inflammation is considered to be a hallmark of cancer (96). In agreement, inflammation in oral cavity increases the risk of oral cancer (97). In the inflammatory oral diseases, such as gingivitis or CP, increased amounts of oPMN are observed (55). This can be due to chemoattractants produced by epithelium, but also due to increased oPMN-derived CCL3 or IL-1 (71). Patients with CP show more than a 2.5-fold increase of oral neutrophil counts, as compared to healthy individuals (71). Such neutrophils are of the pro-survival neutrophil phenotype with a prolonged lifespan (71).

The functionality of oPMN reflects the activation of the immune system and may serve a diagnostic parameter for the disease severity and a prognostic marker. In chronic periodontitis (CP) patients distinct changes of the neutrophil transcriptome during migration to the oral cavity has been shown, with 2,386 downregulated and 1,207 upregulated genes in oPMN, compared to cPMN. The major differences were observed in genes responsible for the regulation of apoptosis, but also Toll-like receptor signaling pathways, chemokines and cytokines (71). These changes have an impact on the survival and functions of oPMN.

Inflammatory diseases are often accompanied by the presence of pathogenic bacteria in the oral cavity. Only in rare cases there is one single pathogen inducing the disease, more often it is a shift of microbiome toward certain pathogenic bacteria. In case of CP, the predominance of *Actinomyces* species, which compose

much of the supragingival and subgingival plaque microbiota, *P. intermedia*, *Bacteroides species*, and *F. nucleatum* are reported (98, 99). oPMN become activated by invading microorganisms, which contributes to tissue damage and disease progression. Decreased amounts of oPMN together with their suppressed function might be responsible for the development of secondary bacterial or fungal infections in such individuals. The changes in oPMN phenotype and functions are well studied in CP, while in other inflammatory conditions data is still scarce. As compared to the healthy state, CP oPMN gain proinflammatory phenotype, characterized with decreased size and granularity together with prominent activation (upregulation of CD63, CD66a, CD10, CD64, CD55, CD11b/CD18) (73). Lakschevitz et al. reported the upregulation of degranulation (CD63) and adhesion (CD11b, CD66, CD66b, CD66c, CD66e) markers on CP oPMN (72).

Electron microscopy shows elevated phagocytosis (measured as an increase of early and late phagosome counts) and greater degranulation (lower number of granules) of oPMN during CP. This is in line with upregulated expression of CD63 while no differences in granule content is observed (73). The enhanced activation of oPMN is reflected in the induction of myeloperoxidase (MPO) (100). Proinflammatory neutrophils in CP demonstrate elevated ROS production, but in contrast to neutrophils in healthy state, they show no increase of ROS production in response to PMA, suggesting an exhausted phenotype (73). In agreement, NET release estimated by MPO and histone citrullination, is also significantly increased in proinflammatory neutrophils in chronic periodontal disease (73).

Thus, the changes of oPMN functions induced by the contact with pathogens during the course of disease may lead to several unfavorable consequences. On the one hand, suppression of neutrophil functions results in pathogen persistence and spread. On the other hand, hyperactivated neutrophils can cause tissue damage due to the release of proteases (neutrophil elastase or matrix metalloproteinase 9), NETs (101) or pro-inflammatory cytokines, such as IL-1 (71).

As already mentioned, inflammation due to tissue damage is a known cancer hallmark. The presence of activated neutrophils in the oral environment may influence the progression of HNC, as TNF $\alpha$  and IL8 released by neutrophils were shown to increase tumor cell line invasion *in vitro* (102). Thus, the changes of oral neutrophil functions in certain inflammatory conditions (including periodontitis, the known risk factor of oral cancer) may be permissive for cancer development and progression.

## ORAL CANCER AND MICROBIOME SHIFT

Oral cancer is a consequence of the multiple factors present locally in the oral cavity as well as systemically. Inflammation transforms oral ecosystem, including microbiome and immune components, and leads to the formation of premalignant and malignant lesions. Later, growing tumor influences the microenvironment by releasing a wide range of biologically active molecules, such as cytokines, chemokines and growth factors.

