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The multi-faceted effects of ethanol exposure on oral dysplastic and cancer cells

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Declaration

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Isabel O'Grady

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Summary

Oral cancers (OC) are cancers that arise in several regions within the oral cavity such as the lips, gums, tongue, lining of the cheeks, and the floor or roof of the mouth. Approximately 400,000 cases were diagnosed worldwide in 2020, with almost 50% of these resulting in the death of the patient. Despite efforts to improve diagnostic and therapeutic methods available, most OC is detected at a late stage and the mortality rate remains high. The five-year survival rate for individuals diagnosed with OC is 68.5%, according to data from 2013-2019 from the National Cancer Institute. OC can be preceded by premalignant disorders of the oral cavity, including oral leukoplakia, which is categorised by the degree of dysplasia and transformation rates. Epithelial dysplasia can be indicative of an early neoplastic process, with severe or high-grade dysplasia having a higher chance of malignant transformation and worse overall prognosis. Due to the late diagnosis of most OC and the dense lymphatic network found in the oral cavity, metastasis is very common. Cervical lymph node metastasis is the prognostic factor most associated with morbidity and poor patient outcomes for OC.

The most important aetiological factors in the development of OC are alcohol and tobacco consumption, and more recently there has been increasing interest in the oral microbiome and its role in OC development. The exact mechanisms by which alcohol consumption influences oral carcinogenesis remain unclear, but it has been postulated to act via multiple pathways including direct DNA damage, inflammation, carcinogenic metabolites, acting as a co-carcinogen, altering hormone regulation and metabolic reprogramming of oral cells and tumours. When alcohol enters the body, it is metabolised by alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) enzymes. The ALDH enzymes have important roles as cancer stem cell (CSC) markers in several types of cancers, including OC, as well as contributing to tumour growth, metastasis, and therapeutic resistance. Additionally, there is evidence in other cancers of bacteria having a direct causal relationship with carcinogenesis. However, no distinct relationship between the oral

microbiome and OC has been defined. Exogenous factors, such as alcohol consumption, can alter the composition of the microbiome, increase host susceptibility to oral dysbiosis, as well as affect immune responses of the oral cavity. Therefore, the overall aim of this research was to investigate the multiple pathways by which alcohol consumption promotes malignant transformation and progression of OC, using oral cell lines, *in vitro* ethanol exposure and commensal oral microbiota as a model of these processes.

In this study, three oral cell lines (dysplastic oral mucosa DOK, gingival squamous cell carcinoma Ca9.22, and buccal squamous cell carcinoma TR146 cells) were characterised as models of alcohol consumption in OC progression and metastasis, via ethanol exposure *in vitro*. The unique ADH and ALDH expression profiles found in these cell lines affected their responses to ethanol and its first metabolite acetaldehyde *in vitro*. A novel finding of this thesis was that chronic ethanol exposure, i.e., representative of heavy or long-term alcohol consumption, overall promoted malignant transformation of dysplastic DOK cells and metastatic processes in cancerous Ca9.22 cells. Chronic ethanol exposure also modulated ALDH enzyme expression and activity in a cell line and isoform dependent manner. While ALDH1A1 was shown to have key roles in cellular pathways such as proliferation and metabolism, the use of siRNA concluded that changes to ALDH expression were a secondary feature to ethanol-induced OC progression.

The data in this thesis demonstrates that ethanol acts via multiple indirect mechanisms, such as augmenting metabolic pathways, or influencing inflammatory signalling. Ethanol was also shown to act synergistically with common oral microbe species, *Candida albicans*, which was heat-inactivated, to promote metastasis of Ca9.22 cells. In combination with the ability of ethanol to affect acetaldehyde production both *in vitro* and *in vivo*, and data suggesting indirect metabolic reprogramming of Ca9.22 and DOK cells, the data presented here provides compelling evidence

that acetaldehyde exposure following alcohol consumption may be the driving factor in transformation and metastasis of OC.

Future work will aim to further investigate the fate of acetaldehyde in OC cells and the oral cavity, as well as compare the effect of ethanol exposure on normal oral cells to dysplasia and established OC, as investigated here. The data presented here provides novel insights into the mechanisms behind ethanol-induced oral carcinogenesis and strengthens the argument for the potential use of microbiome sequencing and selective genetic screening for OC prevention. Changes to policy and practice may help improve earlier diagnosis of OC as well as having potentially significant clinical implications for treatment and overall survival of patients. This thesis also provides a framework for further research questions about the effects of ethanol in oral carcinogenesis.

Abbreviations

ACSS	Acetyl-CoA synthetase
ADH	Alcohol dehydrogenase
ALDH	Aldehyde dehydrogenase
APECED	Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy
APS	Ammonium persulfate
BHI	Brain heart infusion
BSA	Bovine serum albumin
cDNA	Complementary DNA
CSC	Cancer stem cell
DCF-DA	Dichlorodihydro fluorescein diacetate
DEAB	4-Diethylaminobenzaldehyde
DEPC	Diethyl pyrocarbonate
DMEM	Dulbecco Modified Eagle's Medium
DMSO	Dimethylsulfoxide
dNTP	Deoxynucleotide triphosphates
DSF	Disulfarim
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-mesenchymal transition
FACS	Fluorescent activated cell sorting
FBS	Fetal bovine serum
FDA	U.S. Food and Drug Administration
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
HICA	Heat-inactivated *Candida albicans*
HNC	Head and neck cancer
HNSCC	Head and neck squamous cell carcinoma
HPLC	High performance liquid chromatography
HPV	Human papillomavirus
HRP	Horseradish peroxidase
IκB-α	NF-κB inhibitor α
IARC	International Agency for Research on Cancer
IFN	Interferon
IL	Interleukin
LDH	Lactate dehydrogenase
LNM	Lymph node metastasis
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MEM	Minimum Essential Medium
MMP	Matrix metalloproteinase
MOI	Multiplicity of infection
mRNA	Messenger RNA
NER	Nucleotide excision repair

NF-κB	Nuclear factor κ-light-chain-enhancer of activated B cells
NO	Nitric oxide
OC	Oral cancer
OD	Optical density
OED	Oral epithelial dysplasia
OLP	Oral lichen planus
OSCC	Oral squamous cell carcinoma
OSF	Oral submucous fibrosis
OXPHOS	Oxidative phosphorylation
p-HEMA	Poly-hydroxyethyl methacrylic acid
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEI	Polyethylenimine
PET	Polyethylenterephthalate
PI	Propidium iodide
PI3K	Phosphatidylinositol-3-kinase
PLA	Polylactic acid
PMD	Premalignant disorder
PRR	Pathogen recognition receptor
PS	Phosphatidylserine
PVDF	Polyvinylidene fluoride
RA	Retinoic acid
RFU	Relative fluorescent unit
RIPA	Radioimmunoprecipitation assay buffer
ROS	Reactive oxygen species
RPM	Revolutions per minute
RT	Room temperature
RT-PCR	Reverse-transcriptase polymerase chain reaction
SDS-PAGE	Sodium dodecyl-sulphate polyacrylamide gel electrophoresis
siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
SSC	Side scatter
TBST	Tris-buffered saline with Tween® 20
TCA	Tricarboxylic acid cycle
TEMED	Tetramethylethylenediamine
TF	Transcription factor
TIMP	Tissue inhibitor of metalloproteinases
TNF-α	Tumour necrosis factor-α
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling

Publications

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Chapter 1: Introduction

1.1 Oral cancer

Head and neck cancers (HNCs) are a group of cancers affecting the mouth, nose, throat, larynx, sinuses, or salivary glands. Oral squamous cell carcinomas (OSCCs), originating in the squamous cells lining the mucosal surfaces, account for 90% of all head and neck cancers (1). The most common sites for OSCC formation are the floor of the mouth and the lateral/ventral surfaces of the tongue (2), however they can also affect the lips, gingiva, buccal mucosa, and the hard palate. Oral cancer (OC) has an incidence rate of approximately 10.2 cases per 100,000 people in the world, irrespective of age or gender (3). GLOBOCAN, an online database providing statistics worldwide for cancer incidence and mortality, estimated a total of 377,713 new cases of lip and oral cavity cancer in 2020, with a mortality rate of almost 50% (3). It is posited that the thin, non-keratinised mucosal tissues in the mouth are more susceptible to an increased uptake of carcinogens due to their increased permeability, which may lead to higher rates of OSCC (4). The high mortality rate associated with OSCC is largely due to the asymptomatic presentation in the early stages of the disease, and the subsequent delay in patient presentation (5). Late clinical detection therefore lends itself to poor prognosis. In recent years, research into OC has shifted towards the discovery of new biomarkers to aid in early cancer detection. To find these markers, the mechanisms by which risk factors contribute to OC need to be fully understood.

1.1.1 Traditional risk factors of oral cancer

Traditional risk factors of OC include alcohol consumption, human papillomavirus (HPV) infection, tobacco smoking and betel quid chewing (6-9). Concurrent consumption of alcohol and tobacco smoking substantially increase the risk of developing OC (10). There are several premalignant disorders (PMDs) including oral lichen planus (OLP), oral submucous fibrosis (OSF) and chronic candidiasis implicated in the development of OC. It has been demonstrated that OLP patients are

5 times more likely to develop OSCC (11). Chronic candidiasis also lends itself to high malignant transformation rates (12). OC resulting from OSF has been defined as a clinico-pathologically distinct entity in comparison to OC that develops in its absence (13). There is also an entirely different incident rate of HPV-associated OC which mainly contributes to the development of oropharyngeal cancers (14). In addition to this, patients presenting with HPV-associated OC typically lack traditional risk factors such as alcohol and tobacco consumption (9, 15). While it is an important aspect of OC research, the research presented in this thesis will focus on HPV-negative OC only.

1.2 Diagnosis, treatment and metastasis of oral cancer

Early carcinomas tend to go unnoticed because they present as minor symptoms (such as ulceration, induration, or red/white patches) or are asymptomatic. Most patients delay seeking healthcare advice as they believe the symptoms will resolve themselves (5). As the malignancy progresses through tumour, node and metastasis (TNM) staging, the first symptom is usually pain (**Table 1.1**) (16). Other symptoms can include loosening of teeth, halitosis, bleeding, difficulty speaking/swallowing and localised swelling depending on the location of the tumour (17). Abnormalities in the oral cavity are typically identified in routine dental clinic visits. Dentists may contribute to the delay in the diagnostic process by not identifying and/or requesting biopsies of suspicious lesions or by misdiagnosing cancers as benign lesions. Tissue sampling by biopsy and histological examination has been the mainstay of diagnostic techniques for years (18, 19). Some salivary biomarkers have been identified, such as specific cytokines and microRNAs, but these require further validation before they can be used diagnostically (20). By the time patients seek medical assistance, the carcinomas are typically at an advanced stage – approximately two thirds of OSCC are diagnosed at stage III and IV (21). Survival rates of early-stage OC are around 85%, whereas patients with advanced stage disease have survival rates of as low as 40% (22).

Table 1.1 TNM classification and stage grouping of carcinomas of the lip and oral cavity (23).

Tumour	Node	Metastasis
T0 No evidence of primary tumour	**N0** No regional lymph node metastasis	**M0** No distant metastasis
Tis Carcinoma in situ	**N1** Metastasis in a single ipsilateral lymph node ≤ 3 cm	**M1** Distant metastasis
T1 Tumour ≤ 2cm	**N2a** Metastasis in a single ipsilateral lymph node, ≥ 3 cm but ≤ 6 cm	
T2 Tumour ≥ 2cm but ≤ 4cm	**N2b** Metastasis in multiple ipsilateral lymph nodes, ≤ 6 cm	
T3 Tumour ≥ 4cm	**N2c** Metastasis in bilateral or contralateral lymph nodes, ≤ 6 cm	
T4a (lip) Tumour invades through cortical bone, inferior alveolar nerve, floor of mouth, or skin	**N3** Metastasis in a lymph node > 6cm	
T4a (oral cavity) Tumour invades through cortical bone, into deep muscle of tongue, maxillary sinus, or skin of face		
T4b (lip and oral cavity) Tumour invades masticator space, pterygoid plates, or skull base; or encases internal carotid artery		

Stage 0	Stage I	Stage II	Stage III	Stage IVA	Stage IVB	Stage IVC
Tis N0 M0	T1 N0 M0	T2 N0 M0	T1/2 N0 M0 or T3 N0/1 M0	T1/2/3 N2 M0 or T4a N0/1/2 M0	Any T N3 M0 or T4b Any N M0	Any T Any N M1

Initial clinical presentation of OC can consist of visible pre-malignant or potentially malignant lesions such as leukoplakia or erythroplakia (**Figure 1.1A, 1.1B**). These lesions are categorised by appearance, degree of dysplasia and transformation rates. Dysplasia refers to the abnormal growth of cells within a tissue or organ. Oral epithelial dysplasia (OED) can be indicative of an early neoplastic process, as the number of maturing and well-differentiated cells decreases, with a corresponding increase in immature cells within that tissue. Severe or high-grade dysplasia has a higher chance of malignant transformation and worse overall prognosis (24, 25).

Leukoplakia is a white plaque that histologically presents as hyperkeratosis with mild to severe dysplasia. The malignant transformation rate of leukoplakia is anywhere from 1-9% (26, 27). Leukoplakias can be homogenous (uniform in appearance) or non-homogenous (irregular and exophytic in nature). Non-homogenous leukoplakia have a higher malignant transformation rate (26). The most aggressive form of leukoplakia with the highest malignant transformation rate (70.3%) is proliferative verrucous leukoplakia (28). Erythroplakia is a red plaque that is typically well-demarcated and has a 'red velvet' texture. Erythroplakia has a higher malignant transformation rate compared to leukoplakia, and up to 50% are reported to be invasive OSCC (28). OSCC can also present as mixed red and white lesions (erythroleukoplakia), ulcers, exophytic growths or lumps in the mouth (**Figure 1.1C**).

The mainstay treatment for OSCC is primary surgical resection of the tumour, leaving wide tumour-free margins. For more advanced tumours, a multidisciplinary treatment approach is best practice. This can involve pre- or post-operative radiotherapy with cytotoxic therapy as a radiosensitiser e.g., cisplatin, in addition to surgery (29). The most important outcomes for quality of life are the preservation of speech and swallowing. Even with seemingly adequate resection of tumours, the survival rates post treatment of OSCC in relation to stage are: I 84 %, II 71 %, III 36 % and IV 28 % (30).

Metastasis occurs when cancer spreads from its original location in the body to distant sites. This process requires a series of correlated events. Initially cells detach from the primary site, migrate, and spread into neighbouring tissue while avoiding normal cell death programming. Then cells must invade the extracellular matrix (ECM), blood vessels and microvasculature and then finally extravasation occurs, with adherence and colonisation of new secondary tumour niches. Due to the late diagnosis of most OSCC, metastasis is very common and results in a 5-year survival rate of less than 50% (31). The most common sites for metastasis of OC are the hypopharynx, the base of the tongue and the anterior tongue (32). However, metastasis to the cervical lymph nodes in the neck is also very common due to the dense lymphatic network found in the mouth and tongue. More than 30% of patients with squamous cell carcinoma in their tongue can be expected to have cervical lymph node metastases (LNM), even in clinically negative cases (33). Elective neck surgery is recommended to patients following removal of primary lesions, even in the absence of significant incidence of occult cervical metastases (34). Cervical LNM is the prognostic factor most associated with morbidity and poor patient outcomes for OC (35, 36).

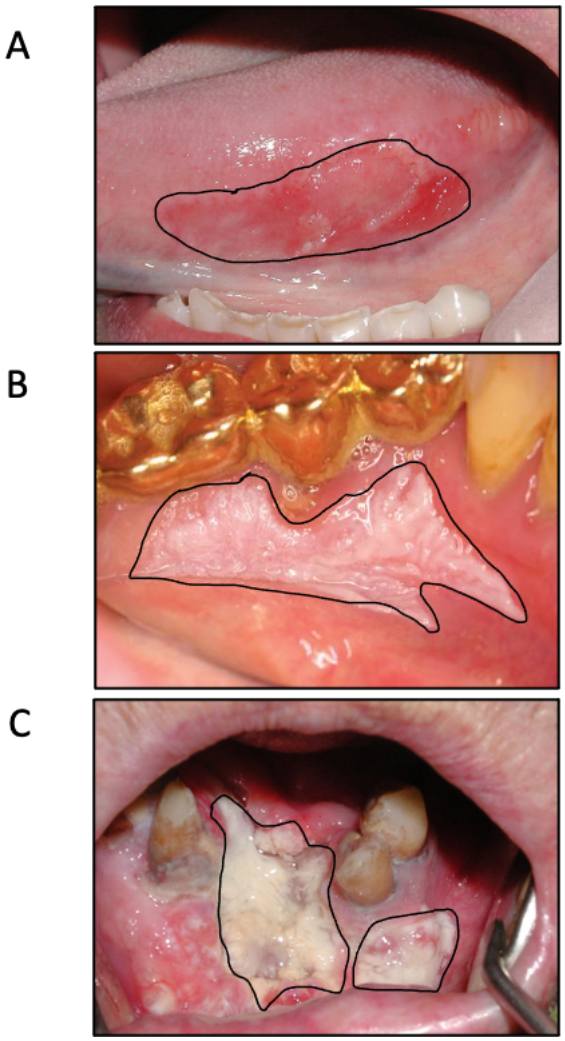


Figure 1.1 (A) Erythroplakia on the lateral border of the tongue. (B) Leukoplakia of the gingiva. (C) Advanced stage exophytic tumour on the floor of the mouth. Affected regions denoted by black outline. Bagan et al., 2010 (17), Parlatescu et al., 2014 (37).

1.3 ALCOHOL AND ORAL CANCER

Alcohol is a major risk factor for numerous cancers, including breast, liver, bowel, and oral cancer. It was found that 4.1% of new cases of cancer in 2020 alone were attributable to alcohol consumption (38). Alcohol can greatly affect the integrity of mucosa via protein-alcohol interactions, hydrogen bonding and subsequent changes in membrane properties. Short-term exposure of oral mucosa to ethanol has been shown to cause increased permeability of the epithelial barrier, by penetrating the membrane and compromising barrier function (39, 40). This effect is seen in the concurrent consumption of alcohol and tobacco, where alcohol acts as a solvent for penetration of carcinogens from cigarette smoke (41). The local permeabilising effects of alcohol could provide enhanced invasion of bacteria across the oral epithelia, as seen in the models of intestinal epithelial cells (42, 43) . Although short-term or acute alcohol exposure increases membrane fluidity, chronic alcohol exposure has been shown to result in the production of fatty acid ethyl esters, changing membrane make-up and ultimately leading to increased rigidity of the membrane (44). Changes to membrane characteristics have the potential to impair intracellular signalling and subsequently affect the inflammatory response. Increased rigidity of cell membranes may delay dissociation of lipid rafts, which are crucial for many immune signalling processes. This could lead to prolonged immune signalling and an increase in pro-inflammatory mediators (45).

1.3.1 ALCOHOL DEHYDROGENASES

Alcohol is metabolised by alcohol dehydrogenase (ADH) into its first metabolite, acetaldehyde (**Figure 1.2**). ADH is a zinc metalloenzyme, with five distinct classes of isozymes (**Table 1.2**). The relative ethanol oxidising ability varies between classes of ADH, with Class I generally requiring a low K_m of ethanol to achieve 'half maximal activity', whereas Class III has a relatively high K_m (i.e., low affinity for ethanol). Class III and Class IV have been shown to be expressed in the oral cavity and in oral tumour cell lines (46, 47).

As well as ADH, the cytochrome p450 class of enzymes are major players in ethanol oxidation in the body. They are chiefly expressed in the liver, in the microsomes of hepatic cells. Cytochrome P450 2E1 (CYP2E1) converts ethanol to acetaldehyde and is inducible by chronic alcohol consumption – this is due to increased stabilisation of the enzyme (48). CYP2E1 has also been shown to be inducible in the oral cavity (49-51) as well as in oral cell lines (52, 53). In the oral cavity, CYP2E1 contributes to a small portion of overall ethanol metabolism and is only posited to be active in chronic alcohol consumers (48). Genetic polymorphisms in CYP2E1 have been linked to increased risk of OC, however the evidence is mixed in the literature (54, 55).

Table 1.2 Alcohol dehydrogenase isozymes

Class	Location	Relative ethanol oxidising ability
I	Liver	High
II	Liver/stomach/intestines	Mid-range
III	Ubiquitous in the body	Low
IV	Upper GI tract	Mid-range
V	Liver/stomach	Mid-range

1.3.2 Oral cavity alcohol dehydrogenase

It has been suggested that the link between alcohol consumption and the oral microbiome in OC is the expression of ADH by certain bacterial species (56-58). When alcohol is consumed it passes transiently through the mouth before reaching the liver and other organs. However, acetaldehyde concentrations of up to 450μM have been found present in saliva following alcohol consumption (57). The highest production of acetaldehyde per gram of tissue occurs in the colonic mucosa, whereas the activity of ADH in oral mucosa is comparatively low (46, 59). When patients were

treated with 4-methylpyrazole (a competitive inhibitor of human ADH) no significant changes were seen in salivary or blood acetaldehyde levels, suggesting that the majority of acetaldehyde production in the oral cavity is of microbial origin (60). This led to numerous studies investigating the ADH activities of specific oral bacteria and yeasts and their contribution to salivary acetaldehyde levels. Notable producers of acetaldehyde in the oral cavity include *Neisseria mucosa* (61), several *Streptococci* species (62) and *Candida albicans* (63). These species are reported to be capable of producing up to 50-270μM, ~135μM and ~215μM of acetaldehyde respectively (61, 63-65).

1.3.3 ACETALDEHYDE

Acetaldehyde was declared a carcinogen by the International Agency for Research on Cancer in 1999 and confirmed as a Group 1 carcinogen to humans in 2009 (66). Acetaldehyde is a genotoxic carcinogen, capable of causing point mutations, chromosomal alterations and forming DNA adducts (67). It interacts directly with DNA to form adducts, breaks, mutations, cross-links and chromosomal aberrations (68). The repair mechanisms shown to alleviate acetaldehyde-mediated DNA damage include nucleotide excision repair (NER), homologous recombination, Fanconi anemia pathway and base excision repair (69). It's also been shown to bind to cell membranes via Schiff's base formation, however this does not seem to affect membrane integrity (70). It is more likely that deleterious effects of acetaldehyde occur as a result of oxidative stress, disruption of mitochondrial function, and triggering of immune responses (71).

Acetaldehyde has also been shown to inhibit O-6-methylguanine-DNA methyltransferase, an enzyme required for repair of DNA adducts from alkylating agents, in a dose-dependent manner (72). The carbonyl carbon group of acetaldehydes can act as an electrophile to interact with DNA directly and the end product is an ethyl-adduct. The most common adduct is N^2-ethyldeoxyguanosine (73). Both O^6- or N^2- adducts can interfere with the fidelity of DNA replication, leading to mutations (74). In Rhesus monkeys chronically exposed to alcohol (4% w/v in water) over

a period of 12 months, levels of acetaldehyde-derived N^2-ethyldeoxyguanosine were found to be increased in oral mucosal DNA (75). Recent studies suggest that DNA damage induced by acetaldehyde is not direct, but rather due to the formation of bulky adducts, which are recognised by the NER pathway and cleaved in a replication independent manner, causing DNA double-stranded breaks (76).

1.3.4 ALDEHYDE DEHYDROGENASES

Acetaldehyde is cleared from the body via aldehyde dehydrogenases (ALDH), a family of 19 isozymes (**Figure 1.3**) (77). ALDH enzymes are a group of $NAD(P)^+$-dependent enzymes that catalyse the conversion of acetaldehyde into acetate. Both ADH and ALDH are expressed in oral mucosa, however ALDH activity of both the oral cavity and the oral microbiome is lower than that of ADH, potentially contributing to a build-up of acetaldehyde (48, 58, 62, 65, 78). As with the isozymes of ADH, the ALDH isozymes possess varying aldehyde metabolising abilities, which will be discussed in more detail later.

ALDH enzymes are not only important in the metabolism of aldehyde but also in retinol metabolism. Substrates of ALDH enzymes include aldehydes, amino acid derivatives, large lipids, and fatty aldehydes (**Table 1.3**). Retinoic acid (RA) signalling in cells is important for regulation of stem cell differentiation, cell maturation, proliferation, and survival. Retinol, or vitamin A, is absorbed by the cells, oxidised to retinal and then irreversibly oxidised into RA by ALDH enzymes. RA is a lipophilic molecule that can diffuse out of the cell. Therefore, signalling via RA receptors and subsequent activation of downstream transcription factors (TFs) can be endocrine or paracrine (79). Increased levels of acetaldehyde can inhibit formation of RA, as it competes as a substrate with retinals on ALDH enzymes. This has been shown in rat oesophagus, where it was determined that differences in retinol metabolism may be a determining factor in organ-specific susceptibility

to cancer due to acetaldehyde exposure (80). RA signalling therefore has important anti-cancer properties (49).

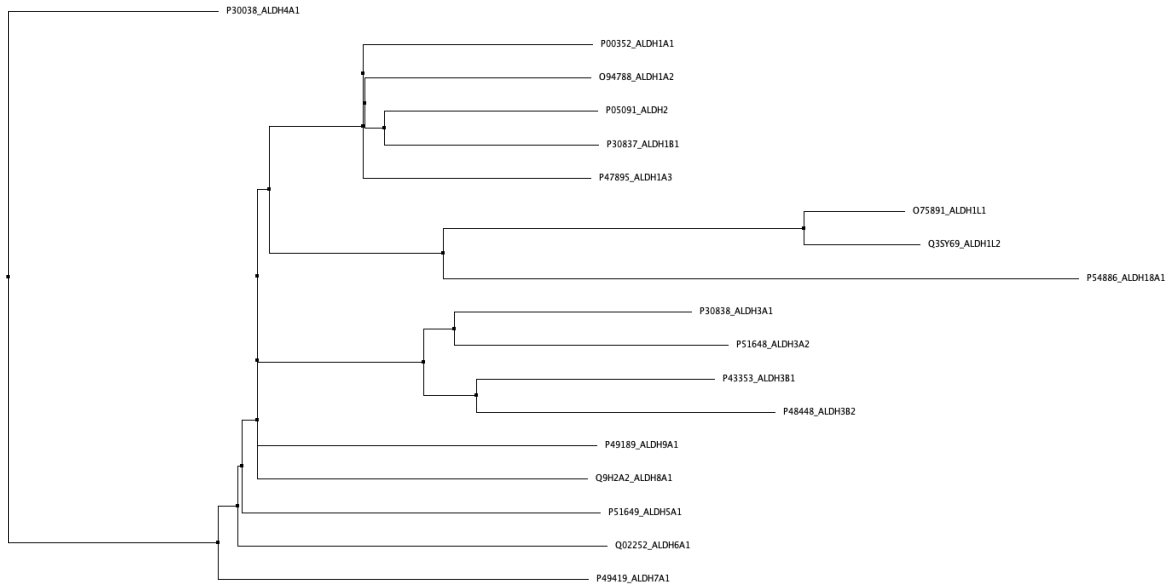


Figure 1.2 Dendrogram showing 18 of the 19 isoforms of ALDH and their relationship in terms of protein sequence similarity. Based on this tree, ALDH1A1, ALDH1A2, ALDH2, ALDH1B1, and ALDH1A3 have very closely related protein sequences. Conversely, ALDH4A1 is an outlier. ALDH16A1 is not included in this tree as it is a pseudo-enzyme and does not possess any catalytic activity. Each branch shows the accession number of the protein according to UniProt, followed by the name of the protein. The dendrogram was generated from a Multiple Sequence Alignment created using JalView software.

ALDH1A1

The ALDH family is of great interest in cancer biology due to its involvement in RA signalling, epithelial-mesenchymal transition (EMT), chemoresistance and its function as a cancer stem cell (CSC) marker. The roles of specific ALDH isoforms and their expression levels are tumour type- and stage-dependent.

ALDH1A1 has a preferred substrate of retinals. The expression of ALDH1A1 is associated with a higher risk of transformation in dysplasia, oral leukoplakia, OLP, as well as other PMDs (81). ALDH1A1 has been shown to increase in expression from normal through dysplastic to cancer cells,

with increased expression also correlating with increased severity of dysplasia (82-84). Expression of ALDH1A1 is also associated with tumour progression, increased risk for LNM as well as unfavourable prognosis in comparison to lower ALDH1A1-expressing tumours (85-87). In contradiction, some literature reports a decrease in expression of ALDH1A1 throughout the disease course of OSCC and no prognostic significance associated with its expression (88, 89).

ALDH1A1 is a putative CSC marker in several cancers, including OSCC. CSCs are a reservoir of self-sustaining pluripotent cells within tumours, with capabilities of self-renewal, differentiation and tumorigenicity. They express several cell surface markers that are indicative of 'stemness' including CD44 and ALDH1A1 (90, 91). They also possess characteristics that separate them from normal stem cells such as loss of growth control. Despite extensive research using ALDH as a CSC marker it has been suggested that ALDH alone is not a suitable marker for CSCs, as it is possible that an isoform specificity exists that depends on cancer type (92, 93).

ALDH1A1 is posited to be upregulated in cancer to fuel alternative metabolic pathways as well as acting as a detoxifying enzyme to enhance survival (92, 94). ALDH1A1 is associated with increased drug resistance, with ALDH inhibitors such as 4-diethylaminobenzaldehyde (DEAB) increasing the efficacy of chemotherapeutics in breast and lung cancers (92, 95-98). Limited data is available on the effect of ALDH inhibition in OSCC, but some enhanced chemosensitivity has been shown in head and neck squamous cell carcinomas (HNSCCs) with inhibition of the ALDH3 isoform (99).

