Universidade do Minho
Escola de Ciências

Shakiba Gholami

Immunoexpression profile of hypoxia-inducible factor (HIF) targets in premalignant and malignant oral lesions: A pilot study

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Master in Molecular Genetics

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Statement of integrity

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

Perfil de imuno-expressão de proteínas alvo do HIF (hypoxia-inducible factor) em lesões orais pré-malignas e malignas: Um estudo piloto

Introdução: O carcinoma da cavidade oral é a décima sexta causa de morte por cancro. Tem um perfil agressivo e é o cancro mais prevalente entre os diferentes subtipos de cancro da cabeça e pescoço. A maioria dos doentes com carcinoma da cavidade oral é diagnosticada com tumores em estádios avançados, e apresentam um prognóstico adverso. Assim, é urgente procurar novos biomarcadores de prognóstico e identificar novas estratégias terapêuticas. A ocorrência de alterações metabólicas é uma dos pilares do cancro. As células malignas são capazes de reprogramar o seu metabolismo, mesmo na presença de oxigénio, aumentando a conversão de glicose em lactato através da via glicolítica, num fenómeno conhecido como "Efeito Warburg". Para tal, várias proteínas relacionadas com o metabolismo glicolítico sofrem um aumento na sua expressão.

Objetivo: Pretendeu-se avaliar a imunoexpressão de GLUT-1, GLUT-3, HK-II, PFK-L, PKM-2, pPDH, LDH-A, MCT-4 e CA-IX em lesões pré-malignas e em amostras de carcinoma de células escamosas (CCE), a fim de identificar possíveis correlações entre a imunoexpressão dos biomarcadores e parâmetros clínico-patológicos e de prognóstico.

Materiais e Métodos: Neste estudo retrospectivo foram recolhidas amostras de CCE de 21 doentes e amostras de lesões orais pré-malignas de 34 doentes, bem como os seus dados clínico-patológicos e de seguimento. Cortes histológicos das amostras fixadas em formol e incluídas em parafina foram submetidos a imunohistoquímica, para identificação das proteínas GLUT-1, GLUT-3, HK-II, PFK-L, PKM-2, pPDH, LDH-A, MCT-4 e CA-IX.

Resultados: Verificou-se que a expressão de CA-IX, MCT-4, LDH-A, PKM-2 e PFK-L nos casos de CCE era significativamente superior à expressão nas amostras de lesões pré-malignas. Foi observada uma tendência de correlação entre o aumento da expressão dos biomarcadores e a agressividade tumoral, sendo a associação significativa para as proteínas HK-II e LDH-A; a expressão aumentada de HK-II e a CA-IX associou-se a uma diminuição das taxas de sobrevivência. Tal foi igualmente notado para as proteínas GLUT-1 e GLUT-3 quando a sua expressão foi observada no compartimento hipóxico das lesões malignas.

Conclusão: As células malignas do CCE apresentam expressão aumentada de proteínas glicolíticas, e tal associa-se com a agressividade tumoral e com um prognóstico adverso dos doentes com CCE. São necessários estudos adicionais que explorem o papel do fenótipo glicólico na carcinogénese oral.

Palavras-chave: Cancro da cabeça e pescoço, transporte de lactato, transportadores de monocarboxilatos, glicólise aeróbia, efeito de Warburg, imunohistoquímica.

Immunoexpression profile of hypoxia-inducible factor (HIF) targets in premalignant and malignant oral lesions: A pilot study

Background: Oral cancer is the sixteenth leading cause of cancer death. It has an aggressive profile and it is the most prevalent cancer among different subtypes of head and neck cancer. The majority of oral cancer patients are diagnosed with advanced stage tumors and display a poor prognosis. Thus, it is urgent to investigate new prognostic biomarkers and identify novel therapeutic strategies. The occurrence of metabolic alterations is one of the hallmarks of cancer. Cancer cells are able to reprogram their metabolism, even in the presence of oxygen, enhancing glucose conversion to lactate through the glycolytic pathway, a phenomenon known as "Warburg effect". For this purpose, several metabolism-related proteins are upregulated.

Objective: The aim of the study was to evaluate the immunoexpression of GLUT-1, GLUT-3, HK-II, PFK-L, PKM-2, pPDH, LDH-A, MCT-4, and CA-IX in premalignant lesions and in oral squamous cell carcinoma samples, in order to identify potential correlations between biomarkers' immunoexpression, clinicopathological and prognostic parameters.

Material and Methods: In this retrospective study, oral squamous cell carcinoma (OSCC) samples from 21 patients and premalignant (PM) oral samples from 34 patients were collected, as well as their clinicopathological and follow up data. The formalin-fixed, paraffin-embedded tissues were stained for GLUT-1, GLUT-3, HK-II, PFK-L, PKM-2, pPDH, LDH-A, MCT-4, and CA-IX by immunohistochemistry.

Results: CA-IX, MCT-4, LDH-A, PKM-2 and PFK-L expressions were significantly increased in OSCC samples when compared to premalignant lesions. A tendency was observed towards increased biomarkers' expression and poor clinicopathological features, being the differences significant regarding HK-II and LDH-A expression; HK-II and CA-IX were additionally correlated with low survival rates. GLUT-1 and GLUT-3 were also significantly associated with a poor outcome when their expression was observed in the hypoxic compartment of the malignant lesions.

Conclusion: Oral cancer cells overexpress glycolysis-related proteins, and this associates with aggressiveness features and a poor outcome of OSCC patients. Further research into a deep understanding of the glycolic phenotype in oral carcinogenesis in needed.

Keywords: Head and neck cancer, lactate transport, monocarboxylate transporters, aerobic glycolysis, Warburg effect, immunohistochemistry.

Table of contents

Introduction 1

1. Cancer metabolism and the Warburg effect 1

1.1. Regulators and effectors of the Warburg effect 3

1.1.1 The main regulator: Hypoxia and hypoxia inducible factor (HIF) 3

1.1.2 The main effectors – HIF downstream targets 5

1.1.2.1. Glucose transporters 5

1.1.2.2. Rate-limiting enzymes of glycolysis 6

1.1.2.3. Pyruvate dehydrogenase complex and lactate dehydrogenase 7

1.1.2.4. Monocarboxylate transporters 8

1.1.2.5. Carbonic anhydrases 9

1.2. Consequences of the Warburg effect: Lactate and microenvironmental acidosis 10

2. Oral Cancer 13

2.1 General overview 13

2.2 Anatomy of the oral cavity 13

2.3 Epidemiology and etiology of oral cancer 14

2.4 Oral Potentially Malignant Disorders 15

2.5 Pathological subtypes, staging and grading of oral cancer 17

2.6 Natural history and molecular pathogenesis of oral cancer 20

2.7 Diagnosis, management and prognosis 22

2.8 Major drawbacks and concerns 22

3 Metabolic alterations in oral cancer 23

Objectives 25

Materials and Methods 26

Patients and tissue samples 26

Immunohistochemistry 29

Evaluation of immunohistochemistry results 31

Statistical analysis 31

Results 32

Prognostic significance of clinicopathological parameters in premalignant and malignant lesions 32

Immunoexpression of metabolism-related biomarkers in premalignant and malignant lesions 32

Clinicopathological and prognostic significance of metabolism-related biomarkers in premalignant and malignant lesions 44

Immunoexpression of metabolism-related biomarkers in normoxic *versus* hypoxic regions of malignant lesions 49

Clinicopathological and prognostic significance of metabolism-related biomarkers in hypoxic regions of malignant lesions 51
Discussion 56
Conclusion 61
References 62
Supplementary data 71

Abbreviations index

[18F]-FDG	2-[18F] fluoro-2-deoxy-d-glucose
ACAC2	Acidic laccase gene 2
Acetyl-coA	Acetyl-coenzyme A
AJCC	American Joint Committee on Cancer
Akt	AKT8 virus oncogene cellular homolog
AMPK	AMP-activated protein kinase
ATP	Adenosine triphosphate
CAFs	Cancer associated fibroblasts
CAs	Carbonic anhydrases
CD	Cluster of differentiation
CDKN2A	Cyclin-dependent kinase inhibitor 2a
CIS	Carcinoma in situ
CREB	C-AMP response element-binding protein
CT	Computed tomography
CXCL	Chemokine (CXC motif) ligand
DAB	3, 3´-diaminobenzidine
DFS	Disease-free survival
DOI	Depth of invasion.
ECs	Endothelial cells
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal growth factor receptor
EMT	Epithelial- mesenchymal transition
ETC	Electron transport chain
FGF-2	Fibroblast growth factor 2
FS	Final score
GLUTs	Glucose transporters
GMCSF	Granulocye-macrophage colony-stimulating factor
GPR81	G protein-coupled receptor 81
HIF-1	Hypoxia-inducible factor 1
HK	Hexokinase
HNC	Head and neck cancer

HPF	High power field
HPV	Human papillomavirus
HRE	Hypoxia-response elements
HRP	Horseradish peroxidase
HUVECs	Human umbilical vein endothelial cells
IARC	International Agency for Research on Cancer
ICVS	Instituto de Investigação em Ciências da Vida e da Saúde - Life and Health Sciences Research Institute
IDH	Isocitrate dehydrogenase
IHC	Immunohistochemistry
IL	Inteleukin
JAK	Janus-family tyrosine kinase
LDH	Lactate dehydrogenase
LEF	Lymphoid enhancer factor
Leuk	Leukoplakia
LOH	Loss of heterozygosity
M	molar
MCTs	Monocarboxylate transporters
Min	minutes
mM	Milimolar
MMPs	Matrix metalloproteinases
MRI	Magnetic resonance imaging
mTOR	Mammalian target of Rapamycin
MYC	Myelocytomatosis oncogene cellular homolog
NADPH	Nicotinamide adenine dinucleotide phosphate
NH	Non-homogenous
NH	Non-homogenous
NHE	Na^+/H^+ exchanger
NOTCH1	Notch homolog 1 genes are translocation-associated
OED	Oral epithelial dysplasia
ON	Overnight
OPMDs	Oral potentially malignant disorders
OS	Overall survival
OSCC	Oral squamous cell carcinoma

OSF	Oral submucous fibrosis
OXPHOS	Oxidative phosphorylation
PBS	phosphate-buffered saline
PDAC	Pancreatic ductal adenocarcinoma
PDC	Pyruvate dehydrogenase complex
PDGF	Platelet-derived growth factor
PDH	Pyruvate dehydrogenase
PDK	Pyruvate dehydrogenase kinase
PET	Positron emission tomography
PFK	Phosphofructokinase
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3
PHD3	Prolyl hydroxylase 3
PI3K	Phosphatidylinositol 3- protein kinase
PK	Pyruvate kinase
PPP	Pentose phosphate pathway
PS	Percentage score
PTEN	Phosphatase and tensin homologue deleted on chromosome 10
PVL	Proliferative verrucous leukoplakia
R-2HG	R enantiomer of 2-hydroxyglutarate
RAS	Rat sarcoma virus oncogenes
RB	Retinoblastoma
ROS	Reactive oxygen species
RT	Room temperature
SLC	Solute carrier
SPSS	Statistical Package for Social Sciences
SS	Strength of staining
STAT	signal transducer and activator of transcription
TCA	Tricarboxylic acid
TCF	T-cell factor
TGF-β	Transforming growth factor-β
TME	Tumor microenvironment
TMs	Transmembrane helices
TNF	Tumour necrosis factor
TNM	Tumor, node, metastasis

TP53	Tumor protein 53
Tris	Trisaminomethane
VEGF	Vascular endothelial growth factor
VHL	Von Hippel-Lindau
WHO	World health organization
Wnt	Wingless-related integration site
α-SMA	α-smooth muscle actin

Figures

Figure 1. Glucose metabolism in cancer cells and normal cells. 2
Figure 2. Hypoxia inducible factor (HIF) pathway. 5
Figure 3. Role of GLUT-1 and GLUT-3 in cancer cell. 6
Figure 4. Rate limiting-enzymes in glycolysis pathway. 7
Figure 5. overview of PDK, PDH and LDH-A roles in cancer cells. 8
Figure 6. Function of MCT-1 and MCT-4 in the metabolic symbiosis between oxidative and glycolytic cancer cells. 9
Figure 7. The role of CA-IX in hypoxic cancer cells. 10
Figure 8. Role of lactic acid in the tumour microenvironment. 12
Figure 9. Anatomy of the oral cavity. 14
Figure 10. Oral cancer distribution in different regions by sex in 2020. 14
Figure 11. Oral Potentially Malignant Disorders (OPMDs). 16
Figure 12. Multiple steps of oral carcinogenesis. 21
Figure 13. Representative images of the immunohistochemical staining reactions for GLUT-1 36
Figure 14. Representative images of the immunohistochemical staining reactions for GLUT-3 37
Figure 15. Representative images of the immunohistochemical staining reactions for HK-II 38
Figure 16. Representative images of the immunohistochemical staining reactions for PFK-L 39
Figure 17. Representative images of the immunohistochemical staining reactions for PKM-2 40
Figure 18. Representative images of the immunohistochemical staining reactions for pPDH 41
Figure 19. Representative images of the immunohistochemical staining reactions for LDH-A 42
Figure 20. Representative images of the immunohistochemical staining reactions for MCT-4 43
Figure 21. Representative images of the immunohistochemical staining reactions for CA-IX in 43
Figure 22. Kaplan-Meier curve demonstrating 3-year disease-free survival of patients with oral squamous cell carcinoma (n=21) based on HK-II immunoexpression status. 45
Figure 23. Kaplan-Meier curve demonstrating 3-year overall survival of patients with oral squamous cell carcinoma (n=21) based on HK-II immunoexpression status. 45
Figure 24. Kaplan-Meier curve demonstrating 3-year overall survival of patients with oral squamous cell carcinoma (n=21) based on CA-IX immunoexpression status. 46
Figure 25. Immunoexpression of GLUT-1, GLUT-3, HK-II, PFK-L, PKM-2, pPDH, LDH-A and MCT-4 in normoxic and hypoxic compartments of oral squamous cell carcinoma cases. 50
Figure 26. Kaplan-Meier curve demonstrating 3-year overall survival of patients with oral squamous cell carcinoma (n=19) based on GLUT-1 immunoexpression status in the hypoxic compartment of the malignant lesions 52
Figure 27. Kaplan-Meier curve demonstrating 3-year overall survival of patients with oral squamous cell carcinoma (n=17) based on GLUT-3 immunoexpression status in the hypoxic compartment of the malignant lesions. 52

Figure 28. Kaplan-Meier curve demonstrating 3-year disease-free survival of patients with oral squamous cell carcinoma (n=11) based on HK-II immunoexpression status in the hypoxic compartment of the malignant lesions. 53

Figure 29. Kaplan-Meier curve demonstrating 3-year disease-free survival of patients with oral squamous cell carcinoma (n=19) based on pPDH immunoexpression status in the hypoxic compartment of the malignant lesions. 53

Tables

Table 1. Different classification systems of oral epithelial dysplasia. Adapted from 17

Table 2. Tumour–Node–Metastasis classification of oral cavity cancer according to AJCC 2016. 19

Table 3. Overview of clinicopathological data in patients with oral premalignant lesions. 27

Table 4. Overview of clinicopathological data in patients with oral squamous cell carcinoma. 28

Table 5. Details of the immunohistochemical procedure. 30

Table 6. Association between the clinicopathological data and the 3-year disease-free survival of oral squamous cell carcinoma patients (n=21). 33

Table 7. Association between the clinicopathological data and the 3-year overall survival of oral squamous cell carcinoma patients (n=21). 34

Table 8. Immunoexpression frequencies for GLUT-1, GLUT-3, HK-II, PFK-L, PKM-2, pPDH, LDH-A, MCT-4 and CA-IX in premalignant lesions and in oral squamous cell carcinoma cases. 35

Table 9. Association between the immunoexpression frequencies of the metabolism-related biomarkers and the 3-year disease-free survival of oral squamous cell carcinoma patients. 47

Table 10. Association between the immunoexpression frequencies of the metabolism-related biomarkers and the 3-year overall survival of oral squamous cell carcinoma patients. 48

Table 11. Association between the immunoexpression frequencies of the metabolism-related biomarkers in the hypoxic compartments of the malignant lesions and the 3-year disease-free survival of oral squamous cell carcinoma patients. 54

Table 12. Association between the immunoexpression frequencies of the metabolism-related biomarkers in the hypoxic compartments of the malignant lesions and the 3-year overall survival of oral squamous cell carcinoma patients. 55

Table S1. Association between the immunoexpression of metabolism-related biomarkers and the clinicopathological data of patients with oral premalignant lesions. 71

Table S2. Association between the immunoexpression of metabolism-related biomarkers and the clinicopathological data of oral squamous cell carcinoma patients 73

Table S3. Association between the immunoexpression of metabolism-related biomarkers in hypoxic regions of the tissue sections and the clinicopathological data of oral squamous cell carcinoma patients. 75

Introduction

1. Cancer metabolism and the Warburg effect

Over the last decades, research has improved our knowledge of cancer disease. Metabolic reprogramming, now recognized as an emerging hallmark of cancer [1], was firstly observed by Otto Warburg in the 1920s [2, 3]. He realized that cancer cells, regardless of oxygen availability, use much higher glucose levels than normal cells, with further fermentation of pyruvate to lactate rather than oxidation in the mitochondria. This particular form of energy metabolism is nowadays termed "Warburg effect" or "aerobic glycolysis" (Figure 1A) [4-6].

In normal cells, under aerobic physiological conditions, glucose breaks down to pyruvate through glycolysis. Then pyruvate is converted into acetyl-coenzyme A (acetyl-coA) that enters the tricarboxylic acid (TCA) cycle and the electron transport chain (ETC) in the mitochondria, to produce 36 moles of adenosine triphosphate (ATP) per unit of glucose. This process is called mitochondrial oxidative phosphorylation (OXPHOS) (Figure 1B) [7, 8]. Unlike normal cells, which mainly rely on OXPHOS to generate the energy for cellular processes, cancer cells, instead of using glycolysis as part of glucose oxidation, use it as the principal pathway of energy production, to produce 2 moles of ATP per unit of glucose. In "aerobic glycolysis", the yield of ATP production is much less in comparison to OXPHOS [9, 10]. Therefore, the question that arises is why cancer cells prefer aerobic glycolysis instead of OXPHOS. This phenomenon can be explained by the fact that cancer cells, through upregulation of glucose transporters and glycolytic enzymes, are enabled to acquire a large amount of net energy. Thus, the amount of ATP generated by cancer cells is much faster and abundant through "aerobic glycolysis" [11]. Another consequence of the Warburg effect is to support uncontrolled cell proliferation. Cancer cells provide a carbon source for anabolic processes via the increased flux of the pentose phosphate pathway (PPP) which is needed for de novo biosynthesis of nucleotides [12] and lipids [13]. In addition, proliferating cells are at more extreme demand of reducing equivalents in the form of nicotinamide adenine dinucleotide phosphate (NADPH) [6, 14]. NADPH is a major cellular antioxidant, which keeps glutathione in a reduced state to secure the redox balance [15]. Also, amino acids such as serine and glycine, required for protein and DNA/RNA synthesis, can be produced from 3-phosphoglycerate, a pyruvate precursor [16]. Moreover, "aerobic glycolysis" causes acidification of the extracellular environment, by the conversion of pyruvate into lactate acid, and its subsequent exportation [17]. This

unique property of cancer cells has downstream consequences which enable them with features of cancer aggressiveness, such as immunosuppression [18], migration, invasion [19], accelerated cell proliferation, angiogenesis [20] and resistance to apoptosis [21].

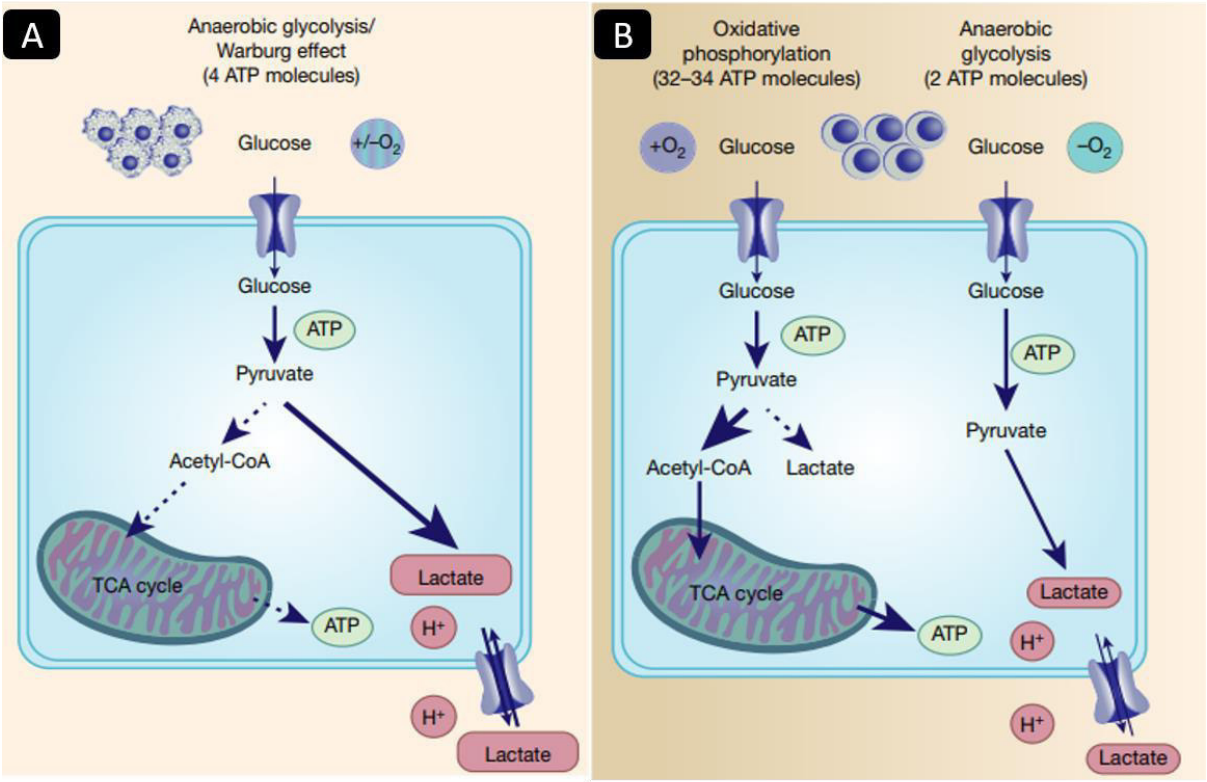

Figure 1: Glucose metabolism in cancer cells and normal cells. (A) Cancer cells rely on "aerobic glycolysis" independently of oxygen availability (The Warburg effect). (B) In normal cells, under normoxic conditions, glucose metabolizes via OXPHOS, while under hypoxia it metabolizes through glycolysis [3]. ATP, adenosine triphosphate; TCA, tricarboxylic acid.

The clinical application of the Warburg effect was implemented by Positron emission tomography (PET) imaging, which is used in the diagnosis and follow-up of malignant neoplasms. In this technique, a radiolabelled analogue of glucose, 2-[18F] fluoro-2-deoxy-d-glucose ([18F]-FDG), is used to detect glucose uptake in tumours. A malignant tumour can be identified by the high rates of glucose uptake by cancer cells compared with the normal tissue [22, 23].