An increased predominance of certain bacteria (e.g., *P. gingivalis*, *F. nucleatum*, *P. intermedia*, *C. gingivalis*, *P. melaninogenica*, *S. mitis*, as well as *Veillonella*, *Actinomyces*, *Clostridium*, *Haemophilus* or *Enterobacteriaceae*) correlates strongly with OSCC (99, 103–106). Such association between changes in oral microbiome and the presence of oral cancer can be explained by different causal links: one hypothesis is, that certain bacteria may cause DNA damage in oral epithelium by secreted endotoxins (107) or induce inflammatory responses supporting cancer development (108). On the other hand, changed immune responses in cancer may promote the expansion of pathological microorganisms in the oral cavity. Moreover, tumor-derived molecules solved in saliva serve as chemoattractants and potent regulators of inflammatory cell function (109). Such factors can influence trafficking and activation of immune cells in the oral cavity.

Bacteria are not the only factors in the oral cavity influencing neutrophil properties. The prevalence rate of HPV in normal human mucosa depend from different sociodemographic variables, sexual behavior and sensitivity of the diagnostic techniques (110). HPV is associated with an increased risk of head and neck cancer (HNC), but the prognosis of HPV-positive tumors is better compared to HPV-negative cases (111, 112). It could be demonstrated that HPV-positive OSCC cells contain secondary changes in genes and pathways involved in activation of the host anti-viral interferon signaling (113). As interferons have been shown to have anti-tumoral capacity (114) and to prime anti-tumor phenotype of neutrophils (8, 10, 115), this phenomenon should be further exploited. Overexpression of HPV proteins in OSCC cells is also associated with an impaired neutrophil infiltration to the tumor, possibly due to the downregulated expression of IL-8 (116). While recently a comprehensive single cell RNA sequencing study investigated the differences between intratumoral immune cells isolated from HPV<sup>+</sup> and HPV<sup>-</sup> HNC [large differences between B-cells, myeloid cells and conventional CD4<sup>+</sup> T-cells, rare differences between regulatory CD4<sup>+</sup> T-cells and CD8<sup>+</sup> T-cells] (117), there is to the best of our knowledge no study investigating the influence of HPV status on oPMN phenotype and function.

## CHANGES IN ORAL CAVITY MICROENVIRONMENT DUE TO ORAL CANCER

Saliva contains various proteins derived from cells populating oral cavity that might attract and activate neutrophils. The levels of such molecules may therefore potentially be used as biomarkers. Saliva contains several chemokines attracting neutrophils. The significant increase of CXCL-8 (118, 119), CXCL-10 and CCL-14 in saliva of patients with head-and-neck carcinoma was reported (120). Moreover, saliva CCL7 levels are shown to correlate positively with lymph node metastasis, tumor size and clinical stage (121). Cytokines and cytokine-coding mRNA in saliva are also shown to be predictors for OSCC progression (51). Goertzen et al. showed that cancer patients have significantly increased pro-inflammatory cytokines, such as

IL-1 $\alpha$ , IL-1 $\beta$ , IL-6, IL-8 and TNF- $\alpha$  in saliva, as compared to controls (122). Salivary levels of IL-6 (118, 119) may serve as oral cancer predictors (123). Higher levels of growth factors in saliva also correlate with oral inflammation and tumor invasion (37). Importantly, the upregulation of all these factors has a significant role in the activation of neutrophils.

Reduced abundance of peptidyl-prolyl cis-trans isomerase A (PPIA, also known as cyclophilin-A) appeared to be a factor that might predict poor prognosis of OSCC patients (124). This cytosolic molecule being released from the cell, is a potent chemoattractant for neutrophils through the receptor, CD147 (125).