ALDH2

ALDH2 is the mitochondrial isoform with acetaldehyde as a preferred substrate (100). Chronic alcohol consumption has been shown to downregulate ALDH2, with low expression levels correlating with significantly poorer survival rates (101). There is a correlation between ALDH2 dysfunction, risk for tumorigenesis and metastasis of established tumours, but the relative expression levels depend on the tumour type (102). For example, in hepatocellular cancer,

oncogenic transformation can be triggered by transcriptional suppression of ALDH1A1 and ALDH2 (102-104). When ALDH2 was induced in hepatocellular carcinoma cells, metastatic properties of the cells were attenuated. This change was independent of changes to EMT genes and of glycolytic pathway genes. The correlation of decreased ALDH2 with increased carcinogenicity is possibly due to accumulating levels of acetaldehyde as a result of lower ALDH2 activity (104).

Despite alcohol consumption being associated with lower ALDH2 levels, and unlike in hepatocellular cancer, ALDH2 expression has been shown to be increased in some OSCC tumours (85, 88). Oesophageal dysplasia is associated with ALDH2 mutants, suggesting potential for acetaldehyde accumulation to contribute to transformation (105). Overexpression of ALDH2 in OSCC highlights the dichotomous role of ALDH enzymes in which they can be both anti-oncogenic by reducing aldehyde exposure and regulating cell energy pathways, but also oncogenic by promoting tumour cell survival and drug resistance.

A common polymorphism in the ALDH2 gene, whereby the mutant enzyme is inactive, can cause severe acetaldehydemia and flushing responses due to acetaldehyde accumulation. Inactive ALDH polymorphisms and therefore decreased clearance of acetaldehyde showed a strong association with the risk of oesophageal and HNCs. This risk is increased multiplicatively in patients with both an inactive ALDH polymorphism and a more active ADH2*1/2*1 allele, increasing overall acetaldehyde accumulation (106-110).

ALDH3A1

Expression of ALDH3A1 has posited roles in the chemoresistance of several cancers (99, 111-113). However, low expression of ALDH3A1 has also been shown to be significantly associated with higher incidence of metastasis and overall worse prognosis of OC patients (114, 115). ALDH3A1 activators have been suggested as a method to enrich yields of stem cells for regenerative therapy of salivary glands for management of post-radiotherapy xerostomia in patients (116, 117). It is

possible that the activity or expression of ALDH3A1 in conjunction with other isoforms in the family is of potential relevance to oral carcinogenesis.

Table 1.3 ALDH isoforms, preferred substrates and possible roles reported in HNSCC.

Isoform	UniProt accession number	Preferred substrates (118, 119)	Implications in head and neck cancers	References
ALDH1A1	P00352	Retinal Acetaldehyde	• Decreased expression in OSCC compared to OLP • Increased expression from OED to OSCC, significant correlation with LNM and reduced survival rate • Higher levels of ALDH1A1^{+} CSC cells associated with worse prognosis • High expression levels in OED compared to normal mucosa	(85, 89, 118, 120-123)
ALDH1A2	O94788	Retinal Acetaldehyde	• Decreased expression in HNSCC, higher expression correlated with better prognosis • Low expression correlated with worse prognosis • Hypermethylation (silencing of gene promoter) worsened prognosis	(124-126)
ALDH1A3	P47895	Retinal	• High expression correlated with poor prognosis • Expression contributed to chemoresistance and	(127, 128)

			tumour relapse after irradiation	
ALDH1B1	P30837	Retinal Acetaldehyde	• High expression correlated with unfavourable prognosis in nasopharyngeal cancer	(129)
ALDH1L1	O75891	10-formyltetrahydrofolate	• Low mRNA expression in primary and metastatic nodes	(130)
ALDH1L2	Q3SY69	10-formyltetrahydrofolate	• High expression in tumours associated with worse prognosis • Single nucleotide polymorphism (SNP) on gene associated with increased risk for nasopharyngeal carcinoma	(115, 131)
ALDH2	P05091	Acetaldehyde	• Inactive polymorphism increased risk for oesophageal and HNSCCs • Increased expression in OSCC compared to OLP • Higher expression associated with better overall survival • Low expression correlated with worse prognosis in HNSCC • Low expression in HNSCC, particularly in heavy drinkers • No difference between controls and	(85, 88, 101, 106, 107, 115, 127, 132)

			OSCC, no influence on prognosis	
ALDH3A1	P30838	Aromatic or aliphatic aldehydes	• Low expression was a marker of poor prognosis • Overexpression inhibited EMT-like changes • Inhibition increased sensitivity of HNSCC to cisplatin • Activation protected salivary stem cells from degeneration post-radiotherapy	(99, 114, 133)
ALDH3A2	P51648	Aliphatic aldehydes	• No difference in expression of RNA between leukoplakia and normal oral mucosa • Rats with induced OSCC/dysplasia showed increased expression in OSCC group	(134)
ALDH3B1	P43353	Octanaldehyde	• High expression in tumours correlated with poor prognosis • Downregulated in late stages of transformation of OED	(127, 135)
ALDH3B2	P48448	Medium and long chain aldehydes	• No significant association between SNPs and risk of oesophageal squamous cell carcinoma	(136)
ALDH4A1	P30038	Glutamate-1-semialdehyde	No significant findings specific to HNSCC	

ALDH5A1	P51649	Succinate semialdehyde	• Upregulated gene but no significant association with OSCC	(137)
ALDH6A1	Q02252	Malonate semialdehyde	No significant findings specific to HNSCC	
ALDH7A1	P49419	α-aminoadipic semialdehyde	• High expression correlated with poor prognosis	(127)
ALDH8A1	Q9H2A2	Retinal	• High expression associated with poorer progression-free survival	(115)
ALDH9A1	P49189	γ-aminobutyraldehyde	• Expression was repressed by areca nut consumption, a major risk factor for HNSCC • Downregulated in HNSCC	(138-141)
ALDH16A1	Q8IZ83	Non-catalytic function	• High expression associated with poorer overall survival	(115)
ALDH18A1	P54886	Glutamic-1-semialdehyde	• High expression correlated with poor prognosis • SNP on gene associated with increased risk for nasopharyngeal carcinoma	(115, 127, 131)

1.4 EPITHELIAL-MESENCHYMAL TRANSITION

Epithelial-mesenchymal transition (EMT) is a process by which epithelial cells lose their apical-basal polarity and cell-cell adhesion, losing epithelial cell characteristics while gaining mesenchymal properties such as increased motility and invasive capacities. Mesenchymal cells are multipotent stem cells that can differentiate into a variety of cell types. This process is essential for tissue repair and embryonic development; however, cancer cells use this process to dedifferentiate, acquire a more aggressive phenotype and initiate metastatic processes. During EMT cells typically lose the so-called 'cobblestone' morphology with compact nuclei and become more spindle-like with elongated nuclei (142). Another feature of EMT is increased cell motility – pathologically this involves epithelium-derived cells invading across basement membrane tissues into connective tissue and migrating or metastasising to an organ separate from the primary tumour. It has been proposed that EMT is a critical mechanism by which normal or dysplastic epithelium becomes tumorigenic and cancer cells become metastatic. Following metastatic dissemination of carcinomas, they undergo the reverse of the EMT process (mesenchymal-epithelial transition or MET) and colonise new tissues.

EMT triggers a loss of E-cadherin with a concomitant upregulation of N-cadherin, vimentin (an intermediate filament that is overexpressed in migrating cells) and release of β-catenin (an adhesion protein that binds E-cadherin to the cytoskeleton) (**Figure 1.3**). The early dysregulation of EMT markers has also been suggested as a prognostic marker in potentially malignant oral disorders. The loss of E-cadherin has been shown to be an early phenomenon in moderate-severe dysplasia and an indicator of higher risk for transformation (143, 144).

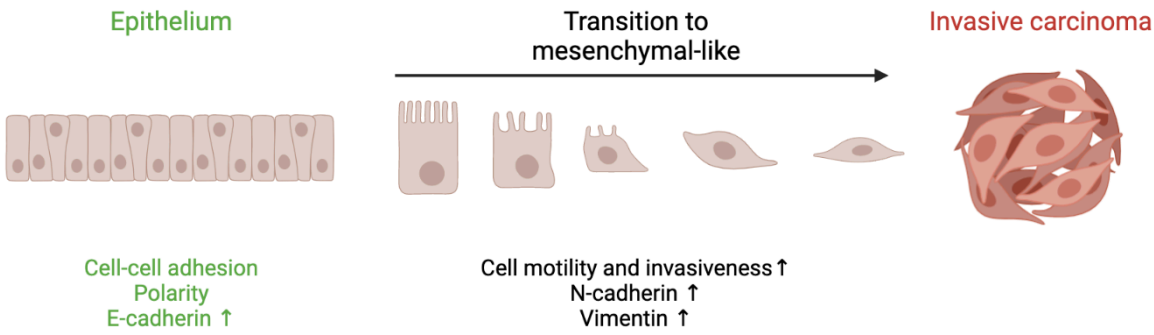


Figure 1.3 Epithelial-mesenchymal transition leads to transformation of normal or dysplastic epithelium as well as promoting metastasis of carcinomas. EMT generates cancer cells from epithelia and can be triggered by numerous factors. Intermediary states of this process are possible, and it also occurs in the reverse (145, 146).

Alcohol consumption has been shown to induce EMT features in oral keratinocytes, liver, colorectal and breast cancers via the regulation of a variety of proteins such as Wnt, β-catenin and Snail (147-150). Moreover, while alcohol consumption can modulate ALDH expression as previously discussed, ALDH expression itself appears to play a role in EMT processes. The isoform and relative expression level of ALDH is cancer specific. For example, human colorectal carcinomas that were overexpressing ALDH1B1 triggered EMT-like characteristics (151). When ALDH2 is induced in hepatocellular carcinoma cells *in vitro*, increased presence of CSCs and EMT-like behaviours is observed (103, 104, 152). Upregulated ALDH3A1 inhibited EMT in OSCC (114). It has also been observed that low expression of ALDH1A1 was shown to be correlated with EMT in large cell neuroendocrine carcinoma (153). Conversely, metastatic tumours of multiple different cancers show the lowest expression of ALDH2 compared to primary and recurrent tumours – demonstrating that expression of ALDH isoforms may fluctuate throughout cancer progression (154).

Downregulation of ALDH isoforms may lead to increased exposure to acetaldehyde in the oral cavity, further enhanced by alcohol consumption. This may also impair RA signalling leading to decreasing differentiation and maturation signalling of cells and advancing oncogenesis. It is worth

noting that the metabolism of acetaldehyde into acetate can be used as an energy source and regulator of cell stress by cancer cells, via acetyl-CoA synthetases (155). This metabolic plasticity demonstrated by cancer cells may be induced by alcohol consumption and further enhance tumorigenicity and chemoresistance (156). It is the hypothesis of this thesis that alcohol uses ALDH modulation to trigger malignant transformation of dysplasia and metastasis of oral carcinomas via both acetaldehyde exposure and EMT processes in oral cells.

More recently, research has suggested that the oral microbiome may influence development and progression of oral cancer. This may be in part due to changes in its basal composition and its influence on inflammatory responses in the mouth through OSCC development (157). Alcohol consumption can modulate microbiome composition, potentially facilitating the growth of pathogenic microbes, while also having an immunomodulatory effect on the oral epithelia, altering immune responses of the host (157). It has also been shown in colorectal cancer that the effect of alcohol on membrane permeability enhanced adhesion and invasion of microbial species (42). Similarly, a synergistic effect of various bacteria and fungi found in the oral cavity can promote the formation of biofilms as well as adhesion and invasion of microbial species into the oral epithelia (158, 159). As previously mentioned, there are an abundance of commensal microbial species that possess ADH enzymes potentially contributing to ethanol metabolism *in vivo*. Furthermore, ADH enzymes possessed by *C. albicans* have been shown to induce differentiation of inflammatory cells, eliciting a host immune response against fungal invasion (160). The specific impact of *C. albicans* on OC will be discussed in greater detail in Chapter 6.

Alcohol consumption, it's metabolism *in vivo* and the oral microbiome are intrinsically linked. This thesis will look at these multi-faceted effects of alcohol on the oral cavity, focusing on its effects on ALDH enzymes as well as it's effect on oral epithelia:microbiome interactions. A better understanding of the mechanisms by which alcohol consumptions exerts its effects in OC may aid

in the identification of potential treatment targets, identify those at higher risk for the development of OC and therefore strengthen the argument for the use of genetic sequencing and screening for the detection and prevention of OC. The overall aims of this thesis are as follows;

1.5 Overall aims

- Characterise three different oral cell lines as models of dysplasia and oral squamous cell carcinoma.
- Investigate the role of alcohol consumption on the characteristics of oral dysplasia and oral squamous cell carcinoma.
- Use chemical inhibitors and siRNA to verify if ALDH modulation is the mechanism behind ethanol-induced carcinogenicity.
- Investigate the role of alcohol consumption on microbial-induced carcinogenesis.

Chapter 2: Materials & Methods

2.1 General methods

All chemicals were obtained from Merck (Ireland), unless otherwise stated.

2.1.1 Measurements of weight

Mass-based measurements in the range of 5g to 200g were obtained using a Mettler B2002-S top-loading balance and weights of <5g were obtained using a Mettler College150 analytical balance.

2.1.2 Measurements of volume

Volumes in the range of 1µL to 5mL were measured and delivered using a set of standard Gilson Pipetman automatic pipettes. All pipettes were regularly calibrated according to the manufacturer's instructions.

2.1.3 Centrifugation

Cells were centrifuged in a benchtop Eppendorf Centrifuge 5910R at room temperature (RT). Samples prepared outside of the laminar flow hood were centrifuged in a benchtop Eppendorf Centrifuge 5415.

2.1.4 Spectrophotometry

Absorbance assays were performed using a Spectra MAX Plus plate reader using clear 96-well plates and SoftMaxPro 6.2.1 software. Fluorescent assays were performed using black 96-well plates.

2.1.5 Thermal Cycling

All thermal cycling reactions for cDNA synthesis and PCR were carried out in a Primus 96 Plus Thermal Cycler.

2.2 CELL CULTURE

Cells were cultured at 37°C in a 5% CO_2 humidified atmosphere in a Thermo Scientific Series 8000DH Incubator. All cell culture work was carried out under an Nuaire™ laminar flow hood using aseptic technique. Once 80-90% confluent, the cells were washed in pre-warmed phosphate buffered saline (PBS) solution and incubated with trypsin-EDTA (0.5% trypsin, 0.02% EDTA) for 5-10 min at 37°C to detach the cells from the surface of the culture flask. Fresh media was added to deactivate the trypsin and the suspended cells were centrifuged (1200 RPM, 5 min). The supernatant was discarded, and the cell pellet was resuspended in suitable media and passaged at an appropriate ratio. Cell confluence was monitored using a Nikon Eclipse TS100 light microscope with 10X and 20X dry objectives. Cancer cell lines were passaged indefinitely, dysplastic cell lines were used up to passage number 25 before lower passage number vials were retrieved from liquid nitrogen storage.

2.2.1 CULTURE OF CA9.22 GINGIVAL SQUAMOUS CELL CARCINOMA CELL LINE

The Ca9.22 oral carcinoma cell line (Health Science Research Resources Bank, Osaka, Japan) was maintained in Minimum Essential Medium (MEM) supplemented with 10% v/v fetal bovine serum (FBS), 1% v/v L-Glutamine (200mM) and 1% v/v penicillin/streptomycin (100μg/ml).

2.2.2 CULTURE OF TR146 BUCCAL SQUAMOUS CELL CARCINOMA CELL LINE

The TR146 oral carcinoma cell line (Health Protection Agency (HPA) Cultures, UK) was maintained in high glucose Dulbecco Modified Eagle's Medium (DMEM) with 10% v/v FBS and 1% v/v penicillin/streptomycin (100μg/ml).

2.2.3 CULTURE OF DOK DYSPLASTIC ORAL MUCOSA CELL LINE

The DOK dysplastic oral cell line (HPA Cultures, UK) was maintained in high glucose DMEM with 10% v/v FBS, 1% v/v penicillin/streptomycin (100μg/ml) and hydrocortisone (5μg/ml).

2.2.4 Culture of HepG2 hepatic carcinoma cell line

The HepG2 hepatic carcinoma cell line (European Collection of Authenticated Cell Cultures, UK) was maintained in high glucose DMEM with 10% v/v FBS, 1% v/v L-Glutamine (200mM) and 1% v/v penicillin/streptomycin (100μg/ml).

2.2.5 Chronic exposure to ethanol or acetaldehyde

To investigate the long-term effects of ethanol or acetaldehyde exposure on the cell lines, cells were treated in ethanol (1% v/v) or acetaldehyde (100μM) for two weeks. Cells were seeded at approximately 60-70% confluency in a T175 flask and three days a week (Mon, Wed, Fri) were trypsinised (Section 2.2) and passaged if required. Chronically treated cells were maintained in normal culture media with fresh ethanol/acetaldehyde added three days a week (Mon, Wed, Fri) at the same time as passaging, if required. For acute ethanol treatment, ethanol was added 24 h prior to the end of the two week timepoint. Cells were harvested by trypsinisation and either centrifuged immediately (1200 RPM, 5 min) or counted (Section 2.2.6) and used for further analysis.

2.2.6 Trypan blue exclusion

Cell viability was determined using the trypan blue exclusion assay. This assay works on the principle that only cells with intact membranes can effectively exclude the dye, whereas dead cells allow entry of the dye to the cytosol through compromised membranes. A viable cell will have a clear cytosol whereas a nonviable cell will have a blue cytosol and will be excluded from the live cell count. Cell suspension was added at a 1:1 dilution with 0.4% trypan blue dye. The mixture (10μl) was loaded onto a LUNA™ Cell Counting Slide and counted using a LUNA-II™ Automated Cell Counter.

2.3 Oral microbe culture

Candida albicans 132A (American Type Culture Collection) was cultured on brain heart infusion (BHI) agar plates. Solid sterile BHI agar was heated in a microwave until it was completely dissolved. Once cool enough to handle, the agar was poured into culture dishes (100mm, 16mm depth) under a laminar flow hood. Plates were stored in the fridge upside down to prevent excess condensation forming on the agar for up to 6 weeks.

2.3.1 Streaking microbial cultures

Cultures were stored in freezing tubes containing 3mm glass beads, cryoprotectant glycerol (30%) and BHI broth at -80°C. Plates were streaked with aseptic technique using an inoculating loop. The loop was used to remove a single bead from the cryovials. The bead was streaked onto one third of the plate to create a reservoir. The loop was sterilized again to maximise isolation of pure colonies and streaked through the edge of the reservoir and the rest of the plate (**Figure 2.1**). Plates were incubated aerobically at 37°C overnight. Plates were monitored for single pure colonies, then stored at 4°C for up to a week before sub-culturing. Single colonies were used for sub-culturing, using the same streaking method.

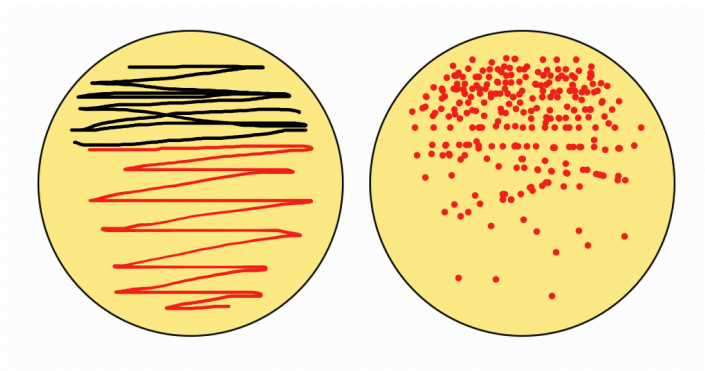


Figure 2.1. Streaking method for microbial cultures. A sterile inoculating loop was used to spread either a glass bead from frozen stocks or a single colony from an existing plate onto one third of a fresh agar plate into a reservoir (black). After sterilising the loop, the edge of the reservoir was streaked through the rest of the plate (red). The plate on the right shows the successful formation of individual colonies (red) from the streaking pattern.

2.3.2 OD_{600} TO DETERMINE CELL COUNT

OD_{600} is a method used to estimate the concentration of microbes in liquid by measuring the optical density of the sample at a wavelength of 600nm using a spectrophotometer. Cultures are grown in liquid broth, centrifuged (14000 RPM, 1 min) and the pellet washed in PBS three times before being resuspended in PBS. Absorbance was read at 600nm using a Spectra MAX Plus Microplate reader, with PBS as a blank measurement. To convert this measurement into an estimate of concentration (cells/ml), a serial dilution of liquid broths was prepared and streaked onto agar plates. Single colonies were counted as one cell (assuming pure colonies arise from a single cell that undergoes binary fission) and converted using the dilution factor. It was calculated that at OD_{600nm} of 1, there is approximately 1 x 10^8 cells/ml of *C. albicans*.

2.4 CO-CULTURE OF ORAL CELL LINES WITH *C. ALBICANS*

For co-culture experiments, *C. albicans* was grown in liquid BHI broth. Cultures were incubated in a shaker overnight (200 RPM, 37°C) with the lids slightly ajar to allow for some aeration while maintaining sterility. Broth was observed for an increase in turbidity of the solution. Cultures were then centrifuged, washed with PBS, placed in a heating block (65°C, 1 h) to inactivate and counted (as described in Section 2.3.2) before use. Heat-inactivated samples were stored at -20°C.

Oral cell lines were cultured as previously described (Section 2.2) and heat-inactivated microbes were added at varying multiplicity of infection (MOI) ratios. MOI is expressed as the ratio of infectious agents to infection targets, i.e., at MOI of 1, there is one microbial particle to one cell and so on.

2.5 ALAMARBLUE ASSAY

AlamarBlue™ (Invitrogen) is a chemical reagent used to analyse cell viability. The active ingredient of AlamarBlue is resazurin, a non-toxic, cell-permeable compound that is blue in colour. Rezasurin

is modified by the environment of living cells to its reduced form resorufin, a compound that is red in colour and highly fluorescent. The colorimetric change in response to cellular respiration can be measured by the intensity changes in absorbance. Cells were seeded onto plates, treated as required and 4 h prior to endpoint AlamarBlue reagent was added (10% v/v). Absorbance was read on a Spectra MAX Plus Microplate reader at wavelengths 570nm/600nm.

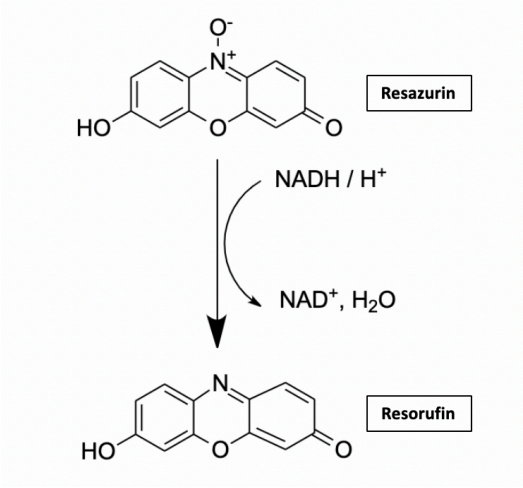


Figure 2.2. AlamarBlue reagents main component is rezasurin. Rezasurin is reduced by living cells into its fluorescent product resorufin, with NADH as a cofactor.

2.5.1 Determination of IC_{50} value

The half maximal inhibitory concentration (IC_{50}) is a quantitative measurement of a substance to inhibit a biological process *in vitro* by 50%. IC_{50} values are determined by constructing a dose-response curve and examining the effect of different concentrations of antagonist against the percentage inhibition of activity. AlamarBlue was used to determine the IC_{50} of ethanol on the viability of cells, to determine a working concentration for other experiments.

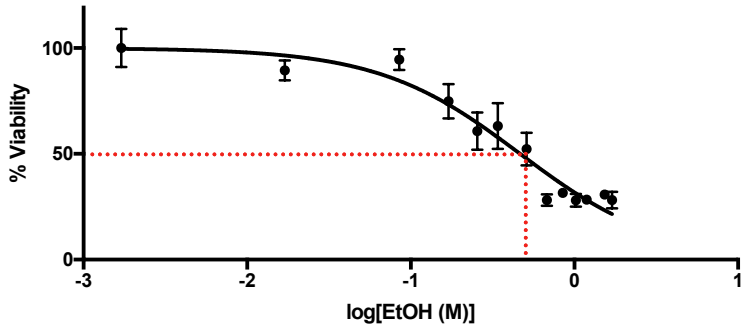


Figure 2.3. Example of IC_{50} curve of Ca9.22 cells treated with ethanol for 24 h. Cells were treated with several concentrations of ethanol over 24 h and AlamarBlue was used to determine viability of the cells (as per **Section 2.5**). The IC_{50} value was determined from the graph (in red). Data shown n=3 ± SEM.

2.6 Reverse transcriptase polymerase chain reaction

Reverse transcriptase polymerase chain reaction (RT-PCR) allows for detection and quantification of mRNA. Total RNA is isolated from samples and transcribed into complementary DNA (cDNA) by reverse transcriptase. Deoxyribonucleotide triphosphates (dNTPs) are included in the reaction mix as building blocks for new DNA strands. This process generates a full-length cDNA molecule from RNA strands.

The resulting cDNA can then be used for PCR reactions. The cDNA sample is denatured to separate the double strand. Specific primers for the gene of interest anneal to the single strand in the presence of dNTPs and *Taq* polymerase. *Taq* polymerase is a thermostable DNA polymerase I used to amplify DNA sequences in PCR reactions as it can withstand the protein denaturing temperatures required during PCR. Once the new strand is elongated by the polymerase, the cycle is repeated 25-30 times via a thermal cycler, amplifying the resulting specified DNA product. Primers for β-actin were used as a positive control. Negative controls contained complete reaction mixes with no template RNA or cDNA.

2.6.1 RNA ISOLATION

Following required treatments cells were washed with PBS and TRIzol (Invitrogen), an RNA isolation reagent, was added to each plate (300µl per $1x10^5$- $1x10^7$ cells). A cell scraper was used to detach and homogenise the cells with TRIzol. Cell lysates were incubated at RT for 5 min to allow complete dissociation of the nucleoproteins complex. Chloroform (60µl) was added as a de-proteinising agent to each sample, vortexed for 15 s and incubated for a further 2-3 min at RT. The samples were then centrifuged (12,000g, 15 min, 4°C). The mixture separates into a lower, red-coloured phenol-chloroform, a cloudy inter-phase, and a colourless upper aqueous phase. The aqueous phase was transferred to a new tube. Isopropanol (150µl) was added to precipitate RNA and samples were incubated at RT for 10 min. The samples were centrifuged (12,000g, 10 min, 4°C) and RNA precipitates formed a gel-like pellet. The RNA pellet was resuspended in 75% ethanol (300µl, made with nucleic acid free water), centrifuged (7,500g, 5 min, 4°C) and the pellet left to air dry for 5-10 min. The pellet was resuspended in nucleic acid free water (25µl). RNA isolation was also carried out using the *EasyPure®* RNA Kit (TransGen Biotech) as per the manufacturer's protocol. RNA was either stored at -80°C or used immediately for PCR.

2.6.2 DETERMINING RNA ISOLATE CONCENTRATION

A Nanodrop ND-1000 Spectrophotometer was used to determine the purity and concentration of RNA. Nucleic acids, RNA, ssDNA, and dsDNA have absorbance maxima at 260nm, whereas proteins have absorbance maxima at 280nm. Based on this principle, the ratio of absorbance at 260/280nm is commonly used to determine purity of DNA/RNA samples. A ratio of spectrophotometric absorbance at 260/280nm between 1.8-2 was accepted as 'pure' for RNA, anything lower indicated poor sample quality and the presence of contaminants such as proteins, so the sample was discarded. The Nanodrop was blanked using nucleic acid free water. Each sample was loaded (1-2µl) and the concentration (ng/µl) and the 260/280nm ratio was recorded.

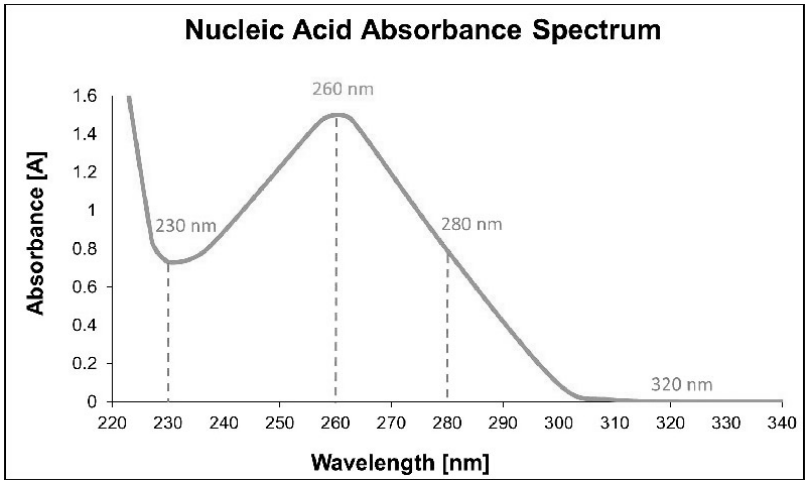


Figure 2.4. Absorbance spectrum of nucleic acids at different wavelengths. Nucleic acids absorbance maxima are at 260nm, whereas proteins have absorbance maxima at 280nm. The ratio of A_{260}/A_{280}nm can be used to determine purity of DNA and RNA samples, where a ratio of 1.8-2 is considered 'pure'. *Image source: DRogatnev/shutterstock.com.*

2.6.3 cDNA SYNTHESIS

cDNA synthesis was carried out using either the PrimeScript™ 1st Strand cDNA synthesis kit (Takara Bio) or Tetro™ cDNA synthesis kit (Meridian Bioscience). Both kits were used as per the manufacturer's protocol. cDNA synthesis was carried out in the thermal cycler, programmed as per **Table 2.1**. Samples were either used immediately for PCR or stored at -20°C for future use.

