Title Page

Title: Analysis of hematological parameters and their relationship with histopathological alterations in a murine model of tongue carcinogenesis induced by 4 nitroquinoline-1-oxide and ethanol.

Isabella Moura Pereira [a], Anaíra Ribeiro Guedes Fonseca Costa[b], Débora de Oliveira Santos[c], Rafael Borges Rosa[d], Emilene Ferreira de Castro[e], Ianca Daniele Oliveira de Jesus[f], Mariana Daiani Costa Silva[g], Adriano Mota Loyola[h] , Paulo Rogério de Faria[i]

[a] Institute of Biomedical Sciences, Federal University of Uberlândia, Uberlândia, Minas Gerais, Brazil, e-mail: isabellamope@gmail.com

[b]Department of Oral and Maxillofacial Pathology, Federal University of Uberlândia, Uberlândia, Minas Gerais, Brazil, e-mail: anairaufu@hotmail.com;

[c]Department of Oral and Maxillofacial Pathology, Federal University of Uberlândia, Uberlândia, Minas Gerais, Brazil, e-mail: debora.oliveira.santos@ufu.br;

[d] Department of Virology and Experimental Therapy (LAVITE), Aggeu Magalhães Institute (IAM), Oswaldo Cruz Foundation (Fiocruz), Recife, Brazil, e-mail:rafael.brosa@ufu.br;

[e] Faculty of Medicine, Federal University of Uberlândia (UFU), Uberlandia, Brazil, e-mail: emicastrorh@gmail.com

[f] Department of Dentistry and Dental Materials, Federal University of Uberlândia - UFU, Uberlândia, Minas Gerais, Brazil, e-mail:iank_oliveira@hotmail.com

[g] Institute of Biomedical Sciences, Federal University of Uberlândia, Uberlândia, Minas Gerais, Brazil, e-mail: marianadaiani@gmail.com

[h] Department of Oral and Maxillofacial Pathology, Federal University of Uberlândia, Uberlândia, Minas Gerais, Brazil, e-mail: loyolaam@gmail.com

[i] Department of Oral and Maxillofacial Pathology, Federal University of Uberlândia, Uberlândia, Minas Gerais, Brazil, e-mail:paulorfaria1976@gmail.com

Corresponding Author

Paulo Rogério de Faria, Department of Oral and Maxillofacial Pathology, School of Dentistry, Federal University of Uberlândia, Av. Pará, 1720, Umuarama, Uberlândia, Brazil, 38.405-302.

Phone:+55-34-3225-8118 Fax: +55-3225-8118 e-mail:paulofaria1976@gmail.com

Data Availability Statement: Data are available upon request.

Funding Statement: This research was financed by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Finance Code 001, by Fundação de Amparo à Pesquisa de Minas Gerais (FAPEMIG), Finance Code #APQ-3020/21, and by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Finance Code #409081-21-3.

Conflict of Interest Disclosure: All authors declare that there are no conflicts of interest related to this study.

Ethics Approval Statement: All procedures followed the institution's ethical standards where the studies were conducted. Ethical approval was obtained from the Ethical Commission in Animal Experimentation (CEUA-UFU, registration n. 020/21).

Acknowledgments: We thank all Rodents Animal Facilities Complex collaborators of the Federal University of Uberlândia (REBIR-UFU).

Abstract

Background: Peripheral blood biomarkers are significant prognostic predictors for several human tumors, as they reflect the dynamics between anti-tumor and tumor-promoting effects of the inflammatory response. Studies have shown that increased levels of neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), and systemic immune-inflammation index (SII) are associated with unfavorable outcomes for oral squamous cell carcinoma (OSCC). This study aimed to analyze the association of tumor inflammation-related hematological alterations to the incidence of epithelial dysplasia (ED) and OSCC in mice treated with tobacco mimicker 4-nitroquinoline-N-oxide (4NQO) and ethanol. **Methods:** 120 C57Bl/6J mice equally divided into males and females were randomly allocated to four groups (n=15): PPG/H2O, treated with vehicle propylene glycol (PPG) for 10 weeks followed by drinking water (H2O) for 15 weeks; PPG/EtOH, treated with PPG for 10 weeks and 8% ethanol (EtOH) for 15 weeks; 4NQO/H2O, treated with 100ug/mL of 4NQO diluted in PPG followed by H2O for 15 weeks; and 4NQO/EtOH, treated with 4NQO for 10 weeks followed by EtOH for 15 weeks (CEUA-UFU, #020/21). At the end of the 25-week treatment period, animals were euthanized and necropsied. Tongues were submitted to histopathological analyses for ED and OSCC diagnoses. Blood samples were collected and processed for red blood cells, white blood cells, and platelet cell parameters analyses. The NLR, PLR, and SII were also calculated. **Results:** Nearly 93% of the males from the 4NQO/H2O and 4NQO/EtOH groups developed OSCC. In females, OSCC was observed in 66.7% and 60% of the 4NQO/H2O and 4NQO/EtOH groups, respectively. This difference between males and females from the 4NQO/EtOH group was significant (p=0.04). Neutrophil count, NLR, and SII increased significantly from the control group to the 4NQO/H2O and

4NQO/EtOH groups containing OSCC-affected male and female mice, while lymphocyte count decreased. Females of the 4NQO/H2O group with ED also had significantly higher neutrophil, NRL, and SII values than the normal mucosa. **Conclusion:** White blood cell counts and NRL changed significantly during epithelial tumorigenesis, reflecting tumor inflammation-related systemic alterations that could be further explored as biomarkers of OSCC development and progression.

Keywords: carcinogenesis, hematological tests, oral cancer, tumor biomarkers, mice.

Introduction

Oral squamous cell carcinoma (OSCC) represents the 6th most common type of cancer worldwide, whose incidence will rise to 40% by 2040, according to the Global Cancer Observatory (GLOBOCAN)[1,2]. Chronic tobacco and alcohol consumption are important extrinsic factors associated with the development of oral potentially malignant disorders (OPMDs) and OSCC proper, of which men present 2 to 3-fold higher risk[1,2]. Unfavorable outcomes in OSCC management are associated with delayed diagnosis, with 70% of the tumors detected in advanced stages[1].

Delayed diagnosis of OSCC is largely due to difficulties in clinical examination and differential diagnosis of OPMDs, as most are asymptomatic[2]. Early detection improves the OSCC 5-year survival rate to 90%[3], which could be achieved with the development of tumor biomarkers of diagnostic and prognostic value, considering the different stages of oral carcinogenesis. In this context, peripheral blood analysis was recently shown to be a noninvasive and low-cost technique with prognostic prediction for OSCC[4].

White blood cell and platelet parameters have been shown to be prognostic factors for OSCC, particularly the neutrophil and lymphocyte counts, the neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), and the systemic immune-inflammation index (SII)[4,5,6]. Differences in peripheral blood and systemic inflammatory biomarkers remain to be explored in the context of OPMD development and malignant transformation to OSCC, as recent evidence has demonstrated higher NLR values in squamous cell carcinoma of the larynx than in benign and precancerous conditions[7].