1.1. Regulators and effectors of the Warburg effect

It is well documented that several factors promote the metabolic reprogramming of cancer. Some oncogenes like Myelocytomatosis oncogene cellular homolog (*MYC*), Rat sarcoma virus oncogenes (*RAS*), and AKT8 virus oncogene cellular homolog (*AKT*), and tumour suppressors such as protein 53 (*TP53*), have a role on this hallmark of cancer. For instance, *MYC, RAS, AKT* and *TP53*, increase the expression and translocation of glucose transporter 1 (GLUT-1); *MYC* and *AKT* activation upregulate hexokinase II (HK-II); the expression level of lactate dehydrogenase (LDH) isoform A and monocarboxylate transporter 1 (MCT-1) is also increased by *MYC* [24, 25]. Epigenetic events have also been reported as effectors of the metabolic reprogramming [26]. As an example, mutations in isocitrate dehydrogenase (IDH) gene (cytosolic IDH1, enzyme responsible for α-ketoglutarate synthesis) cause altered enzymatic activity leading to production of the R enantiomer of 2-hydroxyglutarate (R-2HG), which is involved in cytosine methylation [27]. This mutation leads to hypermethylation of DNA and histones in glioblastomas [28]. Most importantly, among different regulators of the metabolic reprogramming, hypoxia seems to be the main regulator of the Warburg effect in malignancy [29, 30].

1.1.1 The main regulator: Hypoxia and hypoxia inducible factor (HIF)

Hypoxia is a typical feature of solid tumours, mainly caused by abnormal vasculature and by the rapid cellular proliferation [31, 32]. In a growing tumour, blood flow is changeable, with limited access to oxygen perfusion, leading to tumour local hypoxia [33]. In this condition, in order to compensate for oxygen deficiency and adaptation to the new microenvironment, cancer cells use the glycolytic metabolic pathway which activates the expression of the main transcription factor, hypoxia-inducible factor 1 (HIF-1). This transcription factor is composed of HIF-1α and HIF-1β subunits, of which the first one is the oxygen-dependent subunit. Under normoxia, the α subunit is hydroxylated and ubiquitinated by an ubiquitin ligase known as von Hippel-Lindau (VHL) protein, and then finally degraded, while the β subunit, independently from oxygen, is constantly expressed. In the absence of oxygen, HIF-1α stabilizes and dimerizes with HIF-1β; these two subunits then form the HIF-1α/β dimer complex. This complex translocates to the nucleus and binds to target genes through E-box-like hypoxia-response elements (HRE) (Figure 2) [34, 35].

One of the main characteristics of activated HIF-1 signaling pathway is the upregulation of glycolytic gene expression. In this pathway, HIF-1α increases the expression of glucose transporters (GLUTs), such as GLUT-1 and GLUT-3, which results in increased glucose uptake, and monocarboxylate transporter 4 (MCT-4) which is used for lactate release [11, 36]. During glucose metabolism, HIF-1 also facilitates the conversion of glucose to pyruvate by increasing the expression of glycolytic enzymes such as HK-I and HK-II and pyruvate kinase (PK) M2 [36]. Moreover, phosphofructokinase (PFK), mainly PFK-1, is also stimulated by HIF-1 [31]. Furthermore, this transcription factor, in partnership with AMP-activated protein kinase (AMPK) signaling mechanisms, is a main regulator in fatty acid synthesis and metabolism. In human umbilical vein endothelial cells (HUVECs), HIF-1α induces the upregulation of *ALDOC*, gene for aldolase C, and in endothelial cells (ECs), hypoxia also leads to an increased expression of the glycolytic enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3) [33]. HIF-1α can also downregulate mitochondrial function in combination with c-Myc activation, by upregulating pyruvate dehydrogenase kinase (PDK) and thus decreasing the activity of pyruvate dehydrogenase (PDH), which is responsible for converting pyruvate into acetyl-coA for OXPHOS process. Another important enzyme that is upregulated by the activation of these two pathways is LDH-A, which converts pyruvate into lactic acid. Hence, they divert the glycolytic pathway into lactate production, contributing to the development of acidic microenvironments which lead to aggressive features of cancer cells, as it will be explained [11, 35].

Other consequences of HIF-1α activation are the induction of vascular endothelial growth factor (VEGF) expression, a representative molecule involved in angiogenesis, and triggering of several oncogenes such as the phosphatidylinositol 3- protein kinase (PI3K) pathway and its downstream products, which include Akt and mammalian target of Rapamycin (mTOR), favouring tumour survival and therapy resistance [33, 37].

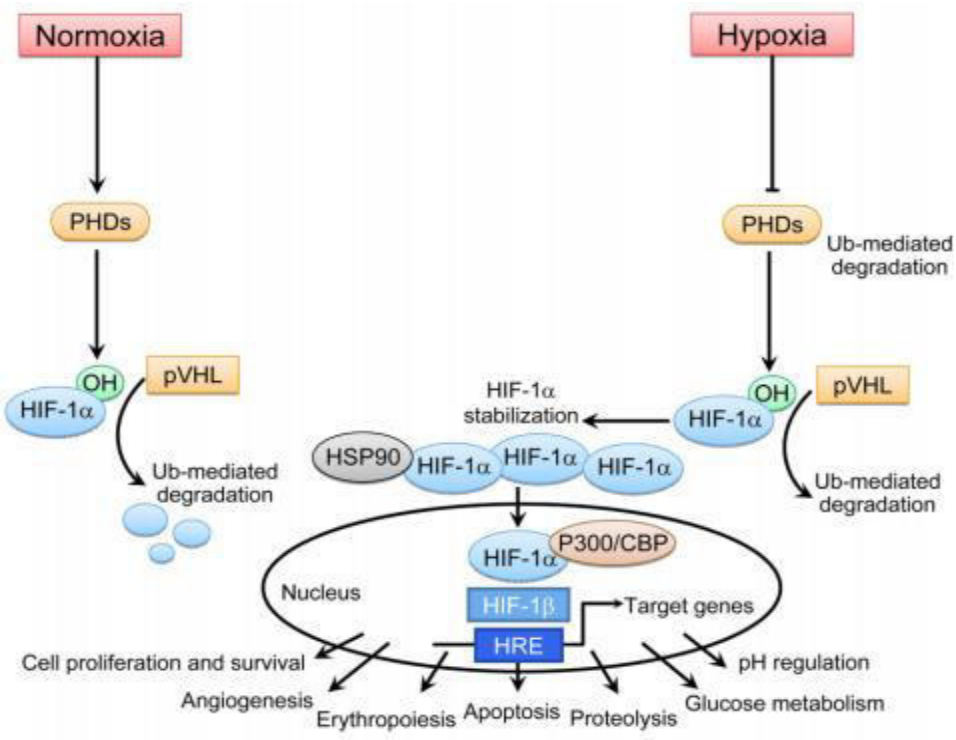

Figure 2: Hypoxia inducible factor (HIF) pathway. In normoxic conditions, HIF-1α is ubiquitinated by the VHL protein, while in hypoxia, HIF-1α stabilizes and form HIF-1α/β complex; this results in cell survival, angiogenesis, altered cellular metabolism and creation of an acidic microenvironment [38]. HIF-1α, hypoxia-inducible factor 1 subunit α; HIF-1β, hypoxia-inducible factor 1 subunit β; HRE, hypoxia response elements; HSP90, heat shock protein 90; VHL, von Hippel-Lindau; PHD, prolyl hydroxylase; p300, histone acetyltransferase; CBP, CREB binding protein.

1.1.2 The main effectors – HIF downstream targets

1.1.2.1. Glucose transporters

Glucose, as the dominant nutrient, is avidly consumed by metabolically-active cancer cells, which require it to increase proliferation. Uptake of glucose is performed by a family of GLUTs, which include fourteen members in humans. According to the distribution of GLUTs in the body, their expression is tissue-specific. These transporters are regulated at the molecular and protein level [39, 40]. From all glucose transporters, GLUT-1 and GLUT-3 have high selectivity for glucose and are known to adjust sugar homeostasis in the blood. HIF-1α has a key role in the upregulation of both GLUT-1 and GLUT-3 isoforms (Figure 3) [41, 42]. For instance, activation of the PI3K-Akt-mTOR signaling pathway through HIF-1α leads to increased GLUT-3 expression in neuronal PC12 cells [43]. Furthermore, other hypoxia-associated

factors including VEGF and calcium channels [44] have been demonstrated as GLUT-1 positive regulators [45]. Overexpression of GLUT-1 has been described in several types of cancer, namely in breast [46] and head and neck cancers [47], being this association implicated in the metabolic reprogramming. As previously mentioned, upregulated GLUT-1 expression to increase glucose uptake is clinically used for the diagnosis of primary malignant diseases, as well as for recurrent lesions and metastasis by FDG-PET scanning [48]. In general, overexpression of GLUT-1 and/or GLUT-3 is associated with poor survival and resistance to therapy [41].

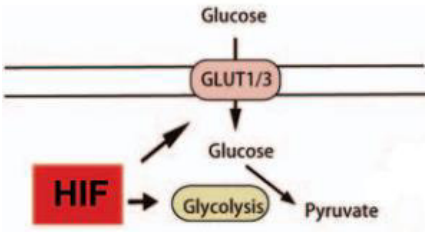

Figure 3: Role of GLUT-1 and GLUT-3 in cancer cell. GLUT-1 and GLUT-3 have high selectivity for glucose and increase glycolytic activity. HIF-1α is the main regulator of GLUT-1 and GLUT-3. Adapted from [49]. GLUT-1 1/3, glucose transporter1/3; HIF-1, hypoxia-inducible factor 1.

1.1.2.2. Rate-limiting enzymes of glycolysis

In the glycolytic pathway, there are important rate-limiting enzymes, namely HK, PFK and PK, that catalyze irreversible reactions (Figure 4). HKs family consists of four isoenzymes (HK I-IV) that are distributed in different tissues. These first rate-limiting enzymes for glycolysis catalyze the conversion of glucose to glucose-6-phosphate. High expression of HK-II, which is under the activation of HIF-1α and PI3K pathways, is related to increased cancer aggressiveness and poor prognosis of the patients [8, 42].

Another important enzyme controlling the glycolytic flux is PFK. During this process, PFK accelerates the conversion of fructose-6-phosphate to fructose-1,6-bisphosphate [20]. This enzyme exists in different oligomeric forms and three isomers of it are present in humans including liver, muscle, and platelet PFK [50]. Several studies have demonstrated that PFK's expression is increased by activation of HIF-1α through PI3K signaling pathway in different cancer types [35].

PK is the last enzyme of glycolysis, catalyzing phosphoenolpyruvate to pyruvate reaction. PK exists in four isoforms, PKL, PKR, PKM-1 and PKM-2. The last one is the major subtype in many malignancies, promoting anabolic pathways, such as PPP and OXPHOS: low-level activity of PK leads to PPP, and high-level activity of it results in OXPHOS occurrence [31, 51]. In the study of *Luo et al.* the authors showed that HIF-1α increased the expression of PKM-2 through the interaction with prolyl hydroxylase 3 (PHD3)

which causes a direct binding to the HIF-1α subunit and activation of HIF-1 target genes [52]. PKM-2 can favor cancer cell survival and invasion, by increasing glucose metabolism and accelerating production of lactate [53].

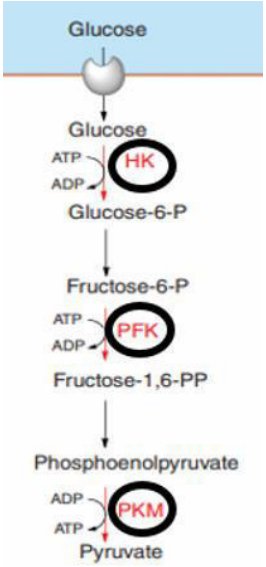

Figure 4: Rate limiting-enzymes in glycolysis pathway. HK, PFK and PKM are important enzymes that forward glycolysis in an irreversible-manner. Adapted from [42]. HK, hexokinase; PFK, phosphofructokinase; PKM, pyruvate kinase M.

1.1.2.3. Pyruvate dehydrogenase complex and lactate dehydrogenase

Pyruvate dehydrogenase complex (PDC) is a multienzyme composed of three active subunits: PDH, dihydrolipoyl transacetylase and dihydrolipoamide dehydrogenase, being PDH responsible for PDC regulation. PDC is an essential mitochondrial multienzyme that converts pyruvate into acetyl-coA which then enters in the TCA cycle to form citrate and subsequently start OXPHOS. That is why PDC, by connecting glycolysis and TCA cycle, is a pivotal complex responsible for the flux of energy production in a cell. However, in cancer cells, PDK, specially PDK1, inhibits PDH in the mitochondria; thus, pyruvate is converted into lactate through lactate dehydrogenase, instead of acetyl-coA conversion (Figure 5). Under hypoxic conditions, PDK expression increases via HIF-1α resulting in mitochondrial dysfunction and OXPHOS disturbance, as it described above [54, 55].

Lactate dehydrogenase is a tetrameric enzyme that exists in different types, of which two of them, LDH-A (muscle) and LDH-B (heart) are the most well-studied forms [56]. LDH is a master regulator of the glycolytic pathway that catalysis pyruvate to lactic acid conversion in combination with the oxidation of NADH to NAD^+. Cancer cells, by converting pyruvate into lactic acid via LDH-A, accelerate the glycolysis pathway for their purposes (Figure 5). Several studies demonstrated that LDH-A's expression and activity is induced by HIF-1α [57] and c-Myc [58] in various types of cancer.

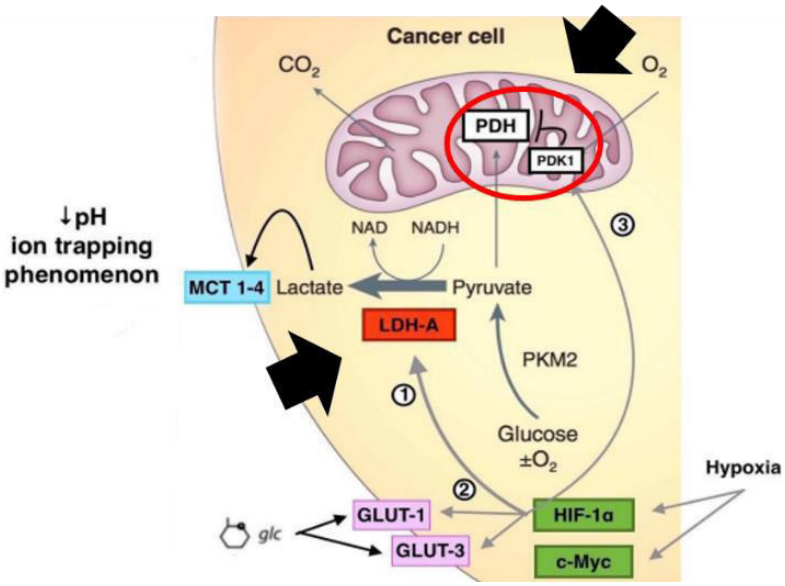

Figure 5: overview of PDK, PDH and LDH-A roles in cancer cells. PDK blocks PDH in mitochondria which favours pyruvate diversion into aerobic glycolysis instead of OXPHOS. LDH-A converts pyruvate to lactate in combination with oxidation of NADH to NAD^+ [56]. PDH, pyruvate dehydrogenase; PDK, pyruvate dehydrogenase kinase; LDH-A, Lactate dehydrogenase A; MCT, Monocarboxylate transporter; GLUT, glucose transporters; HIF-1, hypoxia-inducible factor 1; c-Myc, myelocytomatosis oncogene cellular homolog.

1.1.2.4. Monocarboxylate transporters

MCTs are transmembrane proteins that transport short chain monocarboxylates like pyruvate, L-lactate, D-β-hydroxybutyrate and acetoacetate through the plasma membrane. They have an important role in adjusting essential processes related to key metabolic pathways [59, 60]. The family of monocarboxylate transporters, which is encoded by the solute carrier gene *(SLC)* 16, consists of fourteen different isoforms according to their sequence homology. All members are composed of 12 transmembrane helices (TMs), which are acutely conserved [59, 61, 62]. The best characterized isoforms are MCT-1 and MCT-4, mainly due to its prognostic value in cancer patients [59, 63].

The human MCT-1 is encoded by the *SLC16A1* gene [64]. Depending on the metabolic demands of the cell, it seems to function either in the uptake or efflux of monocarboxylates. The predicted MCT-1 binding site for the substrate opens to the extracellular matrix, when lactate is transported into the cell. At first, a proton attaches to this binding site, followed by lactate. MCT-1 structure switches conformation, transporting H^+ and lactate into the intracellular space. However in this step, lactate release occurs first [59, 64]. Another important monocarboxylate transporter is MCT-4, which is encoded by the *SLC16A3* gene. In some highly glycolytic tissues such as white skeletal muscle fibres, astrocytes, white blood cells, chondrocytes and some mammalian cell lines, MCT-4 is up-regulated at mRNA and protein level, being the main lactate extruder. However, MCT-4 has lower affinity for substrates in comparison to MCT-1 [60,

65]. MCT-1 and 4 roles are particularly relevant in the metabolic symbiosis established between some cancer cells, as it will be detailed further (Figure 6).

MCTs regulation depends on the physiological conditions, and is altered in several pathologies, including cancer [60, 66]. Hypoxia is the main regulator of MCTs expression, mainly MCT-4. In hypoxic conditions and/or in highly glycolytic tissues, HIF-1α activates MCT-4 gene expression [67], while MCT-1 is mainly under Myc regulation [68]. CD147 is an accessory protein required for the proper location of MCT-1 and MCT-4 at the plasma membrane (Figure 6). Similarly to MCTs, CD147 expression is increased in tumour cells, correlating with a malignant phenotype [69].

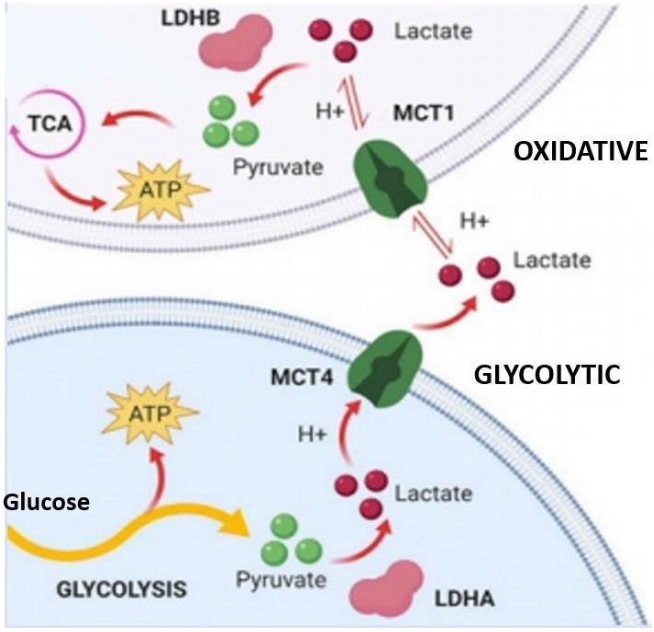

Figure 6: Function of MCT-1 and MCT-4 in the metabolic symbiosis between oxidative and glycolytic cancer cells. In glycolytic cancer cells, lactate produced by aerobic glycolysis is released into the microenvironment through MCT-4, and then enters oxidative cancer cells through MCT-1. Adapted from [63]. MCT1/4, Monocarboxylate transporter1/4; TCA, Tricarboxylic acid; ATP, Adenosine triphosphate; LDH, Lactate dehydrogenase.

1.1.2.5. Carbonic anhydrases

Carbonic anhydrases' (CAs) family consists of fifteen isoforms in human [70]. Classification of CAs according to their subcellular location categorized them into four clusters, namely cytosolic, mitochondrial, membrane-associated and secretory. Carbonic anhydrase IX (CA IX) is a membrane associated glycoprotein [71]. Expression of CA IX increases during hypoxia; it promotes tumor microenvironment (TME) acidification and subsequent maintenance of intercellular pH. Under hypoxic conditions, cancer cells produce lactic acid through "aerobic glycolysis". In mitochondria, a proton in

combination with bicarbonate generates water and carbon dioxide. In the tumour microenvironment, additional CO_2, which diffuses from the cell membrane to the environment, generates HCO_3^- and H^+ through CA IX enzyme, leading to microenvironmental acidosis (Figure 7) [72]. CA IX upregulation, which is characterized as a tumour marker in many types of cancer such as pancreatic ductal adenocarcinoma (PDAC) [73], breast and renal carcinomas [74] , is correlated with migration, invasion, progression and poor prognosis [75].

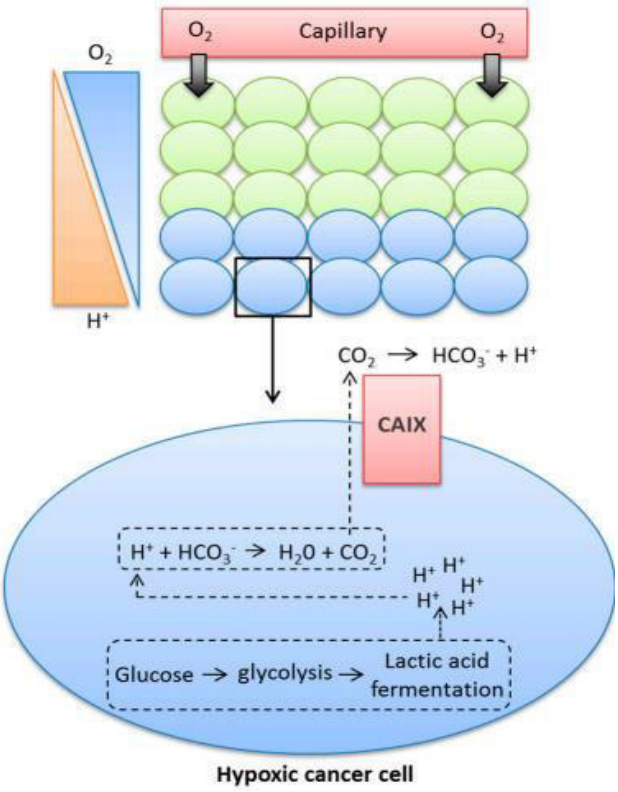

Figure 7: The role of CA-IX in hypoxic cancer cells. Under hypoxic conditions, cancer cells produce lactic acid through aerobic glycolysis. The resulting protons, in combination with HCO_3^-, generate water and CO_2 in the mitochondria. In the microenvironment, CA IX isoform converts external CO_2 to HCO_3^- and H^+ which favours acidosis of the tumour microenvironment [76]. CA IX, carbonic anhydrase IX.

1.2. Consequences of the Warburg effect: Lactate and microenvironmental acidosis

In glycolytic cancer cells, lactate is the final product of pyruvate fermentation. A balance between intracellular and extracellular environments is necessary to maintain this fermentation. The continued production of lactic acid through "aerobic glycolysis" in combination with the hydrolysis of ATP releases H^+, leading to lower intracellular pH in cancer cells. As previously mentioned, in order to sustain the intracellular pH at neutral or alkaline levels, cancer cells upregulate specific MCTs symporters, namely MCT-1 and MCT-4, to export lactic acid in association with a proton to the extracellular space, finally leading to acidification of the TME [17, 37]. This type of transporter is not the only one that causes TME

acidification; several other transmembrane transporters such as the Na^+/H^+ exchanger (NHE) [77], H^+-ATPase [78], $Na^+/HCO3$ cotransporters and also CAs [17], cooperate for pH regulation.