2.6.4 PCR REACTION

The REDTaq® ReadyMix™ PCR Reaction mix was used for PCR reactions. The following mixture was prepared for each sample, as per the manufacturer's guidelines; REDTaq ReadyMix (12.5µl), forward primer (1µl), reverse primer (1µl), template cDNA (1µl) and RNase-free dH_2O (9.5µl). Specific primers were designed as per **Table 2.2** (Eurofins). The tubes were vortexed then briefly centrifuged before being placed into the thermal cycler (**Table 2.3**), and either used immediately for gel electrophoresis or stored at 4°C.

Table 2.1 cDNA thermal cycler programme.

Temperature (°C)	Time (min)	Reaction
30	10	Annealing of primer
42	45	Elongation of cDNA
70	15	Enzyme deactivation
-20	-	Storage of cDNA

Table 2.2. Table of primer sequences and expected amplicon length. Sequences were acquired from OriGene.

	Forward primer '5 – 3'	Reverse primer 5' – 3'	Amplicon length
ADH Class I	ATC CAC ACC TCC ATC AGT CAT TTC C	AGA ATT TGT AAA AAC CCG GAG AGC AAC TAC	462 bp
ADH Class II	CCT TGA CTG TGC AGG TGG ATC T	GTC AAT CCT TTG CTA CCA GCA GC	89 bp
ADH Class III	GTA AAC CCA TCC AGG AAG TGC TC	TGT GAC ATG CCT CAA GTG CTG C	89 bp
ADH Class IV	CCA TCA GTG AGG TGC TGT CAG A	GCA TCT TGG CTG ATG GAG GAA C	136 bp
ALDH1A1	CGG GAA AAG CAA TCT GAA GAG G G	GAT GCG GCT ATA CAA CAC TGG C	147 bp
ALDH2	TTG CCT CCC ATG AGG ATG TGG A	GGT CAC TCT CTT GAG GTT GCT G	104 bp
ALDH3A1	CTC GTC ATT GGC ACC TGG AAC T	CTC GCC ATG TTC TCA CTC AGC T	119 bp
β-actin	TGC GTG ACA TTA AGG AGA AG	CTG CAT CCT GTC GGC AAT	297 bp

Table 2.3 PCR thermal cycler programme. *Annealing temperatures for each set of primers were as follows; ADH Class I 63°C, ADH Class II 55°C, ADH Class III 59°C, ADH Class IV 49°C, ALDH1A1/ALDH2/ALDH3A1 62°C, β-actin 55°C and GAPDH 63°C.

Temperature (°C)	Time (min)	Reaction	Number of cycles
95	1	Initial denaturation	1
94 *Specific to primer pairs 72	1 1 1	Denaturation Annealing of primers Polymerisation	30
72	5	Final extension	1
8	-	Storage	-

2.7 FLOW CYTOMETRY

Flow cytometry was used to analyse distribution of cells within the cell cycle, to detect apoptotic cell death, to determine cell morphology and to detect ALDH1A1.

2.7.1 ANALYSIS OF CELL CYCLE BY PROPIDIUM IODIDE

Propidium iodide (PI) is a fluorescent DNA inter-chelating agent. The dye stains DNA stoichiometrically, allowing differentiation of cells in G_0-G_1, S, and G_2/M phase since the levels of fluorescence measured from each cell correlate to the amount of DNA present. PI also stains RNA so the cells must be treated with an RNase to ensure accuracy. Cells must be permeabilised to allow entry of the dye to the cell, as live cell membrane integrity excludes PI.

Cells were seeded in 6-well plates and treated as required. Cells were trypsinised and media and PBS washes were collected. Samples were centrifuged (1200 RPM, 5 min), the supernatants discarded, and the pellets washed with PBS. Ice-cold ethanol (2ml) was added to each pellet and incubated for 20 min at 4°C to fix the cells. Samples were centrifuged (1000 RPM, 5 min), supernatant discarded, and the pellet air dried. The pellet was resuspended in PBS, RNase (10mg/ml) and PI (1mg/ml). The samples were incubated in the dark for 30 min at 37°C, before vortexing, and transfer to polystyrene round-bottom tubes for fluorescence-activated cell sorting (FACS). The PI was excited using a 488nm laser in a BD FACSCanto™ II flow cytometer and the resulting histograms were generated using FlowJo™ software (TreeStar, Version 10.9).

2.7.2. GATING STRATEGY

Single cells were gated based on their side-scatter height (SSC-H) vs side-scatter area (SSC-A), and doublets excluded from analysis (**Figure 2.6**).

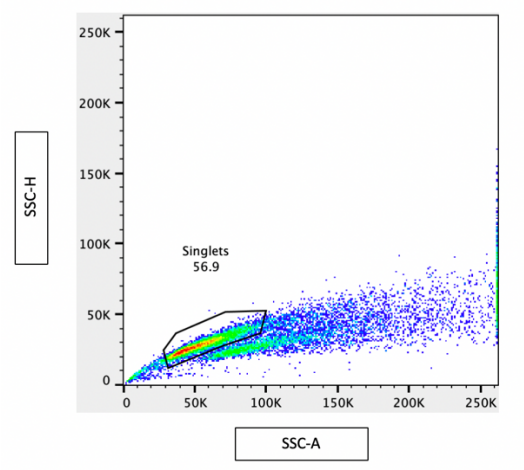


Figure 2.6 Representative example of gating of single cells using FlowJo™. Single cells populations are gated, excluding doublet or clumps of cells from SSC-H vs SSC-A plots.

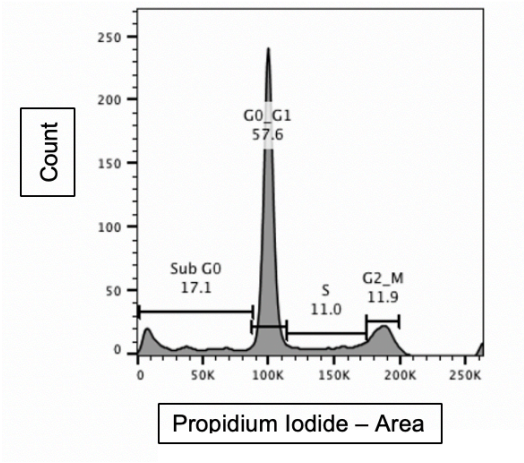


Figure 2.7 Representative example of a histogram of cell cycle distribution in a control cell population. Ca9.22 cell cycle distribution histogram following staining with PI. The percentage of cells distributed within the cell cycle phase is annotated beneath the phase title. Most cells (57.6%) are in the G_0-G_1 phase. Histograms were generated using FlowJo™ software.

2.7.3 Analysis of apoptotic and necrotic cells

An early event in apoptosis is the translocation of phosphatidylserine (PS) in the plasma membrane from the cytoplasmic leaflet to the exoplasmic leaflet. Annexin V binds specifically to PS. Cells are not permeabilised for this assay so viable cells exclude PI, whereas necrotic and late-stage apoptotic cells do not. Necrotic cells do not undergo the same exposure of PS. Using labelled Annexin V (to FITC) in conjunction with PI, FACS analysis can be used to discriminate between live (Annexin V/PI

-/-), early apoptotic (Annexin V/PI +/-), late apoptotic (Annexin V/PI +/+) and necrotic (Annexin V/PI -/+) cells.

Cells were treated as required, trypsinised and centrifuged (1200 RPM, 5 min). Pellets were resuspended in ice-cold Annexin V binding buffer (BD Pharmingen, 500μl) and centrifuged (600g, 5 min, 4°C). The supernatant was discarded, and the pellets were stained by resuspending in binding buffer (100μl), Annexin V- FITC (BD Pharmingen, 5μl) and PI (5μl, 75μM). The samples were incubated on ice for 15 min before adding binding buffer (400μl) and transfer to FACS tubes for analysis. The Annexin V-FITC (485nm excitation, 535nm emission) and PI (494nm excitation, 696nm emission) were excited using a 488nm laser in a BD Accuri C6 flow cytometer. Data compensation for the stains was carried out using an unstained sample, an Annexin V-only stained sample, and a PI-only stained sample. Single cells were gated as described in Section 2.7.2, before gating for live, early apoptotic, late apoptotic and necrotic cells.

2.7.4 Cell morphology analysis

Cell morphology was evaluated according to the relationship between forward scatter (FSC) and side scatter (SSC), cell size and granularity (151). PBS-washed cells were trypsinised, centrifuged (1200 RPM, 5 min) and resuspended in PBS with PI at a final concentration of 0.1mg/ml. Controls for gating included an unstained sample and a dead control (treated with ethanol 100%). Live cells were gated as before (Section 2.7.2), and the peak shift of mean fluorescence intensity of FSC/SSC for live cells only was compared to evaluate any changes in morphology.

2.7.5 ALDH1A1 protein expression

Cells were treated as required, trypsinised, and centrifuged (1200 RPM, 5 min). Cell pellets were washed in PBS then resuspended in ice-cold FACS buffer (1X PBS, BSA 2%, EDTA 2mM) and stained with Green-AIDeSense ALDH1A1 live cell dye (2μM) for 30 min on ice in the dark. Cells were centrifuged (1200 RPM, 5 min) and pellets resuspended in ice-cold FACS buffer. Immediately before

reading, PI was added to the sample (1μg/ml). Cells were excited using a 488nm laser on a BD Accuri C6 flow cytometer and analysis was carried out using FlowJo™ software.

2.8 WESTERN BLOT ANALYSIS

2.8.1 BRADFORD ASSAY

Protein concentrations were estimated using the Bradford assay. A protein standard curve was made from serial dilutions of bovine serum albumin (BSA) ranging from 0-2000μg/ml. Standards and unknowns were added in triplicate to a 96-well plate and Coomassie blue reagent was added. Samples were incubated in the dark for 10 min at RT. Absorbance was read on a Spectra MAX Plus Microplate spectrophotometer at 595nm. The concentrations of the unknown samples were calculated from the BSA standard curve.

2.8.2 SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Following determination of protein concentration, samples of equal protein concentration were combined with Laemmli buffer (4X, Bio-Rad) and heated at 65°C for 15 min. The resolving gel was prepared between glass plates at 12% polyacrylamide as in **Table 2.4**, with 70% ethanol on top to ensure a level surface. Once the resolving gel was set, ethanol was washed off the gel, the stacking gel was prepared (**Table 2.4**), poured on top, and the comb inserted to form loading wells. Once the stacking gel had polymerised, the gels were washed with dH_2O and placed in a vertical gel electrophoresis tank and immersed in running buffer (**Table 2.5**). Protein samples were loaded into each well along with a Spectra multi-colour broad range protein ladder as reference (Thermo Scientific). The gels were run at 90-100V using a Bio-Rad power pack until the protein markers were sufficiently separated and the sample dye had reached near the end of the gel.

2.8.3 WESTERN BLOTTING

SDS-PAGE gels were soaked in cathode buffer for 15 min (**Table 2.5**). A semi-dry transfer technique was used to transfer the proteins from the gel to Immobilon®-polyvinylidene fluoride

(PVDF) membrane, by sandwiching pre-soaked sheets of filter paper in anode and cathode buffer along with the gel and PVDF membrane (activated in methanol for 60 s) as described in Figure 2.8. Proteins were electroblotted in a combination of cathode and anode buffers for 1 h at 9V using a Trans-Blot module (Bio-Rad). Once the transfer was complete, membranes were incubated in 5% skimmed milk powder in TBST (Table 2.5) for 1 h to block non-specific binding sites. The membrane was washed in TBST (3 x 10 min) before addition of the primary antibody at the required dilution (Table 2.6) in 5% skimmed milk for either 2 h at RT or overnight at 4°C. The membrane was washed in TBST (3 x 10 min) before incubating with the relevant secondary antibody for 2 h at RT. The membrane was washed as before in TBST. Antibody labelled proteins were visualised using Enhanced Chemiluminescence (ECL) horseradish peroxidase (HRP) substrate (Millipore). ECL reagents were mixed at a 1:1 ratio and incubated on the membrane for 2 min at RT. Signal was detected using a Chemi-Luminescent gel documentation system (Bio-Rad), using ImageLab software (Version 6). Loading discrepancies were controlled for using densitometry against housekeeping genes β-actin or GAPDH using ImageJ software (Version 1.53).

2.8.4 Membrane stripping

To investigate more than one protein of a similar size on the same blot, membranes were stripped of their primary and secondary antibodies for re-probing. Membranes were incubated in stripping buffer (Table 2.5) (2 x 5 min). Membranes were then washed with PBS (2 x 10 min) before washing in TBST (2 x 5 min). The membranes were then ready for re-blocking in 5% skimmed milk for 1 h at RT before incubating with primary and secondary antibodies as before.

Table 2.4 Components of resolving and stacking gels for SDS-PAGE.

Reagents (ml)	Resolving gel	Stacking gel
dH_2O	3.3	2.1
30% acrylamide mix	4.0	0.5
1.5M Tris (pH 8.8)	2.5	-
1M Tris (pH 6.8)	-	0.38
10% SDS	0.1	0.03
10% APS	0.1	0.03
TEMED	0.005	0.04

Table 2.5. Buffers for SDS-PAGE and Western blotting.

Buffer	Recipe	pH
RIPA	Tris 50mM, NaCl 150mM, 1% Triton x-100, 0.5% Sodium deoxycholate, 0.1% SDS, Protease inhibitor cocktail (Roche)	8
Running buffer	Tris 25mM, Glycine 0.19M, 1% SDS	8.3
TBST	Tris 20mM, NaCl 0.15M, 0.1% Tween-20	7.5
Cathode buffer	Tris 25mM, Glycine 40mM	9.4
Anode 1 buffer	Tris 300mM, 10% MeOH	10.4
Anode 2 buffer	Tris 25mM, 10% MeOH	10.4
Stripping buffer	Glycine 0.2M, 1% SDS, 1% Tween-20	2.2

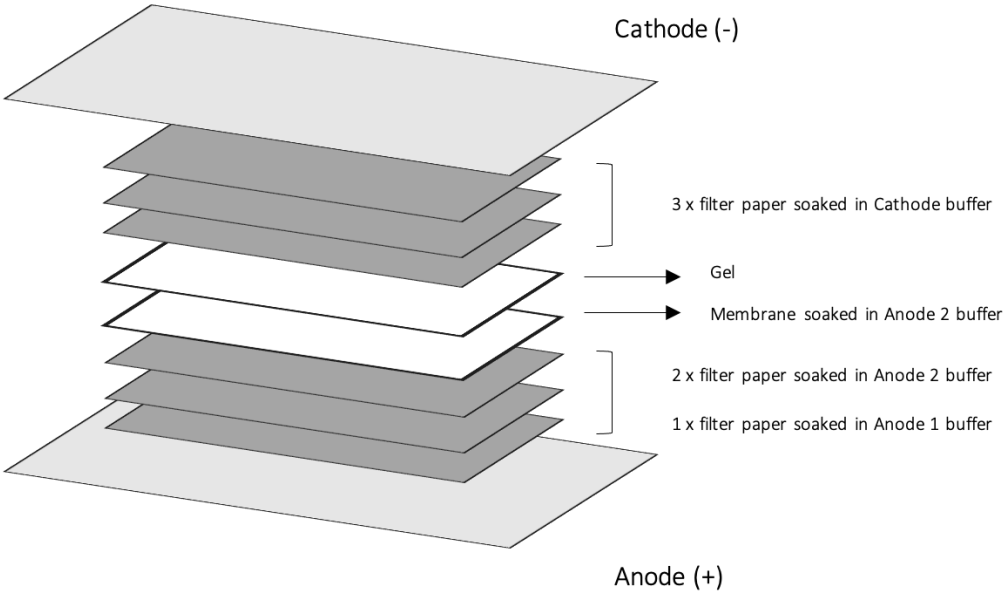


Figure 2.8 Semi-dry transfer apparatus setup.

Table 2.6 Supplier, host species and dilutions for primary and secondary Western blotting antibodies.

	Primary antibodies		
Target	**Supplier, catalogue number**	**Host species**	**Final dilution**
ALDH1A1	OriGene, TA321182	Rabbit	1:500
Caspase p17	Abcam, ab2302	Rabbit	1:500
E-cadherin	Brennan & Co, 3195T	Rabbit	1:1000
GAPDH	Cohesion Biosciences, CPA9067	Mouse	1:10000
IκB-α	Brennan & Co, 4812S	Rabbit	1:1000
N-cadherin	Brennan & Co, 13116T	Rabbit	1:1000
PARP	Abcam, ab32138	Rabbit	1:1,000
Vimentin	Brennan & Co, 5741T	Rabbit	1:1000
β-actin	Merck, A5441	Mouse	1:10000

	Secondary antibodies		
Target	**Supplier, catalogue number**	**Host species**	**Final dilution**
Mouse	Brennan & Co, 7076S	Goat	1:10000
Rabbit	Brennan & Co, 4812S	Goat	1:5000

2.9 Determination of intracellular ROS by DCF-DA assay

To measure intracellular reactive oxygen species (ROS) production, dichlorodihydro fluorescein diacetate (DCF-DA) was used. DCF-DA (Fisher) is cell permeable and upon entry into cells is hydrolysed by intracellular esterases. Within the cell, non-fluorescent DCF-DA can be oxidized by ROS and converted into the fluorescent form, DCF. The oxidation reaction can be mediated by H_2O_2, superoxide or NO. Fluorescent intensity of DCF correlates with levels of ROS in cells.

Cells were seeded in black 96-well clear-bottom plates and following required treatment, washed with PBS. DCF-DA was added to each well (20μM) and incubated for 30 min in the dark. H_2O_2 (10μM) was added as a positive control and PBS as a negative control. Fluorescence was read on a Spectra MAX Plus Microplate plate reader at excitation wavelength 490nm and emission wavelength 529nm.

2.10 Determination of extracellular ROS by Amplex Red assay

The Amplex Red assay was used to measure extracellular H_2O_2 production. In contrast to the DCF-DA assay, Amplex Red is used to detect H_2O_2 that has been released by the cell. In the presence of HRP and H_2O_2, Amplex Red is oxidized to resorufin, a fluorescent red compound. A standard curve was made using known concentrations of H_2O_2. Cells were seeded in black 96-well plates and treated as required. Cells were washed in Krebs buffer (300μM KCl, 14mM NaCl, 2.5mM Tris-HCl, 200μM $MgCl_2$, 200μM $CaCl_2$, 10mM glucose, pH 7.4), before adding Amplex Red (50μM) and HRP (2.5U/ml) in Krebs buffer. The plate was incubated in the dark at 37°C for 1 h before reading on a Spectra MAX Plus Microplate plate reader at excitation wavelength 550nm and emission wavelength 585nm.

2.11 ETHANOL ASSAY

To determine the amount of ethanol present in cell culture wells the Megazyme Ethanol Assay Kit® was used as per the manufacturer's protocol. Ethanol was added to cell culture media (0.5-5% v/v) and assayed from the time of addition (T_0) and at several timepoints thereafter (4 h, 8 h, 24 h, 48 h). Ethanol is oxidised to acetaldehyde by ADH using NAD^+, forming NADH. The equilibrium of this reaction lies in favour of ethanol and NAD^+ so a further reaction step is required to 'trap' the products. The addition of ALDH to the reaction mix catalyses the further oxidation of acetaldehyde to acetic acid. The amount of NADH formed in this reaction pathway is stoichiometric with twice the amount of ethanol and is measured via absorbance on a Spectra MAX Plus Microplate plate reader at 340nm.

2.12 SCRATCH ASSAY

To investigate the proliferation and migratory capabilities of cells, a 'scratch' or 'wound healing' assay was used. A polylactic acid plastic, 3D printed mould was designed and used to create a zone of exclusion in the cell monolayer or a 'wound'. This allowed for the creation of uniform gaps in the cell layer (**Supplementary Figure A, B**). Cells were seeded onto 24-well plates with the mould held in place by rubber bands to prevent it from moving and to allow for a constant interface between the 3D print and the plate surface. Proliferation and migration across the scratch area was measured over a 48 h period or until the scratch area was 100% closed i.e., the cells migrated and 'closed' the gap created in the monolayer. An IncuCyte™ S3 was used to obtain images of the scratches at 2 h intervals. Proliferation/migration was quantified as rate of wound closure using ImageJ software (Version 1.53) (**Figure 2.9**).

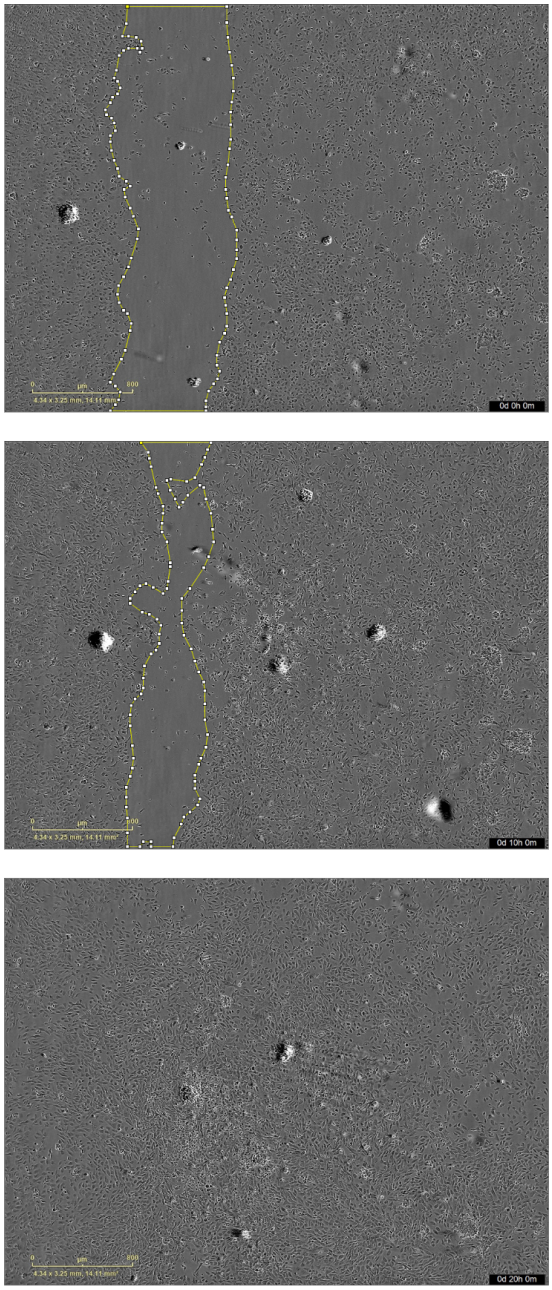


Figure 2.9 Representative images of wound closure rate quantification using ImageJ software. Ca9.22 cells were seeded around a custom designed 3D printed mould to create a zone of exclusion in the cell monolayer. Cells were imaged every two hours using an Incucyte™ S3, the gap size measured using polygonal selection tool in ImageJ software, and the rate of change in gap size calculated using GraphPad Prism.

2.13 ANCHORAGE-INDEPENDENT GROWTH ASSAY

Ca9.22, DOK and TR146 cell lines are adherent cell lines, i.e., cannot exist in suspension but rather, require adherence to a flask. *In vivo*, epithelial cells require adherence to a basement membrane to preserve tissue architecture and cellular function. For such cells to survive in suspension, they must evade a type of cell death known as *anoikis*, which refers to induction of apoptosis upon loss of attachment to the ECM. Cancer cells develop the ability to avoid this type of cell death to circulate the body and metastasise. Therefore, the ability of cells to grow independently of an anchor, such as a flask surface, is indicative of a more cancerous phenotype, and a more aggressive form of tumour. Anchorage-independent growth can be assayed for by coating tissue culture plates with poly-hydroxyethyl methacrylic acid (p-HEMA), an anti-adhesive polymer, and then measuring cell proliferation with AlamarBlue.

24-well plates were coated with 100μl of p-HEMA (12mg/ml) in 95% ethanol or with 95% ethanol alone as a control and dried overnight in a laminar flow hood at RT, twice. Cells were seeded onto the plate and allowed to grow for 24 h. 4h prior to endpoint, AlamarBlue (10% v/v) was added to each well, the plate wrapped in tinfoil and incubated at 37°C. The fluorescence was read on a Spectra MAX Plus Microplate reader at excitation wavelength 530nm and emission 590nm. Values were normalised to cells grown in wells treated with 95% ethanol only as representative of 100% survival.

2.14 FLUORESCENT MICROSCOPY

Coverslips were placed into 12-well plates, washed with ethanol (100%) and the plate/coverslips sterilised under UV light in the laminar flow hood for 15 min. Each coverslip was coated with Poly-D-lysine to promote adhesion of cells onto the coverslip, and the plate incubated at 37°C for 2 h. Coverslips were then washed three times with PBS and either used immediately or stored in the

fridge in PBS. Cells were seeded onto coverslips and once confluent; media was removed, and the cells washed with PBS. Paraformaldehyde (2%) was used to fix the cells for 10 min at RT, then washed three times with PBS. Cells were permeabilised using PBS-0.2% Tween-20 (PBT) (3 x 10 min) then incubated with blocking solution (PBT-0.2%, BSA 5%) for 1 h at RT. Primary antibody (Abcam, ab134188, rabbit recombinant ALDH1A1 antibody, 1:250) was added to cells and incubated overnight at 4°C. Cells were washed in PBT-0.1% (3 x 10 min) and then incubated with secondary antibody (Abcam, ab175471, goat Anti-Rabbit IgG Alexa Fluor® 568, 1:500) in PBT-0.2% and BSA 5% for 3 h at RT in the dark. Cells were washed in PBT-0.1% (3 x 10 min) then stained with DAPI (Merck, 1:1000) in PBS for 10 min at RT in the dark. Cells were then washed once in dH_2O, excess water blotted off with tissue paper and mounted onto microscope slides with the cells facing down using ~5μl VECTASHIELD® Antifade Mounting Medium with DAPI. Edges were sealed using clear nail varnish and slides were either stored at 4°C in the dark or imaged immediately on a Leica SP8 confocal microscope. Fluorescence intensity was quantified using Imaris software (Version 9).

2.15 ADH AND ALDH ENZYME ACTIVITY ASSAY

To assay for ADH and ALDH enzyme activity, cells were seeded in T25 flasks and treated as required. Cells were scraped from the surface of the flask, washed with PBS and centrifuged (1200 RPM, 5 min). The pellets were resuspended in cold assay buffer (0.1M sodium phosphate, 1% Triton-X-100, protease inhibitor cocktail (Roche), pH 8) then centrifuged to remove insoluble material (13,000g, 10 min). Cell lysis was added to a 96-well plate and reaction mix was added — for ADH activity; assay buffer containing 2.4mM NAD, 10% v/v ethanol, and for ALDH activity; assay buffer containing 2.4mM NAD, 10mM acetaldehyde. Absorbance was read at 340nm every 3 min for 30 min total reaction time on a Spectra MAX Plus Microplate reader. For ALDH, the plate was incubated at 30°C while reading absorbance. As a positive control, mouse liver was homogenised in assay buffer and assayed at 5mg/ml.

The previous assays were validated using the ADH or ALDH Activity Assay Kit (Sigma) as per the manufacturer's protocol. The ADH assay kit used isopropanol as a substrate and the ALDH kit used acetaldehyde. NADH standards were prepared as per the protocol and used to plot a standard curve. Absorbance was read on a Spectra MAX Plus Microplate reader at 450nm wavelength every 3 min until the most active samples exceeded the A_{450} of the highest NADH standard (10nM). Following ADH/ALDH assay as per Section 2.15 or using the kit as described here, specific activity was reported as nmole/min/mg based on the protein concentration of each sample, determined by Bradford Assay (Section 2.8.1).

2.16 CYTOKINE ARRAY

To detect inflammatory factors secreted by cells, samples were tested using the Human Inflammation Array C1 (RayBiotech®) according to the manufacturer's protocol. The kit allowed semi-quantitative detection of 20 human proteins – eotaxin I, eotaxin II, GCSF, GM-CSF, IFN-γ, IL-1-α, IL-1β, IL-2, IL-3, IL-4, IL-6, IL-7, IL-8, IL-10, IL-11, IL-12 p40, IL-12 p70, IL-13, I-309 and TIMP-2. Cells were treated as required, before scraping from the surface of the flasks and centrifugation (1200 RPM, 5 min) and the supernatants kept on ice. Membranes were blocked using blocking buffer (30 min, RT) before sample supernatants were pipetted onto the membranes and incubated overnight at 4°C. Samples were aspirated, washed as per kit instructions then incubated with Biotinylated Antibody Cocktail overnight at 4°C. Membranes were washed as before, incubated with HRP-Streptavidin for 2 h at RT, washed again and then transferred onto a plastic sheet. Detection buffer was added on top of the membrane and incubated for 2-5 min at RT before reading on a Chemi-Luminescent gel documentation system (Bio-Rad). Membranes were stored at -20°C for future reference. Signal intensities of blank spots were removed for background subtraction before cytokine spots were normalised to the intensity of positive controls on the same membrane. Data was expressed as fold-increase of signal intensity of the sample compared to the control (media only).

2.17 LACTATE DEHYDROGENASE CYTOTOXICITY ASSAY

CyQUANT™ Lactate Dehydrogenase (LDH) Cytotoxicity assay was used to measure cellular death. LDH is a cytosolic enzyme that is released into culture medium upon damage to cell plasma membranes. Production of extracellular LDH can be quantified via a coupled enzymatic reaction – LDH catalyses the conversion of lactate to pyruvate with NAD^+ as a cofactor which is reduced to NADH. Diaphorase enzymes use NADH to reduce tetrazolium salt to a red formazan product that can be measured at absorbance wavelength 490nm. The level of red formazan is directly proportional to the amount of LDH present. Cells were seeded, treated as required and assayed according to kit instructions. Absorbance was read at 490nm and 680nm as a reference wavelength on a Spectra MAX Plus Microplate reader.