Systemic inflammatory biomarkers in cancer are of clinical interest since they reflect the anti-tumor and tumor-promoting effects of the patient's inflammatory and immune responses[8]. We have recently demonstrated alterations in these parameters in a

murine model of oral cancer, in which the role of inducible nitric oxide synthase, an important component of tumor-associated inflammation and prognostic predictor of OSCC lymph node metastasis, was investigated[9]. Considering that tobacco smoking and alcohol consumption are major factors for tumor extrinsic inflammation and that immunoinflammatory response has gender-specificities, this study aimed to analyze the association between tumor inflammation-related hematological alterations and the incidence of epithelial dysplasia (ED) and OSCC in male and female mice treated with tobacco mimicker 4-nitroquinoline-N-oxide (4NQO) and ethanol.

Materials and methods

Study design and ethical issues

All protocols in this *in vivo* study were approved by the Ethical Commission on Animal Use of the Federal University of Uberlândia (CEUA-UFU, n. 20/21). Our experimental units comprised 60 male and 60 female *Mus musculus* C57Bl/6J mice provided by the Rodents Animal Facilities Complex of the Federal University of Uberlândia (REBIR-UFU). The experimental protocol consisted of a combination of the murine tongue cancer model induced with 4-nitroquinoline-*N*-oxide (4NQO, N8141, Sigma-Aldrich, San Louis, USA) diluted in propylene glycol (PPG, Synth, Diadema, Brazil) and sterilized water (H2O) to the Meadows-Cook chronic alcoholism model with 8% ethanol (EtOH, Êxodo Científica, 95% Pa Acs) diluted in H2O (v/v).

Experimental protocol

To investigate how the association between chronic tobacco and alcohol consumption, individually and in combination, alters the hematological parameters during oral carcinogenesis, we submitted the experimental animals to four distinct treatments (n=15).

The PPG/H2O control group was treated with the vehicle used in 4NQO dilution at 5mg/mL for 10 weeks, followed by H2O for 15 weeks; the PPG/EtOH group was treated similarly, except for the substitution of H2O for 8% ethanol solution; the 4NQO/H2O group was treated with 100ug/mL 4NQO solution for 10 weeks, followed by H2O alone for 15 weeks; and the 4NQO/EtOH group was treated with 4QNO solution for 10 weeks followed by 8% ethanol for 15 weeks. All EtOH-treated groups were initially subjected to a 1-week acclimatization period in which the ethanol concentration was increased at 4% every 3-day interval. Animals were followed along the 25-week experimental period, with the following humane endpoints verified twice daily: ≤ 20% of weight loss, inability to ambulate, labored respiration, dehydration, hunched posture, ocular or nasal discharge, and inability to access food and water. All experimental procedures were conducted at the REBIR-UFU. Groups of five mice were kept in 32 x 20 x 21cm micro-isolator cages at 22ºC, light-dark cycles of 12h, free access to commercial mouse chow, and sterilized water.

Blood sample collection

After general anesthesia with intraperitoneal administration of ketamine (100mg/kg) and xylazine (10mg/kg), blood samples of approximately 1mL were collected from the retro-orbital plexus and by intracardiac punction of the animals into ethylenediaminetetraacetic acid (EDTA) tubes. Animals were then subjected to cervical dislocation and necropsy.

Hematological profiling

Samples were processed in the auto-hematology analyzer Hematoclin 2.8 VET (Quibasa-Bioclin, Belo Horizonte, Brazil). Red blood cell count (RBCC), hemoglobin (Hgb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin

(MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution (RDW), white blood cell count (WBCC), platelet cell count (PCC), and mean platelet volume (MPV) values were obtained. Manual differential leukocyte counts were performed by two calibrated researchers on blood smears stained with the panoptic fast stain kit (LaborClin, Santa Bárbara, Brazil). Systemic inflammation markers based on the blood cell counts were obtained, including the neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), and systemic immune-inflammation index (SII), as previously reported.

Biochemical analyses

Conventional liquid biochemical analyses were conducted on semiautomatic equipment BIO2000 (Bioplus, São Paulo, Brazil). We used the albumin monoreactive kit (#K040-1, Quibasa-Bioclin, Brazil) to colorimetrically measure serum albumin concentration, in which the absorbance was measured at 630 nm.

Histopathological analyses

Tongues were collected at necropsy, fixed in 7% paraformaldehyde for 24 hours, and embedded in paraffin. Two pathologists then analyzed hematoxylin and eosin-stained slides to identify areas of ED and OSCC, as reported elsewhere[10]. Regions of the tongue lined with either atrophic or hyperplastic epithelium showing alterations in the tissue architecture and cell differentiation were considered dysplastic. OSCC was identified in areas of epithelial invasion with broad pushing borders, microinvasion areas with ill-defined basement membranes, or, more frankly, invasive epithelial nests. We considered the most severe lesion in the mouse tongue as the outcome measure.

The depth of invasion (DOI) of all OSCC was measured as proposed by Müller *et al.*[11] using ImageScope software (Leica Biossystems, Nussloch, Germany). In summary, after H&E-stained slides were scanned in 400x magnification with Aperio AT2 (Leica Biosystems, Nussloch, Germany), a line was drawn in parallel to the basement membrane from one healthy margin to the other, and the distance between this line and the deepest part of the tumor was considered the DOI.

Statistical analyses

All analyses were carried out in R software version 4.3.0 (R Core Team) and GraphPad Prism version 8.2.1 (GraphPad, San Diego, USA) with α set at 5%. The incidence of ED and OSCC in the experimental animals was analyzed with the Chi-square test. Survival analyses were conducted using the Mantel-Cox log-rank test. Differences in the hematological parameters, systemic inflammatory markers and biochemical tests among the treatment groups and the identified lesions were verified with Kruskal-Wallis and Dunn's *post hoc* test with adjusted *p*-values. Differences in the DOI between the 4NQO-treated groups were verified with the Mann-Whitney U test. Correlations between DOI and the hematological parameters were verified with Spearman's ρ (rho).

Results

Incidence of epithelial dysplasia and carcinoma

By the end of the 25-week treatment period, no differences in animal survival were observed among sex and treatment groups. The incidence of OSCC in males from the 4NQO/H2O and 4NQO/EtOH groups was 92.9% (n=13/14). One animal from the 4NQO/H2O and 4NQO/EtOH groups died by week 6 and week 5, respectively, due to

aggressive behavior. Also, one animal from each group had developed only ED. On the other hand, the frequency of OSCC in females was 66.7% (n=10/15) in the 4NQO/H2O group and 60% (n=9/15) in the 4NQO/EtOH group. Differences between males and females regarding OSCC frequency were only significant in the 4NQO/EtOH groups (p=0.04).