Neutrophil-derived molecules in saliva may reflect the amount and activation status of oPMN. Proteomic analysis of human saliva and saliva-derived extracellular vesicles from healthy individuals and patients with OSCC revealed a significant overrepresentation of proteins related to acute inflammatory response, regulation of humoral response and regulation of hydrogen peroxide metabolic processes (124). Elevated levels of total protein and neutrophil-derived molecules (lysozyme) in saliva were reported for oral cancer patients (126). During severe oral cancer, levels of TNF- $\alpha$ , IL-1 and RANKL are also elevated (33). In neutrophils, MPO makes up to 5% of the total protein content (127). MPO level in saliva increases proportionally to the number of oPMN (100) and therefore is elevated during oral inflammation (128, 129). High levels of neutrophil-derived defensin-1 in saliva can be a sensitive marker for earlier stages of OSCC, while in other conditions, such as glossodynia or oral discomfort, the levels are comparable with healthy controls (130, 131).

Cortisol levels in saliva are significantly increased in OSCC patients in comparison to controls (132), which may potentially influence neutrophil trafficking (133) and functions (134). Thus, saliva from oral cancer patients contains multiple factors regulating oPMN functions or reflecting their activation status during disease progression.

## ORAL NEUTROPHILS DURING THE COURSE OF ORAL CANCER

Neutrophils present in tumor microenvironment can suppress or potentiate cancer progression (135, 136), depending on their modulation via tumor microenvironment. This can occur locally as well as systemically in distant organs (137). oPMN being in close contact to the tumor site, may influence tumor development, therefore the assessment of their functions may serve an important diagnostic tool. However, only few studies that focus on oPMN in cancer are available.

oPMN isolated from patients with untreated OSCC, demonstrate comparable phagocytic activity, but significantly lower chemotactic capacity to fMLP, as compared to healthy. Moreover, lower superoxide production in response to fMLP and PMA treatment is observed. In agreement, reduced *Candida* killing is observed in such neutrophils (138, 139).

Radio(chemo)therapy is a treatment option for OSCC either in the primary (definitive) setting or as an adjuvant to



Diminished cytotoxic capacity of oPMNs in certain conditions (aging, smoking) (89, 90) may indicate the lack of cytotoxic activity against tumor cells, thus predisposing to tumor progression. Further studies on oPMN functions in HNC are required to verify this.

Early stages of HNC are characterized with increased NET formation by cPMN. Moreover, tumor-derived factors stimulate production of NETs *in vitro* (23). While oPMNs in healthy are reported to produce high amount of NETs (63), and NET formation is even increased in inflammatory conditions (73), there are no data about NET formation by oPMN in HNC. NETs released by neutrophils contribute to tumor spread (147), thus NET formation by oPMN might also be a useful tool in the evaluation of prognosis in HNC.

## CONCLUDING REMARKS

In the recent years, the knowledge about neutrophils and their role in the pathogenesis of various diseases has significantly evolved. Originally characterized as short-living killers, neutrophils are now considered to be important players in the regulation of multiple vital processes. Numerous functions of blood or tumor neutrophils during cancer progression and metastasis have been revealed, including the support of angiogenesis or modulation of the adaptive immune responses. At the same time, very scarce information is available for oral neutrophils.

Here, we are collecting the available evidence that a combination of physiological (aging) and pathological conditions

(smoking, oral inflammation) leads to the disbalance of oral neutrophil functions, resulting in the changes in the oral ecosystem. This may contribute to immune evasion and trigger the pro-cancerous mechanisms in the oral cavity. The available data about neutrophils in the oral cavity suggest the suppression of oPMN activity during oral cancer progression, which could lead to microbial complications and chemoradiotherapy side effects. Such changes of oPMN activity might be monitored, providing a useful diagnostic tool for disease progression.

## AUTHOR CONTRIBUTIONS

MD, JR, EP, and JJ: conceptualization and writing-original draft preparation. MD, JR, EP, CK, JB, SL, and JJ: writing-review and editing. All authors contributed to the article and approved the submitted version.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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