2.18 HUMAN MMP-2 ELISA KIT

Secretion of matrix metalloproteinase-2 (MMP-2) was detected using Human MMP-2 ELISA kit (RayBiotech®) as per the manufacturer's protocol. The kit allowed quantitative detection of MMP-2 from cell culture supernatants. Standards and samples were incubated in the pre-coated ELISA plate for 2.5 h at RT. Biotinylated antibody was added to each well and incubated for 1 h at RT before adding Streptavidin solution and incubating for a further 45 min. 3,3,5,5'-tetramethylbenzidine substrate reagent was added to wells and incubated for 30 min. Stop solution was added to each well and absorbance was read using a Spectra MAX Plus Microplate reader at 450nm wavelength. The concentration of MMP-2 in each sample was calculated using a standard curve generated on GraphPad Prism.

2.19 INVASION ASSAY

The invasion assay is a measure of metastatic potential of cells *in vitro*. Cells that demonstrate an increased capacity to invade through an artificial matrix towards a chemoattractant gradient are

considered to have a better-equipped phenotype for migration, invasion, and metastasis. Invasion assays were performed using 8μM pore-size Corning® Transwell® chambers with a polyethylenterephthalate (PET) membrane in 24-well plates. ECM gel from Engelbreth-Holm-Swarm murine sarcoma was thawed overnight at 4°C, diluted 1:1 with DMEM, added to the inserts and incubated for 1 h at 37°C to allow polymerisation. Cells were seeded onto the inserts in serum-free media with 10% serum media in the bottom chamber to create a chemoattractant gradient. Cells were treated as required and incubated for 24 h. Media was discarded, and inserts washed twice in PBS. Cells were fixed with ethanol (70%, 15 min at RT) then inserts allowed to air dry. Crystal violet (0.5% w/v) was added to inserts and incubated for 10 min at RT, protected from light. Crystal violet stain was removed, inserts washed twice with PBS and cotton buds were used to remove excess dye and any cells that did not invade through the ECM. Photographs of invaded cells stained with crystal violet were taken using a phase-contrast Olympus IX81. Acetic acid (10% v/v) was added to inserts to solubilise crystal violet dye and incubated at RT for 10 min. Elution of acetic acid was added to a 96-well plate in triplicate and absorbance read at 600nm on a Spectra MAX Plus Microplate reader.

2.20 KNOCKDOWN OF ALDH1A1 USING SIRNA

Small interfering RNA (siRNA) molecules were obtained from Cohesion Biosciences and Eurofins Genomics. From Cohesion Biosciences three different target-specific siRNA oligo duplexes of lyophilised siRNA for ALDH1A1 (CRH0151, SwissProt: POO352) and GAPDH (CRH1731. SwissProt: P04406) were resuspended in DEPC water and pooled together to achieve knockdown of the target gene. Negative controls included non-targeting or 'scrambled' versions of the targeted siRNA duplexes. Identical oligonucleotide sequences of siRNAs were obtained from Eurofins Genomics for ALDH1A1 (CGGGAAAAGCAAUCUGAAGAGGG) and GAPDH (AUUCCAUGGCACCGUCAAG) with a fluorescent tag of Cyanine5 at the 5' end. A non-specific negative control was also used, which did

not significantly associate with any human proteins according to a BLAST search (31% GC content, UAAUGUAUUGGAACGCAUA).

Targeting siRNA was transfected using either Lipofectamine™ 2000 or Polyethylenimine (PEI) MAX (1mg/ml). Cells were cultured without penicillin/streptomycin antibiotics. For PEI transfection, PEI (3μl per well in 6-well plates) was diluted in Opti-MEM™ reduced serum media (100μl per well in 6-well plate) and incubated for 5 min at RT. Oligomers of siRNA (50-100nM) or cDNA (pEGFP, 1-4μg) were combined with PEI and incubated for 15 min at RT. PEI:oligomer complexes were added to cells in Opti-MEM™ media and after 4 h, transfection media was replaced with full serum media, and cells were cultured for another 24 h before treatment or analysis. For Lipofectamine™ 2000, siRNA oligomer-Lipofectamine™ 2000 complexes were diluted in Opti-MEM™ reduced serum media and incubated at RT for 20 min, before adding to cells in fresh Opti-MEM™ media (50-100nM siRNA, 5μl Lipofectamine™ 2000 per well in 6-well plates). After 4 h, transfection media was replaced with full serum media, and cells were cultured for another 48-72 h before treatment or analysis.

2.21 STATISTICS

Data are represented as either mean ± SD or ± SEM. The 'n' number refers to number of technical replicates completed. Experiments included triplicates of each sample or condition, unless otherwise stated, and these biological replicates did not contribute to 'n'. Statistical analysis was carried out on GraphPad Prism software (Version 10). Unpaired t-tests were performed to analyse samples of two different groups. One-way ANOVA followed by a Tukey post hoc test was used for multiple comparisons for samples with more than two groups. Two-way ANOVA followed by a Tukey post hoc test was used for multiple comparisons for samples with more than two group that were split into multiple variables.

Chapter 3: Ethanol Modulates ALDH Enzymes and EMT Processes in Oral Cell Lines

3.1 Introduction

OC can affect the lips, gums, tongue, lining of the cheeks, and the floor or roof of the mouth. OC typically affects the squamous cells of the oral cavity. These thin, flat cells are found in the outer layer of epithelium line the oral cavity. Malignant disorders preceding OC can be categorised by the degree of dysplasia – a high degree of dysplasia denotes a decrease in well-differentiated cells while the number of immature or poorly differentiated cells increases (24, 25). In this study, two OSCC cells lines originating from gingiva and buccal tissue (Ca9.22 and TR146 respectively) and a dysplastic cell line (DOK) isolated from the dorsal tongue will be cultured *in vitro* to study the effects of chronic or long-term alcohol consumption on cell characteristics.

While there are a number of studies utilising ethanol exposure *in vitro*, the concentration used varies depending on cell model used. Ranges of 10-100mM ethanol have been used on cell lines in other studies (147, 161-163). Physiological concentrations are difficult to define - *in vivo*, alcohol may evaporate from the oral cavity, be ingested and metabolised, and the corresponding blood alcohol level does not always equate to exact concentration of alcohol consumed (164). *In vitro*, evaporation from cell culture vessels, as well as tolerance of the cell line being utilised must be taken into consideration. Acetaldehyde production may also occur depending on the ADH/ALDH activity of cell lines. *In vivo*, acetaldehyde production would be aided by microbial species that possess active ADH enzymes (157). Therefore, the aims of this chapter are to determine a working concentration of both ethanol and acetaldehyde that maintain enough cell viability for ensuing assays as well as being biologically relevant. The effect of ethanol and acetaldehyde on protein expression and downstream cellular characteristics will also be investigated.

3.2 AIMS

- Characterise the activity and expression profiles of ADH and ALDH isoforms in three different oral cell lines – dysplastic oral mucosa DOK, gingival squamous cell carcinoma Ca9.22 and buccal squamous cell carcinoma TR146.
- Investigate the effect of ethanol or acetaldehyde exposure on expression and activity of these enzymes, cell proliferation, cell death and ROS production.
- Determine the effect of chronic ethanol or acetaldehyde exposure on transformative and metastatic characteristics of oral cells.

3.3 Ethanol and acetaldehyde had dose- and time-dependent effects on oral cell lines

To establish an *in vitro* model of ethanol exposure to oral cell lines, Ca9.22, DOK and TR146 cells were treated with a range of concentrations of ethanol (0.5-5% v/v) for 4, 24 or 48 h and viability was tested using the AlamarBlue assay. In addition, cell culture media was assayed for ethanol at various timepoints to determine if the evaporation of ethanol from cell culture wells was significant. When ethanol was added to flasks with cell culture media only, in the absence of cells there was a significant loss of ethanol by 24 h compared to initial concentration (**** $P < 0.0001$, **Figure 3.1A**). From 24 h to 48 h however, there was no significant decrease, showing that most of the ethanol added evaporated from the flask by 24 h. When ethanol was assayed from culture flasks containing oral cell lines, a similar result was seen (**Figure 3.1B**), confirming that the cell lines do not significantly increase ethanol clearance from culture flasks.

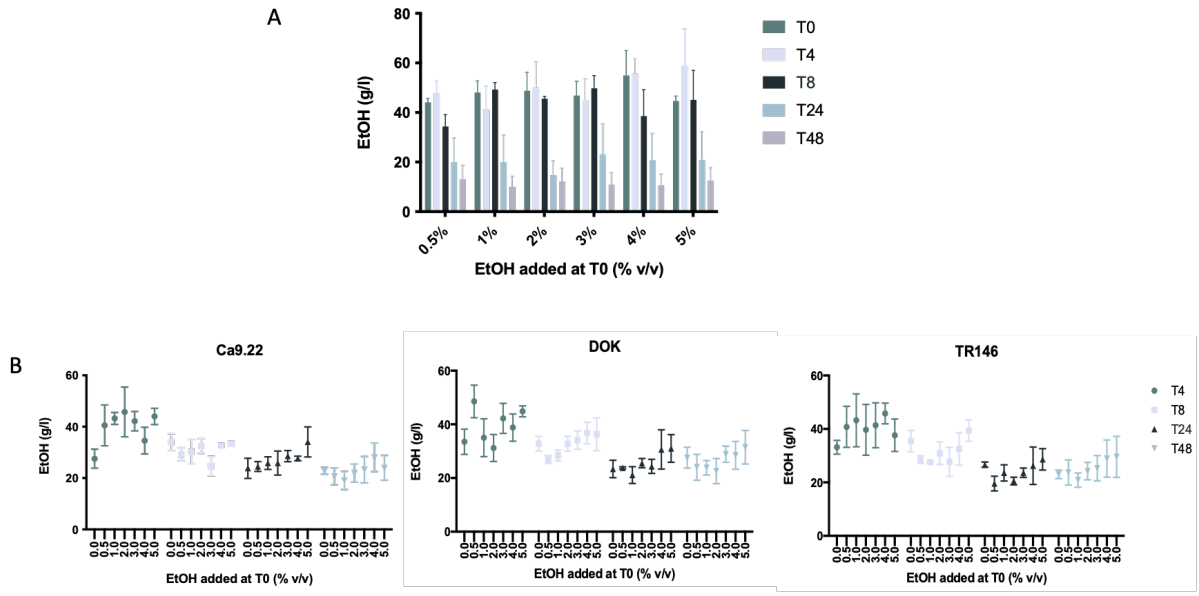


Figure 3.1. (A) Ethanol evaporation from cell culture media only or from (B) Ca9.22, DOK and TR146 cell culture flasks over 48 h. Cell culture media containing ethanol (0-5% v/v) was added to cell culture media alone or to flasks containing oral cell lines. Ethanol content was assayed using Megazyme Ethanol Assay Kit®. Absorbance was read on a Spectra MAX Plus Microplate reader at wavelength 340nm. Statistical analysis was performed by two-way ANOVA followed by Tukey's multiple comparisons tests. Data shown n=3, mean ± SEM.

Ethanol and acetaldehyde affected oral cell viability in a time-, dose- and cell line-dependent manner. Cell lines were treated for 4, 24 and 48 h and AlamarBlue assay was used to measure cell proliferation. Flow cytometry was used to measure cell death via apoptosis. All three cell lines showed significant cell death at ethanol concentrations ≥3% v/v, however DOK cells were the most resistant overall to ethanol treatments. TR146 cells were the most sensitive to ethanol treatments (Figure 3.2).

Oral cell viability in response to acetaldehyde treatments varied greatly. Ca9.22 cells were the most significantly affected by acetaldehyde, with concentrations of ~200μM significantly affecting viability (* $P < 0.05$) and almost no viable cells remaining at concentrations ≥450μM (**** $P < 0.0001$). DOK and TR146 cells were significantly more resistant to acetaldehyde treatments, with decreased viability and significant cell death only occurring at higher concentrations of ≥600μM (Figure 3.2).

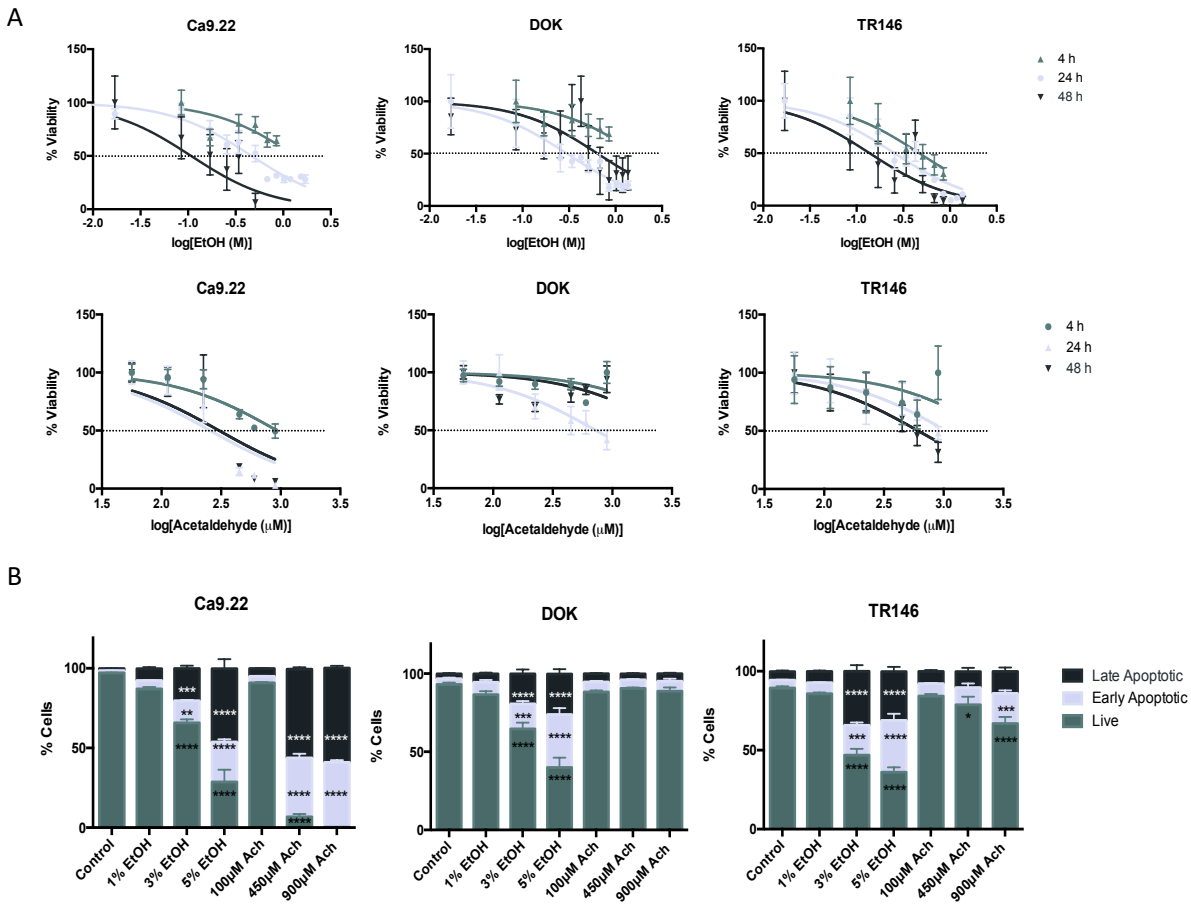
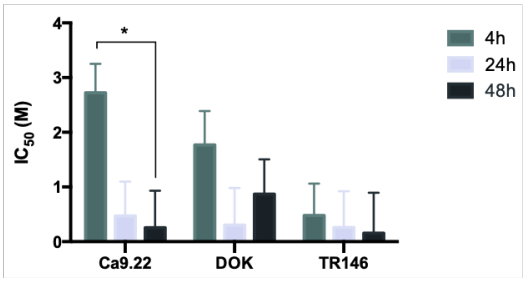


Figure 3.2. Ethanol and acetaldehyde have discrete, time-dependent effects on viability of OC and dysplastic cell lines. (A) Ca9.22, DOK and TR146 cells were treated with ethanol (0-8% v/v) or acetaldehyde (Ach, 50-900μM) for the required timepoints. 4 h prior to endpoint, AlamarBlue reagent was added (10% v/v). Absorbance was read on a Spectra MAX Plus Microplate reader at wavelengths 570nm/600nm. The dotted line represents IC_{50} value determination. Data shown n=3, mean ± SEM. **(B)** Cells were treated as previously stated then stained with Annexin V and PI prior to analysis on a BD Accuri C6 flow cytometer (488nm excitation). Data shown n=3, mean ± SEM. EtOH = ethanol, Ach = acetaldehyde. Significance is shown with respect to the control. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001, ns = not significant.

The previously calculated IC_{50} values (**Figure 3.2A**) confirmed that Ca9.22 cells tolerated high levels of ethanol at short timepoints but demonstrated a significant decrease in IC_{50} at 48 h (1.4% ±1.17) compared to 4 h (16% ±1.19) (**Figure 3.3**). TR146 cells were the most sensitive to ethanol with the lowest IC_{50} at all timepoints used compared to the other two cell lines. DOK cells demonstrated a level of recovery from ethanol treatments, whereby the IC_{50} decreased from 10.4% ±1.38 at 4 h to 1.8% ±1.18 at 24 h, but then increased again to 5% ±1.27 at 48 h. Ca9.22 cells were the most sensitive to acetaldehyde, with the lowest IC_{50} at all timepoints used compared to the other two cell lines. DOK cells were the most resistant to acetaldehyde of the cell lines used. A similar trend was seen for acetaldehyde treatment in DOK cells as with ethanol, where a level of recovery was observed from 24 to 48 h, and IC_{50} increased from 0.65M ±1.27 to 2.91M ±1.36 respectively (**Figure 3.3**).

EtOH

Cell line	4 h			24 h			48 h		
	Moles/L	% v/v	SD	Moles/L	% v/v	SD	Moles/L	% v/v	SD
Ca9.22	2.72	16%	1.19	0.47	2.7%	1.09	0.25	1.4%	1.17
DOK	1.77	10.4%	1.38	0.30	1.8%	1.18	0.87	5%	1.27
TR146	0.48	2.8%	1.30	0.26	1.5%	1.14	0.16	0.9%	1.28



Acetaldehyde

Cell line	4 h		24 h		48 h	
	Moles/L	SD	Moles/L	SD	Moles/L	SD
Ca9.22	0.93	1.15	0.25	1.24	0.29	1.31
DOK	4.97	1.38	0.65	1.27	2.91	1.36
TR146	2.52	1.87	1.01	1.38	0.53	1.33

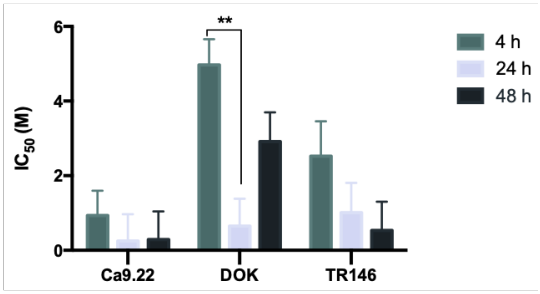


Figure 3.3. Comparison of IC_{50} values for ethanol and acetaldehyde in Ca9.22, DOK and TR146 cell lines. IC_{50} values were extrapolated from **Figure 3.2A** for ethanol and acetaldehyde treatments. Data shown n=3, mean ± SEM. Statistical analysis was performed by two-way ANOVA followed by Tukey's multiple comparisons tests. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001, ns = not significant.

3.4 ETHANOL DID NOT AFFECT ROS PRODUCTION IN ORAL CELL LINES

Cells were treated as before with either ethanol (0-5% v/v) or acetaldehyde (50-900μM) for 0.5, 1, 4, 24 or 48 h and ROS production was measured using DCF-DA assay. Ethanol did not significantly affect production of intracellular ROS in Ca9.22, DOK and TR146 cell lines (**Figure 3.4**). Conversely, acetaldehyde significantly increased ROS production in the initial hour in all three cell lines, before a significant decrease in ROS production was noted. Initial increase in ROS production was observed at concentrations ≥100μM for Ca9.22 and TR146 cells and ≥450μM in DOK cells (**Figure 3.4**). The subsequent decrease in ROS production is likely attributable to cell death occurring, as shown by previous flow cytometry experiments (**Figure 3.2B**).

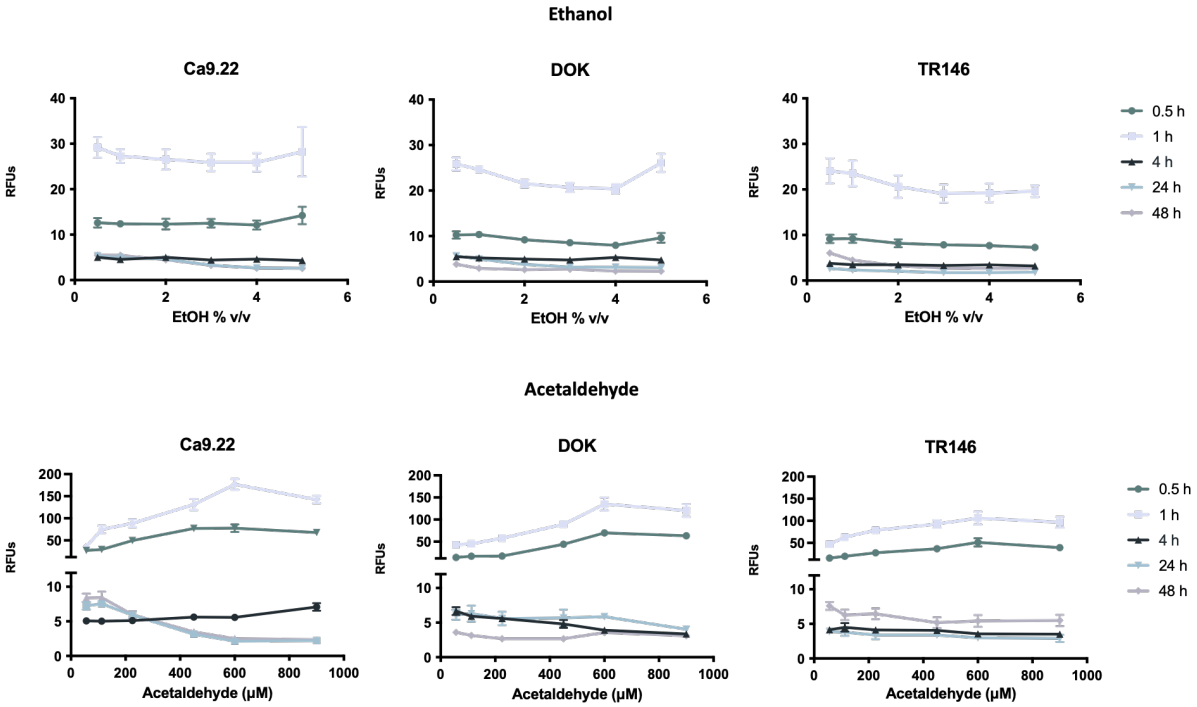


Figure 3.4. Ethanol did not affect ROS production, while acetaldehyde caused a significant initial increase in ROS in Ca9.22, DOK and TR146 cells. Cells were seeded in black plates, treated as required, then stained with DCF-DA before reading fluorescence on a Spectra MAX Plus Microplate reader, at excitation 490nm and emission 529nm. RFUs = relative fluorescent units. Data shown n=4, mean ± SEM. Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparisons tests.

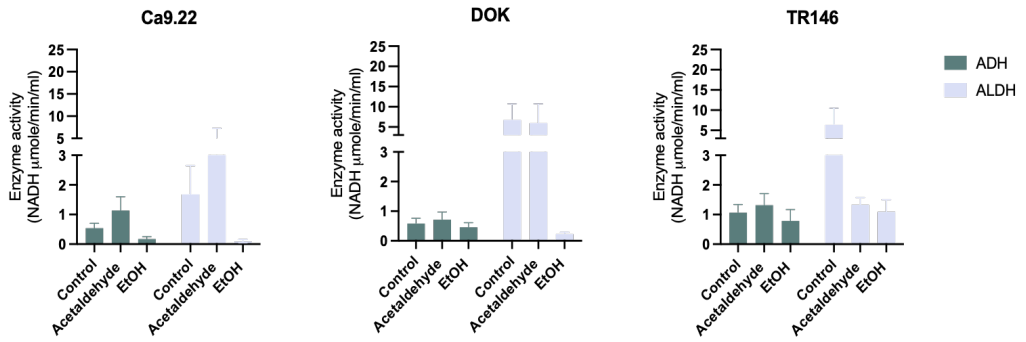
3.5 ETHANOL DECREASED ALDH ACTIVITY IN ORAL CELL LINES

Cells were treated with ethanol (1% v/v) or acetaldehyde (100μM) for 24 h before assaying for ADH and ALDH enzyme activity. All three oral cell lines possess ADH and ALDH activity, with ALDH activity being generally higher (**Figure 3.5A**). Exposure to low levels of acetaldehyde (100μM) did not significantly affect ADH or ALDH activity in Ca9.22 and DOK cells but decreased ALDH activity in TR146 cells. Conversely, ethanol appeared to dramatically decrease ALDH activity in all oral cell lines (**Figure 3.5A**).

To control for the effects of ethanol and acetaldehyde metabolism products, the specific activity of ADH and ALDH for each cell line was used to calculate the approximate maximal concentration of acetaldehyde and acetate that would be produced under experimental conditions. When treated with ethanol 3% v/v, Ca9.22, DOK and TR146 cell lines could hypothetically produce 64.3μM, 76.2μM and 102μM acetaldehyde respectively (**Figure 3.5B**). Therefore, the use of acetaldehyde 100μM as a control means any effect ethanol treatments have on cells is attributable to ethanol itself and not its by-products. Acetate is a mostly harmless molecule to cells, and at the highest concentration of acetaldehyde used (900μM), the production of acetate was calculated to not exceed 91.4μM under the experimental conditions employed (**Figure 3.5B**).

Cells were chronically treated in ethanol (1% v/v, 2 wk) and their viability tested using AlamarBlue assay. Chronic ethanol treatment did not significantly affect viability or proliferation of oral cell lines (**Figure 3.5C**). Protein concentration was determined via Bradford assay and the specific activity of the enzymes calculated. Chronic ethanol exposure decreased the activity of both ADH and ALDH activity in all three oral cell lines (**Figure 3.5D**).

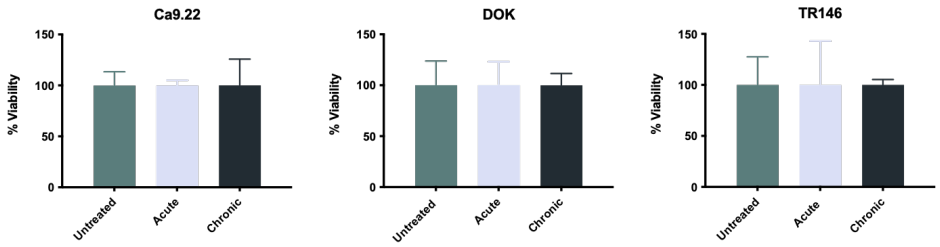
A



B

Cell line	ADH activity (µmol/min/mg protein) mean ± SD	Acetaldehyde (µM) derived from EtOH 3% v/v over 4 h	ALDH activity (µmol/min/mg protein) mean ± SD	Acetate (µM) derived from acetaldehyde 900µM over 4 h
Ca9.22	0.89 ± 0.688	64.3	1.66 ± 3.377	35.9
DOK	1.06 ± 1.156	76.2	3.18 ± 4.588	68.6
TR146	1.42 ± 1.256	102	4.23 ± 6.281	91.4

C



D

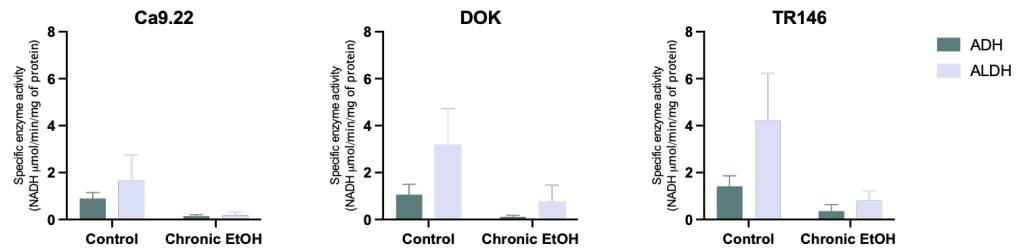


Figure 3.5. (A) The effect of ethanol and acetaldehyde on ADH and ALDH enzyme activity of Ca9.22, DOK and TR146 cells. Cells were treated with ethanol (1% v/v) or acetaldehyde (100µM) for 24 h before lysing in assay buffer (0.1M sodium phosphate, 1% Triton-X-100, protease inhibitor cocktail (Roche), pH 8). Reaction mix, which consisted of assay buffer as before, and for ADH activity 10% ethanol and 2.5mM NAD, or for ALDH activity 10mM acetaldehyde and 2.5mM NAD, was added to each well. Absorbance was read on a Spectra MAX Microplate reader at 340nm every 3 min for 30 min at RT for ADH and at 30°C for ALDH to measure the production of NADH. **(B) Acetaldehyde and acetate produced by oral cell lines when treated with ethanol or acetaldehyde based on specific enzyme activity.** Using specific activity of ADH and ALDH enzymes calculated from **Figure 3.5A**, the approximate maximum amount of acetaldehyde or acetate that would be produced by oral cell lines under experimental conditions was calculated. **(C) Neither acute nor chronic ethanol treatment significantly affected viability and proliferation of oral cell lines.** Cells were treated in ethanol (1% v/v) for 24 h (acute) or 2 wk (chronic) before viability was tested using AlamarBlue assay as previously described. **(D) Chronic ethanol treatment decreased specific activity of ADH and ALDH in Ca9.22, DOK and TR146 cell lines.** Protein concentration was determined via Bradford assay and the specific enzyme activity calculated for ADH and ALDH. Data shown n=3-6, mean ± SEM. Statistical analysis was performed by two-way ANOVA followed by Tukey's multiple comparisons tests.