Among five 4NQO-treated females who did not develop OSCC, three had ED, one had squamous papilloma, and one had no tongue epithelial alteration (Fig 1). All six OSCC-free females from the 4NQO/EtOH developed ED (Fig 2). Regarding the number of tumors observed per animal (Figure 3), most males (69.3%) from the 4NQO/H2O and 4NQO/EtOH groups showed two or more OSCCs. Multiple lesions were observed in 60% and 77,7% of the females from the 4NQO/H2O and 4NQO/EtOH group, respectively. These differences, however, were not significant. Moreover, no macro or microscopic lesions were observed in PPG/H2O and PPG/EtOH mice. The DOI median (range) in males from the 4NQO/H2O and 4NQO/EtOH groups was 174μm (520) and 146μm (926), respectively. The median DOI in females from the same groups was 136μm (358) and 129μm (324). There were no significant differences regarding the DOI when comparing sex and treatment groups.

Hematological profiling of the treated animals

Table 1 describes the red series, hematimetric, and platelet indices obtained from blood samples of the experimental animals according to sex and treatment groups. The RBCC, Hgb, and Hct were significantly high in males from the 4NQO-treated groups. In females, most parameters decreased with 4NQO treatment, except for platelet cell count.

Concerning white blood cell counts, systemic inflammatory markers, and biochemical parameters (Table 2), a significant increase in neutrophil count, NLR ratio, and SII was observed in males and females from the 4NQO-treated groups. In contrast, the lymphocyte count decreased. Only the PLR was significantly higher in females from the PPG/EtOH groups than in the others. Albumin measurements also showed no differences between the groups.

White blood cell and platelet parameters in epithelial dysplasia and carcinoma

There were significant differences in leucocyte counts and systemic inflammatory biomarkers when NM, ED, and OSCC were compared between the 4NQO/H2O females (Figure 4). Lymphocyte count, neutrophil count, NLR, PLR, and SII values significantly differed between NM and OSCC in females from the 4NQO/EtOH group ($p<0.05$). In males from the PPG/H2O and 4NQO/H2O, differences between NM and OSCC were achieved for neutrophil count and NLR ($p<0.05$). No correlation was found between the analyzed blood parameters and the OSCC DOI.

Discussion

In this study, we treated C57Bl/6J mice with tobacco mimicker 4NQO and ethanol to verify whether these agents, individually and in combination, are associated with tumor inflammation-related hematological alterations and tongue-derived ED and OSCC incidence between males and females. The combination of the 4NQO-induced OSCC and the Meadows-Cook chronic alcoholism models did not increase the tumor incidence when compared to the 4NQO/H2O group. However, more tumor-bearing males were observed in the 4NQO/EtOH group than in females of the same group. In other words, females

exposed to similar environmental conditions still had a reduced frequency of OSCC, which contradicts the well-established knowledge that the difference in human OSCC epidemiology concerning biological sex relies on extrinsic risk factors[1,2].

Even though the 4NQO-induced OSCC murine model has been conducted in both male and female mice, studies comparing tumor incidence between the sexes are scarce. In a study by Green et al.[12], a high histopathology score was observed in males from the 4NQO-treated groups, which the authors attributed to differences in hormonal regulation of immunity and gene expression. The role of sex hormones in 4NQO-induced carcinogenesis was reported by Haque *et al.*[13], who demonstrated that the administration of testosterone with or without ovariectomy in female rats increased the number of invasive OSCC. In contrast, the administration of estrogen with orchiectomy in males reduced it. Although the molecular mechanisms underlying such effects remain elusive, they might be associated with sexual dimorphism regarding inflammatory and immune responses. One study revealed that androgens suppress the activity of immune cells, while estrogen improves cell- and humoral-mediated immune responses[14]. Nevertheless, the profiling of tumor-infiltrating leucocytes failed to report sex-related differences in rodent models[12].

The fact that the association of 4NQO and ethanol did not increase OSCC incidence in our study was somewhat unexpected, considering that tobacco and alcohol consumption have a synergistic effect on human OSCC[15]. A similar finding was reported by Osei-Sarfo *et al.*[16], who treated C57Bl/6J male mice with 4NQO at 100ug/mL for 10 weeks, followed by 20% ethanol for 10 weeks. Guo *et al.*[17], on the other hand, demonstrated a significant increase in the number of OSCC-bearing male mice treated with 4NQO alone at

100ug/mL for 8 weeks (20%) when compared to animals that were subsequently treated with 8% ethanol for 16 weeks (43%). Such contrasting findings might be related to differences in the experimental protocols regarding the duration of the treatment and the ethanol concentration. In our experience, C57Bl/6J mice do not tolerate well 20% ethanol in the drinking water *ad libitum* as per the Meadows-Cook model. For such a high concentration, other chronic alcoholism protocols might be considered[18]. The duration of the 4NQO treatment and observational periods also impact the number of lesions identified in the histopathological analysis, with a high incidence of OSCC occurring with 16 weeks of 4NQO treatment followed by an 8-week observation period.

Irrespective of the study design, ethanol has been shown to promote oral carcinogenesis in association with 4NQO by activating 5-lipoxygenase pathway[17], as well as by increasing and reducing p38 MAPK and phosphorylated β-catenin expression levels, respectively[16]. Increased levels of histone epigenetic marks were also observed with the combination of 4NQO and EtOH, such as H3K27ac, H3K9/14ac, H3K27me3, and H3K9me3[19]. It remains to be enlightened how these molecular alterations affect the incidence of tumors between males and females since the previous studies used only male mice as experimental units.

Regarding the hematological profile, we observed that RBCC, Hgb, and Hct were significantly higher in 4NQO/H2O and 4NQO/EtOH-treated males than in the PPG/H2O and PPG/EtOH groups. Such results are akin to those previously published by our research group [10]. Even though increased red blood cell parameters could result from hemoconcentration related to animal dehydration, which is expected during the 4NQO and ethanol treatments[10], serum albumin dosage was not statistically different. Indeed,

hyperalbuminemia would be expected under conditions of low water intake when plasma osmolarity and albumin concentration increase similarly, with the latter being a major determinant of blood colloidal osmotic pressure[20].

In this matter, another hypothesis is that 4NQO would affect hematopoiesis. Micronucleate polychromatic erythrocytes were identified in peripheral blood samples of ICR mice following 4NQO treatment[21], which suggests bone marrow toxicity. These alterations, however, could not be analyzed in our blood samples. How 4NQO affects red blood cell production needs further investigation. Increased MCH and MCHC in males may also be due to RBCC, Hgb, and Hct changes. In contrast, females significantly reduced these parameters, particularly between the PPG/H2O and 4NQO/H2O groups, which could be interpreted as anemia. Indeed, anemia is very common in patients with OSCC[22] as a result of the suppressive effect of cancer in erythropoiesis[23]. Overall, further research needs to clarify the difference in red series parameters between male and female mice.

Concerning platelet cell parameters, 4NQO and ethanol treatment did not affect platelet count, PLR, and SII in male mice. Females from the PPG/H2O and PPG/EtOH groups, on the other hand, had significantly low platelet counts. It is impossible to exclude that this reduction represents pseudo thrombocytopenia due to using EDTA tubes[24], even though no platelet clusters were observed under the microscopic analysis of blood smear slides.