The TME is not a uniform milieu, comprising different stromal cells surrounding the cancer cells, as endothelial cells, pericytes, immune inflammatory cells, cancer-associated fibroblasts (CAFs), and the extracellular matrix [1, 79]. Thus, a heterogeneous population of cancer cells and stromal cells, which creates the TME components, exhibit heterogeneity in metabolic characteristics [80]. This metabolic heterogeneity and plasticity of cancer cells emerges from innate gene dysregulation or from extrinsic signaling in the TME [23]. Cells which are located near the blood vessels have facilitated access to oxygen and nutrients, and are supplied with ATP from glycolysis and subsequent OXPHOS, which lead to enhanced anabolic pathways. After different stimuli, cancer cells increase proliferation, and the ones located distant from blood vessels experience hypoxic conditions that promote them to switch to the aerobic glycolytic pathway. In this condition, lactate, which is previously extruded by glycolytic cancer cells preferentially via MCT-4, is then captured by oxidative cancer cells via MCT-1 to be employed as carbon building blocks for the anabolic pathways [81]. This type of metabolic symbiosis also occurs between cancer cells and the surrounding stroma of cancer-associated cells such as CAFs [82], immune [83] and endothelial cells [84], highly affecting the growth, progression and metastasis of cancer [81]. In this circumstance, the TME selects the cells that have acquired resistance to acidosis, which is a consequence from long term exposure to the acidic microenvironment [16, 26]. Thus, this role of lactic acid as metabolic substrate in cancer cells is one of the main roles of this molecule in the carcinogenesis process (Figure 8).

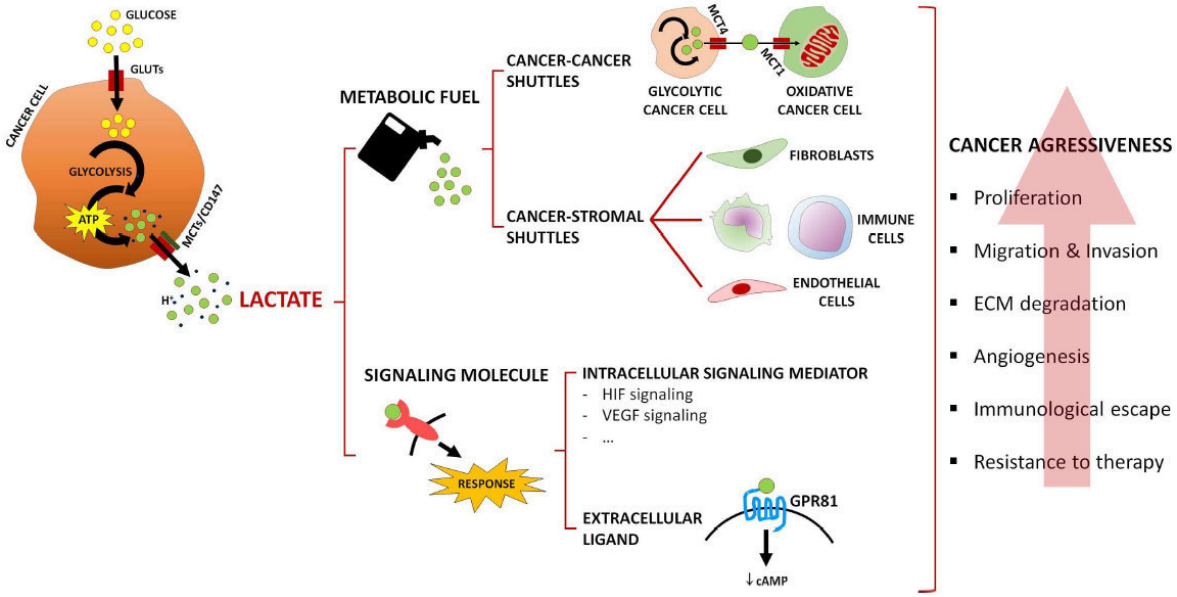

Figure 8: Role of lactic acid in the tumour microenvironment. Lactate is the main precursor for gluconeogenesis and can operate as metabolic fuel between cancer cells and the surrounding tissues. It can also function as a signalling molecule, also stimulating tumour aggressiveness [17]. GLUTs, glucose transporters; MCT1/4, Monocarboxylate transporter1/4; VEGF, vascular endothelial growth factor; HIF, hypoxia-inducible factor; GPR81, G protein-coupled receptor 81; cAMP, Cyclic adenosine monophosphate.

It is well-documented that lactate is the chief messenger in the TME (Figure 8) [85] and, as previously mentioned, its accumulation is correlated with the malignant phenotype. In fact, the extracellular acidic environment, which results in the activation of matrix metalloproteinases (MMPs), the production of hyaluronic acid, and the expression of CD44 chaperone [86], leads to the degradation of extracellular matrix components which promote migration, invasion, and metastasis [87]. Lactate as a signaling molecule is also involved in the tumour escape from the immune system. It can promote polarization of M2 type of macrophages, constitute a barrier to the differentiation of dendritic cells from monocytes, inhibit secretion of cytokines like tumour necrosis factor (TNF) and interleukin-6 (IL-6), and activate the IL-23/IL-17 as a pro-inflammatory pathway. Another consequence of microenvironmental acidosis is that cytotoxic activities dramatically decrease in T-cells, and apoptosis induction occurs within it [88, 89]. Lactate also has a main role in angiogenesis by stabilizing HIF-1α and stimulating VEGF production by endothelial cells [90, 91]. Besides, elevated lactate levels can influence the expression of c-AMP response element-binding protein (CREB), PGC-1α and MCT-1, through the activation of G protein-coupled receptor 81(GPR81). Expression of GPR81 is increased in some types of cancers such as breast carcinoma [92, 93].

2. Oral Cancer

2.1 General overview

Lip and oral cavity malignancies, globally known as oral cancer, are part of the widespread group of head and neck cancers (HNC), which encompasses not only malignant lesions from the lip and oral cavity, but also from larynx, pharynx, nasal cavity and paranasal sinuses, and salivary glands. According to the International Agency for Research on Cancer (IARC), HNC is the seventh most common cancer in both sexes, being more prevalent in men than in women. The 2020 annual incidence rate of HNC was almost 930.000 new cases and over 460.000 deaths have been estimated worldwide. The incidence rates were higher in Southern Asia and the Pacific Islands [94]. It is well-documented that the major etiologic factors for HNC are excessive use of alcohol, tobacco smoking, and papillomavirus (HPV), specially type-16 [95].

2.2 Anatomy of the oral cavity

The anatomy of the oral cavity is determined by the vermillion of the lips to the circumvallate papillae of the tongue and the junction of the soft and hard palate. The oral cavity is divided in seven parts: tongue, lips, the floor of the mouth, hard palate, buccal mucosa, retromolar trigone and soft palate, and gingiva (Figure 9); this terminology is used when classifying oral cancer [96, 97].

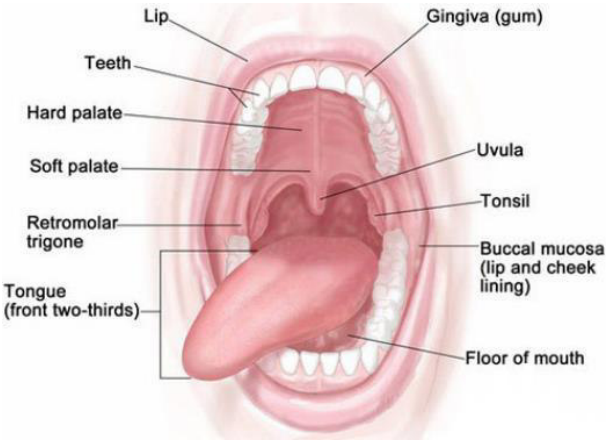

Figure 9: Anatomy of the oral cavity. Oral cavity can be subdivided into seven anatomical areas as the figure shows [98].

2.3 Epidemiology and etiology of oral cancer

According to IARC in 2020, oral cancer was the sixteenth leading cause of cancer death among other cancer types. Almost 377713 new cases and 177,757 oral cancer-related deaths have been estimated worldwide. The occurrence of oral cancer is high in regions of South Central Asia such as India, Sri Lanka and Pakistan, as well as in Melanesia such as Papua New Guinea, with a high rate of incidence in men (Figure 10) [94].

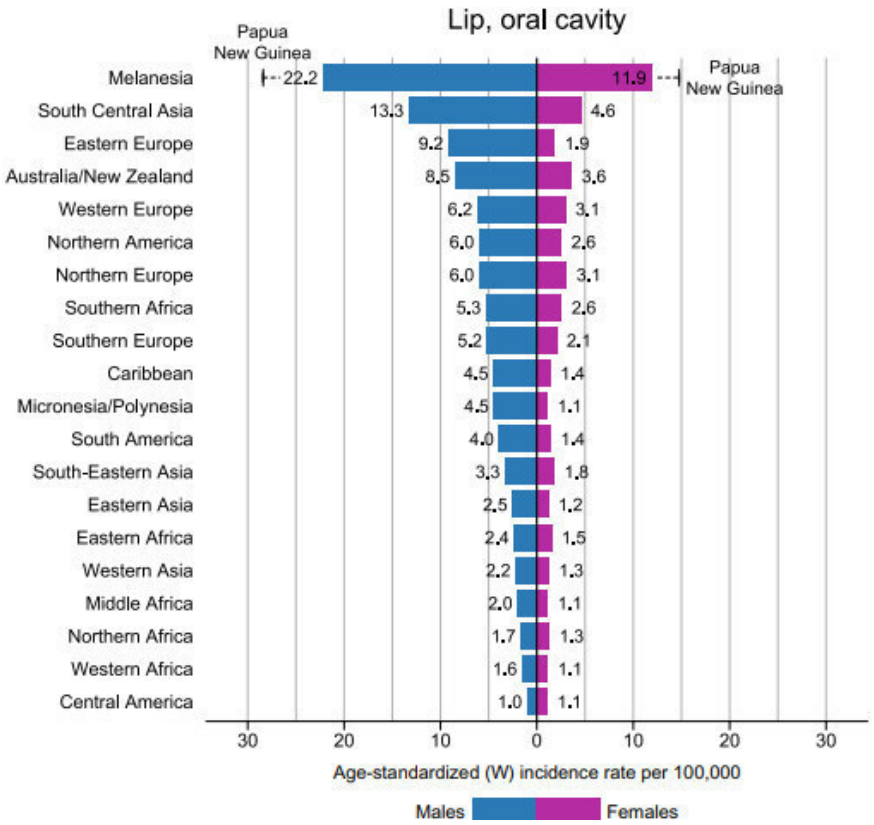

Figure 10: Oral cancer distribution in different regions by sex in 2020 [94].

In terms of aetiology, scientific reports support the role of both hereditary and non-hereditary factors in oral cancer. Regarding hereditary aspects, family history of cancer and personal defective immune system are the known factors. In the case of non-hereditary risk factors, the most important are related to tobacco chewing and smoking, alcohol drinking and HPV virus (type-16). Several studies also highlighted other risk factors such as deficient nutrition, betel quid chewing, excessive sun exposure, male gender and old age [96, 99].

2.4 Oral Potentially Malignant Disorders

According to the World Health Organization (WHO), the combination of precancerous conditions and epithelial lesions, which each of them has the potential of transforming into a malignancy with no clinically or pathologically cause by any other definable lesion, are called oral potentially malignant disorders (OPMDs). The more common consist of leukoplakia, erythroplakia, oral lichen planus, and oral submucous fibrosis (Figure 11) [100, 101].

Leukoplakia is the most common OPMD, and is clinically divided into homogenous and non-homogenous leukoplakia. In regards to the homogenous lesion, leukoplakia has generally demarcated borders and a flat surface, and is white. In the case of the non-homogenous lesion, there are three clinical classifications, namely speckled (erosive) leukoplakia, nodular leukoplakia, and verrucous/ exophytic leukoplakia. Speckled leukoplakia, named erythroleukoplakia, is a subtype of leukoplakia that has mix red and white patches (although white patches are predominant). Nodular leukoplakia is characterized by a small polypoid structure with red or white rounded excrescences; and verrucous or exophytic leukoplakia has wrinkled and/or corrugated surface pattern [100, 102]. Proliferative verrucous leukoplakia (PVL) is a rare subtype of leukoplakia (verrucous/ exophytic) which, at the early stage, is a small, flat and white plaque, but as it progresses becomes multifocal and has a high malignant potential. Erythroplakia, the least common form of OPMDs, is a solitary, irregular and fiery red velvety patch. This type of lesion exhibits a higher malignant potential than leukoplakia and frequently coexists with oral epithelial dysplasia (OED), carcinoma-in-situ (CIS) or oral squamous cell carcinoma (OSCC). It most commonly appears in the soft palate [101, 103].

Oral lichen planus is a chronic inflammatory disease, commonly appearing as a white, reticular and lace-like network plaque on the buccal mucosa, gums and tongue. Oral submucous fibrosis (OSF) is a chronic disorder that affects the oral mucosa, with loose of fibroelasticity, which leads to limited mouth opening, stiffness and tongue rigidity [100, 103].

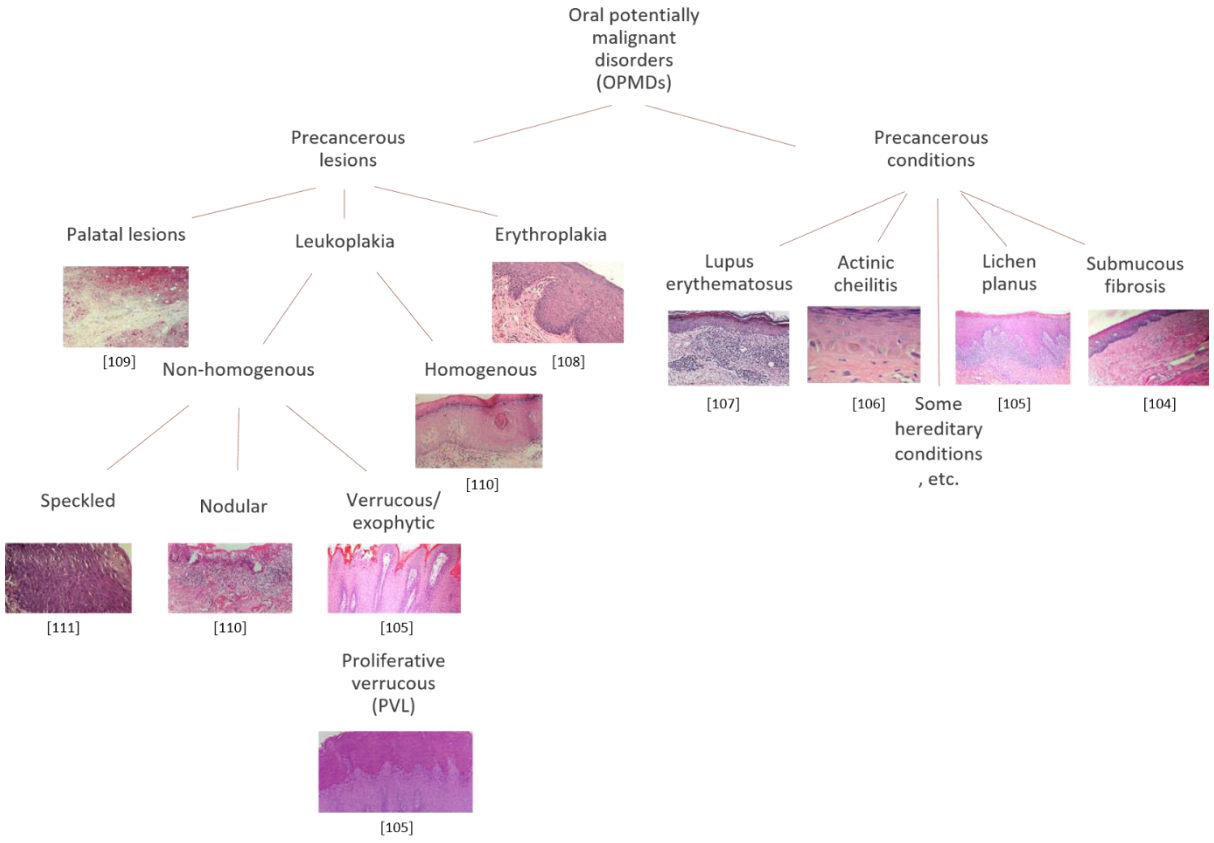

Figure 11: Oral Potentially Malignant Disorders (OPMDs). The two main groups under the umbrella of OPMDs: precancerous conditions and precancerous lesions. Oral submucous fibrosis [104], Lichen planus [105], Actinic cheilitis [106], lupus erythematosus [107], Erythroplakia [108], Palatal lesion [109], Homogenous leukoplakia [110], Verrucous/ exophytic [105], Nodular leukoplakia [110], Speckled leukoplakia [111], Proliferative verrucous leukoplakia PVL [105].

The clinical diagnosis of OPMD commonly involves the histological diagnosis of OED. Cytological (individual cell changes/cytological atypia and architectural/tissue) alterations characterize OED, reflecting the loss of normal maturation and stratification pattern of surface epithelium [112]. Several grading systems have been developed over the last decades to categorize OED. The latest WHO classification, generally used by pathologists, categorizes OED into mild, moderate and severe, which associates with risk of developing malignant disease. These changes can be mild (grade I), showing less than lower third involvement of the epithelium; moderate (grade II), extending into two-thirds of the epithelium thickness; and severe (grade III), involving more than two-thirds of the epithelial tissue (Table 1) [100, 104].

Table 1. Different classification systems of oral epithelial dysplasia. Adapted from [112].

Classification systems					
WHO 1978 classification	Mild dysplasia	Moderate dysplasia	Severe dysplasia		
SIL 1988	Hyperplasia/ keratosis	SIL I (low grade)	SIL II (High grade)		
Ljubljana classification 2003	Squamous cell (simple) hyperplasia	Basal/ parabasal cell hyperplasia	Atypical hyperplasia	Carcinoma in situ	
SIN 2005	SIN 1 low grade dysplasia	SIN 2 High grade dysplasia	SIN 3 High grade dysplasia		
WHO 2005 classification	Squamous hyperplasia	Mild dysplasia	Moderate dysplasia	Severe dysplasia	Carcinoma in situ
Binary system 2006	Low risk	High risk			
OIN/CIS (JSOP) system 2010	Reactive atypical epithelium	Oral epithelium dysplasia	OIN/CIS (JSOP)		
WHO 2017 classification	Mild dysplasia	Moderate dysplasia	Severe dysplasia		

SIN, Squamous Intraepithelial Neoplasia; SIL, Squamous Intraepithelial Lesion; OIN, Oral Intraepithelial Neoplasia; CIS, Carcinoma *in situ*; JSOP, Japanese Society for Oral Pathology; WHO, World Health Organization.

2.5 Pathological subtypes, staging and grading of oral cancer

Oral cancer is the most prevalent cancer among different subtypes of HNC and the tongue is the most common site for its appearance. The most common histological type is squamous cell carcinoma,

which is originated from epithelial cells. Other cellular origins like neoplasms of mesenchymal and neural origin are less common [113].

Based on the American Joint Committee on Cancer (AJCC), the Tumour-Node-Metastasis (TNM) system is the most commonly used clinical classification for oral cancer. TNM staging clinically and/or pathologically describes the anatomic extent of cancer and determines its stage. The TNM name stands for extension of the primary tumour (T), involvement of lymph nodes (N) and the presence of distant metastasis (M) throughout the body (Table 1) [96, 97]. In general, there are four stages of oral cancer according to the eighth version of TNM, which consist of stage I (T1) and stage II (T2), as early stages, identified by small tumours without lymph node involvement; stage III (T3, N1) and stage IV a, b, c (M1) are late stages, involving invasion of neighbouring tissues or lymph node involvement, and distant metastases. In the latest edition of this classification, tumour depth of invasion in the oral cavity was also considered (Table 1) [97, 114].

Regarding grading systems, there are three histological grades of oral cancer according to the WHO classification, namely highly, moderately and poorly differentiated tumours, that consider the assessment of keratinization, mitotic activity, cellular and nuclear pleomorphism, pattern of invasion, and host response. However, this classification is not sufficient for optimal prognostication and there is still lack of some factors such as tumor growth pattern and dissociation, stromal reactions and tumor-stroma ratio [115, 116]. The classification system established by *Anneroth et al* is more cost-effective and available with less limitations. According to this grading system, six parameters are considered, namely degree of keratinization, nuclear polymorphism, number of mitosis/ high power field (HPF), pattern of invasion, stage of invasion and lympho-blasmo-cystic infiltration as morphological parameters. This grading system is accepted as a standard method with highly predictive power for prognosis [115, 117].

Table 2. Tumour–Node–Metastasis classification of oral cavity cancer according to AJCC 2016. Reviewed in [95, 118].

Tumour	
T1	Tumour size ≤2 cm and DOI ≤5 mm
T2	Tumour size 2-4 cm and DOI ≤10 mm or tumour size ≤2 cm and DOI 5-10 mm
T3	Tumour size >4 cm or tumour of any size and DOI >10 mm
T4a	Locally advanced tumour (extrinsic tongue muscle infiltration now deleted) Tumour invades adjacent structures only e.g., through cortical bone of the mandible/maxilla, or involves the maxillary sinus or skin of the face
T4b	Very advanced tumour Tumour invades masticator space, pterygoid plates, or skull base and/or encases internal carotid artery
Node	
N1	Metastasis to a single ipsilateral lymph node, ≤3 cm in greatest dimension, without extranodal extension
N2a	Metastasis to a single ipsilateral node, >3 cm but <6 cm in greatest dimension, without extranodal extension
N2b	Metastases to multiple ipsilateral lymph nodes, none >6 cm in greatest dimension, without extranodal extension
N2c	Metastases to bilateral or contralateral lymph nodes, none >6 cm in greatest dimension, without extranodal extension
N3a	Metastasis to a lymph node, >6 cm in greatest dimension, without extranodal extension
N3b	Metastases to one or more lymph nodes, with clinically overt extranodal extension
Metastasis	
M1	Distant metastases

DOI, depth of invasion.

2.6 Natural history and molecular pathogenesis of oral cancer

The process of oral carcinogenesis, like in other carcinomas, is a multistep and multifactorial process at the molecular level, being characterized by high levels of genomic instability and mutations in several genes (Figure 12). If the accumulation of random genetic mutations is sufficient, the transformation of normal mature oral keratinocytes initiates with disturbance of multiple physiological events that include the control of the cell cycle, DNA repair process, terminal differentiation and apoptosis. This creates unstable keratinocytes and promotes pre-malignancy, with inheritance capability to their next generation. Also, the surrounding microenvironment has an important role in selecting cells from the heterogeneous clones which have the ability to adapt, survive and proliferate in this conditions [119, 120].