3.6 CHRONIC ETHANOL TREATMENT MODULATED ACETALDEHYDE METABOLISM IN DYSPLASTIC ORAL CELL LINES

To examine the effect of chronic ethanol treatment on acetaldehyde metabolism, cells were treated in chronic ethanol as before (1% v/v, 2 wk) and then treated with several concentrations of acetaldehyde (50-900μM) and proliferation determined using AlamarBlue assay. Ca9.22 cells pre-treated with chronic ethanol remained very sensitive to acetaldehyde and viability decreased in a dose-dependent manner. TR146 cells pre-treated with chronic ethanol also showed very little change in their responses to acetaldehyde. However, DOK cells pre-treated with chronic ethanol proliferated significantly more in the presence of acetaldehyde (** $P < 0.01$, **Figure 3.6**).

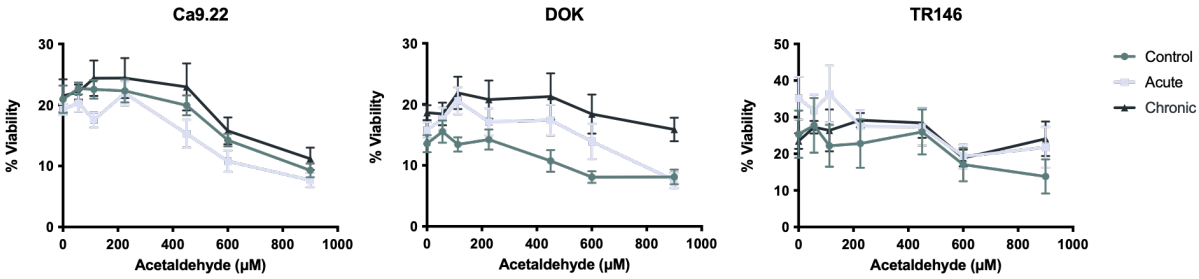
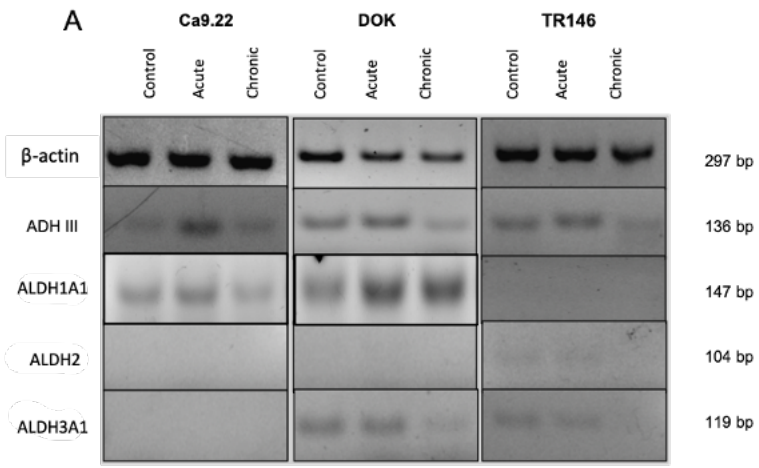


Figure 3.6. Proliferation and viability of Ca9.22, DOK and TR146 cells treated with acetaldehyde (50-900μM) following chronic (2 wk) ethanol exposure. Cells were chronically treated in ethanol (1% v/v, 2 wk) before treatment with acetaldehyde (50-900μM) for 24 h and viability measured using AlamarBlue as previously described. Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple comparisons tests. Data shown n=3, mean ± SEM.

3.7 ORAL CELL LINES POSSESSED UNIQUE EXPRESSION PROFILES OF ADH AND ALDH

RT-PCR was used to detect expression of ADH class I, II, III, IV and ALDH1A1, ALDH2 and ALDH3A1. Ca9.22, DOK and TR146 cell lines were all found to express the ubiquitous ADH class III, whereas ADH class I, II and IV were not detected (data not shown). Each oral cell line was found to have unique expression profiles of ALDH isoforms (**Figure 3.7A, 3.7B**). Of the ALDH isoforms investigated, Ca9.22 cells were shown to express ALDH1A1 only, DOK cells expressed ALDH1A1 and ALDH3A1 and TR146 cells expressed ALDH2 and ALDH3A1. The mRNA of ALDH2 and ALDH3A1 was shown to decrease following acute and chronic ethanol exposure in both DOK and TR146 cells. Conversely, in both cancer cell lines Ca9.22 and dysplastic cell line DOK, the mRNA of ALDH1A1 was shown to be increased following ethanol exposure (**Figure 3.7C**).



B

	Location in oral cavity	ADH III	ALDH1A1	ALDH2	ALDH3A1
Ca9.22	Gingiva	✓	✓	✖	✖
DOK	Tongue	✓	✓	✖	✓
TR146	Buccal	✓	✖	✓	✓

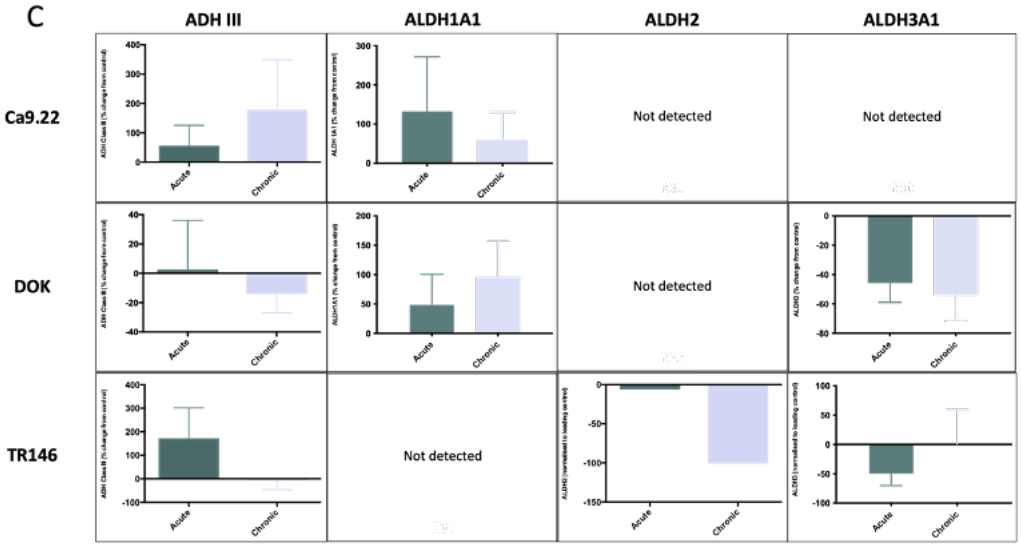


Figure 3.7 (A) Agarose gel electrophoresis of PCR amplified products using specific primers for ADH class III, ALDH1A1, ALDH2 and ALDH3A1. Cells were treated in ethanol (1% v/v) either acutely (24 h) or chronically (2 wk) before RT-PCR was carried out using specific primers for ADH Class I, II, III, IV and ALDH1A1, ALDH2 and ALDH3A1. Amplified DNA products were separated using agarose gel (2%) and β-actin was used as a loading control. No cell lines were found to express ADH class I, II or IV (results not shown). Blot images shown are representative of experiments done in triplicate. **(B) Summary of ADH/ALDH mRNA expression profiles of Ca9.22, DOK and TR146 cells and their tissue of origin within the oral cavity. (C) The percentage change from control levels of mRNA detected in ethanol treated Ca9.22, DOK and TR146 cells following acute or chronic ethanol exposure.** Densitometry was used to normalise mRNA levels to the loading control and expressed as percentage increase or decrease from the untreated control levels. The change in mRNA following acute or chronic ethanol treatment was not significant in any cell line. Data shown n=3-4, mean ± SEM.

3.8 Chronic ethanol treatment modulated protein expression of ALDH1A1 in Ca9.22 and DOK cells

Based on mRNA data of ALDH1A1 in Ca9.22 and DOK cells, further analysis of protein expression was examined using immunofluorescence (IF), flow cytometry and western blot. ALDH1A1 was initially examined by IF microscopy in DOK cells and demonstrated that expression of ALDH1A1 was significantly reduced with chronic ethanol treatment (* $P < 0.05$) compared to untreated and acutely treated cells (**Figure 3.8C**). However, these IF microscopy experiments yielded low throughput results, likely due to relatively low expression of ALDH1A1 in the cell lines used. IF microscopy only allowed for a small number of cells to be examined per experiment (approximately 25 cells per image), so following investigation of ALDH1A1 via IF in DOK cells (with hepatocellular carcinoma cell line HepG2 used as a positive control, **Figure 3.8A**), subsequent experiments focused on higher throughput experiments like flow cytometry (**Figure 3.9A, 3.9B**).

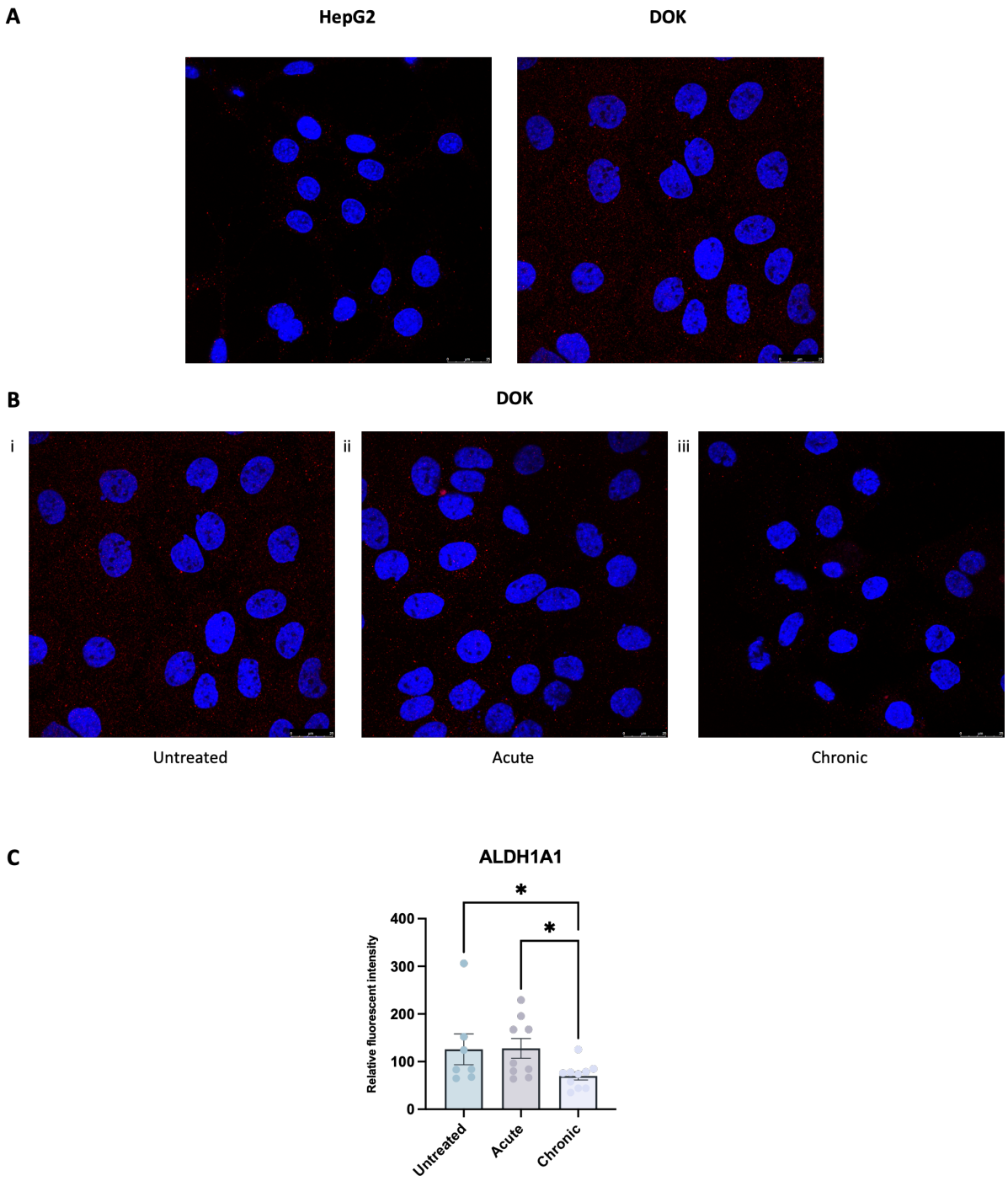


Figure 3.8 (A) Positive staining for ALDH1A1 (red) in HepG2 cells and DOK cells. (B) ALDH1A1 immunofluorescence in (i) untreated DOK cells (ii) DOK cells treated with ethanol for 24 h (acute exposure) or (iii) 2 wk (chronic exposure). DAPI was used to stain the nuclei blue, cytosolic ALDH1A1 was stained red using Alexa Fluor® 568 and the images merged. Images are representative of an experiment done in triplicate. **(C) ALDH1A1 expression decreased with chronic ethanol exposure in DOK cells.** Fluorescent intensity of images from **(B)** were quantified using Imaris software (Version 9) and graphed. Data shown n=3, mean ± SD. * $P < 0.05$.

Flow cytometry was used to look at ALDH1A1 protein expression in both Ca9.22 and DOK cells using a live cell dye. Chronic ethanol exposure significantly decreased expression of ALDH1A1 in DOK cells (* $P < 0.05$, **Figure 3.9B**), which confirmed previous findings from IF microscopy (**Figure 3.8C**). Conversely, chronic ethanol exposure significantly increased expression of ALDH1A1 in Ca9.22 cells (** $P < 0.01$, **Figure 3.9A**). Chronic exposure had a more significant impact on expression of ALDH1A1 compared to acute exposure, showing a time-dependent change in expression levels.

Western blot analysis was also used to look at expression of ALDH1A1. While no significant changes were detected via densitometry, an increase in ALDH1A1 expression was observed in Ca9.22 cells (**Figure 3.9C**). No significant changes were detected via Western blot in DOK cells. This may be because of overall lower expression levels of this protein (**Figure 3.9D**).

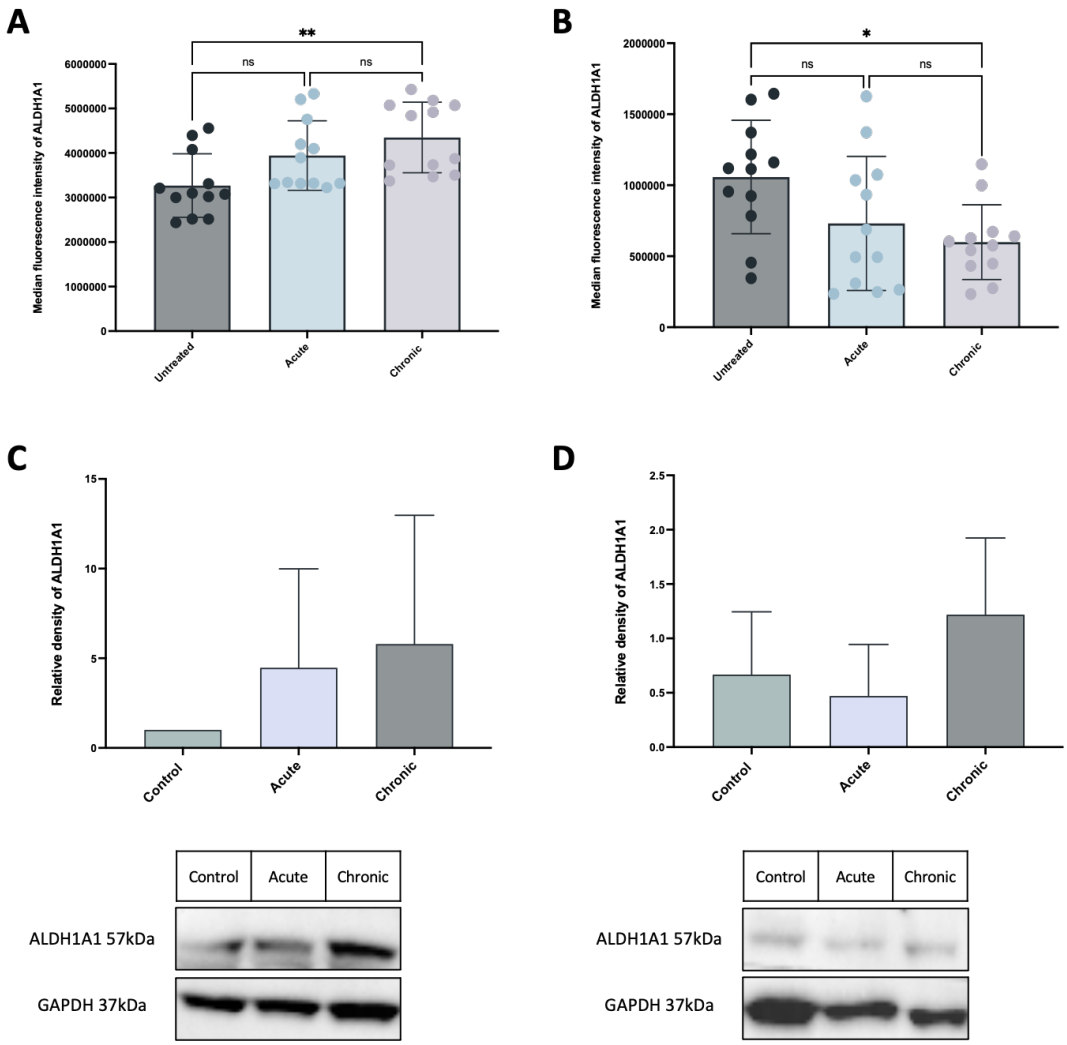


Figure 3.9 (A) Chronic ethanol treatment significantly increased fluorescent intensity of ALDH1A1 protein expression via flow cytometry in Ca9.22 cells (B) and significantly decreased fluorescence of ALDH1A1 protein expression in DOK cells. Cells were treated in ethanol as previously described, stained with Green-AIDeSense ALDH1A1 live cell dye before analysis on a BD Accuri C6 flow cytometer (488nm excitation). Live cells were gated and median fluorescent intensity of ALDH1A1 was graphed. Data shown n=4, mean ± SD. **(C) Chronic ethanol treatment appeared to increase expression of ALDH1A1 via Western blot in Ca9.22 cells (D) while no significant changes were observed in DOK cells.** Densitometric analysis was carried out using ImageJ software and normalised to the loading control GAPDH. Overall, Ca9.22 cells showed a higher expression of ALDH1A1 compared to DOK cells. Data shown n=3, mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns = not significant.

3.9 CHRONIC ETHANOL TREATMENT DID NOT AFFECT MORPHOLOGY OF ORAL CELL LINES

To further examine the effect of chronic ethanol exposure on oral cells, cells were imaged using an Olympus IX81 phase-contrast microscope following chronic ethanol treatment as before. Approximate cell size and granularity were measured using forward-scatter (FSC) and side-scatter (SSC) on a flow cytometer. Neither acute nor chronic ethanol treatment affected morphology of Ca9.22, DOK or TR146 cells according to visual examination, or cell size and granularity as measured by relationship of FSC to SSC (**Figure 3.10**).

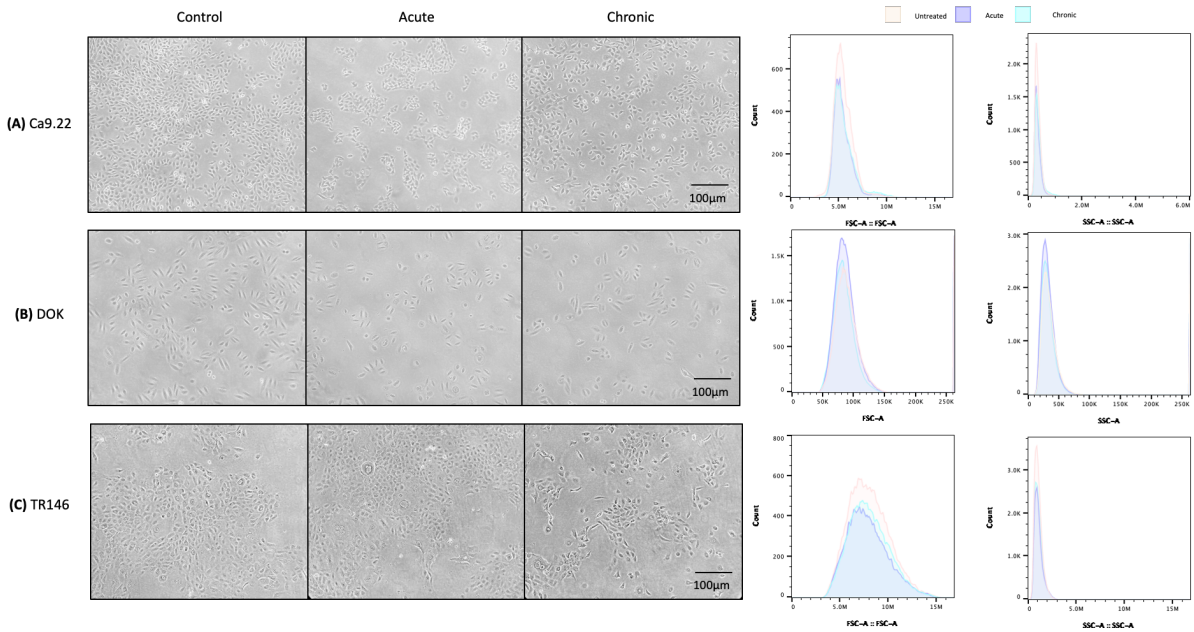


Figure 3.10. Chronic ethanol treatment did not affect morphology of oral cell lines. (A) Ca9.22, **(B)** DOK and **(C)** TR146 cells were chronically treated in ethanol (1% v/v, 2 wk), imaged using an Olympus IX81 phase-contrast microscope and subsequently stained with PI before analysis on a flow cytometer. Both the representative images and the overlapping mean fluorescence intensity peaks of FSC/SSC show that no changes to cell size or granularity were observed between control and ethanol-treated cells. Data shown n=3.

3.10 Chronic ethanol significantly increased proliferation and migration of Ca9.22 and DOK cells in scratch assays

Oral cell lines were tested for their proliferative and migratory capacities using scratch assays. Chronic ethanol treatment significantly increased the rate of wound closure in both Ca9.22 and DOK cells compared to untreated controls (**Figure 3.11A, 3.11B**). The same effect was not noted in TR146 cells, where chronic ethanol treatment significantly decreased the rate of wound closure, likely due to cell death, as they were previously shown to be the most sensitive to ethanol treatments (**Figure 3.3, Figure 3.11C**). Overall, Ca9.22 cells had the fastest rate of wound closure, with untreated cells closing the gap in the cell monolayer after ~30 h. DOK and TR146 cells did not always completely close the monolayer gap over the 48 h period analysed.

To confirm that the results of the scratch assays were from ethanol exposure and not due to production of acetaldehyde, oral cell lines were tested for their proliferative and migratory capacities following short term exposure to acetaldehyde (30-180μM, 24 h) using scratch assays. Overall, short term exposure to acetaldehyde did not affect the rate of wound closure of Ca9.22 or DOK cells (**Figure 3.12A, 3.12B**). However, at ≤90μM acetaldehyde the rate of wound closure of both Ca9.22 and TR146 cells was significantly increased (**Figure 3.12A, 3.12C**). Higher concentrations did not affect Ca9.22 cells but significantly decreased the rate of wound closure in TR146 cells, likely due to cell death occurring.

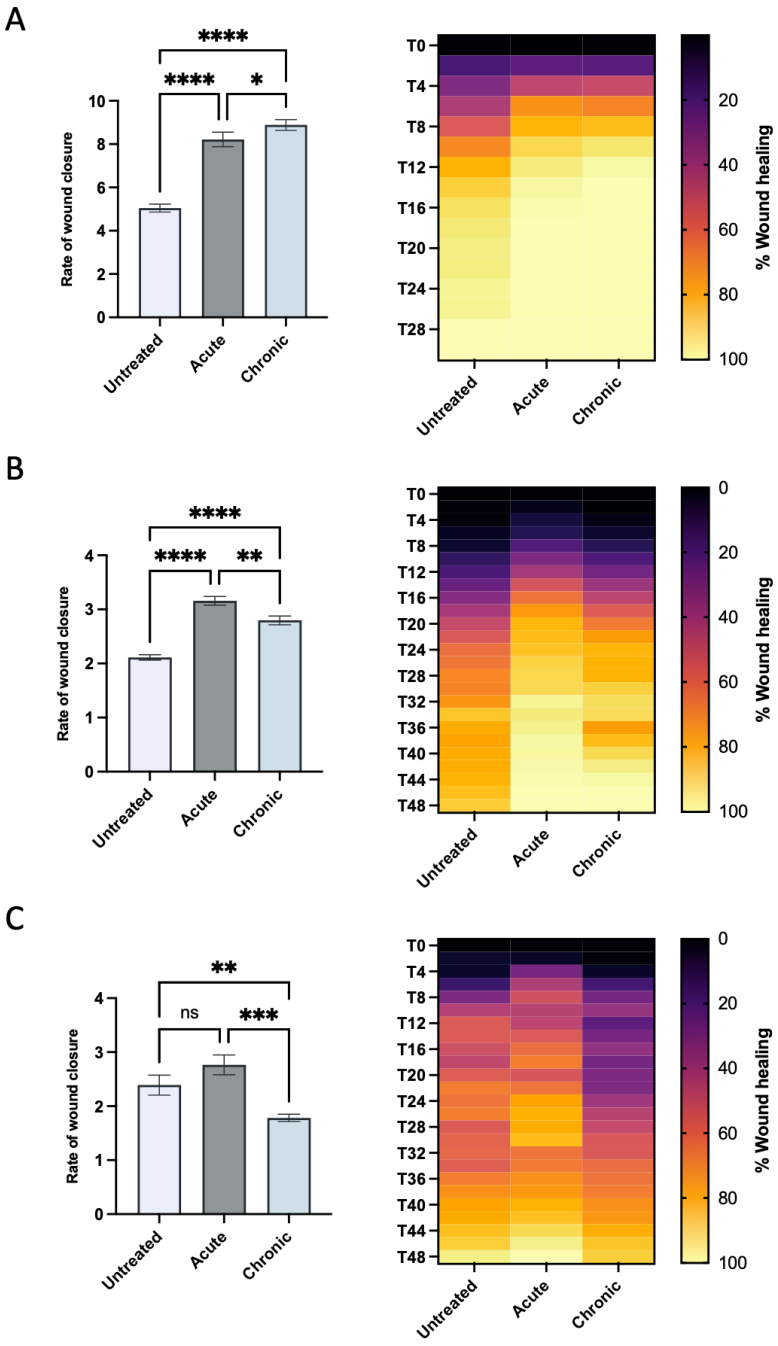


Figure 3.11. Chronic ethanol treatment significantly increased the rate of wound closure in scratch assays in both (A) Ca9.22 cells and (B) DOK cells, but significantly decreased the rate of wound closure in (C) TR146 cells. Cells were treated with ethanol (1% v/v) either acutely (24 h) or chronically (2 wk) before seeding around a 3D printed mould to create a zone of exclusion in the monolayer. The rate of closure of the monolayer 'wound' was quantified and the rate of closure calculated. Data shown n=3, mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns = not significant.

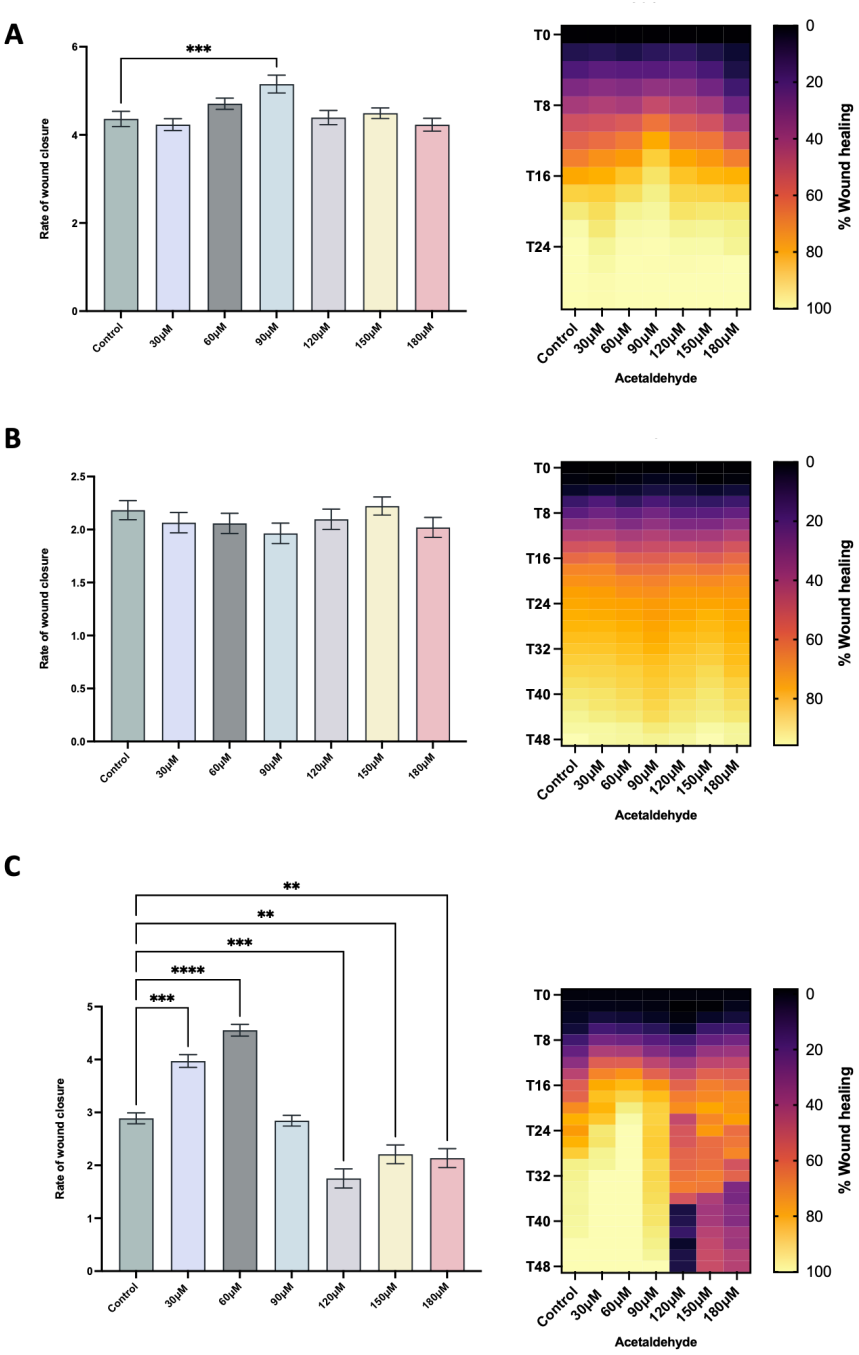


Figure 3.12 (A) Acetaldehyde (90μM) significantly increased the rate of wound closure in Ca9.22 cells. (B) Acetaldehyde (30-180μM) did not affect the rate of wound closure in DOK cells. (C) Acetaldehyde (≤90μM) significantly increased the rate of wound closure in TR146 cells, but higher concentrations significantly impaired rate of wound closure. Cells were treated with a range of concentrations of acetaldehyde (30-180μM) before seeding around a 3D printed mould to create a zone of exclusion in the monolayer. The rate of closure of the monolayer 'wound' was quantified and the rate of closure calculated. Data shown n=3, mean ± SD. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001, ns = not significant.