In cancer development, platelets might secrete pro-inflammatory and anti-inflammatory cytokines and growth factors, promoting angiogenesis and facilitating metastasis [25.] In a

recent study, Hasegawa (2020)[26] reported an association between high PLR and poorer survival rates in oral cancer. Although significant differences were found for platelet count, PLR, and SII between NM and OSCC in females, further studies should be carried out to clarify platelet function in oral carcinogenesis, considering the methodological issues that could alter platelet-related parameters in this study. The use of sodium citrate blood collection tubes might address this issue.

Our data analysis for white blood cell parameters revealed an increase in neutrophils in 4NQO-treated animals while lymphocyte count decreased. These findings are similar to a recent study published by our research group[10]. Neutrophilia and lymphocytopenia are common features of cancer-associated chronic inflammation, so the NLR can be applied to measure the tumorigenic effect of neutrophil-mediated innate immunity and the anti-tumor effect of lymphocyte-mediated adaptive immune response[27].

Despite the cancer-related alterations of white blood cell counts, it is essential to consider the direct effect of 4NQO treatment in these parameters. Sahu *et al.*[28] demonstrated the cytotoxicity and genotoxicity of 4NQO against B and T lymphocytes and a significant reduction of neutrophils in the peripheral blood of 4NQO-treated animals. These results were slightly different from our study. However, an interesting finding in their work was a reduction and increase in lymphoid and myeloid cells from dysplastic to OSCC-affected animals, suggesting significant differences in circulating inflammatory cells between preneoplastic and neoplastic conditions[28.]

When analyzing the different stages of oral carcinogenesis, changes in lymphocyte and neutrophil counts, NLR, and SII were already detected between normal mucosa and

epithelial dysplasia of females from the 4NQO/H2O groups. These data suggest systemic hematological alterations could be detected in peripheral blood samples in the early stages of tumor development. Indeed, previous studies have compared hematological inflammatory parameters between precancerous and malignant laryngeal lesions, especially the NLR and PLR, whose values were high compared to benign and normal tissue[7]. In the context of OPMDs and OSCC, a study by Ram *et al.*[29] revealed that NLR and PLR significantly increased from healthy subjects to OPMDs- and OSCC-harboring patients. These alterations, according to Yang *et al.*[30], could be detected even decades before the diagnosis of head and neck cancer.

Considering the benefits that hematological analyses could offer for early diagnosis of OSCC and risk assessment for patients with OPMDs, more studies are necessary. Regarding animal models, a few limitations should be considered. The first is related to the amount of blood samples that can be collected from animals, as large volumes could only be obtained prior to euthanasia. Although certain analyses could be performed with a small blood volume, multiple samples should be collected throughout the experimental period better to characterize the hematological changes during multistep oral carcinogenesis, significantly impacting animal welfare.

Another important aspect of 4NQO-induced OSCC in rodents is the incidence of multiple lesions, some of which are mixed in nature, with animals developing ED and OSCC at the same time and in different regions of the tongue. The small number of animals with ED was a limitation of our study since it hindered statistical comparisons among the male groups. With this in mind, 4NQO concentration, treatment period, and/or time of euthanasia should be adjusted so a high number of ED-bearing mice can be obtained.

Evaluating these parameters with longer observation periods following 4NQO and ethanol treatment is also recommended to evaluate larger and more invasive tumors. Our study showed no significant differences in DOI between sex and treatment groups, possibly resulting from a short 4NQO treatment period with less invasive lesions induced. Consequently, no correlations were found between DOI and the hematological parameters analyzed. Additionally, since the carcinogen comes into contact not only with the oral mucosa but also with the esophageal region, potentially leading to carcinoma in that area, it is not possible to definitively determine whether the alterations observed in this experiment are either caused by oral lesions or influenced by lesions in other regions of the upper digestive tract.

In conclusion, our results indicate that neutrophil and lymphocyte counts and the derived neutrophil-to-lymphocyte ratio are significantly altered during epithelial tumorigenesis in mice. These findings suggest that tumor inflammation-related systemic changes could be explored as biomarkers for OSCC development and progression. Sex-related differences in tumor-related immunoinflammatory response should be further investigated regarding the differential incidence of OSCC between male and female mice submitted to 4NQO and ethanol treatment.

Figure captions

Fig 1.

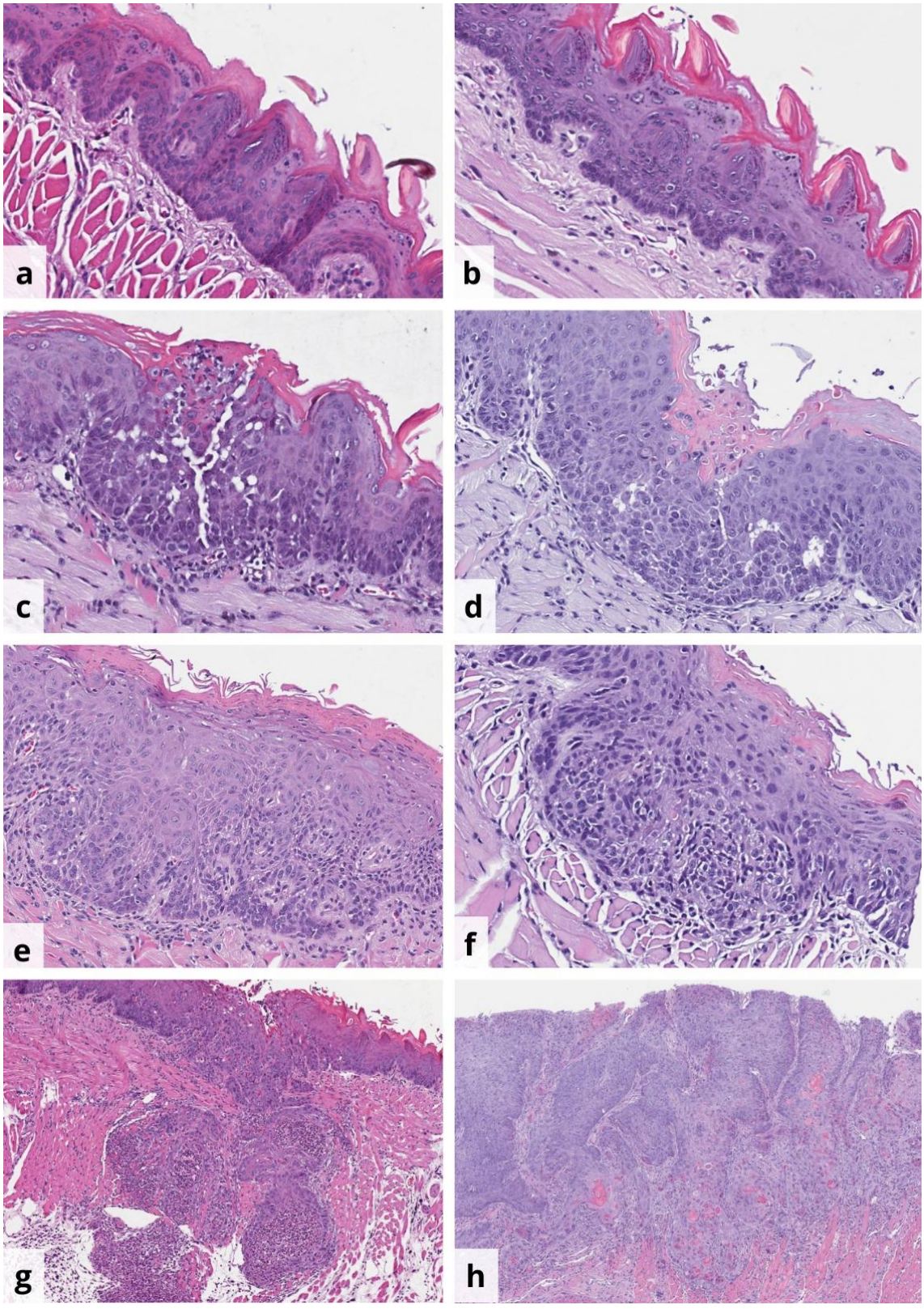


Fig 2.