There are several pathways involved in oral carcinogenesis, of which the most important are the TP53/ retinoblastoma (RB), PI3K/Akt/mTOR, Notch homolog 1, translocation-associated (*Drosophila*) (NOTCH1), Epidermal growth factor receptor (EGFR), Janus-family tyrosine kinase (JAK)/ signal transducer and activator of transcription (STAT) and Wingless-related integration site (Wnt) signalling pathways [121]. In the TP53/RB pathway, inactivation of RB functionality, which is a cell cycle regulatory protein, in combination with p53 mutations, are associated with uncontrolled proliferation and its downstream consequences like therapy resistance [122]. In PI3K/Akt/mTOR signalling cascade, the loss of the negative regulator phosphatase and tensin homologue deleted on chromosome 10 (PTEN) and p16 induce Akt phosphorylation, which leads to the upregulation of several cellular functions such as growth, proliferation and survival [123]. *NOTCH1*, which is a tumour suppressor gene, is a strong inducer of keratinocyte differentiation and its loss of function is associated with increased β-catenin levels that contribute to carcinogenesis and poor prognosis. Moreover, molecular crosstalk between the NOTCH1 and WNT signalling can be responsible for recurrence and metastasis [114, 124]. EGFR is a transmembrane receptor tyrosine kinase in which ligand binding leads to tyrosine kinase activation and downstream signalling cascades. It can also function as a transcription factor, translocating to the nucleus and regulating cell-cycle progression, invasion and metastasis. As an example, *CCND1*, which encodes Cyclin D1 protein, is one of the EGFR targets that controls cell-cycle progression. Mutations that lead to EGFR overexpression have been reported in oral cancer, correlating with poor prognosis and resistance to radiotherapy. The JAK-STAT pathway is also implicated in oral carcinogenesis, and STAT3 overexpression has been reported in oral cancer patients and not identified in healthy individuals. This pathway, in combination with EGFR signalling, is responsible for cancer cell survival, angiogenesis and

resistance to treatments [124, 125]. Finally, the canonical Wnt pathway (or Wnt/β-catenin pathway), through the accumulation of β-catenin in the cytoplasm and its eventual translocation into the nucleus, where it acts as a transcriptional coactivator of transcription factors that belong to the TCF/LEF (T-cell factor/lymphoid enhancer factor) family, plays a key role in the progression of oral cancer at different stages, and it is also responsible for recurrence, metastasis and poor prognosis of patients [126].

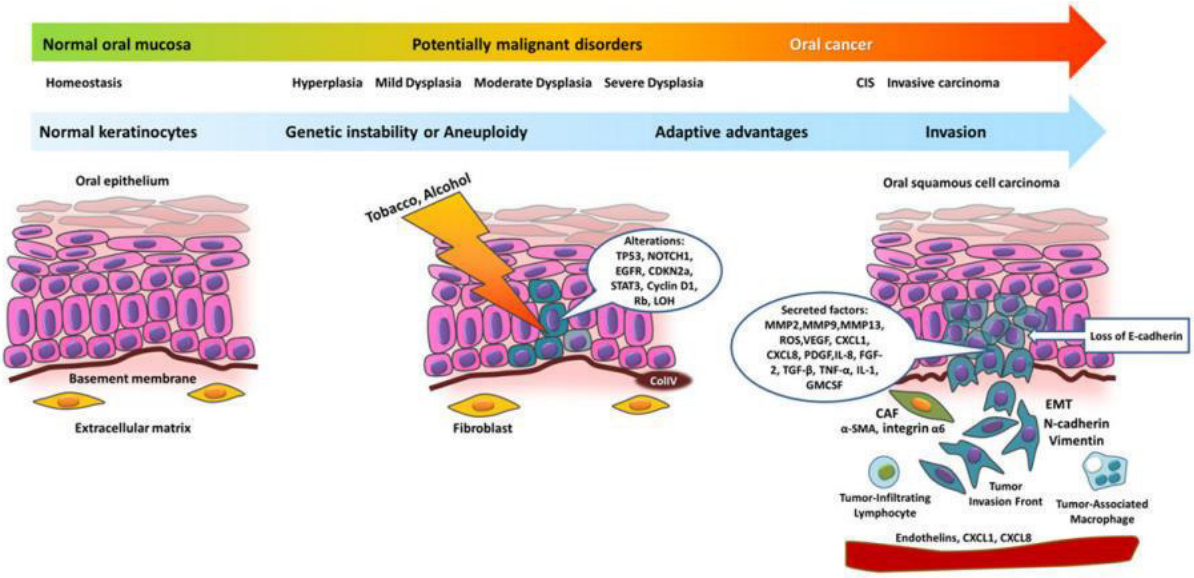

Figure 12: Multiple steps of oral carcinogenesis [119]. Oral carcinogenesis is a multistep process in which the transformation of normal oral keratinocytes begins with some mutations that cause instability of the cells by modifying their proliferation, cell growth, differentiation and apoptosis. In this condition, the surrounding microenvironment enhances the instability of cells and selects the ones which have the ability to adapt, survive and proliferate. TP53; NOTCH1, Notch homolog 1, translocation-associated (*Drosophila*); EGFR, epidermal growth factor receptor; *CDKN2A*, cyclin-dependent kinase inhibitor 2a; genes STAT3, signal transducer and activator of transcription 3; Rb, retinoblastoma; LOH, loss of heterozygosity; MMP2, matrix metalloproteinase 2; MMP9, matrix metalloproteinase 9; MMP13, matrix metalloproteinase 13; ROS, reactive oxygen species; VEGF, vascular endothelial growth factor; CXCL1, chemokine (CXC motif) ligand 1; CXCL8, chemokine (CXC motif) ligand 8; PDGF, platelet-derived growth factor; IL-8, inteleukin 8; FGF-2, fibroblast growth factor 2; TGF-β, transforming growth factor-β; TNF-α, tumour necrosis factor-α; IL-1, inteleukin 1; GMCSF, granulocye-macrophage colony-stimulating factor; EMT, epithelium mesenchymal transition; CAFs, tumour-associated fibroblasts; α-SMA, α-smooth muscle actin.

2.7 Diagnosis, management and prognosis

Oral cancer at the earlier stages is usually asymptomatic. Therefore, early detection of malignant lesions is difficult [127]. However, in advanced stages symptoms are obvious and lesions are mostly huge, exophytic and ulcerated. They also bleed with minor provocation and may spread to regional lymph nodes which can easily be detected by touching the lymphadenopathies [120]. For early detection of oral cancer, there are different types of biomarkers that are still in a pre-clinical stage, such as salivary biomarkers (L-phenylalanine), but also markers of angiogenesis occurrence such as Cluster of differentiation factor 34 (CD34), and genomic (integrins $\alpha 3$ and $\beta 4$) and proteomic (acidic laccase gene 2, *ACAC2*) biomarkers [127]. The use of toluidine blue staining can also facilitate early diagnosis of oral cancer, in which dark parts reveal malignancy and a light stain appears in normal tissue [120]. However, the gold standard for clinical diagnosis of oral cancer is based on biopsy, which includes punch biopsy, core needle biopsy, or fine-needle aspiration, followed by histopathological evaluation. Advanced imaging techniques might be used to assess other tumor features and consist of computed tomography (CT) for assessment of bone and neck lymph nodes, magnetic resonance imaging (MRI) for complementary evidence of extending tumor to soft tissue and perineural areas, and PET scan for evaluation of distant metastases. The successful treatment strategy for an oral cancer patient at an early stage is surgery followed by radiotherapy, and in an advanced stage surgery is combined with cisplatin chemotherapy, which increases survival of the patient [96, 120]. However, it is notable that, despite advancements in diagnostic and treatment in the past few decades, the overall survival rate for oral cancer patients is estimated at 5 years, and this has remained unchanged over the last thirty years. Hence, the search for prognostic biomarkers which can improve the outcome of the patients, increasing disease-free survival (DFS) and overall survival (OS) rates and contributing to the patients' quality of life is urgently needed [128, 129].

2.8 Major drawbacks and concerns

The oral cavity is an important anatomical region that is responsible for critical functions such as speech, chewing and swallowing, also having aesthetic implications. Thus, the management of oral cancer is complex, as it encompasses aspects related to the physical and psychologic patients' quality of life [97]. Despite advancements in oral cancer treatment, the main challenge is that oral cancer is

generally not detected at early stages, which would allow successful treatments, and still implies a poor prognosis for the patients, being the overall rate of survival estimated at approximately 5 years [130, 131]. In addition, conventional treatments have significant medical costs and some subsequent drawbacks. Some of the consequences of oral cavity radiotherapy are mucositis, a painful inflammation and ulceration of the mucous membranes lining the oral cavity, and xerostomia, that causes loss of salivary function and dehydration. Other general side effects include hair loss, alterations in skin color and irritation of the skin. Regarding diagnosis, a few progress has been made in the field of oral cancer biomarkers, although some limitations remain. As an example, the level of salivary biomarkers is variable, and in inflammatory conditions, there is the need for validation. Moreover, the lack of standard procedures for sampling and storage of saliva, make it difficult to use it as a biomarker. Therefore, it is necessary to look for new diagnostic and prognostic biomarkers, as well as therapeutic targets [127, 129].

3 Metabolic alterations in oral cancer

Oral cancer cells, like other types of cancer cells, consume much more glucose to produce energy than non-cancer cells. Therefore, they overexpress glycolytic enzymes and related transporters to favor them to achieve this goal. Regarding glucose metabolism, HIF-1 seems to be the main regulator of the Warburg effect in oral cancer [132-134].

In the study of *VC Angadi* and P*V Angadi;* GLUT-1 was upregulated in response to HIF-1α in OSCC [135]. Also, it was shown that GLUT-1 expression was significantly increased in dysplastic tissues, which suggests it as a potential biomarker for the identification of premalignant lesions [136]. In OSCC cell lines, cell proliferation and glycolysis metabolism was upregulated by GLUT-1 overexpression [137], while its knockdown inhibited cell proliferation [138]. Moreover, under hypoxic conditions, GLUT-1 deletion enhanced the sensitivity of oral cancer cells to cisplatin [139].

Lactate transporters such as MCT-1 and MCT-4 seem to be overexpressed in epithelial and stromal compartments of OSCC [140]. A higher expression of MCT-1 and 4 in neoplastic compared to non-neoplastic oral tissues was observed [141]. In the same study CD147, the chaperone of MCTs, was also upregulated as a reflex of increased glycolytic metabolism [141]. MCT-1 silencing significantly impaired

organoid formation in OSCC cells [142]. Under hypoxic conditions, MCT-4 was shown to be a direct target of HIF-1α, being upregulated in OSCC [143].

In a study conducted by *Tanaka F et al*, PKM-2 was upregulated in oral cancer cells and had a pivotal role in promoting cancer cell progression in human OSCC in a glycometabolism-independent way [144]. An increased expression of LDH has also been observed in OSCC, being associated with a poor survival of OSCC patients [145].

CA-IX was shown to be upregulated in OSCC, correlating with a poor prognosis; this transmembrane enzyme was co-expressed with HIF1-α [146, 147]. CA-IX levels increased during malignant transformation of OED in the study of *Zhang X et al.* [148].

As demonstrated above, metabolic reprogramming is a unique feature of oral cancer cells, and hypoxia-related proteins are closely associated with this feature [132, 133]. As TNM classification system and grading alone cannot predict the aggressive potential of OSCC from pre-malignancy to malignancy, prognostic biomarkers are a valuable option that may ultimately favour patients suffering from this disease [149, 150]. Additionally, proteins activated under hypoxic conditions can potentially be used as predictive markers of the conversion of oral premalignant lesions into cancer. Strategies that aim at identifying metabolic biomarkers in cancer cells through immunohistochemistry (IHC) have shown great potential as diagnostic and prognostic approaches [132]. The main advantage of the identification of prognostic factors in OSCC by IHC is the establishment of a pattern of expression which can offer valuable insights into the process of tumour progression [151]. Hypoxia-related proteins have been studied for years. GLUT-1 [135], HK-II [136], PKM-2 [144], LDH-A [145], MCT-4 [143], GLUT-3 [152] and CA-IX [146] are well-known hypoxic biomarkers that have potential as prognostic markers in oral cancer, although studies have focused mainly in cancer cells. To the best of our knowledge, the expression of other hypoxia-related glycolytic enzymes such as PFK-L and PDH during the carcinogenesis process has not been described yet in this type of cancer.

Hence, it is important to invest in the search for valid diagnostic and prognostic biomarkers that can accurately predict the course of the disease from pre-malignancy to malignancy and ultimately improve patients' outcome and increase their quality of life. In this study, we aimed to evaluate the immunoexpression of HIF-1α-targeted metabolism-related proteins by using the immunohistochemistry technique in malignant and premalignant oral lesions.

Objectives

1. Evaluate the immunoexpression of GLUT-1, GLUT-3, HK-II, PFK-L, PKM-2, phospho-PDH (pPDH), LDH-A, MCT-4, and CA-IX in oral squamous cell carcinoma and in premalignant lesions – white oral lichen planus, leukoplakia without dysplasia, leukoplakia with low grade dysplasia and leukoplakia with high grade dysplasia.

2. Identify potential correlations between biomarkers' immunoexpression, clinicopathological and prognostic parameters.

Materials and Methods

Patients and tissue samples

Ethical approval for this retrospective clinical study was obtained under the permission of the Galician Autonomous Committee of Ethics (reference 2019/271). A total of fifty-five formalin-fixed paraffin-embedded oral cavity lesions, composed of 34 premalignant and 21 malignant cases, were obtained from the Department of Oral Medicine, Oral Surgery and Implantology, University Hospital Complex of Santiago de Compostela, from patients admitted at that institution from October 1997 to February 2020 and from January 1992 to Jun 2019, respectively. Clinicopathological information was retrieved from the medical records of the patients. Only cases diagnosed with premalignant and malignant oral lesions were collected for this study. Regarding patients with premalignant lesions, their median age was 59 years (range 29-85); eighteen (52.9%) were male and sixteen (47.1%) were female. The median age of OSCC patients was 67 years (range 50-95); eleven (52.4%) were male and ten (47.6%) were female. Classification of premalignant lesions and oral cancer specimens was performed according to WHO [153] and to the last version of AJCC staging system [118], respectively. Clinical data collected from the records of patients with premalignant lesions included age, gender, lesion location, lesion size, smoking and alcohol consumption habits, lesion type and clinical presentation, biopsy type, type of treatment and follow-up. Regarding OSCC patient's clinicopathological parameters, these included age, gender, tumor location, tumor size, smoking and alcohol consumption habits, TNM stage (clustered into four categories), grade and follow-up data (disease recurrence and occurrence of death). All data are shown in Table 3 and 4. For the OSCC patients, the follow-up period was calculated from the date of admission until the last follow-up appointment; it ranged from one to twenty-eight months, with a mean and median follow-up of 14.6 and 16.7 months, respectively. DFS was defined as the time from the first treatment until recurrence; this occurred in ten patients (47,6%). OS was defined as the length of time from the first treatment until the last follow-up assessment or patient death; this occurred in eleven patients (52,4%).

Table 3. Overview of clinicopathological data in patients with oral premalignant lesions.

		n	%
Age			
	<59	17	50
	≥59	17	50
Gender			
	Male	18	52.9
	Female	16	47.1
Biopsy location			
	Jugal mucosa	15	44.1
	Soft palate	3	8.8
	Hard palate	1	2.9
	Tongue	11	32.4
	Mouth floor	1	2.9
	Alveolar rim	1	2.9
	Keratinized gum	2	5.9
Lesion size			
	<0.8	15	44.1
	≥0.8	19	55.9
Smoking habits			
	Non smoker	15	44.1
	Ex-Smoker	13	38.2
	Smoker	6	17.6
Alcohol consumption			
	No	21	61.8
	Yes	13	38.2
Lesion type			
	White oral lichen planus	12	35.3
	Leuk without dysplasia	11	32.4
	Leuk with low grade dysplasia	8	23.5
	Leukoplakia with high grade dysplasia	3	8.8
Clinical presentation			
	White oral lichen planus	12	35.3
	Leuk Homogeneous	13	38.2
	Leuk NH Verrucous	6	17.6
	Leuk NH Nodular	2	5.9
	Erythroleukoplakia	1	2.9
Biopsy type			
	Incisional	29	85.3
	Excisional	5	14.7
Treatment			
	Follow-up	18	52.9
	Corticosteroids	4	11.8
	Full exeresis	4	11.8
	Laser	8	23.5
Follow-up			
	Cure	5	14.7
	Stable disease	24	70.6
	Recurrence	3	8.8
	Progression to OSCC	2	5.9

Leuk, Leukoplakia; NH, Non-homogenous; OSCC, oral squamous cell carcinoma.

Table 4. Overview of clinicopathological data in patients with oral squamous cell carcinoma.

		n	%
Age			
	<67	10	47.6%
	≥67	11	52.4%
Gender			
	Male	11	52.4%
	Female	10	47.6%
Biopsy Location			
	Jugal mucosa	3	14.3%
	Soft palate	1	4.8%
	Hard palate	1	4.8%
	Tongue	5	23.8%
	Maxilla	3	14.3%
	Mouth floor	3	14.3%
	Alveolar rim	3	14.3%
	Lower lip	2	9.5%
Lesion Size			
	<2.5	10	47.6%
	≥2.5	11	52.4%
Smoking Habits			
	Non smoker	11	52.4%
	Ex-Smoker	4	19%
	Smoker	6	28.6%
Alcohol			
	No	14	66.7%
	Yes	6	28.6%
	Ex	1	4.8%
TNM Stage			
	I	5	23.8%
	II	5	23.8%
	III	1	4.8%
	IV	10	47.6%
Grade			
	Well differentiated	10	47.6%
	Moderately differentiated	10	47.6%
	Poorly differentiated	1	4.8%
Recurrence			
	No	11	52.4%
	Yes	10	47.6%
Death			
	No	10	47.6%
	Yes, from causes other than OSSC	3	14.3%
	Yes, from OSCC	8	38.1%

TNM: (T) primary tumor, (N) regional lymph nodes and (M) distant metastasis; OSCC, oral squamous cell carcinoma.

Immunohistochemistry

IHC is a technique which is commonly used to visualize the distribution and localization of specific cellular components such as proteins within cells of different parts of a biological tissue. It is also widely used as diagnostic technique in the field of tissue pathology and identification of biomarkers for diagnosis, prognosis and targeted therapy [154]. IHC was performed on the collected human samples. For that, the dissected tissues, fixed with 4% formalin and embedded in paraffin, were cut into 4μm-thick sections for IHC staining. The tissue sections were deparaffinised with xylene and rehydrated through a graded ethanol series. For antigen retrieval, the sections were heated in 10mM sodium citrate buffer (pH 6.0) at 98 °C for 20 minutes in a water bath (unless otherwise specified, Table 5). After that, sections were cooled and washed with phosphate-buffered saline (PBS), then treated with 3% hydrogen peroxide reagent for 10 minutes to eliminate endogenous peroxidase activity. Following washing steps, sections were blocked with a normal serum solution to avoid non-specific background staining. The Thermo Scientific™ Lab Vision™ UltraVision™ Large Volume Detection System: anti-Polyvalent, Horseradish peroxidase (HRP) (Thermo Fisher Scientific), based on the streptavidin-biotin peroxidase principle, was used for PKM-2, PFK-L, GLUT-1 and HK-II detection. The Thermo Scientific™ Lab Vision™ UltraVision™ ONE Detection System: HRP Polymer (Thermo Fisher Scientific), based on the polymeric method principle, was used for LDH-A, pPDH, CA-IX, MCT-4 and GLUT-3 detection. Details can be found in Table 5. Color development was achieved with the Thermo Scientific™ DAB (3, 3´-diaminobenzidine) colorimetric substrate, for 10 minutes at RT. For the subsequent steps, slides were counterstained with hematoxylin, dehydrated and mounted. Images were captured by light microscopy in an Olympus BX61 microscope.

Table 5. Details of the immunohistochemical procedure.

Biomarker	Primary antibody		Positive Control	Antigen retrieval
	Dilution, conditions	Reference, company		
GLUT-1	1:500, ON, RT	ab15309, AbCam	Colorectal carcinoma	Citrate Buffer (0.01M, pH=6.0), 98°C, 20 min
GLUT-3	1:75, ON, RT	ab41525, AbCam	Colorectal carcinoma	Tris + EDTA (1 mM, pH=8,0), 98°C, 20 min
HK-II	1:800, ON, RT	ab104836, AbCam	Gastric carcinoma	Citrate Buffer (0.01M, pH=6.0), 98°C, 20 min
PFK-L	1:100, ON, RT	ab37583, AbCam	Liver	Citrate Buffer (0.01M, pH=6.0), 98°C, 20 min
PKM-2	1:100, ON, RT	ab38237, AbCam	Liver	Citrate Buffer (0.01M, pH=6.0), 98°C, 20 min
pPDH	1:1000, ON, RT	ab92696, AbCam	Colorectal carcinoma	Citrate Buffer (0.01M, pH=6.0), 98°C, 20 min
LDH-A	1:1000, ON, RT	sc-137243, Santa Cruz Biotechnology®	Colorectal carcinoma	Citrate Buffer (0.01M, pH=6.0), 98°C, 20 min
MCT-4	1:250, ON, RT	sc-50329, Santa Cruz Biotechnology®	Bladder carcinoma	Citrate Buffer (0.01M, pH=6.0), 98°C, 20 min
CA-IX	1:2000, ON, RT	ab15086, AbCam	Gastric carcinoma	Citrate Buffer (0.01M, pH=6.0), 98°C, 20 min

EDTA, ethylenediaminetetraacetic acid; Tris, trisaminomethane; M, molar; min, minutes; mM, milimolar; ON, overnight; RT, room temperature.

Evaluation of immunohistochemistry results

For the evaluation of immunohistochemistry results, stained tissues were scored semi-quantitatively by two independent pathologists (ALF and JMP), being discordant cases re-evaluated and classified by consensus. The grading system used considered the percentage score (PS), which reflects the concentration of target proteins, being grouped into 0: 0% of immunoreactive cells, 1: <5% of immunoreactive cells, 2: 5-50% of immunoreactive cells and 3: >50% of immunoreactive cells; the strength of staining (SS) was graded as 0: negative, 1: weak, 2: intermediate, and 3: strong. The percentage and strength scores were summed to obtain the final score (FS = PS + SS) and clustered as negative (score 0-2 or 0-3) and positive (score 3-6 or 4-6), depending on the biomarker. Thus, the considered FS were the ones which allowed us to obtain the most informative results regarding clinicopathological and prognostic implications for each studied biomarker. Protein localization, namely cytoplasmic, nuclear and/or plasma membrane location, was also assessed in this study. Oral premalignant tissue lesions were evenly assessed for protein expression, and OSCC sections were evaluated in their normoxic and hypoxic compartments, i.e., areas close to and distant from the blood microvessels, respectively, as previously described [155]. A blood microvessel was considered when a group of endothelial cells around a patent lumen was observed; identification was facilitated by the presence of red blood cells in the lumen.

Statistical analysis

Data analyses were performed using the Statistical Package for Social Sciences (SPSS version 25; IBM Company, Chicago, USA). Correlations of biomarkers' expression in premalignant and malignant oral lesions, and relationship with the clinicopathological parameters, were conducted using Pearson's chi-square ($\chi 2$) test and Fisher's exact test. OS rates were assessed by the Kaplan-Meier method, and analysis was compared using Log-Rank or Breslow tests. A value of $p< 0.05$ was considered to indicate a statistically significant difference.

Results

Prognostic significance of clinicopathological parameters in premalignant and malignant lesions

All patients with premalignant oral lesions were alive at their last clinical assessment, as expected. 91.7% of patients with white oral lichen planus had stable disease, while the two patients in which progression to carcinoma occurred had leukoplakia with dysplasia lesions ($p<0.008$). Regarding OSCC patients, females ($p=0.020$) and patients with poorly differentiated lesions ($p=0.034$) showed a significantly lower DFS. Seven of the eight patients (87.5%) who had died from OSCC had developed prior recurrent disease ($p=0.009$). Smokers had a lower propensity to develop recurrence ($p=0.033$) and a higher overall survival ($p=0.058$); this is intriguing and may be due to the low number of cases in analysis. Detailed results are depicted in Tables 6 and 7.