Using previously calculated concentrations of acetaldehyde produced by cells (**Figure 3.5B**) and based on the increase in scratch closure rates in Ca9.22 at 90μM (**Figure 3.12A**), cells were treated in chronic acetaldehyde (100μM, 2 wk). Viability and scratch assay rates were investigated in cells treated in chronic acetaldehyde.

In Ca9.22 cells, chronic acetaldehyde (100μM, 2 wk) had no significant effect on viability or rate of wound closure (**Figure 3.13A**). In DOK cells, viability was not affected but the rate of wound closure was significantly slower when treated with acetaldehyde; **** $P < 0.0001$ for acute exposure and * $P < 0.05$ for chronic exposure (**Figure 3.13B**). Therefore, the increase in wound closure rates observed following chronic ethanol treatment (**Figure 3.11**) was not because of exposure to acetaldehyde.

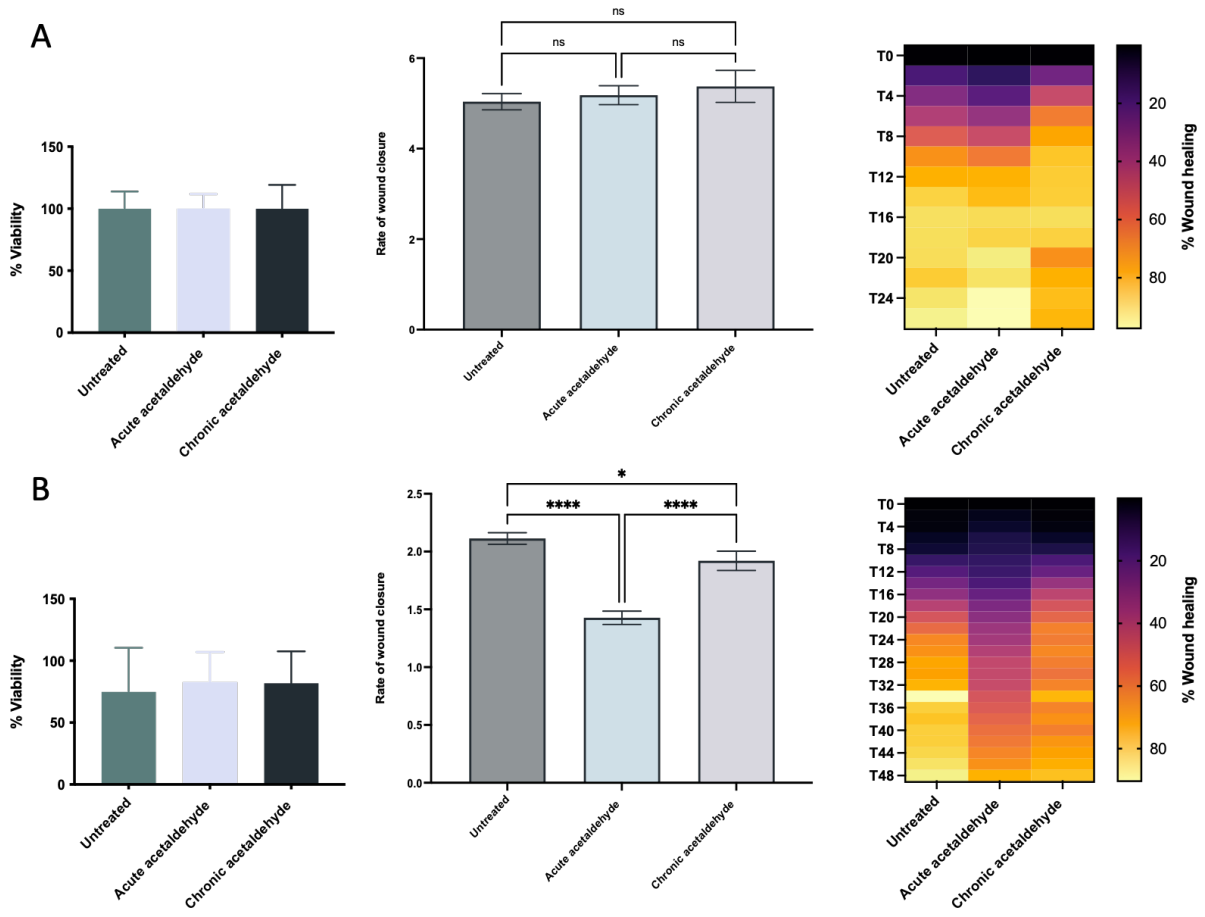


Figure 3.13. Acute and chronic acetaldehyde did not affect viability of (A) Ca9.22 or (B) DOK cells. Acetaldehyde had no effect on the rate of wound closure in (A) Ca9.22 cells, but significantly decreased rate of wound closure in (B) DOK cells. Ca9.22 and DOK cells were treated in acetaldehyde (100μM) acutely (24 h) or chronically (2 wk). Viability was tested using AlamarBlue assay as previously described. Cells were then seeded around a 3D printed mould to create a zone of exclusion in the monolayer. The rate of closure of the monolayer 'wound' was quantified and the rate of closure calculated. Data shown n=3, mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns = not significant.

3.11 CHRONIC ETHANOL INCREASED THE CAPACITY OF DOK CELLS FOR ANCHORAGE-INDEPENDENT GROWTH

In vivo, cells typically undergo apoptosis upon losing attachment from their substratum or extracellular matrix (ECM). The ability of cells to evade this cell death pathway and grow independently of attachment to a surface or anchor, i.e., a tissue culture flask coated for adherent cells, is indicative of a more cancerous or metastatic phenotype. The acquired trait of survival without an anchor is essential for cells to transform and metastasise. Based on increased rate of wound closure in the presence of chronic ethanol treatment, Ca9.22 and DOK cells were tested for their ability to grow independently of an anchor. Ca9.22 cells had overall better survival without an anchor (~50% survival) compared to DOK cells (~41% survival) (**Figure 3.14C, 3.14D**). However, neither ethanol or acetaldehyde, acute or chronic, affected Ca9.22 cell's ability to grow independently of an anchor. Conversely, in DOK cells, chronic ethanol significantly increased their capacity for anchorage-independent growth. This effect was not attributable to acute or chronic acetaldehyde (**Figure 3.14D**).

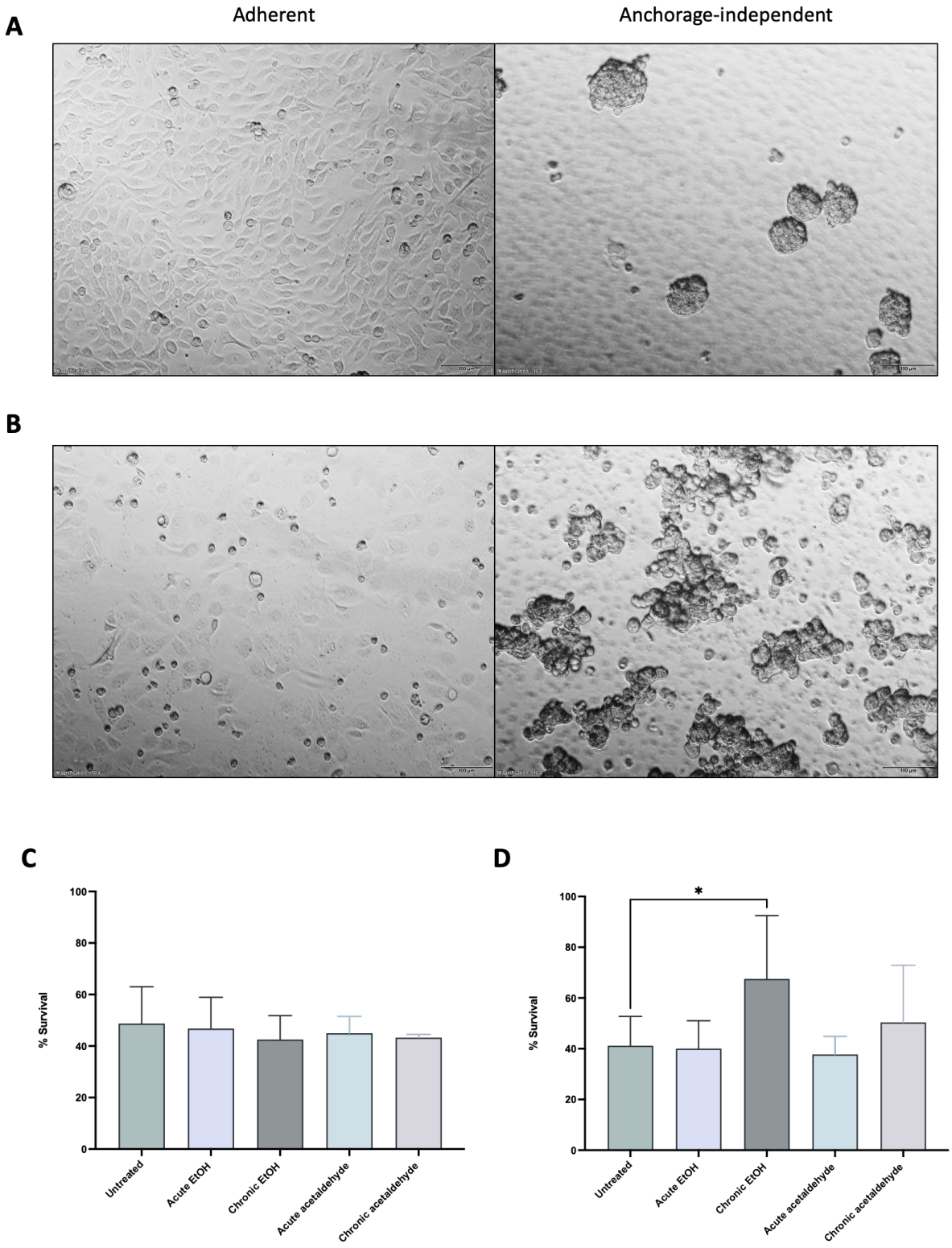


Figure 3.14. Representative images of (A) Ca9.22 and (B) DOK cells under normal adherent conditions (left) and under anchorage-independent growth conditions (right). (C) Neither acute or chronic ethanol (1% v/v) or acetaldehyde (100μM) affected anchorage-independent growth in Ca9.22 cells. (D) Chronic ethanol exposure significantly increased anchorage-independent growth in DOK cells. p-HEMA was added to tissue culture plates to remove adherent coating before seeding cells. Images were taken using an Olympus IX81 phase-contrast microscope. Viability was measured using AlamarBlue assay as previously described. Data shown n=3-9, mean ± SEM. * $P < 0.05$.

3.12 Chronic ethanol decreased expression of E-cadherin in Ca9.22 cells

Western blot analysis was used to detect the differential expression of EMT-related proteins E-cadherin, N-cadherin, and vimentin in Ca9.22 and DOK cells following chronic ethanol treatment. The expression of E-cadherin and vimentin was detected in both cell lines, whereas N-cadherin was only detected in DOK cells. Chronic ethanol treatment was shown to significantly decrease the expression of E-cadherin in Ca9.22 cells. No significant changes to any EMT-related protein expression were detected in DOK cells (**Figure 3.15**).

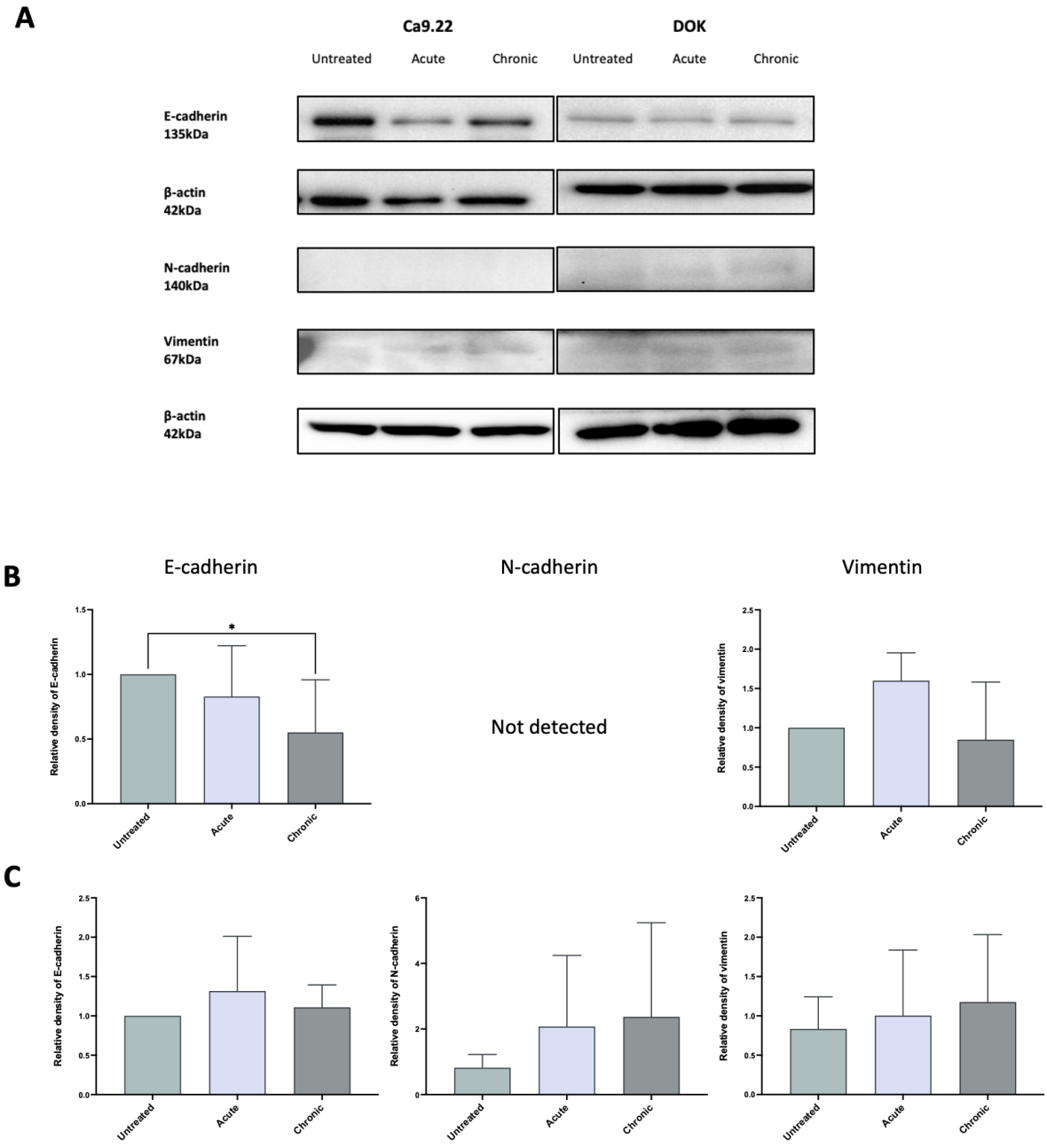


Figure 3.15. (A) Western blot analysis of E-cadherin, N-cadherin, and vimentin in Ca9.22 and DOK cells. N-cadherin and vimentin were probed for the on the same blot and share the β-actin loading control. Data shown is a composite image representative of n=4. **(B) Chronic ethanol significantly decreased expression of E-cadherin in Ca9.22 cells.** N-cadherin expression was not detected in Ca9.22 cells and no significant changes to vimentin expression were observed. **(C) No significant changes to E-cadherin, N-cadherin, and vimentin protein expression occurred following chronic ethanol treatment in DOK cells.** Densitometric analysis was completed using ImageJ software and normalised to the loading control β-actin. Data shown n=4, ± SD. * $P < 0.05$.

3.13 Chronic ethanol increased invasive characteristics of Ca9.22 and DOK cells

Invasion assays were utilised to determine the chemotactic capacity of cells to invade through an artificial ECM. Both acute and chronic exposure to ethanol increased the invasive capacity of Ca9.22 cells, with chronic ethanol having a more significant effect (**Figure 3.16**). In DOK cells, both acute and chronic exposure to ethanol significantly increased invasion of cells, but with no significant difference between exposure times (**Figure 3.16**).

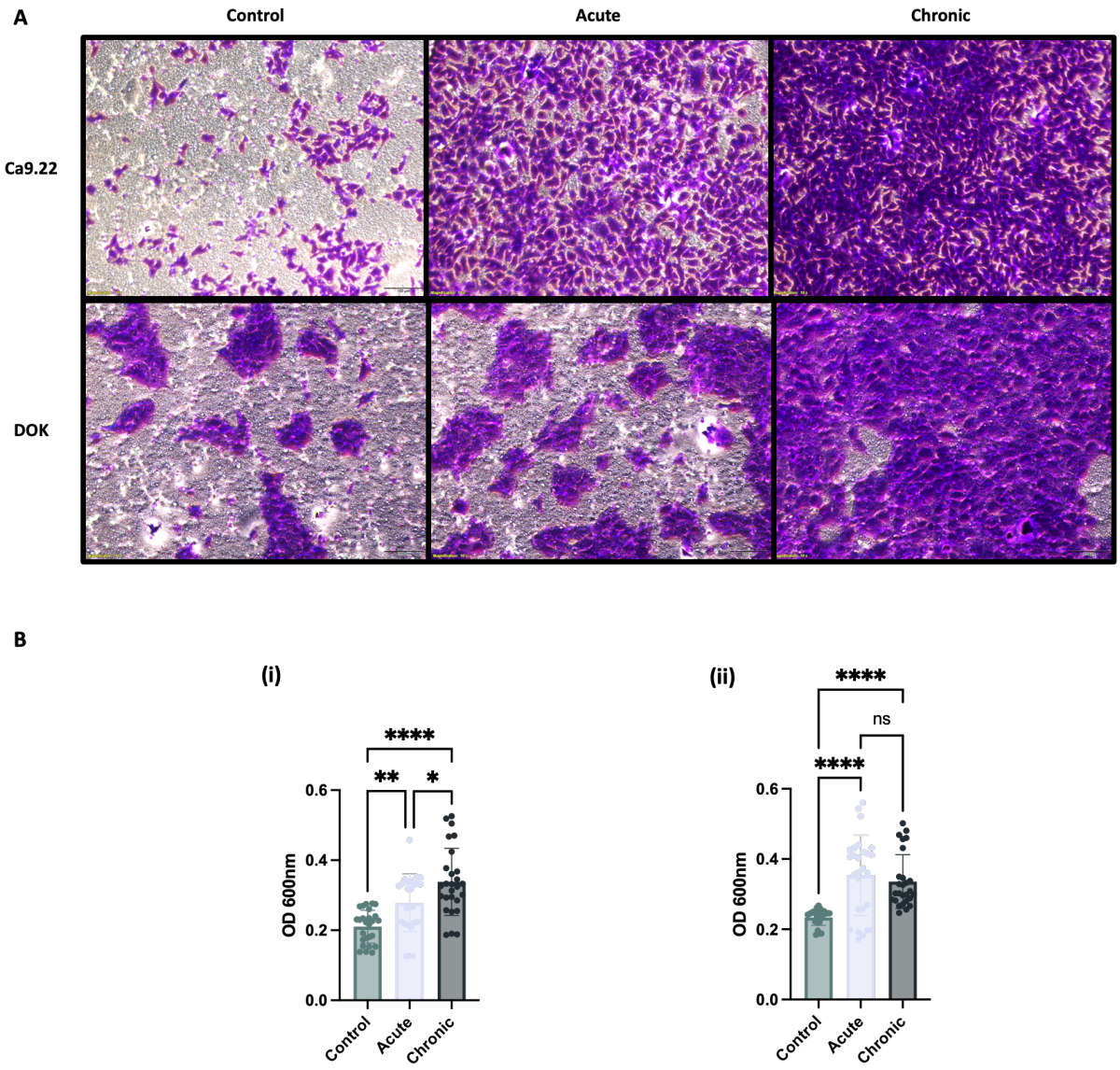


Figure 3.16. Chronic ethanol increased invasive capacities of Ca9.22 and DOK cells. (A) Representative images of crystal violet stained Ca9.22 and DOK cells that invaded into ECM-coated inserts. (B) (i) Acute and chronic exposure to ethanol increased invasion of Ca9.22 cells, with chronic exposure having a greater effect. (ii) Acute and chronic exposure to ethanol increased invasion of DOK cells, with no significant difference between exposure time. Transwell® inserts (8.0μM pore size) were coated with ECM gel and cells were grown on the inserts in serum-free media. Media with 10% serum was placed in the well below to create a serum-gradient. After 24 h cells were fixed with ethanol, stained with crystal violet, and imaged using an Olympus IX81 phase-contrast microscope. Crystal violet dye was eluted using 10% acetic acid and the absorbance read on a Spectra MAX Plus Microplate reader at wavelength 600nm. Data shown n=3, mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns = not significant.

3.14 Discussion

Initial assays determined to what extent oral cell lines could be exposed to ethanol *in vitro*, to study the effects elicited by ethanol while maintaining enough viable cells for analysis. Some published studies employed protocols to maintain constant ethanol content in cell culture plates over longer time-periods, via the use of sealed containers and ethanol reservoir chambers (161, 162). While this method of constant ethanol exposure has been shown to induce the epithelial-mesenchymal transition (EMT) in gingival keratinocytes, alcohol exposure in the oral cavity is transient as it is ingested and metabolised into acetaldehyde (149). Furthermore, induction of EMT-like characteristics from chronic ethanol exposure has been shown in hepatocytes without the need for use of constant exposure methods (147). *In vitro*, 20mM ethanol represents a blood alcohol level of 0.08% (165). In this study, 1% v/v was chosen for chronic ethanol exposure which is equivalent to 170mM – while this is quite a high concentration, it may be taken as representative of local level of alcohol in the oral cavity as opposed to absorbed levels in the bloodstream. Following alcohol consumption, the concentration in the saliva is higher than in blood plasma, and dissipates approximately 30 min following consumption (164). It was demonstrated by ethanol assays that the majority of ethanol evaporates from culture media after 24 h. Therefore, this timepoint was considered 'acute' exposure, and for 'chronic' exposure, media was replenished three times a week over the course of two weeks. For *in vitro* studies, although tolerance of different cell models to alcohol may vary, 10-100mM is considered to be physiologically relevant, with 25mM equating to the consumption of approximately 4 alcohol units (163). By increasing exposure time, the 'chronic' model used here was taken as representative of regular and/or long-term alcohol consumers (164).

Rodent models have been used for decades to study the effects of excessive alcohol consumption on numerous pathologies. In rodents, alcohol can be administered via oral gavage or by voluntary self-administration. However, the innate tendencies of rodent models is to not consume amounts

of alcohol to be overtly intoxicated or to increase blood alcohol levels over legal limits (166). Other models include rat genotypes modified for a high alcohol preference, alcohol deprivation, dependence/withdrawal models and schedule-induced polydipsia (166). The main limitations of cell culture systems used here are the concentrations of ethanol that the cells can withstand while still high enough to see an effect, and the simplicity of the system itself. While the ease of manipulation of cell culture systems is a major advantage, *in vitro* exposure cannot recapitulate the full complexity of *in vivo* alcohol consumption. Some organoid models have also been developed to better replicate the cellular complexity of *in vivo* tissues compared to singular cell monolayers (167).

The three oral cell lines had different tolerance levels to ethanol and acetaldehyde treatments, in a time- and dose-dependent manner (Figure 3.2, 3.3). Overall, Ca9.22 cells were the most tolerant to ethanol and TR146 cells were the least tolerant to ethanol. DOK cells demonstrated an ability to recover from ethanol injury, whereby the IC_{50} for ethanol concentration showed an increase from 24 to 48 h. The same effect was observed in the case of acetaldehyde treatments. Conversely, Ca9.22 cells were the most sensitive to acetaldehyde treatments, with the most cell death occurring at lower concentrations compared to TR146 and DOK cell lines. The varying tolerance of the cell lines to ethanol and acetaldehyde exposure prompted further investigation into their characteristics as models of ethanol-induced carcinogenesis.

Despite different effects on oral cell line viability, ROS production by the cell lines was similar when treated with ethanol or acetaldehyde. Ethanol did not significantly affect ROS production in any of the cell lines, whereas acetaldehyde caused an early significant increase in ROS production. Therefore the effects of ethanol on cell viability are not attributable to ROS production. Despite the role of ROS in normal cell function, increased ROS can promote proliferation and tumourigenesis via the activation of pro-proliferative and angiogenic pathways such as VEGF, PI3K/Akt and MAPK

signalling, as reviewed by Galadari et al. (168). However, no increase in ROS production was observed in the cell lines used here upon exposure to ethanol (**Figure 3.4**). The DCF-DA assay used has a wide recognition of total radicals, including intracellular H_2O_2, superoxide or NO. Cancer cells typically produce abundant ROS due to their hyperproliferative and metabolic nature in comparison to normal cells. The effect of ethanol exposure on cells with an already high basal level of ROS production may be negligible, therefore the sensitivity of the assay may not have detected any changes that occurred.

Acetaldehyde caused an initial spike in ROS production in all three cell lines in a dose-dependent manner, which peaked 1 h after treatment (**Figure 3.4**). Acetaldehyde is an extremely volatile compound at room temperature. When looking at the effects of longer treatment times (>1h) of acetaldehyde on cells, it is likely at this timepoint that acetaldehyde has evaporated from culture media and that cell death would have started to occur. The production of ROS is transient, again making it difficult to determine if it is the cause of any downstream effects of acetaldehyde treatments. This is especially pertinent when most assays looked at cellular responses to acetaldehyde at least 24 h post exposure. One of the proposed mechanisms of ethanol-induced carcinogenesis is via impaired antioxidant defence systems and enhanced production of ROS. Acetaldehyde also increases the production of ROS indirectly, via damage to mitochondria and scavenging of anti-oxidant molecules such as glutathione (169). Major contributors to ethanol-induced ROS production are the CYP2E1 enzymes, which are induced 10-20 fold by chronic alcohol consumption (48, 170). It is possible that while ROS production is a factor in ethanol-induced carcinogenesis, it's role in OC may be to a lesser extent due to a lack of these enzymes in the oral cavity. The expression of these enzymes in the oral cell lines was not investigated but may be useful in future studies. Therefore, the cell lines used here are not exemplary of ROS-related ethanol-induced carcinogenesis.

The three oral cell lines all possessed ADH and ALDH activity, as determined by enzyme activity assays (**Figure 3.5**). To test if enzyme activity would change when exposed to ethanol or acetaldehyde, the cells were treated for 24 h prior to assaying. The overall activity of these enzymes was unchanged by acetaldehyde, however, ethanol decreased ALDH activity in all three cell lines. To control for the effect of acetaldehyde produced endogenously by cells when treated with ethanol, the specific enzyme activity was calculated and the approximate maximal acetaldehyde concentration that could be produced by cells was determined (**Figure 3.5B**). For context, in the oral cavity there are microbe species that possess ADH capable of producing up to 200μM acetaldehyde in the presence of ethanol *in vitro*, and up to 450μM acetaldehyde has been found in saliva directly following alcohol consumption (63, 157). Comparatively high ALDH activity tissues like colonic and gastric cells cultured *in vitro* produced 0.25mM and 1.75mM acetaldehyde in the presence of ethanol, respectively (171). The concentrations of acetaldehyde used in this project, prior to determination of enzyme specific activity, ranged from 50-900μM. The concentrations used throughout the literature are varied – for example 0.1% v/v was used on gastric epithelial cells (172), 100μM on OSCC lines (65), and as low as 10μM on primary neurons (173). The specific activity of ADH in oral cell lines determined that the maximum amount of acetaldehyde produced by the highest concentration ethanol treatment (3% v/v) over 4 h would be 64.3μM, 76.2μM and 102μM by Ca9.22, DOK and TR146 cells respectively. This satisfied the criteria of using 100μM acetaldehyde as a control for the effects of ethanol on cell viability and in further experiments.