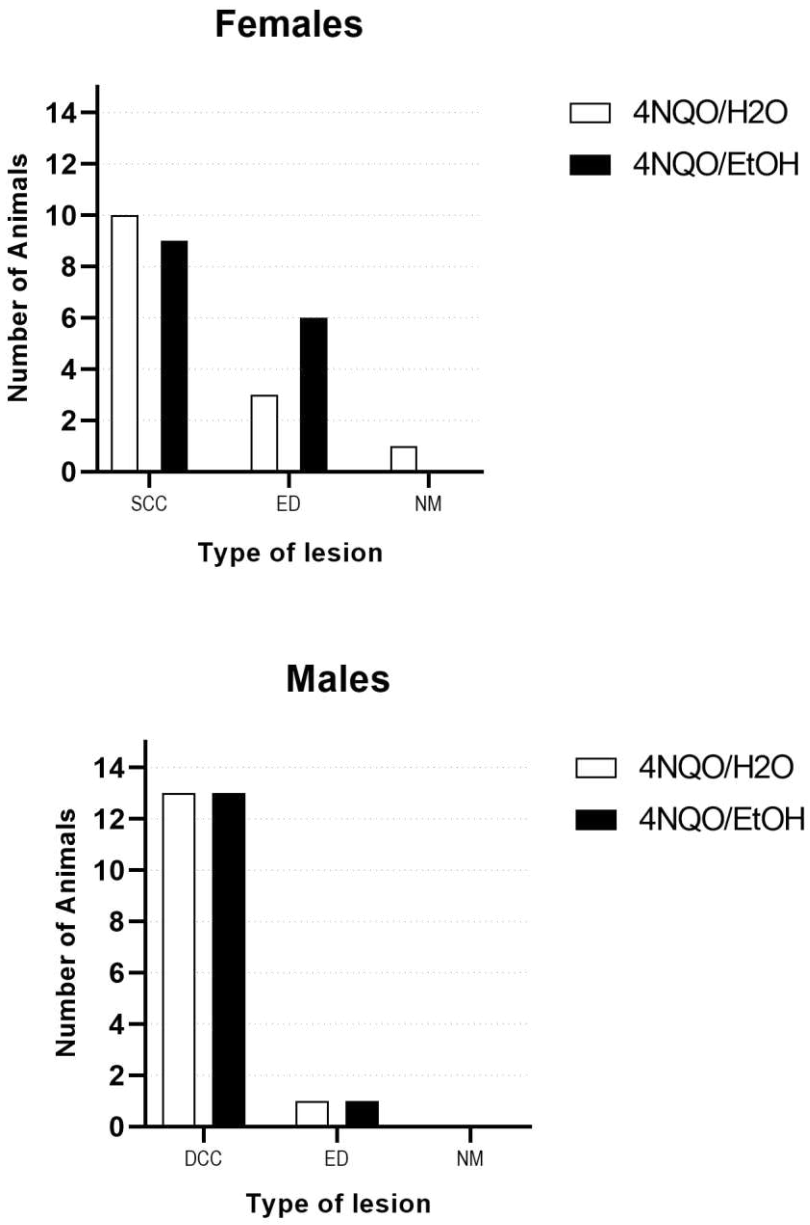
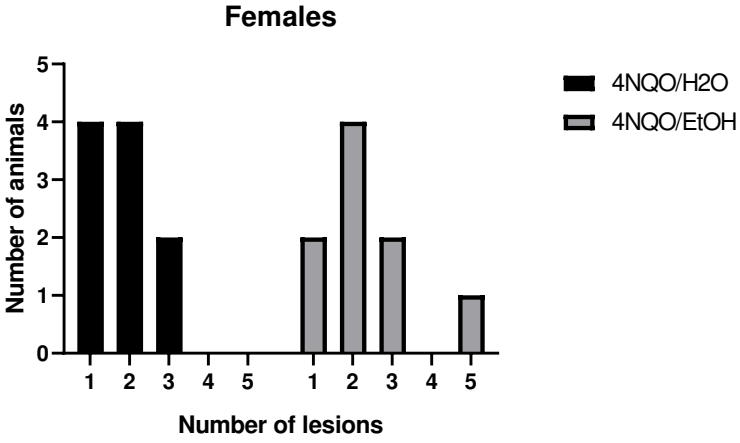

Females
Number of Animals
14
12
10
8
6
4
2
0
SCC
ED
NM
Type of lesion
4NQO/H2O
4NQO/EtOH
Males
Number of Animals
14
12
10
8
6
4
2
0
DCC
ED
NM
Type of lesion
4NQO/H2O
4NQO/EtOH

Fig 3.


Males
Number of animals
0
2
4
6
8
1 2 3 4 5 1 2 3 4 5
Number of lesions
4NQO/H2O
4NQO/EtOH

(a)


Females
Number of animals
0
1
2
3
4
5
1 2 3 4 5 1 2 3 4 5
Number of lesions
4NQO/H2O
4NQO/EtOH

(b)

Fig 4.