Immunoexpression of metabolism-related biomarkers in premalignant and malignant lesions

A cohort of fifty-five oral cavity tissue sections, composed of thirty-four premalignant and twenty-one malignant lesions, was analyzed for GLUT-1, GLUT-3, HK-II, PKM-2, PFK-L, pPDH, LDH-A, MCT-4 and CA-IX immunoexpression. Representative images of the immunohistochemical staining reactions in the different types of lesions, as well as the positive controls for each biomarker, are depicted in Figures 13 to 21. Regarding the final staining scores for each biomarker, positivity was considered as ≥ 3 for GLUT-1, GLUT-3, PFK-L, pPDH, LDH-A, MCT-4 and CA-IX staining, and ≥ 4 for PKM-2 and HK-II staining, as these were the scores that allowed us to obtain the most informative results. Detailed information of the immunoexpression frequencies of the biomarkers is shown in Table 8. The expression analysis of these proteins revealed that there were significant differences when comparing the immunoexpression frequencies of CA-IX ($p<0.001$), MCT-4 ($p<0.011$), LDH-A ($p=0.012$), PKM-2 ($p=0.037$) and PFK-L ($p=0.041$) in oral premalignant (even assessment of protein expression) and OSCC lesions (assessment of protein expression in the normoxic areas), with a higher percentage of positive cases for these biomarkers being observed in OSCC samples. HK-II and pPDH were mostly expressed by premalignant lesions, although the differences did not reach statistical significance. No differences were observed regarding GLUT-1 and GLUT-3 immunoexpression. Expression of LDH-A, PKM-2, PFK-L, HK-II and pPDH was noted at the cytoplasm of stained cells, while CA-IX and GLUT-1 expression was mostly membranous. MCT-4 and GLUT-3 were seen in both membrane and cytoplasmic regions. Regarding PKM-2 expression,

occasional nuclear staining was observed in a few premalignant and malignant cases. A positive immunoexpression in endothelial cells was seen with PKM-2 and pPDH biomarkers, although this was only observed in premalignant cases.

Table 6. Association between the clinicopathological data and the 3-year disease-free survival of oral squamous cell carcinoma patients (n=21).

Variables				**Disease-Free Survival**	
		Total number	Number of events	Months	*p**
Age					.686
	<67	10	6	12.513± 2.596	
	≥67	11	4	18.009± 4.077	
Gender					**.020**
	Male	11	3	19.190± 2.291	
	Female	10	7	8.753± 3.784	
Smoking Habits					**.033**
	Non smoker	11	8	8.771± 3.189	
	Ex-Smoker	4	1	15.475± 4.467	
	Smoker	6	1	21.647± 1.926	
Lesion size					.403
	<2.5 cm	10	4	14.667± 2.591	
	≥2.5 cm	11	6	13.809± 3.945	
TNM stage					.083
	I	5	3	10.647± 4.055	
	II	5	4	5.031± 2.409	
	III	1	1	13.633± 0.000	
	IV	10	2	22.613± 3.575	
Grade					**.034**
	Well differentiated	10	7	9.771± 2.516	
	Moderately differentiated	10	2	22.680± 3.519	
	Poorly differentiated	1	1	0.000± 0.000	

p values from Log-rank or Breslow tests. *p* values < 0.05 are shown in bold. TNM: (T) primary tumor; (N) regional lymph nodes and (M) distant metastasis.

Table 7. Association between the clinicopathological data and the 3-year overall survival of oral squamous cell carcinoma patients (n=21).

Variables				Overall Survival	
		Total number	Number of events	Months	*p**
Age					.250
	<67	10	4	17.021± 2.161	
	≥67	11	7	14.645± 3.306	
Gender					.187
	Male	11	4	17.667± 2.674	
	Female	10	7	14.633± 3.161	
Smoking Habits					.058
	Non smoker	11	9	12.915± 2.691	
	Ex-Smoker	4	1	16.167± 4.619	
	Smoker	6	1	20.728± 2.805	
Lesion size					.423
	<2.5 cm	10	4	15.371± 2.665	
	≥2.5 cm	11	7	16.239± 3.030	
TNM stage					.454
	I	5	1	17.653± 3.441	
	II	5	4	13.493± 4.356	
	III	1	1	16.767± 0.000	
	IV	10	5	17.377± 3.497	
Grade					.366
	Well differentiated	10	6	16.532± 3.191	
	Moderately differentiated	10	4	19.687± 3.500	
	Poorly differentiated	1	1	5.833± 0.000	
Recurrence					.223
	No	11	4	20.115± 3.333	
	Yes	10	7	15.127± 3.132	

p values from Log-rank or Breslow tests. *p* values < 0.05 are shown in bold. TNM: (T) primary tumor; (N) regional lymph nodes and (M) distant metastasis.

Table 8. Immunoexpression frequencies for GLUT-1, GLUT-3, HK-II, PFK-L, PKM-2, pPDH, LDH-A, MCT-4 and CA-IX in premalignant lesions and in oral squamous cell carcinoma cases.

	n	GLUT-1 (positive)	*p*	n	GLUT-3 (positive)	*p*	n	HK-II (positive)	*p*
Premalignant lesions	34	34 (100%)	.358	34	5 (14.7%)	1.00	34	14 (41.2%)	.375
OSCC	19	18 (94.7%)		17	2 (11.8%)		19	5 (26.3%)	
	n	**PFK-L (positive)**	***p***	**n**	**PKM-2 (positive)**	***p***	**n**	**pPDH (positive)**	***p***
Premalignant lesions	34	0 (0.0%)	**.041**	34	0 (0.0%)	**.037**	33	23 (74.2%)	.078
OSCC	19	3 (15.8%)		18	3 (16.7%)		19	8 (42.1%)	
	n	**LDH-A (positive)**	***p***	**n**	**MCT-4 (positive)**	***p***	**n**	**CA-IX (positive)**	***p***
Premalignant lesions	31	0 (0.0%)	**.012**	34	0 (0.0%)	**.011**	34	2 (11.8%)	**<.001**
OSCC	17	4 (23.5%)		18	4 (22.2%)		19	15 (88.2%)	

p values from Pearson Chi-square or Fisher's exact tests, for the comparison between premalignant and oral squamous cell carcinoma lesions. *p* values < 0.05 are shown in bold. OSCC, oral squamous cell carcinoma.

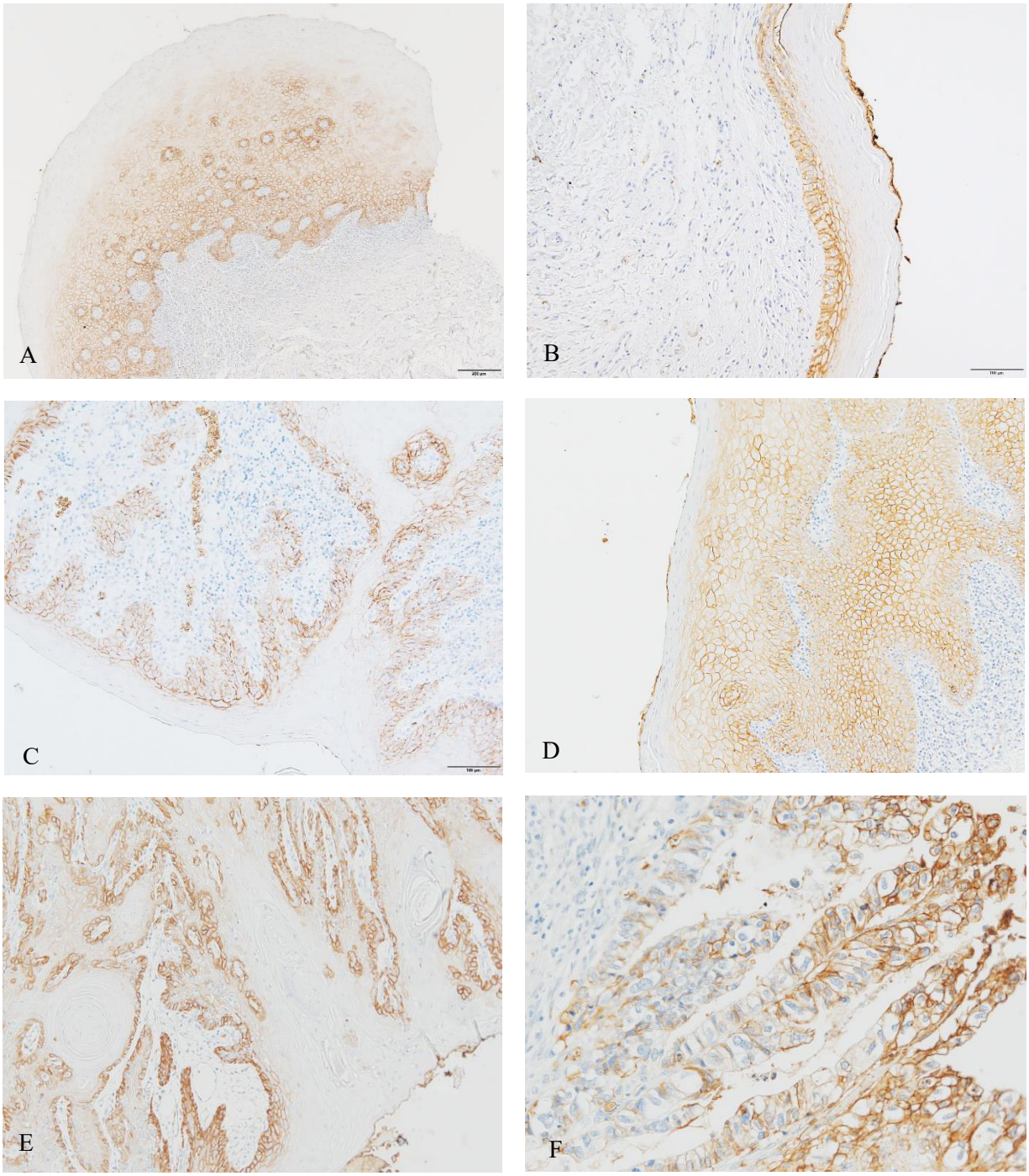

Figure 13: Representative images of the immunohistochemical staining reactions for GLUT-1 in white oral lichen planus (A), leukoplakia without dysplasia (B), leukoplakia with low grade dysplasia (C), leukoplakia with high grade dysplasia (D) and oral squamous cell carcinoma (E), as well as the positive control for GLUT-1 (gastric carcinoma, F). Original magnifications of 40X (A and E), 100X (B, C, D) and 200X (F).

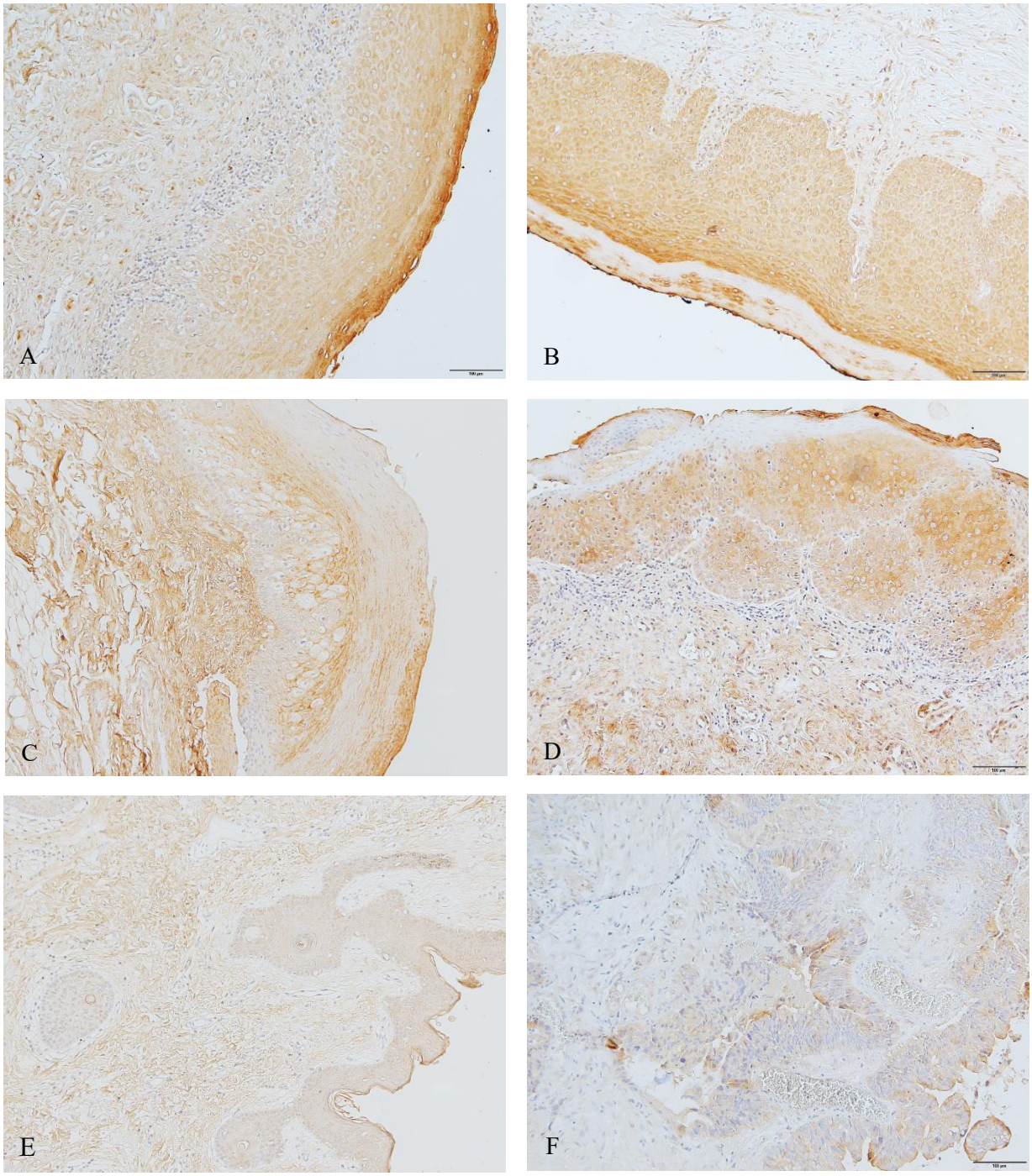

Figure 14: Representative images of the immunohistochemical staining reactions for GLUT-3 in white oral lichen planus (A), leukoplakia without dysplasia (B), leukoplakia with low grade dysplasia (C), leukoplakia with high grade dysplasia (D) and oral squamous cell carcinoma (E), as well as the positive control for GLUT-3 (colorectal carcinoma, F). Original magnifications of 100X.

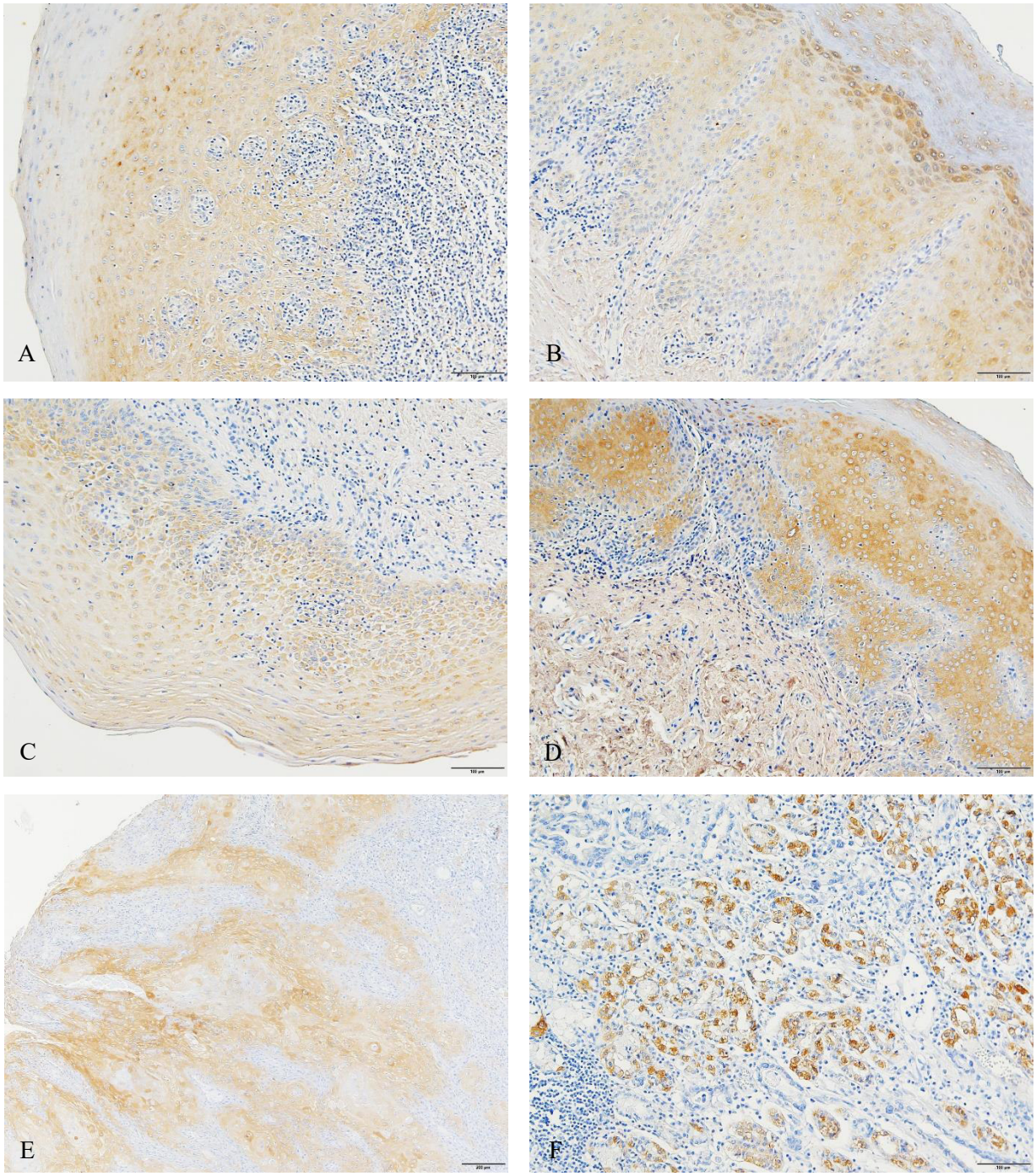

Figure 15: Representative images of the immunohistochemical staining reactions for HK-II in white oral lichen planus (A), leukoplakia without dysplasia (B), leukoplakia with low grade dysplasia (C), leukoplakia with high grade dysplasia (D) and oral squamous cell carcinoma (E), as well as the positive control for HK-II (gastric carcinoma, F). Original magnifications of 40X (F) and 100X (A, B, C, D, E).

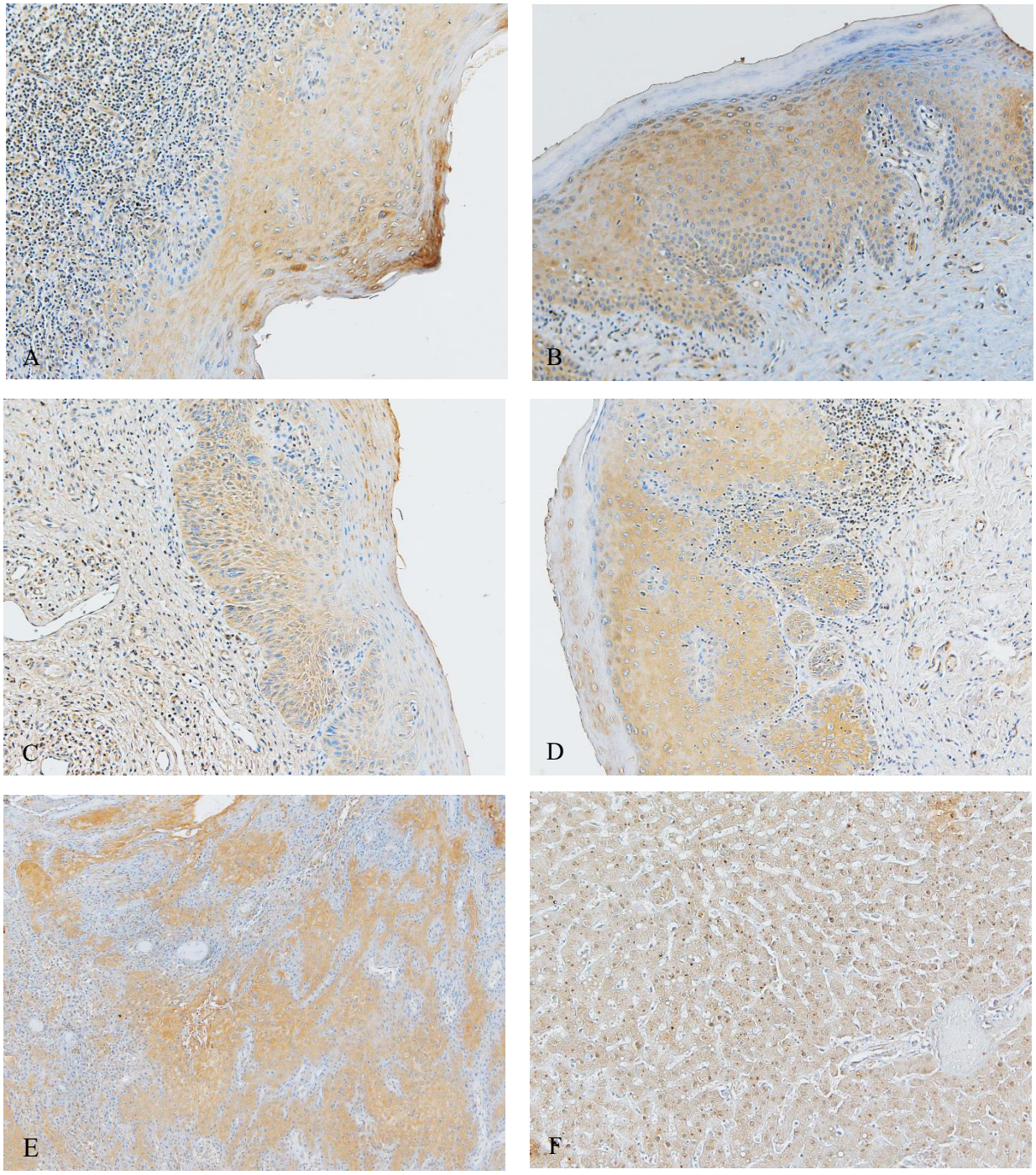

Figure 16: Representative images of the immunohistochemical staining reactions for PFK-L in white oral lichen planus (A), leukoplakia without dysplasia (B), leukoplakia with low grade dysplasia (C), leukoplakia with high grade dysplasia (D) and oral squamous cell carcinoma (E), as well as the positive control for PFK-L (liver, F). Original magnifications of 100X.