By establishing an oral cell culture model looking at the effects of long-term ethanol exposure, the aim of this study was to represent the oral cavities of those who are regular and/or long-term alcohol consumers. It was shown that all three cell lines could tolerate ethanol (1% v/v) over 2 week periods with no significant effect on cell viability. This chronic ethanol exposure was also shown to overall decrease both ADH and ALDH activity. Low ALDH activity has been posited as a main contributor to oral carcinogenesis. Particularly, mutations in the ALDH2 isoform may contribute to

accumulating acetaldehyde, increasing the risk for cellular DNA damage and the likelihood of mutations to occur. This is noted both *in vitro* and in populations with the ALDH2 polymorphism (105, 106). To examine the effect this overall lower ALDH activity would have on oral cell lines, *after* chronic ethanol treatment, cells were subjected to acetaldehyde treatment. In Ca9.22 and TR146 cancer cell lines, no difference in viability was observed following acetaldehyde treatments. However, in dysplastic DOK cells, those that were pre-treated in chronic ethanol showed significantly increased viability/proliferative rate in the presence of acetaldehyde compared to untreated controls (**Figure 3.6**). This increase in proliferation following acetaldehyde exposure is an interesting and novel finding, as it was unique to the dysplastic cell line. The enzyme activity assay used to measure ALDH utilises acetaldehyde as a substrate, and was carried out at 30°C as a compromise between enzyme optimum temperature and the volatile nature of the compound (174). The differences in cell viability and proliferation are possibly due to other isoforms compensating for enzyme activity –although many isoforms in the family have *preferred* substrates, most have multiple possible substrates (**Table 1.3**). Therefore, it is possible that DOK cells can be induced to have a higher expression of more active ALDH isoforms that metabolise acetaldehyde compared to Ca9.22 and TR146 cells. Conversely, chronically treated DOK cells may be more efficient at using acetaldehyde converted into acetate as a metabolic fuel source (156).

RT-PCR was used to identify mRNA expression of ADH Class III and unique expression profiles of ALDH1A1, ALDH2 and ALDH3A1 in the oral cell lines (**Figure 3.7**). It is not unusual for ALDH expression profiles to vary amongst human populations, as well as across different types of cancers (115). ALDH1A1 has been reported as not typically expressed in healthy oral mucosa (82), and previous papers have also reported no ALDH1A1 in primary human buccal tissues, normal oral cell lines or malignant oral cell lines (175). However, the expression of ALDH1A1 is highly associated with tumours in the oral cavity, as well as worse overall prognosis (176-178).

Ca9.22 cells lacked expression of ALDH2 and ALDH3A1 – isoforms with the lowest K_m for aldehydes. Ca9.22 cells were previously shown to be the most sensitive of the three cell lines to acetaldehyde treatments, and this is likely due to a greater build-up of acetaldehyde compared to DOK and TR146 cells, leading to increased cell death. Conversely, TR146 cells were particularly sensitive to ethanol treatments, and lack ALDH1A1 compared to Ca9.22 and DOK cells. Since different isoforms have preferred substrates, ALDH expression profiles must contribute to tolerance of ethanol or acetaldehyde in cell culture media. It is important to note that mRNA expression does not correlate to protein expression, and likewise protein expression does not necessarily correlate to enzyme activity. It has been shown that expression of ALDH enzymes progresses from normal tissue, through dysplasia and cancer tissues (82-84). The different profiles of the three oral cell lines presented an opportunity to study the role of ALDH expression and distinct patient expression profiles of ALDH that may be encountered in a clinical setting, and subsequently the influence this would have on alcohol-induced oral carcinogenesis and metastasis.

The novel finding that Ca9.22 and DOK cell lines both possess the ALDH1A1 isoform and lack expression of ALDH2 was of considerable interest. As mentioned previously, ALDH2 polymorphisms that render the enzyme inactive or less active than wild-type genes contribute greatly to the increased risk of developing HNCs (**Table 1.3**). ALDH1A1 is a highly researched CSC, prognostic and chemoresistance marker for OC tumours. Furthermore, chronic ethanol treatment was shown to decrease ALDH2 and ALDH3A1 mRNA expression in both DOK and TR146 cells, but increased mRNA expression of ALDH1A1 in Ca9.22 and DOK cells. In DOK cells, this biphasic increase in ALDH1A1 and simultaneous decrease in ALDH2/3A1 may suggest a mechanism promoting malignant transformation of dysplasia. By increasing acetaldehyde exposure via downregulation of ALDH2 and ALDH3A1 (isoforms preferring aldehydes), the oral cavity may be at inherent risk for more mutations. Simultaneous upregulation of ALDH1A1 expression may infer an increased capacity for detoxification, enhancing survival, as well as affecting downstream signalling pathways of ALDH1A1

including cell cycle, cell proliferation, stem cell differentiation and tumour morphology (83). Due to the lack of ALDH1A1 expression by TR146 cells as well as their relatively poor tolerance to ethanol, further experiments focused on ALDH1A1-expressing cell lines, Ca9.22 and DOK.

However, protein expression of ALDH1A1 did not correlate to the changes observed in mRNA expression. Using both microscopy and flow cytometry, it was shown that despite an observed increase in mRNA expression of ALDH1A1 in both Ca9.22 and DOK cell lines following chronic ethanol treatment, increased protein expression was only observed in Ca9.22 cells. In DOK cells, overall protein expression of ALDH1A1 was reduced by chronic ethanol exposure (Figure 3.8, 3.9). It has been reported in bacteria, cell-free culture systems, hepatic cell lines and mouse models of cancer-related cachexia that ethanol can have direct effects on translation machinery within the cell (179-182). Alcohol induces a reduction in global protein synthesis rates that may be caused by impaired translation. This could potentially explain the discrepancy in mRNA vs protein expression of ALDH1A1. Increased metabolic rates are a hallmark of cancer cells, which is linked to an increased rate of protein synthesis to support hyperproliferation of cells (183). Protein synthesis is also proportional to cell size – larger cells proliferate slower than smaller cells (184). DOK cells are partially transformed dysplastic cells, and they are on average ~18μM in size in comparison to ~14μM Ca9.22 cells. Taking this into account, the effect of ethanol on translation rates may be occurring in both cell lines but only detectable in DOK cells due to their slower proliferation and protein synthesis rates compared to Ca9.22 cells.

Modulation of ALDH protein expression in HNCs has been shown to contribute to increased chemoresistance, increased migratory potential of cells and progression of EMT processes (99). Moreover, previous studies have shown that increased ALDH1A1, alongside decreased ALDH2 and ALDH3A1 correlate with overall worse survival and prognosis across five different cancers; although a high level of variation was noted in HNCs (115). Expression of ALDH1A1 in oral PMDs conferred a

3-11 fold increased risk for malignant transformation, while the expression of ALDH1A1 in OSCC patients ranges widely, with estimates from 13.5-70% (83). Increased expression of ALDH1A1 in dysplasia is also associated with increased grade of histological severity (83). The expression of ALDH1A1 in primary OSCC tumours is significantly correlated with expression at distant LNM (89, 176, 185, 186). It is also of note that ethanol has been shown to promote the process of oral carcinogenesis in mice-models induced with 4-nitroquinoline 1-oxide (4NQO), increasing incidence of OSCC by 20% (187). Chronic ethanol exposure has also been shown to increase stemness features in in oral cancer cell lines via regulation of glycolysis (165). Based on the finding that chronic ethanol treatment affected activity and expression of ALDH1A1 in a dysplastic, pre-cancerous cell line and an established OC cell line, the effects this would have on carcinogenesis and metastatic cell characteristics were investigated.

As previously described, EMT processes are important in the transformation of dysplastic epithelium and in the metastasis of cancer cells (**Figure 1.3**). To determine if chronic ethanol was influencing EMT processes in oral dysplasia or OC, cell morphology and the expression of EMT markers were investigated in Ca9.22 and DOK cell lines. The phenotypic plasticity observed during EMT mostly results in easily quantified, separate epithelial- and mesenchymal-state cells, recognisable by their morphology (142). It is important to note that these changes are not binary, and it is possible to observe hybrid state phenotypes throughout transitions of cells (188). Neither acute nor chronic ethanol exposure affected morphology of Ca9.22 or DOK cell lines. Previous studies have shown that overexpression of ALDH1B1 was correlated with altered cell morphology (151), and $ALDH1A1^{+ve}$ lung cancer cells have been shown to have a more spindle-shaped morphology compared to $ALDH1A1^{-ve}$ cells (189). Inhibition of ALDH1A1 in tissue-engineered *ex vivo* oral mucosa led to phenotypic changes consisting of more 'normal' differentiation patterns – basal cells were more compact, uniform and organised with more viable cellular layers (82). Similarly, ethanol has been shown to induce morphology changes indicative of EMT in immortalised human

gingival keratinocytes following chronic ethanol treatment lasting 9 weeks (149). Chronic ethanol (14-21 days) given to hepatocellular carcinoma cell lines and clinical specimens, alongside a mouse model fed ethanol (2% v/v) drinking water for 2 months also showed morphology and genetic changes related to EMT processes (147), overall resulting in promotion of carcinogenesis. The oral cavity is generally quite robust as it is continually exposed to both mechanical trauma as well as chemical agents *in vivo*. It is possible that oral cells would require a longer or higher concentration of ethanol exposure to observe morphological changes *in vitro*.

While ethanol did not appear to affect cell morphology via flow cytometry, this method is a rudimentary way of looking at EMT processes (**Figure 3.10**). To establish if EMT was occurring, the expression of protein markers E-cadherin, N-cadherin and vimentin were investigated via Western blot analysis (**Figure 3.15**). During EMT, expression of E-cadherin typically decreases while expression of N-cadherin increases. Vimentin, an intermediate filament, is typically overexpressed during cancer metastasis and therefore used as an additional marker of EMT (190). These canonical changes in protein expression are hallmarks of EMT occurring in cells, but these changes are not fixed – some heterogeneity or intermediary genotypes may be observed (146). The loss of epithelial surface marker E-cadherin may be observed without acquisition of mesenchymal marker N-cadherin. Chronic ethanol exposure has been shown to increase expression of EMT-related protein markers in hepatocellular carcinoma cell lines (191). The expression of these proteins varied between the two cell types; for example, – cancerous Ca9.22 cells expressed E-cadherin but had no detectable N-cadherin. Conversely, DOK cells expressed overall lower levels of E-cadherin compared to Ca9.22 cells, but simultaneously expressed both N-cadherin and vimentin. This genotypic plasticity is not unexpected in a dysplastic cell line, where lower E-cadherin expression and more N-cadherin expression is observed compared to both normal epithelium and to OSCC (192, 193). No changes to these protein markers were observed in DOK cells with ethanol treatment at any timepoint, demonstrating that ethanol (24 h/2 wk) does not influence EMT processes at the genome level in dysplasia.

Conversely, chronic ethanol exposure significantly decreased expression of E-cadherin in Ca9.22 cells (Figure 3.15). E-cadherin has been shown *in vivo* to decrease in expression from normal oral mucosa, through mild to severe dysplasia and OSCC, contributing to the early process of oral carcinogenesis (194). In established tumours, the expression of E-cadherin at the membrane is reduced, but higher in the cytoplasm, potentially indicating a more aggressive tumour (195). This higher cytoplasmic E-cadherin is associated with the invasive front of tumours, and clinically associated with poorer prognosis and higher incidence of LNM for patients (196-198). It is typically thought that cadherin 'switching', i.e., loss of E-cadherin and *de novo* expression of N-cadherin expression, is a hallmark characteristic of EMT. However, while E-cadherin expression has been implicated in OC as previously discussed, N-cadherin expression was not found to have a statistically significant correlation to patient prognosis, nor was it shown to have any correlation to histological grade of OSCC (193, 199). One study showed that overexpression of N-cadherin increased motility, invasion, and MMP-9 expression in OC cell lines, completely independently of E-cadherin expression levels (200). Therefore, the canonical cadherin 'switch' is not necessary for the progression of OSCC (201). The loss of E-cadherin in Ca9.22 cells, without detectable expression of N-cadherin, still suggests EMT processes are occurring. The use of longer timepoints may enhance the effects of ethanol on EMT protein marker expression. Nevertheless, chronic ethanol contributing to the decrease in expression of E-cadherin may suggest a mechanism for ethanol-induced metastasis. While there is no research directly establishing a link between alcohol consumption and OC metastasis risk, there is evidence of a direct effect of ethanol on the metastasis of colorectal cancer (202). Alcohol may also play a role in metastasis of oral cancers via the expression of MMP proteins (203). It will be shown in Chapter 6 that ethanol reduces secretion of MMP-2 in Ca9.22 cells (Figure 6.6C).

E-cadherin in the cell is complexed with β-catenin, providing both a structural role in cell-cell contacts as well as playing a role in signal transduction networks. When E-cadherin is

downregulated, β-catenin is released, accumulating in the cytoplasm and this increase in expression can be detected via Western blot. Detection of β-catenin expression in Ca9.22 cells to confirm the results of downregulated E-cadherin expression would be pertinent. It may also be of use to identify the compartments of the cell where E-cadherin expression is localized via fluorescent labelling and microscopy. As mentioned previously, increased cytoplasmic E-cadherin as opposed to membranous expression is associated with higher grade of histological severity, and this could potentially reveal more in-depth information about the nature of Ca9.22 cells following ethanol exposure (195).

There is limited research linking EMT and ALDH proteins in oral cancer. Knockdown of ALDH1A1 in tissue-engineered *ex vivo* oral mucosa was shown to decrease proliferation markers, slow hyperproliferation and led to normal differentiation patterns of cells (82). This would suggest that increased ALDH1A1 expression in oral tissues promotes carcinogenic processes. The results of this study are in line with the data shown in this project – chronic ethanol exposure increases ALDH1A1 protein expression in Ca9.22 cells, simultaneously decreasing E-cadherin expression, suggesting a more aggressive or metastatic phenotype emerging via EMT processes. Conversely, Zhang et al. found a positive correlation of ALDH1A1 with an epithelial phenotype of cells in neuroendocrine carcinoma (shown by E-cadherin/N-cadherin expression) suggesting that ALDH1A1 could inhibit EMT-like processes (153). However, since no changes in EMT markers were noted in DOK cells despite decreased ALDH1A1 expression, the influence of alcohol and ALDH on EMT processes are likely dependent on cell-type and tumour-type.

Throughout EMT processes, as normal or dysplastic cells transform into squamous cell carcinoma and as cancer cells progress to metastatic, several cell characteristics are acquired that impart a more tumourigenic and aggressive phenotype. The migratory, invasive, and proliferative capacities of cells increase throughout malignant transformation. A ‘scratch’ or ‘wound healing’ assay allows

quantification of proliferation and migratory rates of cells *in vitro*. Increased migration and motility are characteristic of transforming or metastatic cells, as well as indicative of a hyperproliferative state. Typically, the wound-healing assay is carried out by scraping the cell monolayer with a pipette tip. Inconsistencies in scratch size/area are difficult to avoid as different angles or pressure applied by the individual can create varying size scratches and can cause excess damage to the cell monolayer, subsequently affecting the rate of closure and confounding data analysis (204).

To overcome these inherent variability issues, 3D printed mould prototypes were custom designed and tested specifically for the 24-well plates used in our lab. Initial versions were designed to fit the same dimensions as tissue culture plate lids, with holes cut over each well wide enough to fit a p200 pipette tip, so that it could reach the cell monolayer but only to a fixed depth. This was to eliminate bias in the angle and pressure being applied by the pipette tip to the cell monolayer (Supplementary Figure A). Later versions of the mould were based off the work of Boyer et al. (205), where designs were created to produce a zone of exclusion upon seeding the cells, as opposed to creating a gap in an established cell monolayer. This reduced variability at the wound edge, the potential for cell death and the potential damage to coating on the surface of the tissue culture plate, which may lead to excess cell detachment (Supplementary Figure B).

Overall, cancer cell line Ca9.22 had faster rates of scratch closure, compared to DOK and TR146 cells. Both acute and chronic ethanol exposure significantly increased the rates of wound closure in Ca9.22 and DOK cells (Figure 3.11). When treated with low levels of acetaldehyde (90μM), a significant increase in the migration rates of Ca9.22 cells was observed, but not to the same extent as ethanol (Figure 3.12). Therefore the increase in migration rates following ethanol exposure may be in part due to the increased availability of acetaldehyde as metabolic fuel, although the impact of this is minor compared to the direct effects of ethanol (156). Conversely, the exposure of Ca9.22 and DOK cells to chronic acetaldehyde (100μM), did not increase migration rates (Figure 3.13). The

ability of cells to use short-term exposure of acetaldehyde to increase migration rates, as opposed to chronic exposure, further suggests it may be shuttled into metabolic pathways.

While ethanol has opposite effects on ALDH1A1 expression in Ca9.22 and DOK cells, it produces a similar increase in migratory capacities of both cell lines. This could suggest that alcohol consumption increases the risk for both transformation of dysplasia and the metastasis of oral cancer, independent of its link to ALDH expression. Ethanol exposure (25-100mM) has previously been shown to increase migratory rates in other oral cell lines (206). Increase in migration rates of nasopharyngeal cancer cells, but not normal cells, was shown to be via activation of chloride channels by Wei et al. (207). This same study also suggested that long-term exposure to ethanol may increase the incidence of metastasis. This is reflected in the data of this project, where chronic ethanol exposure significantly increased the migration of Ca9.22 cells compared to both acute ethanol exposure and untreated controls. The relationship of ALDH expression and migration rates of cells is more tenuous. Knockdown of ALDH1A1 and ALDH3A1 was shown to reduce migration rate in oesophageal cancer, while overexpression of ALDH1B1 increased migration (151). In OC cell lines, increased expression of ALDH3A1 decreased proliferation and migration in scratch assays, and decreased invasive capacities of the cells (114). It is worth noting that neither Ca9.22 or DOK cell lines possess the ALDH2 isoform, which has been heavily linked to an increased risk for developing HNC (106). Knockdown of this isoform has been shown to decrease migration in NSLC cell lines, while its induced expression inhibited these properties in hepatocellular carcinoma (208, 209). Therefore, induction of ALDH2 expression in Ca9.22 and DOK cells may mitigate these increasing migratory rates induced by ethanol. Further manipulation of ALDH expression may be warranted in future studies, either via transfection with a DNA vector or small molecule ALDH activators such as Alda-1 (210).

One of the hallmarks of cancer is the evasion of cell death (211), and this can be assayed by measuring the ability of a cell to grow independently of an appropriate attachment matrix or 'anchor'. Normal epithelia are adhered to basement membranes and neighbouring cells, providing growth and survival signals. Programmed cell death occurs upon detachment and is a critical mechanism in preventing dysplastic cell growth (212). Detachment is also the initial step of metastasis, meaning cancer cells must avoid attachment-related programmed cell-death to migrate and invade blood and lymphatic circulation. Metastatic tumour cells are far more resistant to this type of cell-death, termed '*anoikis*' (213).

The addition of ethanol or acetaldehyde at any timepoint did not affect anchorage-independent cell growth in Ca9.22 cells (**Figure 3.14**). Metastasis has distinct stages, beginning with detachment, followed by migration and invasion, avoidance of cell death, intravasation/extravasation and then the colonisation of new sites, requiring the cells to re-attach to new tissues (214). While ethanol appears to be promoting metastatic processes, as shown by reduced E-cadherin expression (**Figure 3.15**) and increased migration rates, the length of exposure time may result in a stage of metastasis in Ca9.22 cells that does not elicit or require increased anchorage-independent growth. Conversely, chronic ethanol significantly increased the ability of dysplastic DOK cells to grow independently of an anchor, avoiding *anoikis*. This effect has been noted across multiple cell types, and at varying concentrations and exposure times of ethanol. Chronic ethanol exposure (2 wk, 100mM) has been shown to induce a transformed phenotype in hepatocytes, increasing anchorage-independent growth and colony formation, another measure of adhesion-independent growth, via regulation of stem cell and EMT markers (191). Chronic ethanol exposure (up to 90mM for 9 wk) was also shown to initiate transformation of oral keratinocytes, increasing their ability to grow independently of an anchor via modulation of tumour suppressor genes (149). Chronic ethanol (100mM, 3 months) was also shown to increase anchorage-independent growth in two OC cell lines via modulation of glycolysis (165), suggesting that the use of longer exposure times might result in the same effect in

Ca9.22 cells as observed in DOK cells. Ethanol induced activation of transcription factor NFAT signalling to increase cancer stemness and glycolysis in these oral cell lines (165). Of note, the NFAT family of TFs require calcium signalling for activation, and acetaldehyde exposure has been shown to promote intracellular calcium influx which may potentiate this effect (71).

Ethanol has been shown to promote anchorage-independent growth multiple times throughout the literature, but the role of ALDH in this capacity is less clear. Previous studies have shown that oesophageal squamous cell carcinoma cells with relatively high levels of ALDH1A1 expression have higher levels of adhesion-independent growth as shown by colony formation assays (215). This is contradictory to the data shown here, where chronic ethanol decreased ALDH1A1 expression in DOK cells but increased anchorage-independent growth. In addition, chronic ethanol increased ALDH1A1 expression in Ca9.22 cells but had no effect on anchorage-independent growth. Multiple studies show the effects of overexpression of different ALDH isoforms in various tumour types on anchorage-independent growth. For example, overexpression of ALDH1B1 reduced anchorage-independent growth in colorectal cancer cells (151), overexpression of ALDH1A1 increased anchorage-independent growth in NSLC cells (216), overexpression of ALDH3A1 decreased adhesion-independent growth in OSCC cell lines (114), overexpression of ALDH2 decreased adhesion-independent growth in hepatocellular carcinoma cells (208) and high expression of ALDH1A1 increased adhesion-independent growth in ovarian cancer cells (217). The roles of ethanol and ALDH in promoting anchorage- or adhesion-independent growth do not appear to be linked.

Following detachment, avoidance of *anoikis* and migration, cancer cells must invade into new environments to establish new tumours in distant metastatic niches in the body. Invasive migration is a key process in normal development and immune responses; however, it also plays a significant role in pathological events like cancer development and metastasis. The invasion assay imitates this

process by measuring the ability of cells to invade an artificial ECM, by creating a chemoattractant gradient to encourage the cells to migrate (218). Cells that successfully migrate through the matrix can be fixed, stained, and quantified via microscopy and spectrophotometry.

Ethanol treatment, both acute and chronic, increased invasion of Ca9.22 and DOK cells (**Figure 3.16**). Chronic ethanol has been shown to increase invasive capacities of cells across multiple cancer types including hepatocellular, colorectal and breast cancer (147, 202, 219-221). While alcohol consumption has a clear association with invasive oral cancers (222, 223), there are no studies to the best of our knowledge directly investigating the mechanism of this effect. No conclusive argument can be made for the involvement of ethanol in tumour invasiveness, in any type of cancer, as the concentration and duration of alcohol exposure varies greatly across studies, affecting the outcomes observed (224). However, the expression of ALDH1 in OC patients is associated with greater depth of invasion by tumours in the oral cavity (84). Similarly, when EMT-related transcription factor SNAIL was induced in OC cell lines, ALDH1 expression was increased as well as invasive capacities of the cells (96). Since chronic ethanol had opposite effects on ALDH1A1 expression in the cell lines used here, it is unlikely that invasive capacities are related to modulation of this isoform.

There is a disparity between the causal link of ALDH1A1 and other ALDH isoforms expression to OC and their clinical relevance which remains to be addressed. A high level of heterogeneity has been observed between patient samples, with ALDH1A1 expression cited to vary from 13.5-70% in OSCC (83). In addition, epigenetic changes in combination with genetic alterations should be considered for their potential effect on pathogenesis and prognosis for OSCC patients. Specific methylation signatures including ALDH enzymes have been recognized as a prognostic marker in HNCs (225, 226). Furthermore, alcohol, ALDH enzymes and acetate can affect methylation patterns of genes (227, 228). If ethanol-induced carcinogenesis was driven by epigenetic changes, it may explain the

variability of acute and chronic exposure on Ca9.22 and DOK cells. Only chronic ethanol exposure was shown to influence expression of ALDH1A1 and EMT proteins. However, this temporal difference is not entirely unexpected as protein synthesis is a multiple step process, and depends on mRNA stability, transcription rates and translation rates. Conversely, epigenetic switches are much more rapid. Acute and chronic ethanol exposure had significantly different effects on rates of wound-healing in scratch assays, anchorage-independent growth, and invasion assays. It is possible that these effects are partly mediated via epigenetic changes influenced by ethanol exposure, both directly and indirectly (228).

In conclusion, ethanol exposure affected the two cell lines differently across multiple assays, including anchorage-independent growth, scratch, and invasion assays. The results presented here showed that while ethanol is contributing to transformative processes in dysplastic cells and metastatic processes in carcinoma cells, the mechanisms appear to be completely independent of each other. Whether or not these mechanisms are linked to ALDH expression remains to be elucidated. To investigate the influence of ALDH1A1 in these observed changes, the use of both chemical inhibitors and siRNA will be investigated in the following chapters.

Chapter 4: 4-Diethylaminobenzaldehyde as an ALDH Inhibitor

4.1 Inhibitors of ALDH

The previous chapter demonstrated that chronic ethanol exposure enhanced oral carcinogenicity in Ca9.22 and DOK cells via potentially independent mechanisms. Both Ca9.22 and DOK cells possess the ALDH1A1 isoform, which has been of great interest in cancer research. Given DOK cells represent pre-cancerous oral cells and Ca9.22 cells are an established cancer cell line, the modulation of ALDH1A1 along with ethanol-induced increased carcinogenic phenotypes led to the hypothesis that ethanol may be acting via modulation of ALDH1A1 protein expression.

ALDH exists as a paradox in cancer whereby the downregulation of certain isoforms may be conducive to enhanced tumorigenicity by increasing exposure to acetaldehyde (48, 105). Despite this, ALDH inhibitors have been utilised to treat both alcoholism and as adjuvants to cancer treatments (119). For over 70 years Disulfarim (DSF), also known as Antabuse™, has been used as an FDA-approved drug to treat the symptoms of alcoholism and help maintain sobriety (229). DSF and other related members of the dithiocarbamate drug family were initially discovered as inhibitors of NF-κB (230). Its putative mechanism of action was originally believed to be the inhibition of ALDH, giving the patient the same phenotypic reaction to alcohol as the ALDH2 polymorphism common within the Asian population. This includes the aforementioned flushing response, headaches, nausea, and dizziness following alcohol consumption (77). However, DSF has been more recently cited as inhibiting CYP2E1 (231), ALDH1A1 (216), ALDH2 (77) and was also shown to inhibit EMT (232). The mechanism of action of DSF has since been shown not to be through the inhibition of ALDH as previously thought, but via the *in vivo* metabolite formed from DSF. The effects of DSF are potentiated by complexing with copper (229, 233) and its anti-cancer

action is purported to be a result of the inhibition of NPL4, an importer for nuclear-tagged proteins and an exporter for poly(A) RNA tails destined for addition to mRNA to increase stability (233).

Natural inhibitors of ALDH2 are derived from the root and flowers of Kudzu plants – namely daidzin and daidzein, the structure of which have been used to design more potent inhibitors of ALDH2 (234, 235). Recent developments of novel selective inhibitors for ALDH isoforms have yielded promising results, with efforts to avoid overlapping functions of other ALDH family members using prodrugs or DSF derivatives (236-239). However, bioavailability, toxicity and selectivity continue to be issues surrounding ALDH inhibition for use in clinical settings.

4.2 4-DIETHYLAMINOBENZALDEHYDE

4-Diethylaminobenzaldehyde (DEAB) is a commonly used and referenced broad-spectrum, reversible inhibitor of ALDH (**Figure 4.1**). DEAB has been shown to increase the efficacy of chemotherapeutics in both breast and lung cancers (97, 98). DEAB is also used as the positive control in the long-established Aldefluor™ assay to identify and isolate cells with high overall ALDH activity – typically used in the isolation of CSCs (240). In oral cell lines, including dysplastic DOK cells used in this research, the Aldefluor™ assay and DEAB have been used to show increasing ALDH activity from normal cells, through dysplasia and to OSCC (91).

Extensive research has shown that DEAB is both a substrate and mechanism-based inhibitor for members of the ALDH family in an isoform-dependent manner. For example, DEAB has been shown to be a substrate for ALDH3A1 (K_m 5.6 ± 0.7μM, K_i 38.8 ± 3.3μM) but with such a slow turnover that it is effectively an inhibitor as it competes with other substrates (241). It was shown to be a potent inhibitor and slow substrate of ALDH1A1 (IC_{50} 57 ± 5nM, K_i 9.8 ± 3.1nM) and an irreversible, covalent inhibitor of ALDH1A2 (IC_{50} .1.2 ± 0.1μM) and ALDH2 (IC_{50} .0.16 ± 0.03μM)(241). DEAB was also

shown to be an irreversible inhibitor of the ALDH7A1 isoform (K_i 100 ± 36μM)(242). The mechanisms of DEAB are isoform-dependent and some studies have suggested that DEAB possesses anti-cancer activity. DEAB was shown to increase apoptosis and eradicate human pancreatic cancer cells via accumulation of toxic aldehydes (243). In human fetal islet-epithelial cells of the pancreas, DEAB was shown to increase apoptosis and inhibit differentiation of stem cells, via reduction of RA signalling (244). The effect of DEAB was also shown to be reversed by the addition of all-trans retinoic acid, an exogenous source of substrate for ALDH enzymes to allow continued RA signalling (244). In addition, DEAB and DEAB analogues were shown to elicit anti-proliferative activity against prostate cancer cells, with increased efficacy when combined with chemotherapeutic docetaxel (245). Other than Aldefluor™ assays utilising DEAB as a control, there is very little research using DEAB as an ALDH inhibitor in OC.

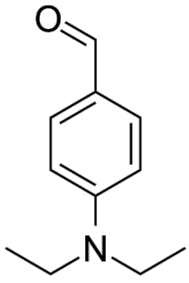


Figure 4.1. Structure of DEAB. The accepted mechanism of action of ALDH enzymes is nucleophilic attack by the catalytic cysteine on the aldehyde, with NAD^+ as a cofactor, and production of an acyl-enzyme intermediate. The proposed mechanism of action for DEAB is stabilisation or stalling of this acyl-enzyme intermediate to varying degrees. Structural features within ALDH isoform active sites stabilise this structure, preventing its hydrolysis into its carboxylic acid product and the regeneration of the native enzyme (241, 242).

4.3 AIMS

- Optimisation of DEAB as a broad spectrum ALDH inhibitor in oral cell lines Ca9.22 and DOK.
- Utilise DEAB to verify if ALDH modulation is the mechanism behind ethanol-induced carcinogenicity.