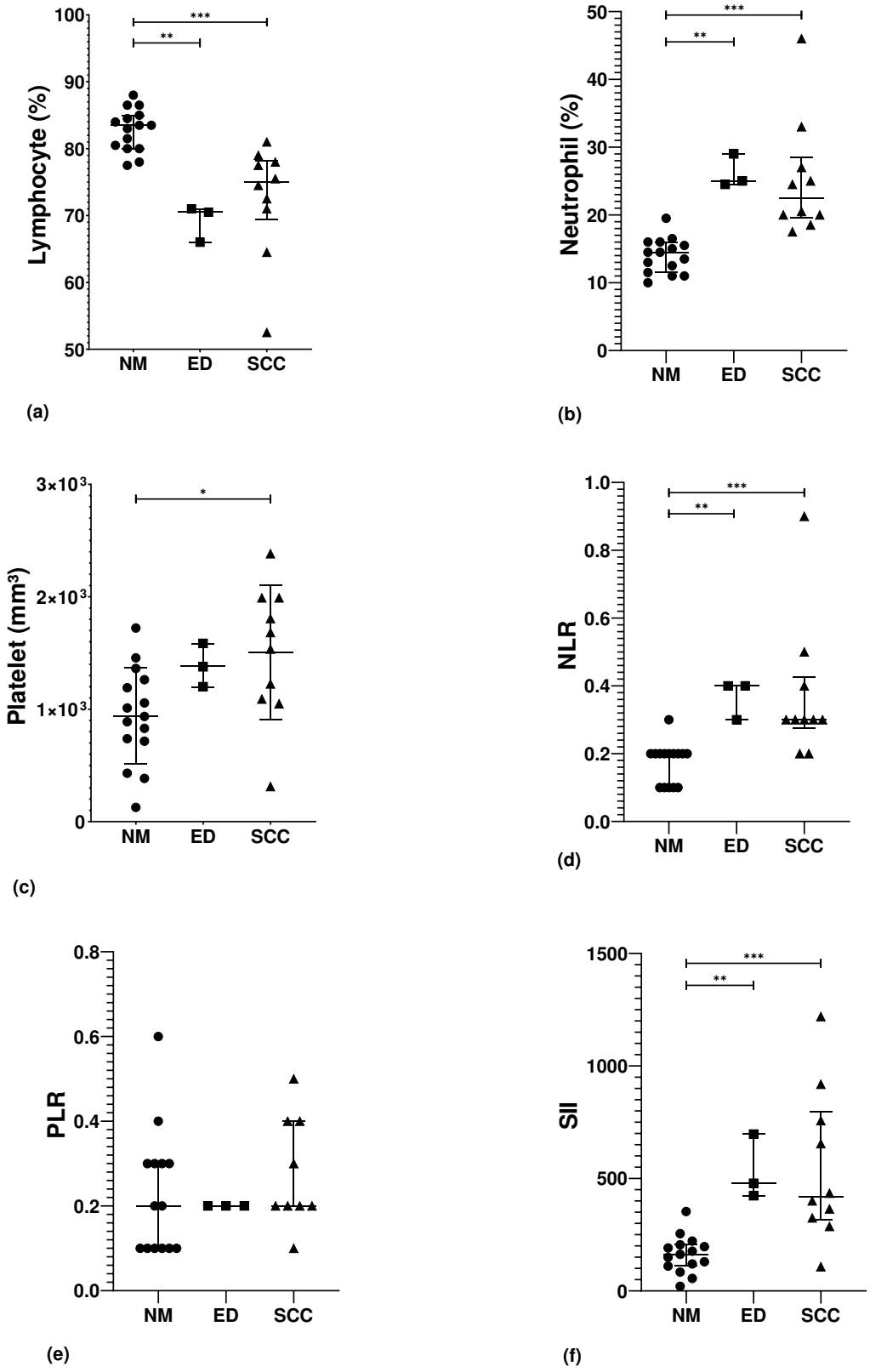

Lymphocyte (%)
100
90
80
70
60
50
***
**
NM
ED
SCC
(a)
Neutrophil (%)
50
40
30
20
10
0
***
**
NM
ED
SCC
(b)
Platelet (mm³)
3×10³
2×10³
1×10³
0
*
NM
ED
SCC
(c)
NLR
1.0
0.8
0.6
0.4
0.2
0.0
***
**
NM
ED
SCC
(d)
PLR
0.8
0.6
0.4
0.2
0.0
NM
ED
SCC
(e)
SII
1500
1000
500
0
***
**
NM
ED
SCC
(f)

Table 1.

Parameters	Experimental groups, median (range)									
	Males					Females				
	PPG/H2O[a]	PPG/EtOH[b]	4NQO/H2O[c]	4NQO/EtOH[d]	*p*[e]	PPG/H2O[a]	PPG/EtOH[b]	4NQO/H2O[c]	4NQO/EtOH[d]	*p*[e]
RBCC[f] (10^6/uL)	9,6 (4,2)^{A}	9,5 (1,9)^{A}	10,7 (4,6)^{B}	10,6 (2,9)^{A}	*	11,8 (5,2)^{A}	10,4 (4,3)^{AB}	9,9 (7,1)^{B}	10,4 (3,6)^{A}	*
Hemoglobin (g/dL)	13,4 (3,6)^{A}	13,7 (2,8)^{A}	15,6 (7,5)^{B}	15,5 (4,6)^{B}	***	17,3 (6,3)^{A}	15,4 (5,8)^{AB}	14,2 (7,9)^{B}	15,2 (3,4)^{A}	**
Hematocrit (%)	42,1 (19,6)^{AB}	42,7 (9,3)^{A}	47 (16,3)^{B}	46,6 (11,9)^{AB}	*	52,7 (16,9)^{A}	46,9 (20)^{A}	43,4 (16,9)^{B}	45,8 (15,2)^{A}	***
MCV[g] (fL)	44,1 (2,8)^{A}	44,8 (4,3)^{A}	44,1 (4,4)^{A}	44,1 (4,6)^{A}	ns	44,2 (8,6)^{AB}	44,8 (5,2)^{A}	43,5 (32,2)^{B}	44,1 (4,9)^{AB}	*
MCH[h] (pg)	14,1 (4)^{A}	14,4 (1,5)^{AC}	14,7 (1,8)^{BC}	14,5 (1,7)^{BC}	**	14,6 (2)^{AB}	15 (2,6)^{A}	14,3 (7,1)^{B}	14,5(3,2)^{AB}	*
MCHC[i] (g/dL)	32 (9,5)^{A}	32,1 (3,2)^{A}	33,4 (4,1)^{B}	32,9 (2,3)^{AB}	****	33 (2,1)^{A}	33,4 (5,2)^{A}	32,8 (21,4)^{A}	32,8 (7,3)^{A}	ns
RDW[j] (%)	15,8 (3,3)^{A}	15,3 (3)^{AB}	15 (4,6)^{B}	15 (3,2)^{B}	**	15,3 (16)^{A}	15 (1,4)^{AB}	14,9 (14,6)^{B}	15 (4,2)^{AB}	*
Platelets (/mm³)	2032 (1803)^{A}	2177 (1759)^{A}	1923 (1800)^{A}	2057 (1512)^{A}	ns	936 (1595)^{A}	1220 (1322)^{AB}	1535 (2071)^{BC}	1600 (2043)^{C}	***
MPV[k]	4,5 (1,1)^{A}	4,5 (0,6)^{A}	4,3 (1,4)^{A}	4,5 (1)^{A}	ns	4,7 (1,9)^{AB}	5,2 (1,5)^{A}	4,6 (2)^{B}	4,5 (1,1)^{AB}	***

[a]PPG/H2O: propylene glycol and water; [b]PPG/EtOH: propylene glycol and ethanol; [c]4NQO/H2O: 4-nitroquinoline-*N*-oxide and water; [d]4NQO/EtOH: 4-nitroquinoline-*N*-oxide and etanol; [e]Kruskal-Wallis with corrected Dunn's *post-hoc* test, $p<0.05$, similar uppercase letters in a row denotes the absence of statistical differences, and different uppercase letters represent statistical significance, ns: not significant; [f]RBCC: red blood cell count; [g]MCV: mean corpuscular volume; [h]MCH: mean corpuscular hemoglobin; [i]MCHC: mean corpuscular hemoglobin concentration; [j]RDW: red blood cell width; [k]MPV: mean platelet volume.