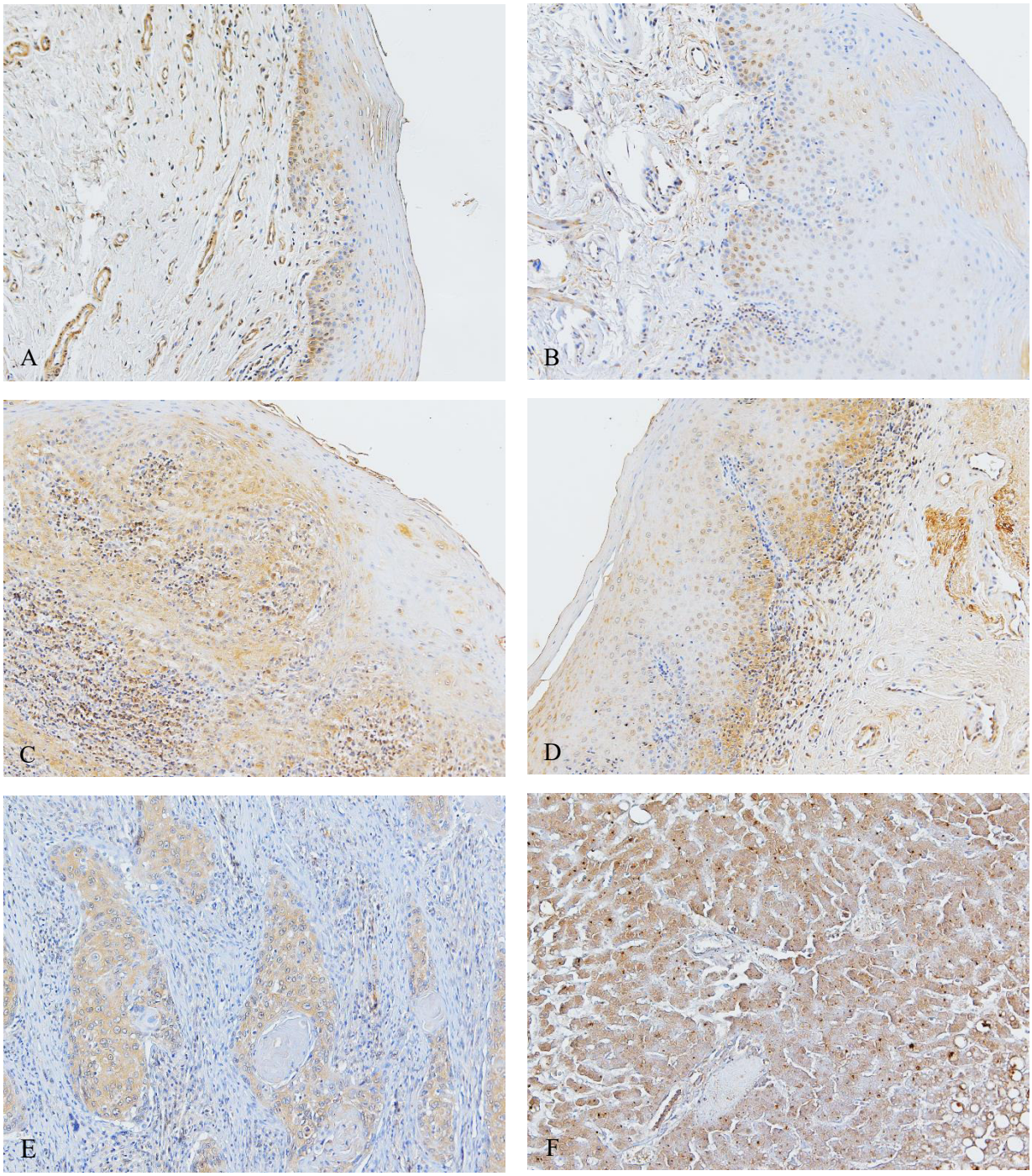

Figure 17: Representative images of the immunohistochemical staining reactions for PKM-2 in white oral lichen planus (A), leukoplakia without dysplasia (B), leukoplakia with low grade dysplasia (C), leukoplakia with high grade dysplasia (D) and oral squamous cell carcinoma (E), as well as the positive control for PKM-2 (liver, F). Original magnifications of 100X.

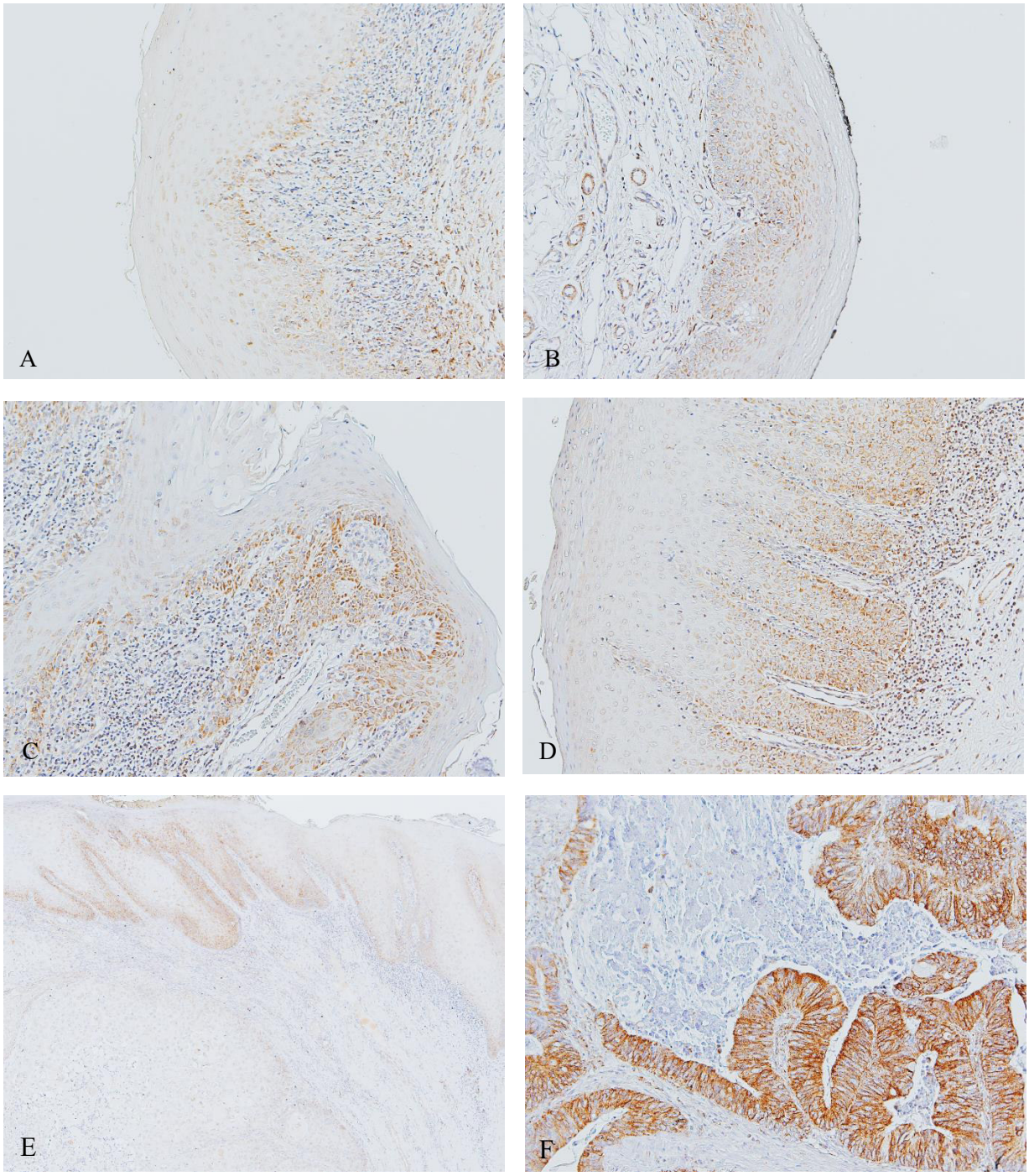

Figure 18: Representative images of the immunohistochemical staining reactions for pPDH in white oral lichen planus (A), leukoplakia without dysplasia (B), leukoplakia with low grade dysplasia (C), leukoplakia with high grade dysplasia (D) and oral squamous cell carcinoma (E), as well as the positive control for pPDH (colorectal carcinoma, F). Original magnifications of 100X.

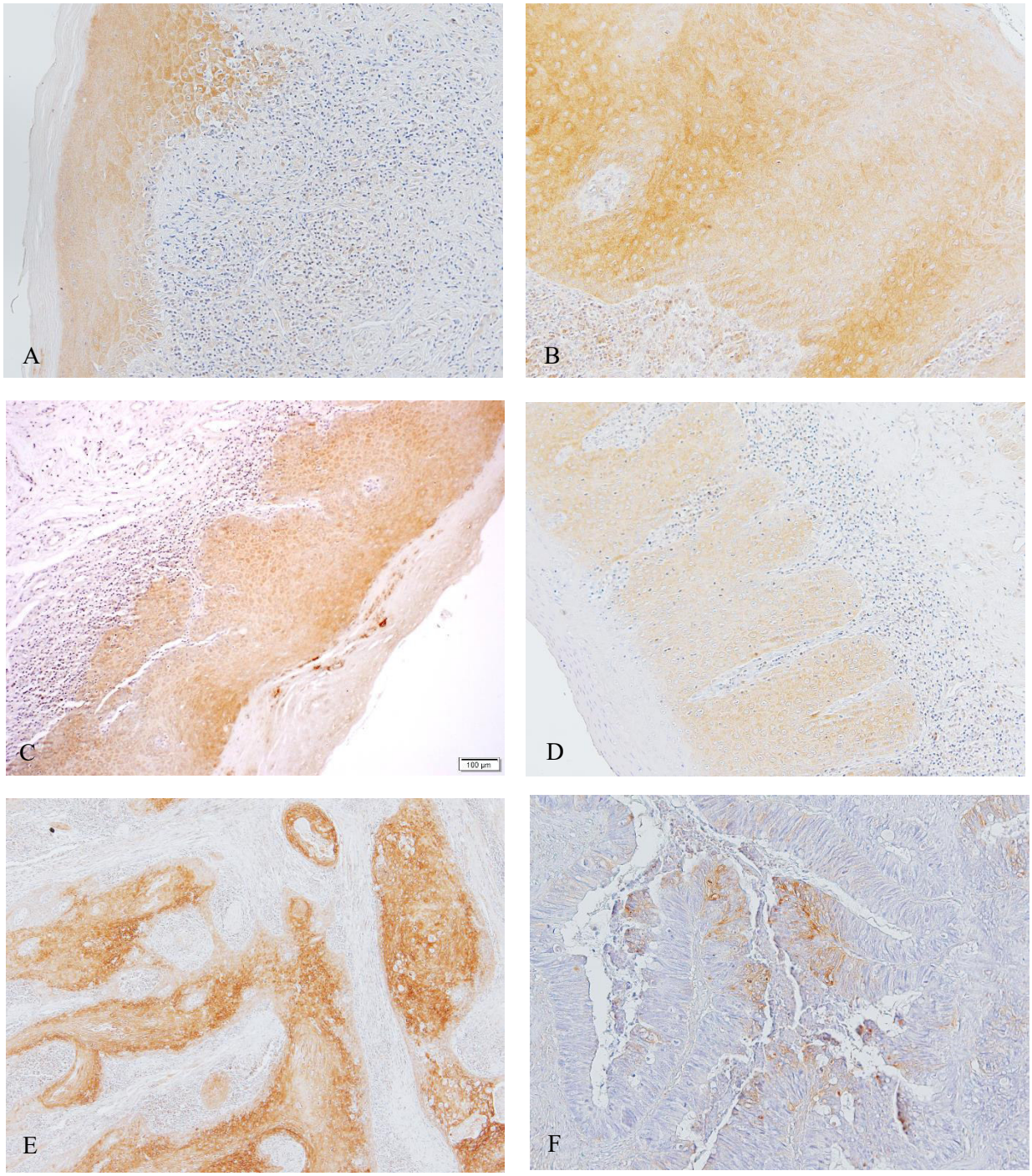

Figure 19: Representative images of the immunohistochemical staining reactions for LDH-A in white oral lichen planus (A), leukoplakia without dysplasia (B), leukoplakia with low grade dysplasia (C), leukoplakia with high grade dysplasia (D) and oral squamous cell carcinoma (E), as well as the positive control for LDH-A (colorectal carcinoma, F). Original magnifications of 100X (A, C, D, E, F) and 200X (B).

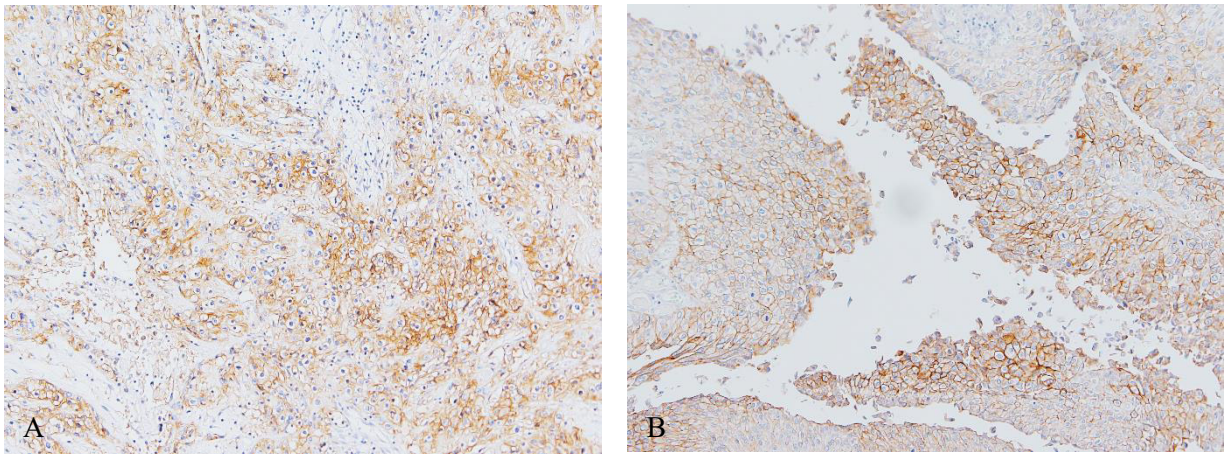

Figure 20: Representative images of the immunohistochemical staining reactions for MCT-4 in oral squamous cell carcinoma (A) and the positive control for MCT-4 (bladder carcinoma, B). Original magnifications of 100X.

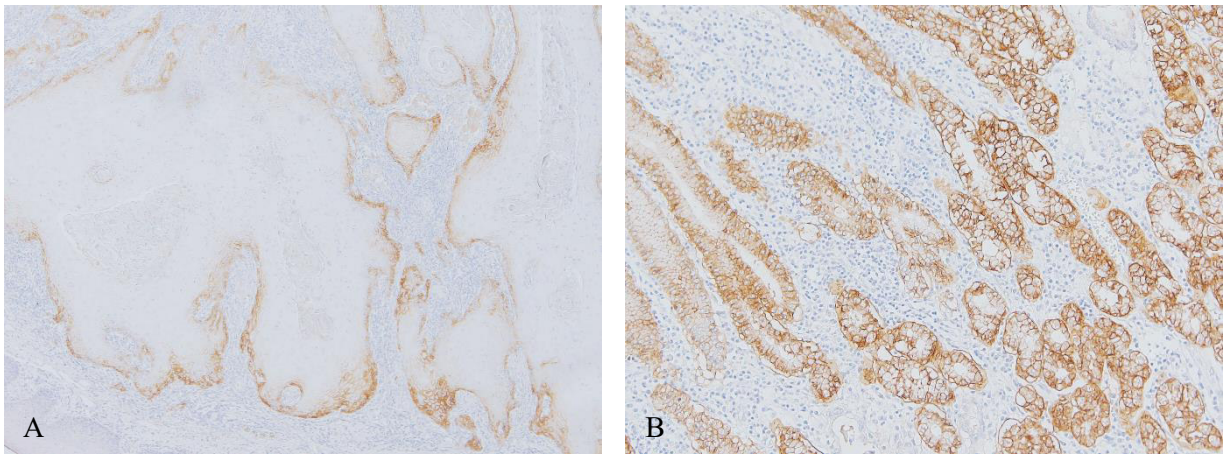

Figure 21: Representative images of the immunohistochemical staining reactions for CA-IX in oral squamous cell carcinoma (A) and the positive control for CA-IX (gastric carcinoma, B). Original magnifications of 100X.

Clinicopathological and prognostic significance of metabolism-related biomarkers in premalignant and malignant lesions

Associations between immunoexpression frequencies of the metabolism-related biomarkers and the clinicopathological parameters of the patients were assessed in the cohort. Detailed analysis may be found in Tables S1 and S2 (Supplementary Data). Regarding the premalignant lesions, 45,5% (5/11) leukoplakia without dysplasia lesions were positive for GLUT-3, while all of the remaining premalignant lesions were negative for this biomarker (p=0.005). HK-II immunoexpression was significantly associated with older age (p=0.013). pPDH was strongly associated with smoking habits, with 92.3% (12/13) of ex-smokers and 66.7% (4/6) of smokers exhibiting pPDH positivity (p=0.046). Regarding the OSCC cases (normoxic regions), a significant association was observed among HK-II immunoexpression and older age (p=0.033); moreover, all of the lesions sized higher than 2.5cm were positive for HK-II (p=0.033). The vast majority of well differentiated and moderately differentiated lesions were negative for PFK-L (p=0.074) and PKM-2 expression (p=0.078), while the single poorly differentiated tumor present in the cohort was positive; the same tendency was observed with increasing TNM stage, although the differences were not significant or near significant. Additionally, LDH-A expression was significantly associated with loss of differentiation (p=0.047), and a tendency was observed on LDH-A positivity being higher in larger tumors (p=0.082); MCT-4 expression increased with increasing TNM stage (not significant differences) and loss of differentiation (p=0.069). All of the tumors from non-smoker patients (9/9) expressed CA-IX (p=0.040); on the other hand, a trend (although not significant) was observed regarding CA-IX positivity with increasing TNM stage.

Survival analysis of the OSCC patients regarding the immunoexpression of the biomarkers revealed that HK-II high expression was significantly correlated with a worse DFS (p=0.007; Figure 22) and near significantly associated with worsened OS (p= 0.060; Figure 23); the same was observed regarding CA-IX and OS rate correlation (p=0.071, Figure 24). Detailed data may be found in Tables 9 and 10.

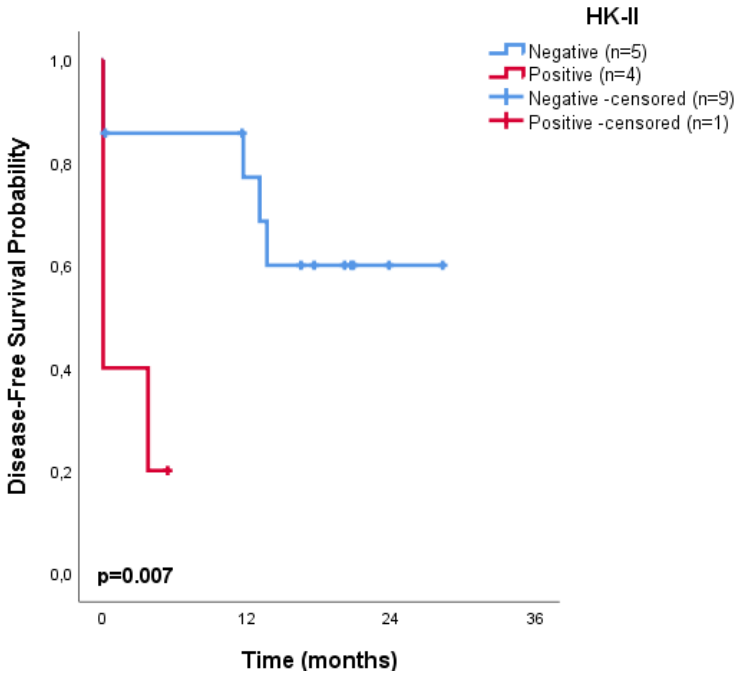

Figure 22. Kaplan-Meier curve demonstrating 3-year disease-free survival of patients with oral squamous cell carcinoma (n=21) based on HK-II immunoexpression status.

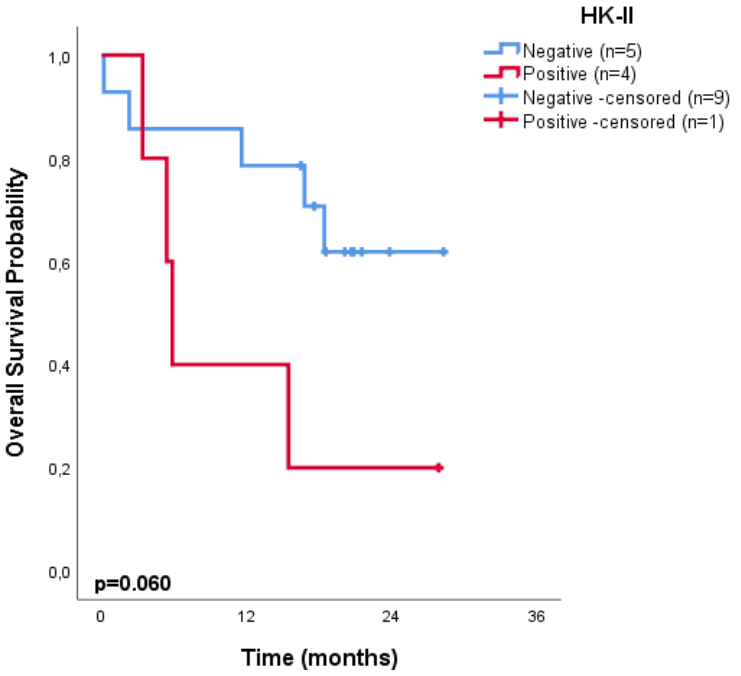

Figure 23. Kaplan-Meier curve demonstrating 3-year overall survival of patients with oral squamous cell carcinoma (n=21) based on HK-II immunoexpression status.

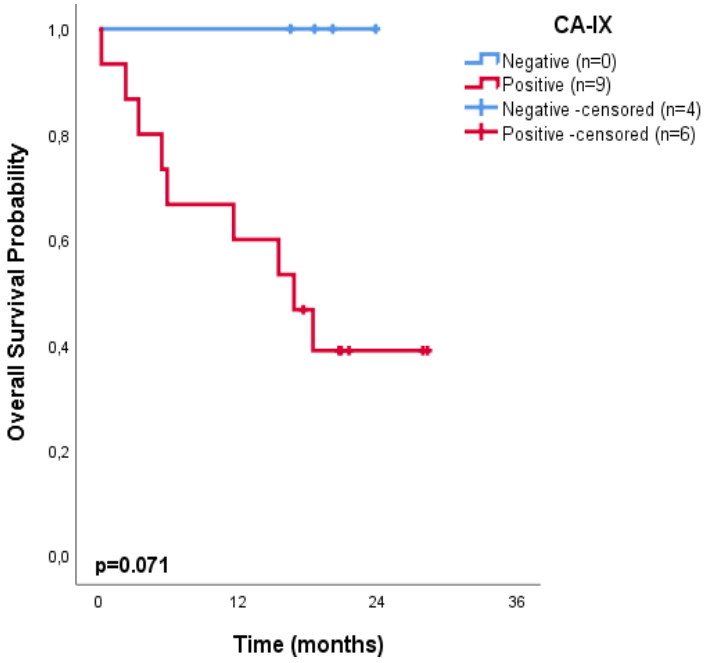

Figure 24. Kaplan-Meier curve demonstrating 3-year overall survival of patients with oral squamous cell carcinoma (n=21) based on CA-IX immunoexpression status.

Table 9. Association between the immunoexpression frequencies of the metabolism-related biomarkers and the 3-year disease-free survival of oral squamous cell carcinoma patients.