Table 2.

Parameters	Experimental groups, median (range)									
	Males					Females				
	PPG/H2O[a]	PPG/EtOH[b]	4NQO/H2O[c]	4NQO/EtOH[d]	p[e]	PPG/H2O[a]	PPG/EtOH[b]	4NQO/H2O[c]	4NQO/EtOH[d]	p[e]
Lymphocytes (%)	84 (20)^{A}	82 (7)^{A}	73 (23)^{B}	76 (27)^{B}	****	83.5 (11.5)^{A}	81 (33)^{A}	75 (23.5)^{B}	72 (24.5)^{B}	****
Neutrophils (%)	13 (17)^{A}	16 (5)^{A}	20 (30)^{B}	21 (32)^{B}	***	14.5 (9.5)^{A}	14.5 (32.5)^{A}	24.5 (28.5)^{B}	22 (27)^{B}	****
Monocytes (%)	2.5 (1.4)^{A}	2.9 (0.9)^{A}	2.7 (1.9)^{A}	2.7 (1.4)^{A}	ns	3 (4.5)^{AB}	2 (6.5)^{A}	2.2 (4.5)^{A}	3 (4.5)^{A}	ns
Albumin	1.8 (1.2)^{A}	1.9 (1)^{A}	1.8 (1.9)^{A}	2.4 (3.1)^{A}	ns	2.65 (4)^{A}	3.5 (3.7)^{A}	3.6 (3.7)^{A}	2.9 (5)^{A}	ns
NLR[f]	0.16 (0.32)^{A}	0.20 (0.1)^{A}	0.28 (11.4)^{B}	0.27 (0.9)^{B}	***	0.17 (0.14)^{A}	0.17 (0.67)^{A}	0.34 (0.65)^{B}	0.31 (5.1)^{B}	****
PLR[g]	0.19 (0.24)^{A}	0.21 (0.14)^{A}	0.22 (4.9)^{A}	0.23 (2.2)^{A}	ns	0.17 (0.57)^{A}	0.37 (0.55)^{B}	0.23 (0.42)^{C}	0.25 (3.6)^{C}	*
SII[h]	330 (694)^{A}	376.9 (398.5)^{AB}	527.2 (26260)^{B}	603.4 (1846)^{B}	*	162.5 (321.9)^{A}	185.7 (321.2)^{A}	434 (1111)^{B}	449.8 (14572)^{B}	****

[a]PPG/H2O: propylene glycol and water; [b]PPG/EtOH: propylene glycol and ethanol; [c]4NQO/H2O: 4-nitroquinoline-*N*-oxide and water; [d]4NQO/EtOH: 4-nitroquinoline-*N*-oxide and etanol; [e]Kruskal-Wallis with corrected Dunn's *post-hoc* test, $p<0.05$, similar uppercase letters in a row denotes the absence of statistical differences, and different uppercase letters represent statistical significance, ns: not significant; [f]NLR: neutrophil-to-lymphocyte ratio; [g]PLR: platelet-to-lymphocyte ratio; [h]SII: systemic immune-inflammation index.

Figure 1. Microscopic features of epithelial lesions induced by 4NQO and ethanol in male (left column) and female (right column) C57Bl/6J mice. (a and b) Normal mucosa of PPG/EtOH treated mice without microscopic alterations. (c and d) Epithelial dysplasia in 4NQO/H2O treated mice. (e and f) Oral squamous cell carcinoma in 4NQO/EtOH treated mice. Hematoxylin and eosin, 200x original magnification. (g and h) Frank invasive oral squamous cell carcinoma in 4NQO/EtOH treated mice (Hematoxylin and eosin, 100x original magnification)

Figure 2. Number of animals in the 4NQO/H2O and 4NQO/EtOH groups that developed each type of lesion.

Figure 3. Number of tumors observed per animal in the 4NQO/H2O and 4NQO/EtOH groups. (a) Males (b) Females.

Figure 4. Comparative analyses in leucocyte parameters and inflammatory biomarkers in the NM, ED, and OSCC of females from the 4NQO/H2O group. (a) limphocytes; (b) neutrophill; (c) platelet; (d) NLR; (e) PLR; (f) SII.

Table 1. Red blood cell and platelet parameters according to animal gender and treatment groups.

Table 2. White series indices, biochemical parameters, and systemic inflammatory markers according to animal gender and treatment groups.

References

1. Tranby EP, Heaton LJ, Tomar SL, Kelly AL, Fager GL, Backley M, Frantsve-Hawley J. 2022. Oral Cancer Prevalence, Mortality, and Costs in Medicaid and Commercial Insurance Claims Data. Cancer Epidemiol Biomarkers Prev;31(9):1849-1857. doi: 10.1158/1055-9965.EPI-22-0114. PMID: 35732291; PMCID: PMC9437560.
2. Tan Y, Wang Z, Xu M, Li B, Huang Z, Qin S, Nice EC, Tang J, Huang C. 2023. Oral squamous cell carcinomas: state of the field and emerging directions. Int J Oral Sci. 15(1):44. doi: 10.1038/s41368-023-00249-w. PMID: 37736748; PMCID: PMC10517027.
3. Zheng J, Chen K, Cai L, Pan Y, Zeng Y. A Potential biomarker for the early diagnosis of OSCC: saliva and serum PrPC. J Cancer 2024; 15(6):1593-1602. doi:10.7150/jca.92489. https://www.jcancer.org/v15p1593.htm
4. Yang, Y., Liu, R., Ren, F., Guo, R., & Zhang, P., 2018. Prognostic and clinicopathological significance of neutrophil-to-lymphocyte ratio in patients with oral cancer. Biosci. Rep. 38(6), BSR20181550. https://doi.org/10.1042/BSR20181550
5. Tangthongkum M, Tiyanuchit S, Kirtsreesakul V,Supanimitjaroenporn P, Sinkitjaroenchai W. 2017. Platelet to lymphocyte ratio and red cell distribution width as prognostic factors for survival and recurrence in patients with oral cancer. Eur Arch Otorhinolaryngol.274(11):3985-3992. doi:10.1007/s00405-017-4734-1
6. Diao P, Wu Y, Ge H, *et al.* 2019. Preoperative circulating platelet, neutrophil, and lymphocyte counts predict survival in oral cancer. Oral Dis.25(4):1057-1066. doi:10.1111/odi.13049
7. Kum RO, Ozcan M, Baklaci D, Kum NY, Yilmaz YF, Gungor V, Unal A. 2014. Elevated neutrophil-to-lymphocyte ratio in squamous cell carcinoma of larynx

compared to benign and precancerous laryngeal lesions. Asian Pac J Cancer Prev;15(17):7351-5. doi: 10.7314/apjcp.2014.15.17.7351.