Variables				Disease-Free Survival	
		Total number	Number of events	Months	*p*
GLUT-1					.505
	Negative	1	1	13.033± 0.000	
	Positive	18	8	16.641± 3.075	
GLUT-3					.509
	Negative	15	8	15.221± 3.189	
	Positive	2	1	0.083± 0.059	
HK-II					**.007**
	Negative	14	5	20.246± 2.932	
	Positive	5	4	1.820± 1.023	
PFK-L					.569
	Negative	16	8	13.664± 2.575	
	Positive	3	1	18.844± 7.693	
PKM-2					.495
	Negative	15	8	14.549± 3.218	
	Positive	3	1	13.756± 5.616	
pPDH					.197
	Negative	11	7	12.918± 3.744	
	Positive	8	2	16.523± 2.657	
LDH-A					.917
	Negative	13	7	14.974± 3.422	
	Positive	4	2	11.250± 4.738	
MCT-4					.448
	Negative	14	7	15.534± 3.438	
	Positive	4	1	15.625± 4.511	
CA-IX					.209
	Negative	4	1	21.108± 2.331	
	Positive	15	8	13.942± 3.390	

p values from Log-rank or Breslow tests. *p* values < 0.05 are shown in bold.

Table 10. Association between the immunoexpression frequencies of the metabolism-related biomarkers and the 3-year overall survival of oral squamous cell carcinoma patients.

Variables				Overall Survival	
		Total number	Number of events	Months	*p*
GLUT-1					.403
	Negative	1	0	.	
	Positive	18	9	.	
GLUT-3					.809
	Negative	15	8	18.364± 2.628	
	Positive	2	1	10.833± 7.542	
HK-II					.060
	Negative	14	5	21.434± 2.665	
	Positive	5	4	11.573± 4.095	
PFK-L					.606
	Negative	16	8	18.280± 2.627	
	Positive	3	1	20.789± 6.106	
PKM-2					.622
	Negative	15	8	18.080± 2.770	
	Positive	3	1	15.700± 4.028	
pPDH					.623
	Negative	11	5	20.477± 2.825	
	Positive	8	4	11.900± 3.699	
LDH-A					.333
	Negative	13	6	19.153± 3.021	
	Positive	4	3	11.817± 3.242	
MCT-4					.801
	Negative	14	6	19.590± 2.893	
	Positive	4	2	13.217± 3.809	
CA-IX					.071
	Negative	4	0	.	
	Positive	15	9	.	

p values from Log-rank or Breslow tests.

Immunoexpression of metabolism-related biomarkers in normoxic *versus* hypoxic regions of malignant lesions

A detailed analysis on the tissue sections of OSCC lesions regarding the expression of the biomarkers in normoxic *versus* hypoxic regions was performed. The results may be found in Figure 25. It was not possible to distinguish between normoxic and hypoxic compartments regarding CA-IX immunoexpression. There was a significant concordance in absence or presence of immunoexpression of HK-II, PFK-L, PKM-2, pPDH, LDH-A and MCT-4 in both normoxic and hypoxic regions. In opposition, GLUT-1 and GLUT-3 expression in the two compartments was discordant.

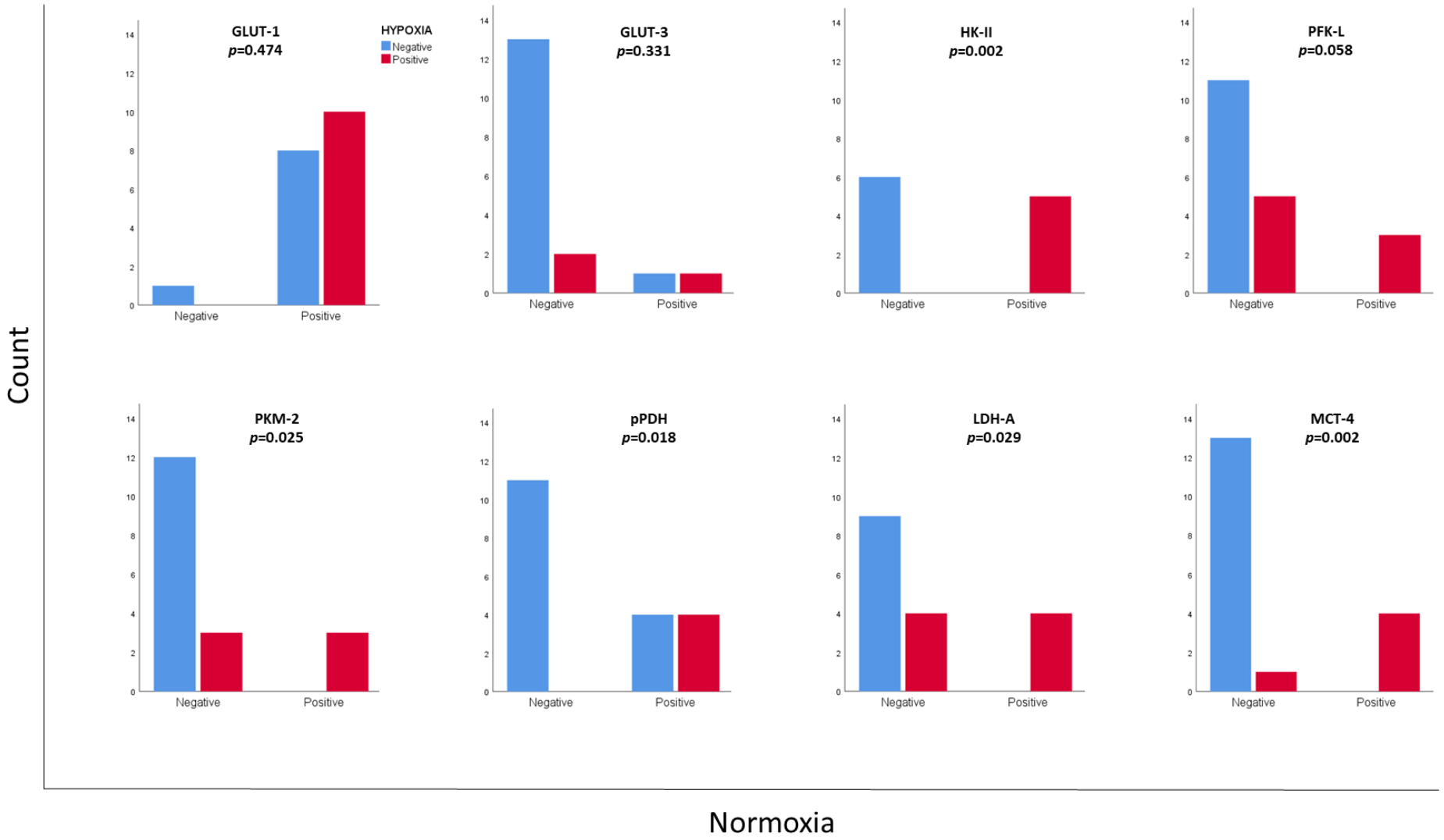

Figure 25. Immunoexpression of GLUT-1, GLUT-3, HK-II, PFK-L, PKM-2, pPDH, LDH-A and MCT-4 in normoxic and hypoxic compartments of oral squamous cell carcinoma cases.

Clinicopathological and prognostic significance of metabolism-related biomarkers in hypoxic regions of malignant lesions

Associations between the clinicopathological parameters and immunoexpression frequencies of the biomarkers in the hypoxic compartment of the OSCC cases were assessed. Detailed results may be found in Table S3 (Supplementary Data). Once concordance was obtained regarding HK-II, PFK-L, PKM-2, pPDH, LDH-A and MCT-4 immunoexpression in both normoxic and hypoxic regions, associations with the clinicopathological parameters were generally similar in the two conditions, as expected. Thus, HK-II (p=0.061) and PFK-L (p=0.001) positivity was mostly seen in larger tumors; additionally, near significant associations were obtained between HK-II expression and increasing age (p=0.061), and PFK-L expression and increasing TNM stage (p=0.074). All of the tissue sections from tumors larger than 2.5 cm were negative for pPDH expression (p=0.033). Significant (or near significant) associations were obtained between MCT-4 expression, TNM stage (p= 0.083) and grade of differentiation (p= 0.044); the same tendency was also observed regarding LDH-A positivity and TNM stage (p= 0.072). GLUT-1 and GLUT-3 immunoexpression did not associate with the clinicopathological parameters, but significant associations were observed between positivity of these biomarkers in the hypoxic compartment of the tumors and a low overall survival rate (p=0.044 and p=0.006, respectively; Figures 26 and 27). HK-II expression was significantly associated with a low disease-free survival (p=0.009, Figure 28), while pPDH expression was near significantly associated with a higher DFS rate (p=0.060, Figure 29). Detailed results may be found in Tables 11 and 12.

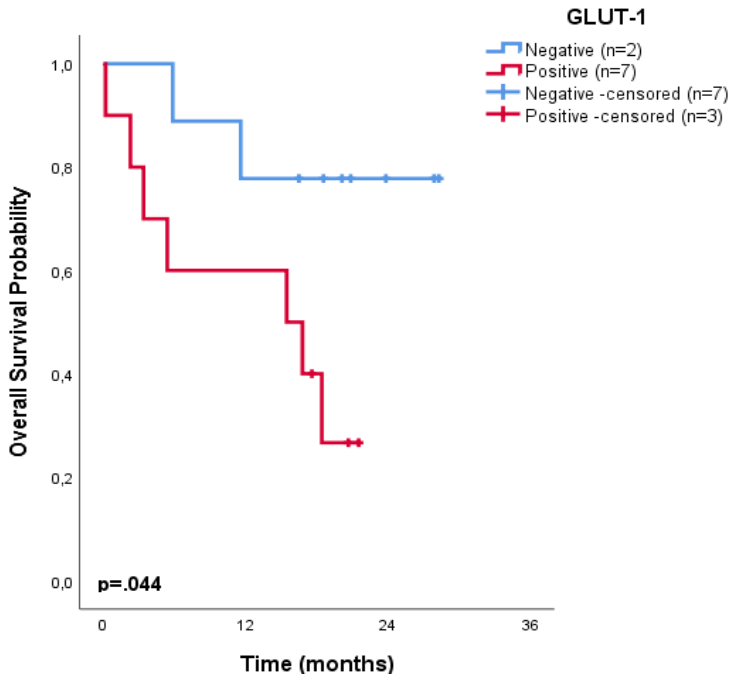

Figure 26. Kaplan-Meier curve demonstrating 3-year overall survival of patients with oral squamous cell carcinoma (n=19) based on GLUT-1 immunoexpression status in the hypoxic compartment of the malignant lesions.

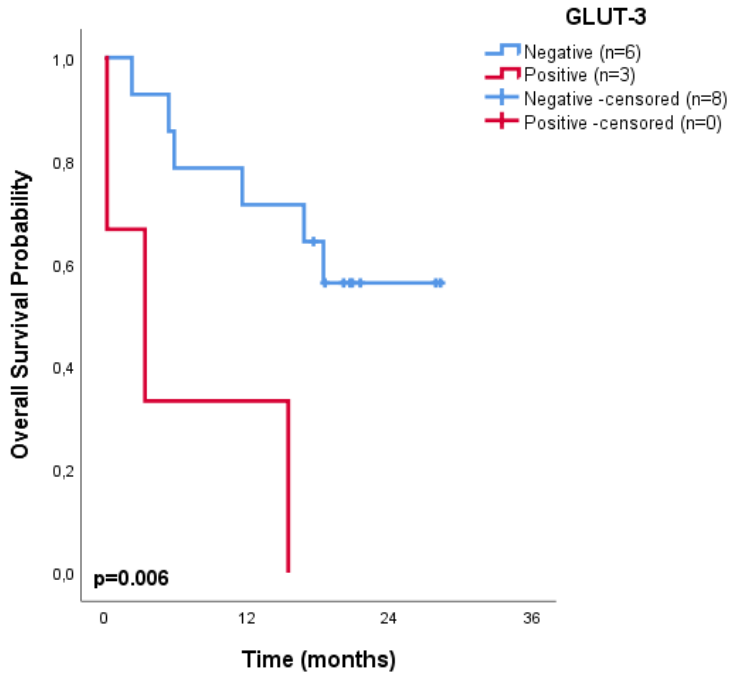

Figure 27. Kaplan-Meier curve demonstrating 3-year overall survival of patients with oral squamous cell carcinoma (n=17) based on GLUT-3 immunoexpression status in the hypoxic compartment of the malignant lesions.

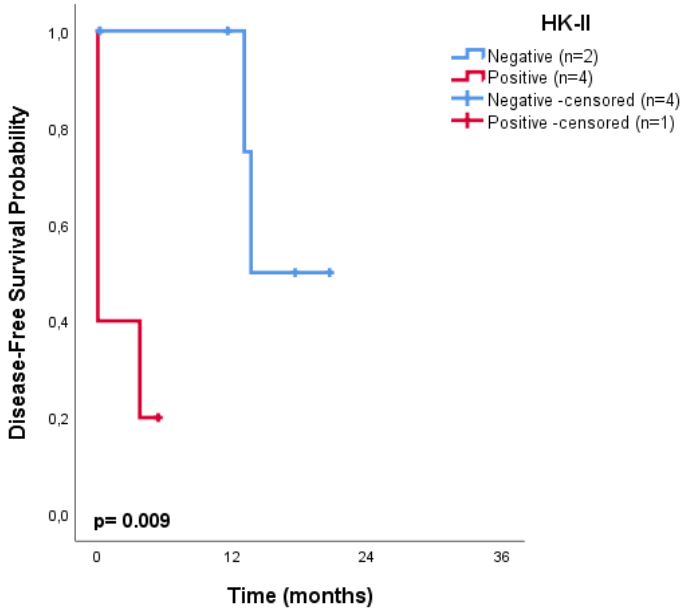

Figure 28. Kaplan-Meier curve demonstrating 3-year disease-free survival of patients with oral squamous cell carcinoma (n=11) based on HK-II immunoexpression status in the hypoxic compartment of the malignant lesions.

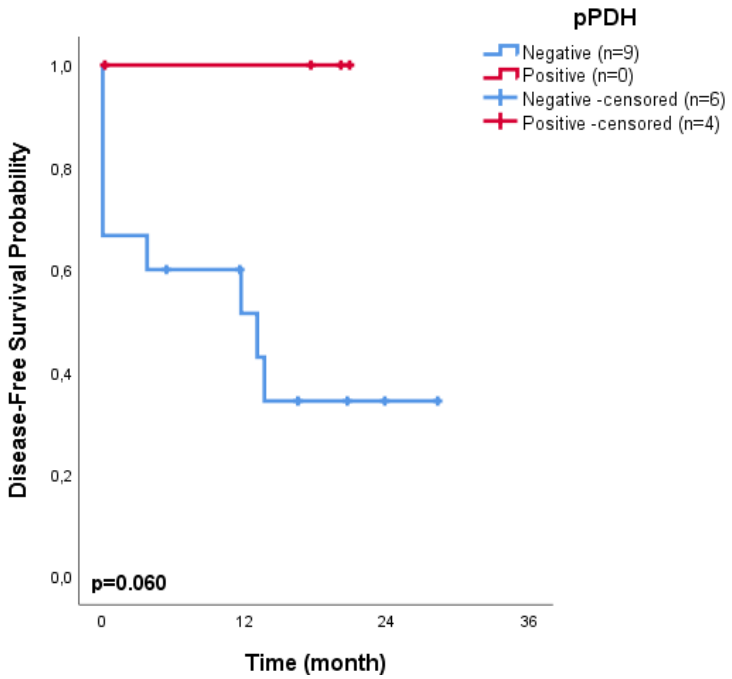

Figure 29. Kaplan-Meier curve demonstrating 3-year disease-free survival of patients with oral squamous cell carcinoma (n=19) based on pPDH immunoexpression status in the hypoxic compartment of the malignant lesions.

Table 11. Association between the immunoexpression frequencies of the metabolism-related biomarkers in the hypoxic compartments of the malignant lesions and the 3-year disease-free survival of oral squamous cell carcinoma patients.

Variables				Disease-Free Survival	
		Total number	Number of events	Months	*p*
GLUT-1					.194
	Negative	9	3	20.010± 3.995	
	Positive	10	6	10.143± 2.774	
GLUT-3					.195
	Negative	14	7	16.042± 3.310	
	Positive	3	2	2.489± 1.437	
HK-II					**.009**
	Negative	6	2	16.983± 1.828	
	Positive	5	4	1.820± 1.023	
PFK-L					.190
	Negative	11	4	17.026± 2.828	
	Positive	8	5	11.833± 4.485	
PKM-2					.850
	Negative	12	6	16.221± 3.472	
	Positive	6	3	10.939± 3.990	
pPDH					.060
	Negative	15	9	.	
	Positive	4	0	.	
LDH-A					.936
	Negative	9	5	14.757± 3.732	
	Positive	8	4	10.883± 3.541	
MCT-4					.234
	Negative	13	7	12.627± 2.957	
	Positive	5	1	22.613± 5.056	

p values from Log-rank or Breslow tests. *p* values < 0.05 are shown in bold.

Table 12. Association between the immunoexpression frequencies of the metabolism-related biomarkers in the hypoxic compartments of the malignant lesions and the 3-year overall survival of oral squamous cell carcinoma patients.

Variables			Overall survival		
		Total number	Number of events	Months	*p*
GLUT-1					**.044**
	Negative	9	2	23.919± 2.749	
	Positive	10	7	16.641± 3.075	
GLUT-3					**.006**
	Negative	14	6	20.364± 2.636	
	Positive	3	3	6.322± 4.648	
HK-II					.261
	Negative	6	3	15.067± 3.028	
	Positive	5	4	11.573± 4.095	
PFK-L					.386
	Negative	11	4	17.851± 2.644	
	Positive	8	5	16.650± 3.602	
PKM-2					.239
	Negative	12	5	20.586± 2.965	
	Positive	6	4	11.878± 2.969	
pPDH					.469
	Negative	15	8	18.280± 2.628	
	Positive	4	1	15.667± 4.474	
LDH-A					.940
	Negative	9	5	18.026± 3.566	
	Positive	8	4	17.683± 3.785	
MCT-4					.824
	Negative	13	6	18.696± 2.987	
	Positive	5	2	19.200± 4.966	

p values from Log-rank or Breslow tests. *p* values < 0.05 are shown in bold.

Discussion

Oral cancer is one of the deadliest type of cancer and its incidence rate is increasing annually according to the latest report of the Global Cancer Statistics [94]. As previously mentioned, oral cancer has an aggressive profile and it is the most prevalent cancer among different subtypes of HNC. The majority of oral cancer patients display advanced stage tumors at the time of diagnosis and their outcome is poor, which confirms the urgent need to investigate new prognosis biomarkers and identify useful therapeutic options. Metabolic reprogramming of cancer has been recently considered as a potential field of research. Through aerobic metabolism, cancer cells increase glucose consumption and overexpress proteins involved in the glycolytic pathway, being able to increase production of energy and metabolic intermediates, and ultimately to survive and proliferate. Thus, a deep characterization of the metabolic phenotype of cancer cells is urgently needed. Evaluation of the expression pattern of metabolism-related proteins in oral lesions has been reported in some studies [135, 136, 156], although those were mainly focused in OSCC and not in premalignant conditions.

In the present study, we sought to investigate the expression pattern of cancer metabolism-related biomarkers in the carcinogenesis of oral cancer. For that, a pilot study with 34 premalignant and 21 malignant cases was conducted, and immunohistochemistry was performed to evaluate the immunoexpression of GLUT-1, GLUT-3, HK-II, PFK-L, PKM-2, pPDH, LDH-A, MCT-4, and CA-IX in the tissue sections. As previously mentioned, these biomarkers are involved in the glycolytic metabolism, being direct targets of HIF-1α, the main regulator of the Warburg effect in oral cancer. We observed that LDH-A, PKM-2, PFK-L, HK-II and pPDH were mainly expressed at the cytoplasm, while GLUT-1, GLUT-3, CA-IX and MCT-4 were predominant at the plasma membrane, which is in accordance with their cellular location and function. A hyperglycolytic metabolism, supported by the overexpression of CA-IX, MCT-4, LDH-A, PKM-2 and PFK-L in malignant over premalignant lesions, was observed in our casuistic, as it has been reported by others [135, 136, 141, 148, 157, 158] and reinforced by the PET scanning images in oral cancer [159, 160]. No significant immunoexpression differences were observed between premalignant and malignant lesions regarding GLUT-1, GLUT-3, HK-II and pPDH. In other studies of oral carcinogenesis, a gradual increase in GLUT-1 [148] CA-IX [147, 148] and HIF-1α [161] was observed from pre-malignancy to malignancy under hypoxic condition. Regarding the discrimination between hypoxic and normoxic parts, a significant immunoexpression concordance was obtained for most of the biomarkers, with GLUT-1 and GLUT-3 being the exceptions, as it will be detailed further.

Cancer cells enhance glucose consumption when compared to normal cells, which is accomplished by upregulation of glucose transporters. GLUT-1 is essential in mediating the glycolytic energy metabolism, favoring cancer cells' proliferation and survival [162]. Various studies demonstrated a significant correlation among GLUT-1 expression and increased glucose uptake [163], proliferation [136], degree of dysplasia [148] and loss of differentiation [135], which indicates that an aggressive biological behavior of OSCC associates with GLUT-1 overexpression. These authors suggested that GLUT-1 is an important prognosis biomarker. The prognostic value of this biomarker is not only reported in oral cancer but also in other types of malignancies such as breast [164] and hepatocellular carcinoma [165], non-small cell lung cancer [166] and carcinomas of the pancreatobiliary tract [167]. In our cohort, most malignant cases as well as premalignant samples overexpressed GLUT-1, which suggests an essential role of this biomarker since pre-malignancy. However, when its expression was observed in the hypoxic compartment of the cancer samples, it was significantly associated with a lower overall survival. GLUT-1 has been proposed by several authors as an endogenous marker for hypoxia in solid malignancies such as oral cancer [152]. In the central parts of the lesions which suffer from inadequate energy supply due to the distance from the blood vessels, GLUT-1 expression increases to compensate for this condition. In fact, hypoxia stimulates GLUT-1 expression in those central parts, causing areas of squamous differentiation and/or keratinization in oral carcinomas [135, 152]. As previously mentioned, upregulated GLUT-1 expression and increased glucose uptake is clinically used for the diagnosis of primary malignant lesions in oral cancer [160].

GLUT-3 protein is a target gene of HIF1-α that, similarly to GLUT-1, mediates glucose transport and facilitates glycolysis influx. There are few studies about GLUT-3 expression in oral cancer [152] and other head and neck carcinomas [163]. In the study by *Zhou* et al., GLUT-3 gene expression in HNC was significantly higher when compared to non-malignant cases, and this was correlated with lymph node metastasis occurrence in these patients [163]. In another study, GLUT-3 expression correlated with the clinical stage of OSCC, being overexpressed in inflammatory cells and associating with a low DFS [152]. Positive staining for GLUT-3 in larynx cancer cells associated with poor survival [168]. In gastric cancer GLUT-3 positive cases also showed a worse prognosis [169]. In the present study, GLUT-3 had a restricted expression in premalignant and malignant lesions; similarly to GLUT-1 results, although no important participation of GLUT-3 was evident in oral carcinogenesis nor any association was found regarding the clinical data, GLUT-3 expression in the hypoxic compartment of cancer sections significantly associated with a poor overall outcome.