8. Li B, Zhou P, Liu Y*, et al*. 2018. Platelet-to-lymphocyte ratio in advanced cancer: review and meta-analysis. Clin Chim Acta. 483:48-56. doi:10.1016/j.cca.2018.04.023
9. Servato JPS, Ueira-Vieira C, de Faria PR, Cardoso SV, Loyola AM. The importance of inducible nitric oxide synthase and nitrotyrosine as prognostic markers for oral squamous cell carcinoma. J Oral Pathol Med. 2019;48(10):967-975. doi:10.1111/jop.12942
10. Santos, D. O., Costa, A. R. G. F., Duarte, P. V. S., Pitorro, T. E. A., Fonseca, L. C., Cardoso, S. V., de Faria, P. R., & Loyola, A. M. 2024. Depth of invasion of oral squamous cell carcinoma in Nos2-knockout mice correlated to alterations in systemic inflammatory markers following 4NQO treatment. Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology, 53(3), 208–216. https://doi.org/10.1111/jop.13523
11. Müller, S., Boy, C., Day, A., Magliocca, R., Richardson, S., Sloan, P., Tilakaratne, M., Zain, B., Thompson, R. 2019. Data Set for the Reporting of Oral Cavity Carcinomas: Explanations and Recommendations of the Guidelines From the International Collaboration of Cancer Reporting. Archives of pathology & laboratory medicine, 143(4), 439–446. https://doi.org/10.5858/arpa.2018-0411-SA
12. Green, M., Ciancio, J., Goral, J., *et al*. 2021. Dietary fat and male sex increase histopathological changes in a mouse model of oral cancer. Oral Dis. https://doi.org/10.1111/odi.13542
13. Haque, J., Katakuta, A., Kamiyama, I., Takagi, R., Shibahara, T., Noma, H. 2007. Effects of Sex Hormones on Rat Tongue Carcinoma Induced by 4-

Nitroquinoline 1-Oxide (4NQO), The Bulletin of Tokyo Dental College, Volume 48, Issue 1, Pages 9-17, https://doi.org/10.2209/tdcpublication.48.9,

14. Irelli, Azzurra, Sirufo, M., D'Ugo, C., Ginaldi, L., Martinis, M. 2020. Sex and Gender Influences on Cancer Immunotherapy Response. *Biomedicines* 8, no. 7: 232. https://doi.org/10.3390/biomedicines8070232
15. Mello, F.W., Melo, G., Pasetto, J.J. *et al.* 2019. The synergistic effect of tobacco and alcohol consumption on oral squamous cell carcinoma: a systematic review and meta-analysis. Clin Oral Invest . https://doi.org/10.1007/s00784-019-02958-1
16. Kwame, O-S., Tang, X. Urvalek, A., Scognamiglio, T., Gudas, L. 2013. The molecular features of tongue epithelium treated with the carcinogen 4-nitroquinoline-1-oxide and alcohol as a model for HNSCC, *Carcinogenesis*, Volume 34, Pages 2673–2681, https://doi.org/10.1093/carcin/bgt223
17. Guo, Y., Wang, X., Zhang, X., Sun, Z., & Chen, X. 2011. Ethanol promotes chemically induced oral cancer in mice through activation of the 5-lipoxygenase pathway of arachidonic acid metabolism. Cancer prevention research (Philadelphia, Pa.), 4(11), 1863–1872. https://doi.org/10.1158/1940-6207.CAPR-11-0206
18. Brandon-Warner, E. *et al*. 2012. Rodent models of alcoholic liver disease: Of mice and men, Alcohol, Volume 46, Issue 8, Pages 715-725, ISSN 0741-8329, https://doi.org/10.1016/j.alcohol.2012.08.004.
19. Urvalek, M., Osei-Sarfo, K., Tang, H., Zhang, T., Scognamiglio, T., Gudas, J. 2015. Identification of Ethanol and 4-Nitroquinoline-1-Oxide Induced Epigenetic and Oxidative Stress Markers During Oral Cavity Carcinogenesis. Alcohol Clin Exp Res, 39: 1360-1372. https://doi.org/10.1111/acer.12772
20. Roopenian, C., Low, E., Christianson, J., Proetzel, G., Sproule, J., Wiles, V. 2015. Albumin-deficient mouse models for studying metabolism of human albumin and

pharmacokinetics of albumin-based drugs. MAbs. doi: 10.1080/19420862.2015.1008345.

21. Lu, J., Baxter, R., Wang, Y., Harper, L., Tasaka, F., Kohda, K., 1990. Induction of covalent DNA modifications and micronucleated erythrocytes by 4-nitroquinoline 1-oxide in adult and fetal mice. Cancer Res. 50(19):6192-8.
22. Spanier, G., Böttcher, J., Gerken, M., Fischer, R., Roth, G., Lehn, P., Klingelhöffer, C., Meier, J.K., Fraccaroli, A., Tischer, J., Ettl, T., Klinkhammer-Schalke, M., Reichert, T.E., Spoerl, S., 2020. Prognostic value of perioperative red blood cell transfusion and anemia on survival and recurrence in oral squamous cell carcinoma. Oral Oncol. 107:104773. https://doi.org/10.1016/j.oraloncology.2020.104773
23. Dicato, M., Plawny, L., Diederich, M., 2010. Anemia in cancer. Ann. Oncol. 21(7):vii167–vii172. https://doi.org/10.1093/annonc/mdq284
24. Lippi G, Plebani M. 2012. EDTA-dependent pseudothrombocytopenia: further insights and recommendations for prevention of a clinically threatening artifact. Clin Chem Lab Med. 50(8):1281-5. doi: 10.1515/cclm-2012-0081.
25. Zhou L, Zhang Z, Tian Y, Li Z, Liu Z, Zhu S. 2023. The critical role of platelet in cancer progression and metastasis. 2023 Sep 28;28(1):385. doi: 10.1186/s40001-023-01342-w. PMID: 37770941; PMCID: PMC10537080.
26. Hasegawa, T., Iga, T., Takeda, D. *et al.* 2020. Neutrophil-lymphocyte ratio associated with poor prognosis in oral cancer: a retrospective study. BMC Cancer 20, 568. https://doi.org/10.1186/s12885-020-07063-1
27. Howard, R., Kanetsky, P.A., Egan, K.M. 2019. Exploring the prognostic value of the neutrophil-to-lymphocyte ratio in cancer. Sci Rep. 9:19673. doi:10.1038/s41598-019-56218-z

28. Sahu, S. R. *et al*. 2023. 4-nitroquinoline 1-oxide induces immune cells death to onset early immunosuppression during oral squamous cell carcinoma development. Frontiers in immunology vol. 14 1274519. doi:10.3389/fimmu.2023.1274519

29. Ram, B., Chalathadka, M., Dengody, P.K. *et al.* 2023. Role of Hematological Markers in Oral Potentially Malignant Disorders and Oral Squamous Cell Carcinoma. Indian J Otolaryngol Head Neck Surg 75, 2054–2062. https://doi.org/10.1007/s12070-023-03803-4

30. Yang, Y. , Liang, Y. , Sadeghi, F. , Feychting, M. , Hamar, N., Fang, F., Zhang, Z., Liu, Q. 2023. Risk of head and neck cancer in relation to blood inflammatory biomarkers in the Swedish AMORIS cohort. Frontiers in Immunology. Doi: 10.3389/fimmu.2023.1265406