HK-II is the first rate-limiting enzyme in the glycolysis pathway, being upregulated in multiple cancer types [170]. There are some studies regarding HK-II expression in oral cancer [136, 171, 172]. One of these studies demonstrated a key role of HK-II in oral carcinogenesis and its correlation with poor survival of oral cancer patients [136]. In the study by *Li* et al. a high level of HK-II expression was detected in HNC tissues [172]. In other types of malignancies such as gastric cancer, HK-II positive cases also displayed aggressiveness features and a poor prognosis [173]. In hepatocellular carcinoma high expression of HK-II was an independent predictive marker for OS and it was also associated with TNM stage [174]. In accordance with those studies, our results showed a significant association of HK-II positivity with older age and large tumor size. Moreover, HK-II expression had a significant impact on prognosis, lowering the DFS rate in OSCC patients, which indicates its potential as a prognostic factor.

PFK is found in three isoforms (PFK-L, PFK-M, PFK-P) but among them, PFK isoform L is an important enzyme controlling the glycolytic flux and it is the only type whose expression is directly affected by HIF-1α [175]. The study by *Grimm* et al. showed overexpression of PFK in OSCC, but correlations with clinical and prognostic features were not assessed [136]. In colorectal cancer, PFK-L high expression correlated with an increased glucose metabolism and glycolysis flux [176]. In our study, a few cancer sections expressed PFK-L while no expression was observed in premalignant cases. Moreover, PFK-L high expression showed a strong correlation with worse clinicopathological parameters (like tumor size, advanced TNM stage and loss of differentiation).

The glycolytic protein PKM-2 is another HIF-1α target favoring cancer cell survival and invasion through aerobic metabolism [53]. Some reports indicated that PKM-2 expression was significantly associated with oral cancer progression [144, 177, 178]. Moreover*, K-Shimomura* et al. found that PKM-2 expression was strongly correlated with the clinical stage [178]. PKM-2 also enhances VEGF-A expression [179], which is a direct target of HIF-1α and has a main role in angiogenesis, tumor progression and poor survival of oral cancer patients [180]. In another investigation, *Luo* et al. showed that PKM-2 expression was significantly associated with advanced stage, identifying this biomarker as an independent prognostic factor for OSCC patients [177] which is aligned with *Yuan C* findings [181] . The prognostic value of PKM-2 has also been demonstrated in other types of malignancies, namely hepatocellular carcinoma [182], gastric cancer [183] and breast cancer [184]. In our study, PMK-2 expression was observed in cancer samples, while no expression was noted in premalignant tissue sections, and there was a tendency for higher expression in poorly differentiated advanced tumors.

PDH enzyme is responsible for converting pyruvate to acetyl-coA which then enters in the TCA cycle to form citrate and subsequently start OXPHOS. This enzyme is a direct target of HIF1-α and its inhibition by PDK originates phospho-PDH which can divert pyruvate into aerobic glycolysis instead of OXPHOS. To the best of our knowledge, no studies on PDH expression were conducted in OSCC setting, and the studies are also scarce in other malignancies [185], although there are some investigations in which targeting of PDH complex for therapeutic goals was conducted [186, 187]. The results of the present study showed that when considering the hypoxic compartment of the OSCC sections, pPDH expression was lower in larger tumors and its negative expression associated with a lower DFS rate. This is intriguing, since in large tumors cancer cells located distant from the blood vessels experience hypoxic conditions that promote them to switch to the aerobic glycolytic pathway, therefore we could expect an increase in pPDH expression and not a decrease.

LDH-A is a metabolism-related protein that converts pyruvate to lactate. In the study by *Grimm* et al., LDH-A was highly expressed in tumor sections, and associated with adverse prognostic factors for oral cancer patients [188]. In another study, LDH-A increased expression clearly associated with oral carcinogenesis [136]. Several reports showed that LDH-A activity is increased in the serum of patients with leukoplakia and oral cancer [189, 190], as well as in the tumor tissue of these patients [191]. Some authors suggested the use of LDH-A as a salivary biomarker in oral cancer patients [192]. In nasopharyngeal carcinoma LDH-A expression was associated with TNM stage and worse prognosis [193]. In our cohort, LDH-A expression was only observed in the cancer tissues. This biomarker was also associated with aggressiveness features (TNM stage and grade), confirming its importance as a mediator of oral carcinogenesis and cancer aggressiveness.

MCT-4 is a transporter protein that assists cancer cells in balancing their internal acidosis. Studies evaluating MCT-4 expression in oral cancer found a significant association with a large lesion size and worse prognosis, suggesting it as a potential biomarker to predict the aggressiveness of OSCC and the prognosis of the patients [141, 143, 158]. The prognostic value of this biomarker is also reported in the investigation by *Meijer TWH* et al. in adenocarcinomas of non-small cell lung [194]. In the study by *Afonso J.* et al., a significant correlation was found between MCT-4 expression in hypoxic parts of bladder cancer and aggressive feature of the tumors, also associating with a lower overall survival of the patients [155]. In line with those reports, the results of this study showed that MCT-4 expression was predominant in cancer tissues in comparison with premalignant lesions. Furthermore, MCT-4 expression was significantly associated with poorly differentiated and higher TNM staged tumors. As MCT-1 has an important role in

anabolic pathways displayed by oxidative cancer cells and in the metabolic symbiosis that occurs between cancer cells and the surrounding stroma of cancer-associated cells, the study of MCT-1 expression would be interesting to explore in this cohort.

CA-IX transporter is one of the direct targets of HIF-1α which is induced by aerobic glycolysis in order to allow the cancer cells to adapt to the acidic microenvironment. CA-IX high expression level is also known as an intrinsic marker of hypoxia [195]. Accordingly, this protein associates with tumor progression, metastasis [196] and poor prognosis [197] in oral cancer patients. Several studies showed that CA-IX might act as a predictive hypoxia marker for malignant conversion in oral cancer [148, 195-197]. In the present study we observed that CA-IX was overexpressed in malignant tissues when compared with premalignant lesions, which suggests the diagnostic potential of this biomarker and its putative role in oral carcinogenesis. Moreover, CA-IX high expression strongly associated with increasing TNM stage and predicted a worse outcome. However, it was not possible to distinguish CA-IX expression in normoxic and hypoxic compartments.

It is notable that this study had some limitations, namely the small sample size (as this is a pilot study), the semi-quantitative method used for evaluation of immunohistochemistry results, and absence of normal oral tissue samples that would be useful to compare with premalignant and malignant lesions in terms of biomarkers' expression. Treatment modalities, namely chemotherapy regimens undertaken by the patients, were not known, which would be interesting in order to study possible correlations among metabolic reprogramming and chemotherapy resistance, as mentioned in several studies [41, 155, 198]. Supplementary research with a large cohort is required to confirm the role of HIF-1α-targeted metabolic biomarkers as indicators of oral cells' malignant transformation and poor prognosis of OSCC patients. These studies will be essential to conduct further research on targeting these proteins as potential therapeutic strategies for OSCC patients. A few studies with "in vitro" and "in vivo" models have been conducted in this setting, namely studies aiming to explore GLUT-1, CA-IX [148], HK-II, LDH-A [136, 199, 200], PKM-2 [178] and MCT-4 [143, 201] roles in OSCC. GLUT-1, CA-IX, HK-II and LDH-A expression was observed in OSCC cell lines under hypoxic conditions, similarly to what was found in clinical samples [136, 148]. Increased PKM-2 expression was observed in OSCC cells compared to adjacent non-malignant cells in OSCC tissues; its downregulation in OSCC cell lines decreased proliferation, invasion and apoptosis induction [178]. Similarly, MCT-4 downregulation decreased cell proliferation, migration and invasion in OSCC cell lines [143]. HKII was overexpressed under hypoxia in OSCC cell lines, and its downregulation decreased glucose consumption and lactate production, and autophagy, migration,

invasion and EMT abilities [200]. Genetic or pharmacological disruption of LDHA decreased proliferation, migration, invasion and EMT of OSCC cell lines, and inhibited tumor growth “in vivo” [199]. In other “in vivo” study, MCT4 was suggested as a driver of oral malignant progression, highlighting the potential use of MCT4 inhibitors [201]. The results obtained from these studies are beginning to elucidate the important role of the glycolytic metabolism in OSCC, but more research is needed in order to independently validate the prognostic potential of those glycolytic biomarkers as well as their clinical use as therapeutic targets.

Conclusion

This study demonstrates that oral cancer cells overexpress glycolysis-related proteins, and this associates with aggressiveness features, which supports a hyperglycolytic phenotype in this type of cancer. We highlight the potential of HK-II as a prognostic biomarker. We also endorse that it might be useful to look for GLUT-1 and GLUT-3 immunoexpression in the hypoxic compartment of OSCC sections, as these seem to have prognostic value in that tumour section.

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Supplementary data

Table S1. Association between the immunoexpression of metabolism-related biomarkers and the clinicopathological data of patients with oral premalignant lesions.

	GLUT-1			GLUT-3			HK-II			PFK-L			PKM-2		
	n	**Positive**	***p***	**n**	**Positive**	***p***	**n**	**Positive**	***p***	**n**	**Positive**	***p***	**n**	**Positive**	***p***
Age			.			1.00			**.013**			.			.
< 59	34	17 (50.0%)		17	2 (11.8%)		17	3 (17.6%)		17	17 (100%)		17	17 (100%)	
≥ 59	34	17 (50.0%)		17	3 (17.6%)		17	11 (64.7%)		17	17 (100%)		17	17 (100%)	
Gender			.			1.00			.738			.			.
Male	18	18 (100%)		18	3 (16.7%)		18	8 (44.4%)		18	18 (100%)		18	18 (100%)	
Female	16	16 (100%)		16	2 (12.5%)		16	6 (37.5%)		16	16 (100%)		16	16 (100%)	
Tumor size			.			.634			1.00			.			.
<0.08 cm	15	15 (100%)		15	3 (20.0%)		15	6 (40.0%)		15	15 (100%)		15	15 (100%)	
≥0.08 cm	19	19 (100%)		19	2 (10.5%)		19	8 (42.1%)		19	19 (100%)		19	19 (100%)	
Smoking Habits			.			.075			.053			.			.
Non-Smoker	15	15 (100%)		15	0 (0.0%)		15	9 (60.0%)		15	15 (100%)		15	15 (100%)	
EX-smoker	13	13 (100%)		13	3 (23.1%)		13	2 (15.2%)		13	13 (100%)		13	13 (100%)	
Smoker	6	6 (100%)		6	2 (33.3%)		6	3 (50.0%)		6	6 (100%)		6	6 (100%)	
Lesion type			**.**			**.005**			.350			**.**			**.**
Oral lichen Planus	12	12 (100%)		12	0 (0.0%)		12	4 (33.3%)		12	12 (100%)		12	12 (100%)	
Leukoplakia without dysplasia	11	11 (100%)		11	5 (45.5%)		11	3 (27.3%)		11	11 (100%)		11	11 (100%)	
Leukoplakia with dysplasia low grade	8	8 (100%)		8	0 (0.0%)		8	5 (62.5%)		8	8 (100%)		8	8 (100%)	
Leukoplakia with dysplasia high grade	3	3 (100%)		3	0 (0.0%)		3	2 (66.7%)		3	3 (100%)		3	3 (100%)	

		pPDH			LDH-A			MCT-4			CA-IX	
	n	**Positive**	*p*	n	**Positive**	*p*	n	**Positive**	*p*	n	**Positive**	*p*
Age			1.00			.			.			.485
< 59	17	12 (70.6%)		15	15 (100%)		17	17 (100%)		17	0 (0.0%)	
≥ 59	16	11 (68.8%)		16	16 (100%)		17	17 (100%)		17	2 (11.8%)	
Gender			.126			.			.			1.00
Male	18	15 (83.3%)		15	15 (100%)		18	18 (100%)		18	1 (5.6%)	
Female	15	8 (53.3%)		16	16 (100%)		16	16 (100%)		16	1 (6.3%)	
Tumor size			1.00			.			.			.492
<0.08 cm	14	10 (71.4%)		13	13 (100%)		15	15 (100%)		15	0 (0.0%)	
≥0.08 cm	19	13 (68.4%)		18	18 (100%)		19	19 (100%)		19	2 (10.5%)	
Smoking Habits			**.046**			.			.			1.00
Non-Smoker	14	7 (50.0%)		13	13 (100%)		15	15 (100%)		15	1 (6.7%)	
EX-smoker	13	12 (92.3%)		12	12 (100%)		13	13 (100%)		13	1 (7.7%)	
Smoker	6	4 (66.7%)		6	6 (100%)		6	6 (100%)		6	0 (0.0%)	
Lesion type			.136			.			.			.765
Oral lichen Planus	11	6 (54.5%)		12	12 (100%)		12	12 (100%)		12	1 (8.3%)	
Leukoplakia without dysplasia	11	7 (63.5%)		11	11 (100%)		11	11 (100%)		11	0 (0.0%)	
Leukoplakia with dysplasia low grade	8	8 (100%)		7	7 (100%)		8	8 (100%)		8	1 (12.5%)	
Leukoplakia with dysplasia high grade	3	2 (66.7%)		1	1 (100%)		3	3 (100%)		3	0 (0.0%)	

p values from Pearson Chi-square or Fisher's exact tests. *p* values < 0.05 are shown in bold.

Table S2. Association between the immunoexpression of metabolism-related biomarkers and the clinicopathological data of oral squamous cell carcinoma patients.

	GLUT-1			GLUT-3			HK-II			PFK-L			PKM-2		
	n	**Positive**	***p***	**n**	**Positive**	***p***	**n**	**Positive**	***p***	**n**	**Positive**	***p***	**n**	**Positive**	***p***
Age			.			1.00			**.033**			1.00			.559
< 67	9	9 (100%)		8	1 (12.5%)		9	0 (0.0%)		9	1 (11.1%)		8	2 (25.0%)	
≥ 67	10	10 (100%)		9	1 (11.1%)		10	5 (50.0%)		10	2 (20.0%)		10	1 (10.0%)	
Gender			.			1.00			.141			.582			1.00
Male	10	9 (100%)		8	1 (12.5%)		10	1 (10.0%)		10	1 (10.0%)		9	2 (22.2%)	
Female	9	10 (100%)		9	1 (11.1%)		9	4 (44.4%)		9	2 (22.2%)		9	1 (11.1%)	
Tumor size			.			.206			**.033**			.211			1.00
<2.5cm	9	9 (100%)		8	2 (25.0%)		9	0 (0.0%)		9	0 (0.0%)		8	1 (12.5%)	
≥2.5cm	10	10 (100%)		9	0 (0.0%)		10	5 (50.0%)		10	3 (30.0%)		10	2 (20.0%)	
Smoking Habits			.			.088			.351			.554			1.00
Non-Smoker	9	9 (100%)		9	0 (0.0%)		9	4 (44.4%)		9	2 (22.2%)		9	1 (11.1%)	
EX-smoker	4	4 (100%)		4	2(50.0%)		4	0 (0.0%)		4	1 (25.0%)		4	1 (25.0%)	
Smoker	6	6 (100%)			0 (0.0%)		6	1 (16.7%)		6	0 (0.0%)		5	1 (20.0%)	
TNM stage			**.**			.324			.404			.387			.471
I	5	5 (100%)		4	1 (25.0%)		5	0 (0.0%)		5	0 (0.0%)		4	0 (0.0%)	
II	4	4 (100%)		4	1 (25.0%)		4	2 (50.0%)		4	0 (0.0%)		4	0 (0.0%)	
III	1	1 (100%)		1	0 (0.0%)		1	0 (0.0%)		1	0 (0.0%)		1	0 (0.0%)	
IV	9	9 (100%)		8	0 (0.0%)		9	3 (33.3%)		9	3 (33.3%)		9	3 (33.3%)	
Grade			.			.529			.480			.074			.078
Well differentiated	9	9 (100%)		8	0 (0.0%)		9	2 (22.2%)		9	0 (0.0%)		8	0 (0.0%)	
Moderately differentiated	9	9 (100%)		8	2 (25.0%)		9	2 (22.2%)		9	2 (22.2%)		9	2 (22.2%)	
Poorly differentiated	1	1 (100%)		1	0 (0.0%)		1	1 (100%)		1	1 (100%)		1	1 (100%)	

	pPDH			LDH-A			MCT-4			CA-IX		
	n	**Positive**	***p***	**n**	**Positive**	***p***	**n**	**Positive**	***p***	**n**	**Positive**	***p***
Age			.650			.576			1.00			1.00
< 67	9	3 (33.3%)		8	1 (12.5%)		8	2 (25.0%)		10	7 (40.0%)	
≥ 67	10	5 (50.0%)		9	3 (33.3%)		10	2 (20.0%)		10	8 (80.0%)	
Gender			.170			.515			.582			.087
Male	10	6 (60.0%)		10	3 (30.0%)		9	3 (33.3%)		10	6 (60.0%)	
Female	9	2 (22.2%)		9	4 (44.4%)		9	1 (11.1%)		9	9 (100%)	
Tumor size			.370			.082			1.00			.303
<2.5cm	9	5 (55.6%)		8	0 (0.0%)		9	2 (22.2%)		9	6 (66.7%)	
≥2.5cm	10	3 (30.0%)		9	4 (44.4%)		9	2 (22.2%)		10	9 (90.0%)	
Smoking Habits			.290			1.00			.154			**.040**
Non-Smoker	9	2 (22.2%)		9	2 (22.2%)		8	1 (12.5%)		9	9 (100%)	
EX-smoker	4	2 (50.0%)		4	1 (25.0%)		4	0 (0.0%)		4	3 (75.0%)	
Smoker	6	4 (66.7%)		4	1 (25.0%)		6	3 (50.0%)		6	3 (50.0%)	
TNM stage			.767			.812			.251			.146
I	5	2 (40.0%)		4	0 (0.0%)		5	0 (0.0%)		5	2 (40.0%)	
II	4	1 (25.0%)		4	1 (25.0%)		3	0 (0.0%)		4	4 (100%)	
III	1	0 (0.0%)		1	0 (0.0%)		1	0 (0.0%)		1	1 (100%)	
IV	9	5 (55.6%)		8	3 (37.5%)		9	4 (44.4%)		9	8 (88.9%)	
Grade			1.00			**.047**			.069			.666
Well differentiated	9	4 (44.4%)		8	0 (0.0%)		8	0 (0.0%)		9	6 (66.7%)	
Moderately differentiated	9	4 (44.4%)		8	3 (37.5%)		9	3 (33.3%)		9	8 (88.9%)	
Poorly differentiated	1	0 (0.0%)		1	1 (100%)		1	1 (100%)		1	1 (100%)	

p values from Pearson Chi-square or Fisher's exact tests. *p* values < 0.05 are shown in bold.

Table S3. Association between the immunoexpression of metabolism-related biomarkers in hypoxic regions of the tissue sections and the clinicopathological data of oral squamous cell carcinoma patients.

	GLUT-1			GLUT-3			HK-II			PFK-L			PKM-2		
	n	**Positive**	***p***	**n**	**Positive**	***p***	**n**	**Positive**	***p***	**n**	**Positive**	***p***	**n**	**Positive**	***p***
Age			.370			.206			.061			.170			1.00
< 59	9	6 (66.7%)		8	0 (0.0%)		4	0 (0.0%)		9	2 (22.2%)		9	3 (33.3%)	
≥ 59	10	4 (40.0%)		9	3 (33.3%)		7	5 (71.4%)		10	6 (60.0%)		9	3 (33.3%)	
Gender			1.00			1.00			.242			.370			.638
Male	10	5 (50.0%)		8	1 (12.5%)		5	1 (20.0%)		10	3 (30.0%)		8	2 (25.0%)	
Female	9	5 (55.6%)		9	2 (22.2%)		6	4 (66.7%)		9	5 (55.6%)		10	4 (40.0%)	
Tumor size			1.00			1.00			.061			**.001**			.152
<0.08 cm	9	5 (55.6%)		8	1 (12.5%)		4	0 (0.0%)		9	0 (0.0%)		8	1 (12.5%)	
≥0.08 cm	10	5 (50.0%)		9	2 (22.2%)		7	5 (71.4%)		10	8 (80.0%)		10	5 (50.0%)	
Smoking Habits			.604			1.00			.416			.136			1.00
Non-Smoker	9	5 (55.6%)		9	2 (22.2%)		6	4 (66.7%)		9	6 (66.7%)		9	3 (33.3%)	
EX-smoker	4	3 (75.0%)		4	1 (25.0%)		2	0 (0.0%)		4	1 (25.0%)		4	1 (25.0%)	
Smoker	6	2 (33.3%)		4	0 (0.0%)		3	1 (33.3%)		6	1 (16.7%)		5	2 (40.0%)	
TNM stage			.754			.400			1.00			.074			.295
I	5	2 (40.0%)		4	0 (0.0%)		1	0 (0.0%)		5	0 (0.0%)		4	0 (0.0%)	
II	4	3 (75.0%)		4	2 (50.0%)		3	2 (66.7%)		4	3 (75.0%)		4	1 (25.0%)	
III	1	1 (100%)		1	0 (0.0%)		1	0 (0.0%)		1	0 (0.0%)		1	0 (0.0%)	
IV	9	4 (44.4%)		8	1 (12.5%)		6	3 (50.0%)		9	5 (55.6%)		9	5 (55.6%)	
Grade			.484			1.00			1.00			.790			.186
Well differentiated	9	4 (44.4%)		8	1 (12.5%)		5	2 (40.0%)		9	3 (33.3%)		8	1 (12.5%)	
Moderately differentiated	9	6 (66.7%)		8	2 (25.0%)		5	2 (40.0%)		9	4 (44.4%)		9	4 (44.4%)	
Poorly differentiated	1	0 (0.0%)		1	0 (0.0%)		1	1 (100%)		1	1 (100%)		1	1 (100%)	

	pPDH			LDH-A			MCT-4		
	n	**Positive**	***p***	**n**	**Positive**	***p***	**n**	**Positive**	***p***
Age			1.00			.637			1.00
< 67	9	2 (22.2%)		8	3 (37.5%)		8	2 (25.0%)	
≥ 67	10	2 (20.0%)		9	5 (55.6%)		10	3 (30.0%)	
Gender			.087			1.00			1.00
Male	10	4 (40.0%)		8	4 (50.0%)		9	3 (33.3%)	
Female	9	0 (0.0%)		9	4 (44.4%)		9	2 (22.2%)	
Tumor size			**.033**			.153			1.00
<2.5 cm	9	4 (44.4%)		8	2 (25.0%)		9	2 (22.2%)	
≥2.5 cm	10	0 (0.0%)		9	6 (66.7%)		9	3 (33.3%)	
Smoking Habits			.063			.399			.273
Non-Smoker	9	0 (0.0%)		9	4 (44.4%)		8	2 (25.0%)	
EX-smoker	4	2 (50.0%)		4	1 (25.0%)		4	0 (0.0%)	
Smoker	6	2 (33.3%)		4	3 (75.0%)		6	3 (50.0%)	
TNM stage			1.00			.072			.083
I	5	1 (20.0%)		4	0 (0.0%)		5	0 (0.0%)	
II	4	1 (25.0%)		4	2 (50.0%)		3	0 (0.0%)	
III	1	0 (0.0%)		1	0 (0.0%)		1	0 (0.0%)	
IV	9	2 (22.2%)		8	6 (75.0%)		9	5 (0.0%)	
Grade			.666			.218			**.044**
Well differentiated	9	1 (11.1%)		8	2 (25.0%)		8	0 (0.0%)	
Moderately differentiated	9	3 (33.3%)		8	5 (62.5%)		9	4 (44.4%)	
Poorly differentiated	1	0 (0.0%)		1	1 (100%)		1	1 (100%)	

p values from Pearson Chi-square or Fisher's exact tests. *p* values < 0.05 are shown in bold.