4.4 DEAB DID NOT INHIBIT ALDH ACTIVITY IN CA9.22 CELLS

DEAB was tested for its ability to inhibit ALDH activity in Ca9.22 cells using the enzyme activity assay previously described. Ca9.22 cells were treated with DEAB (50-250μM) for up to 24 h, or with a vehicle control of DMSO. DEAB did not significantly affect ALDH activity in Ca9.22 cells compared to the untreated control at any timepoint tested (0.5-24 h) (**Figure 4.2A**). LDH assays were used to determine cytotoxicity of DEAB to Ca9.22 cells. At concentrations ≥250μM in the initial 6 h of treatment, DEAB was significantly cytotoxic to Ca9.22 cells (**Figure 4.2B**).

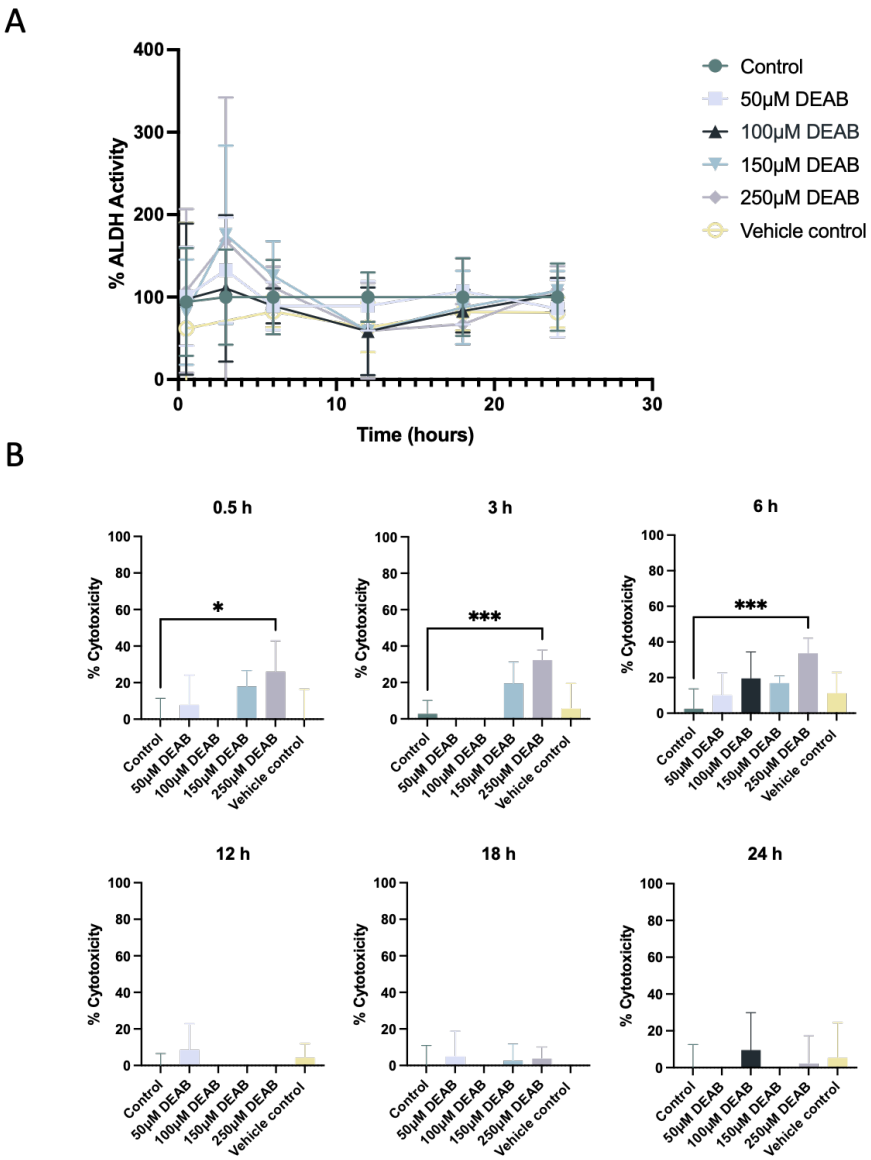


Figure 4.2. (A) DEAB did not inhibit ALDH activity in Ca9.22 cells. Cells were treated with DEAB (50-250µM, 0.5-24 h) before lysing in assay buffer (0.1M sodium phosphate, 1% Triton-X-100, protease inhibitor cocktail (Roche), pH 8). ALDH activity was determined by adding reaction mix containing assay buffer as before, 10mM acetaldehyde and 2.5mM NAD. Absorbance was read on a Spectra MAX Microplate reader at 300/340/360nm every 3 min at 30°C to follow the production of NADH. Data shown n=2-5, mean ± SD. **(B) DEAB ≥250µM is significantly cytotoxic to Ca9.22 cells.** CyQUANT™ LDH Cytotoxicity assay was used to measure cell death as per the manufacturer's protocol. Data shown n=2-3, mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns = not significant.

4.5 DEAB INHIBITED ALDH ACTIVITY IN DOK CELLS BUT THIS EFFECT WAS SHORT-LIVED

DOK cells were treated with DEAB (50-250μM) for up to 24 h, or with a vehicle control of DMSO. Maximal inhibition of ALDH was observed at 12 h following DEAB treatment in DOK cells (**Figure 4.3A**). At 6 h, DEAB 50-100μM inhibited ALDH activity, however at 12 h, 100-250μM produced the greatest inhibition of ALDH activity. The highest concentration of DEAB (250μM) reduced ALDH activity by ~66% after 12 h treatment (**Figure 4.3B**). DEAB was not found to be cytotoxic to DOK cells at any timepoint or concentration used (**Figure 4.3C**).

The ALDH activity of DOK cells recovered to control levels 18 h after treatment with DEAB (**Figure 4.3B**). To determine if DEAB inhibition of ALDH activity was sustainable without increasing cytotoxicity, DOK cells were re-dosed with DEAB using two alternative processes. Initially, at the 12 h timepoint when ALDH inhibition was maximal, media was removed and fresh media with DEAB was added to cells ('Redose'). The activity of ALDH was tested at 24 h from the time of first DEAB addition. This method did not increase cytotoxicity to cells (**Figure 4.4A (ii)**), however, the activity of ALDH recovered to control levels by 24 h, despite the additional DEAB (**Figure 4.4A (i)**).

Next, at the 12 h timepoint, additional DEAB was added, without changing culture media ('Addition redose'). This method led to lower levels of ALDH activity detected at 24 h compared to the previous method (**Figure 4.4B (i)**). 'Addition redose' of DEAB 250μM reduced ALDH activity by 46% compared to the control, however this activity was still higher compared to the 12 h timepoint alone, and was not statistically significant (**Figure 4.4B (i)**, **Figure 4.3B**). Moreover, addition of DEAB on top of existing cell culture media increased the cytotoxicity of the compound, with 250μM causing ~17% cytotoxicity, compared to ~8% cytotoxicity with 'Redose' methods, and no cytotoxicity observed at the 12 h timepoint alone (**Figure 4.4B (ii)**, **Figure 4.4A (ii)**, **Figure 4.3C**).

Based on this observation, it is possible that sustained ALDH inhibition is due to cell death as opposed to increased inhibition of the protein activity.

In summary, the 'addition redose' method of adding DEAB on top of existing culture media significantly decreased ALDH activity compared to cells treated for 24 h consecutively. However, this 'addition redose' inhibition did not reach the same magnitude as the 12 h timepoint. ALDH inhibition reached 46% with 'addition redose' and 74% at the 12 h timepoint alone, with no statistical significance with respect to each other (Figure 4.4C). Similarly, ALDH activity was not significantly reduced with respect to the control (Figure 4.4C). The ability of DEAB to inhibit ALDH activity in DOK cells was exhausted after 12 h and could not be sustained for longer periods of time.

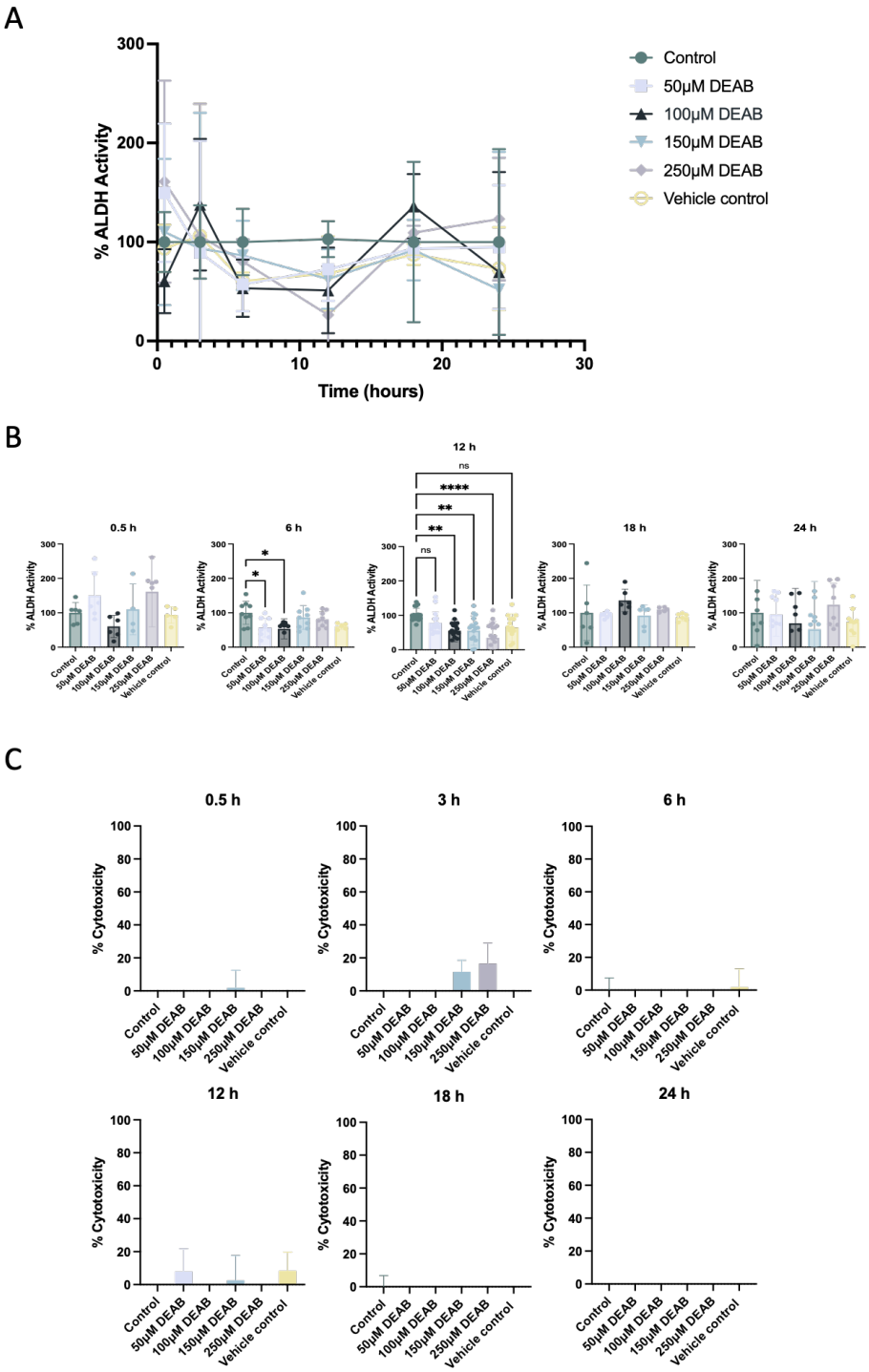


Figure 4.3. (A) DEAB inhibited ALDH activity maximally at 12 h in DOK cells. (B) DEAB (100-250µM) significantly inhibited ALDH activity after 12 h in DOK cells. Cells were treated with DEAB (50-250µM, 0.5-24 h) before lysing in assay buffer (0.1M sodium phosphate, 1% Triton-X-100, protease inhibitor cocktail (Roche), pH 8). ALDH activity was determined by adding reaction mix containing assay buffer as before, 10mM acetaldehyde and 2.5mM NAD. Absorbance was read on a Spectra MAX Microplate reader at 300/340/360nm every 3 min at 30°C to follow the production of NADH. Data shown n=2-6, mean ± SD. **(C) DEAB up tp 250µM was not cytotoxic to DOK cells**. CyQUANT™ LDH Cytotoxicity assay was used to measure cell death as per the manufacturer's protocol. Data shown n=2-6, mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns = not significant.

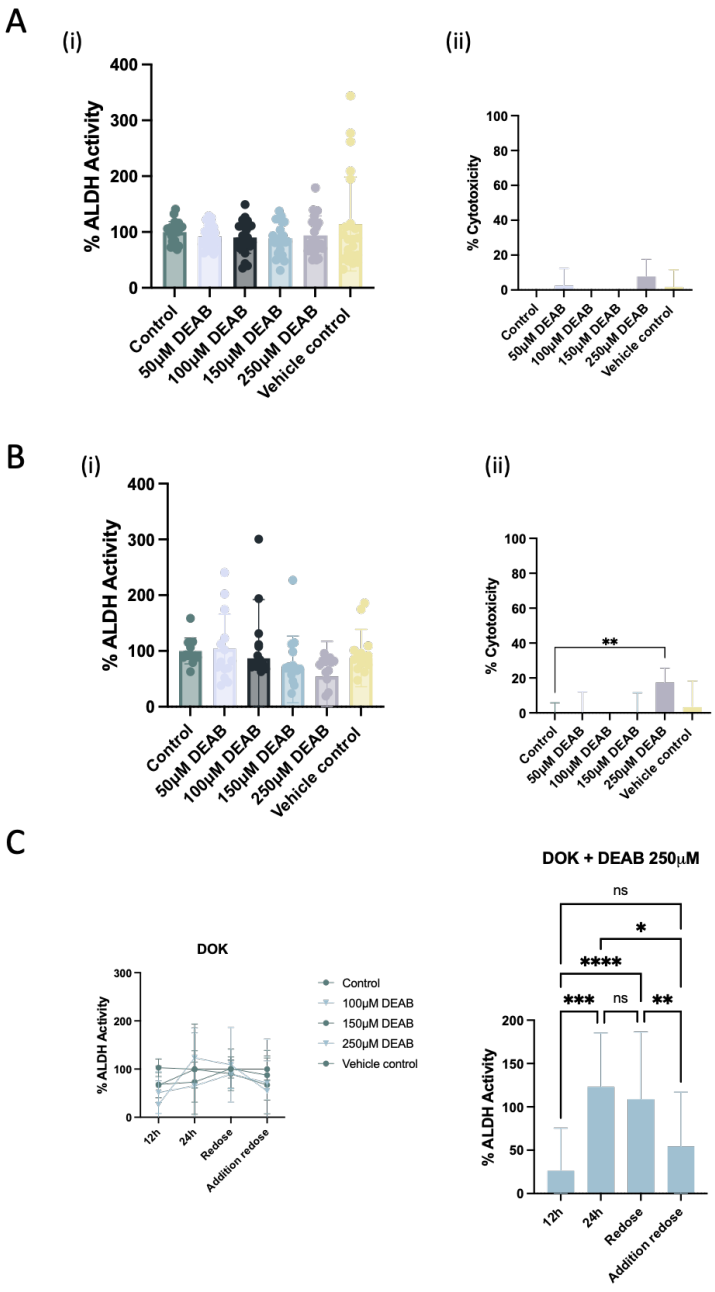


Figure 4.4. (A) 'Redose' of DOK cells with DEAB did not sustain ALDH inhibition. DOK cells were treated with DEAB for 12 h, then media changed and fresh DEAB added for an additional 12 h ('Redose'). ALDH activity was tested at the end of the 24 h. **(i)** This method did not sustain ALDH inhibition in DOK cells over 24 h, **(ii)** and did not increase cytotoxicity of the compound to DOK cells. **(B) 'Addition redose' of DOK cells with DEAB increased duration of ALDH inhibition but increased cytotoxicity to cells.** DOK cells were treated with DEAB for 12 h, then additional DEAB was added to existing cell culture media ('Addition redose') and ALDH activity was tested at the end of the 24 h. **(i)** This method in comparison to 'redose' yielded lower ALDH activity at 24 h but **(ii)** increased cytotoxicity of the compound to DOK cells. **(C) The 'addition redose' method of DEAB treatment resulted in significantly lower ALDH activity at 250µM compared to both 24 h alone and 'redose' methods but was still not as effective as 12 h treatments.** Data shown n=4-6, mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns = not significant.

4.6 DEAB DECREASED PROLIFERATION AND MIGRATION OF DOK CELLS IN SCRATCH ASSAYS BUT DID NOT AFFECT ANCHORAGE-INDEPENDENT GROWTH

Despite the limited ability of DEAB to inhibit ALDH activity in DOK cells, proliferation and migration was tested in the presence of DEAB over 24 h via AlamarBlue and scratch assays. AlamarBlue assays showed no change in viability of cells in the presence of DEAB (**Figure 4.5A**). DOK cells treated with DEAB and subjected to scratch assays showed significantly reduced rates of wound closure compared to untreated controls (**Figure 4.5B**). The rate of wound closure significantly decreased with increasing DEAB concentration (**Figure 4.5C**). However, the vehicle control of DMSO also significantly decreased proliferation and wound closure of DOK cells and is likely a contributing factor to the decreased rates observed in DEAB-treated cells.

To further explore the effects of DEAB on DOK cell behaviour, the ability of DOK cells to grow independently of an anchor was examined. DEAB did not affect the ability of DOK cells to grow without an anchor at any concentration, and neither did the vehicle control (**Figure 4.6**).

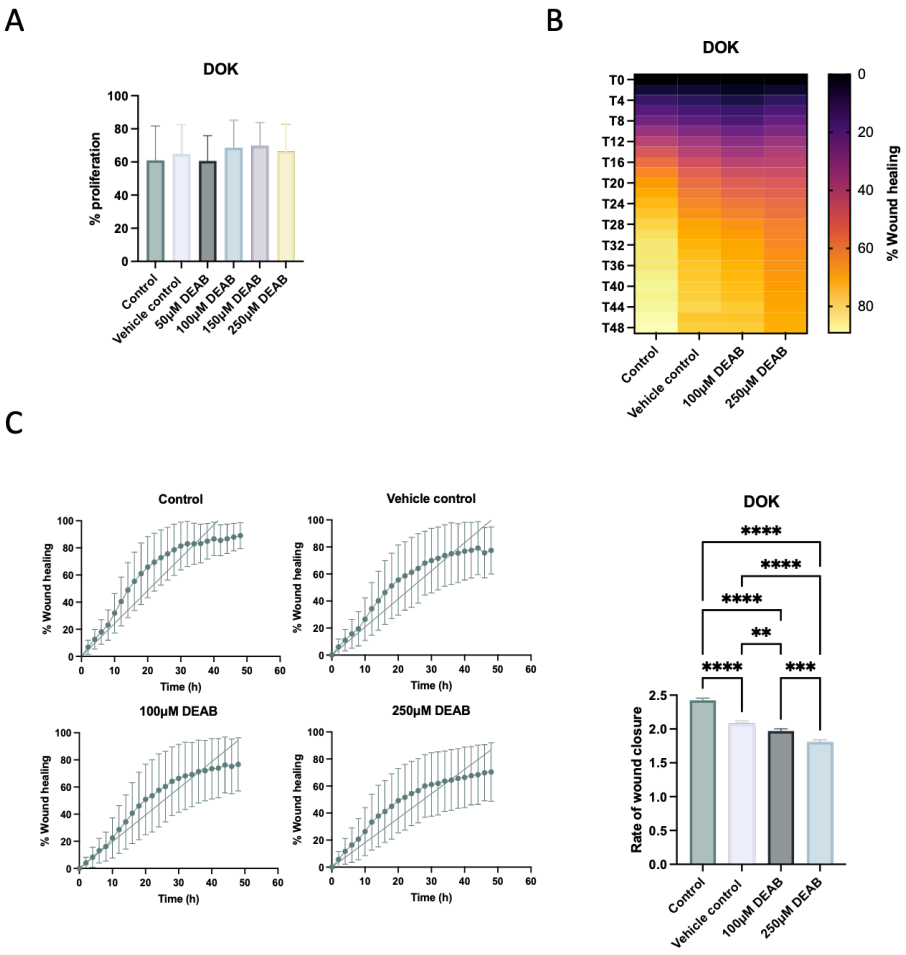


Figure 4.5. (A) DEAB did not affect viability of DOK cells via AlamarBlue assay. Cells were treated as required and 4 h prior to endpoint, AlamarBlue reagent was added (10% v/v). Absorbance was read on a Spectra MAX Microplate reader at 570/600nm. **(B) Heat map showing wound closure of DOK cells following DEAB treatment. (C) The rate of wound closure in scratch assays was significantly decreased by DEAB, as well as by the vehicle control of DMSO.** Cells were seeded with a 3D printed mould in place to create a zone of exclusion. The rate of closure of the monolayer 'wound' was monitored using an IncuCyte™ S3 and rate of closure calculated using ImageJ software. Data shown n=4, mean ± SD. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001, ns = not significant.

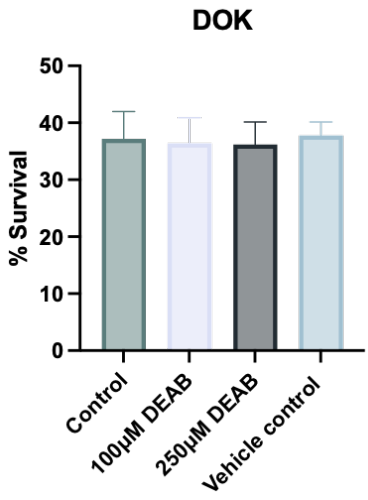


Figure 4.6. DEAB did not affect anchorage-independent growth of DOK cells. Tissue culture plates were coated with p-HEMA to remove adherent surfaces before seeding cells. Viability was measured using AlamarBlue assay as previously described. Data shown n=4, mean ± SD.

4.7 DISCUSSION

ALDH modulation is of great interest in cancer research. Previous work has suggested that the expression of specific ALDH isoforms is associated with an increased risk of OC development, as well as being clinical and prognostic markers (110, 114, 176, 246, 247). Expression of ALDH is also indicative of the presence of CSC populations in OC, and these isoforms can contribute to metastasis and cancer reoccurrence rates (188). Various ALDH expression profiles have been implicated in oral carcinogenesis, with no single isoform standing out as responsible for the development of OC, or even correlating to alcohol or tobacco consumption status of OC patients (83). In **Chapter 3** it was shown that chronic ethanol exposure modulates overall ALDH activity and specific expression of ALDH1A1 in oral cell lines. It was therefore the aim of this chapter to investigate this effect using DEAB, a well-known ALDH inhibitor.

The Aldefluor™ assay was initially purported to detect cells with high ALDH1A1 activity, and hence it was assumed that DEAB was principally an ALDH1A1 inhibitor. Numerous studies have since elucidated the mechanisms of action of DEAB on different members of the ALDH family, demonstrating a broad range of activities, specificities and inhibitory capacities depending on the isoform (248). In the oral cell lines used here, DEAB was shown to have a very limited capacity to inhibit ALDH activity. In the Aldefluor™ assay, cells are typically treated with DEAB (10-20μM) for 30 min before detection of ALDH activity via flow cytometry. Ca9.22 and DOK cells were treated with concentrations of DEAB ranging from 50-250μM, for 0.5-24 h before analysis. In Ca9.22 cells, DEAB at the highest concentration exhibited a high level of cytotoxicity, but at no concentration or timepoint used did it significantly affect ALDH activity of cells (**Figure 4.2**). Conversely, DEAB 100-250μM inhibited DOK ALDH activity significantly after 12 h (**Figure 4.3**). This concentration range is significantly higher than the typical concentrations used in routine Aldefluor™ assays,

demonstrating a weaker ability of DEAB to inhibit ALDH activity in DOK cells compared to other cell lines.

Cell lines that test positive for high ALDH activity in Aldefluor™ assays see a significant reduction in activity with DEAB, however cell lines with overall lower positive rates for Aldefluor™ activity don't see a significant reduction in ALDH activity with DEAB, regardless of concentration used (15-100μM) (249). Duan et al. reviewed Aldefluor™ activity, ALDH isoforms and their significance in different cancers and noted that optimal concentration and timepoints for use of DEAB in different cell lines would be necessary for more specific use in future research (249). The development of selective inhibitors for ALDH isoforms is still in progress, and continues to be hampered by a high degree of structural and sequence homology observed between ALDH isoforms – for example, ALDH1A1 and ALDH2 share up to 70% sequence homology despite having different substrate specificities (250).

It was unexpected that DEAB did not show any significant reduction in ALDH activity in Ca9.22 cells. Ca9.22 cells possess ALDH1A1, the isoform with a preferred substrate of retinals or acetaldehyde, while DOK cells possess both ALDH1A1 and ALDH3A1, the latter having a preferred substrate of aromatic aldehydes. DEAB has been shown to be a slow substrate for both these isoforms (241). The enzyme assay used to detect ALDH activity uses acetaldehyde as the substrate, for which DEAB should be able to outcompete and inhibit ALDH1A1 and ALDH3A1 as concentration increases. The same experimental conditions were tested within our lab on the hepatocellular carcinoma HepG2 cell line, a relatively high ALDH activity cell line, and significant reductions in ALDH activity were noted at 30 min, as per the protocol used in the Aldefluor™ assay, as well as up to 18 h, with DEAB concentrations as low as 50μM (Supplementary Figure C). It is worth noting that DEAB is observed to be an irreversible inhibitor of ALDH2, the isoform with the highest affinity for acetaldehyde, and that neither Ca9.22 or DOK cells express ALDH2, while HepG2 cells do (251). Together, these findings confirmed that the efficiency of DEAB inhibition of ALDH activity is isoform dependent.

To determine if the modest ALDH inhibition observed in DOK cells was enough to mimic the effects of chronic ethanol treatment, cell behaviour in scratch assays and anchorage-independent growth assays were tested in the presence of DEAB. Maximal ALDH inhibition was observed at 12 h, however these assays require cells to grow for 24-48 h. Since it was not possible to sustain ALDH inhibition any longer, this was taken into account for interpretation of data. Chronic ethanol exposure was previously shown to significantly increase the rate of wound closure in DOK cells, and significantly increased the ability of DOK cells to grow independently of an anchor (**Figure 3.11**, **Figure 3.14**). DEAB demonstrated a modest but significantly decreased rate of wound closure in DOK cells and had no effect on anchorage-independent growth (**Figure 4.5**, **4.6**). Since chronic ethanol exposure was previously shown to decrease overall ALDH activity in DOK cells, and specifically decrease the expression of ALDH1A1 in DOK cells, DEAB is clearly not a suitable means of validating the mechanisms involved in ethanol-induced carcinogenesis.

DEAB produced the opposite effect on cell behaviour compared to chronic ethanol exposure. Of note, a study using primary oral keratinocytes and tissue-engineered *ex vivo* oral mucosa reported that DEAB (100μM, 3 days) slowed cell cycle progression and suppressed hyperproliferation as shown by decreased expression of proliferation marker Ki-67, leading to the development of a normal differentiation pattern (82). The proposed mechanism of action of DEAB was determined to be via ALDH1A1 inhibition, as demonstrated by RNAi-silencing. However, the authors concede that off-target pharmacological effects are possible, since no specific ALDH inhibitor exists that does not affect the other isoforms expressed. It is possible that in DOK cells, DEAB also confers some off-target effect, potentially via other ALDH isoforms, leading to decreased proliferation and therefore affecting wound closure rates. DOK cells may also be able to upregulate other ALDH isoforms to compensate for the inhibition caused by DEAB, which could be examined by using qRT-PCR for all ALDH isoforms. While the efficiency of DEAB inhibition of ALDH activity is clearly cell type and ALDH isoform-dependent, it's effect on Ca9.22 and DOK cells could be further examined by

looking at complete expression profiles of all 19 ALDH isoforms in the cell lines used, followed by purification and assaying of each isoform specifically. Assay conditions would have to be optimised for each isoform, including substrate and NAD concentration – as previously detailed by Morgan et al. (241).

In conclusion, DEAB is not a suitable model for ALDH inhibition in Ca9.22 or DOK cell lines. Using DEAB as an ALDH inhibitor, it was not possible to determine if modulation of ALDH activity and expression by chronic ethanol exposure contributes to its effects on carcinogenesis processes in oral cell lines.

Chapter 5: Inhibition of ALDH1A1 by siRNA in Oral Cell Lines

5.1 siRNA-MEDIATED KNOCKDOWN

siRNA-mediated gene silencing is a useful tool to investigate the role of specific proteins and cellular pathways. Many of the ALDH inhibitors available are broad-spectrum and in **Chapter 4**, DEAB was determined to be an ineffective method to examine the effect of ALDH inhibition on oral cell lines. Therefore, to examine the effect of ALDH1A1 expression on oral carcinogenesis, siRNA was employed to specifically target and knockdown expression of ALDH1A1 isoforms in Ca9.22 and DOK cell lines. For effective knockdown of protein expression, the siRNA must be delivered to the cells via transfection. It must also be optimised for maximum delivery, uptake, specificity, and efficacy of gene silencing.

5.2 AIMS

- Optimise siRNA transfection conditions for Ca9.22 and DOK cells.
- Utilisation of siRNA to knockdown ALDH1A1 in Ca9.22 and DOK cells and investigate the downstream effect on cell proliferation, cell death, transformation of dysplasia and metastasis of cancer cells.
- Determine if ALDH1A1 modulation is the mechanism behind ethanol-induced carcinogenesis.

5.3 Optimisation of siRNA transfection conditions for Ca9.22 and DOK cells

To optimise conditions for transfection of Ca9.22 and DOK cells, two transfection methods were examined. Both polyethylenimine (PEI) MAX® and Lipofectamine™ 2000 were tested to maximise transfection efficiency but minimise cytotoxicity to cells. A simple way to test for uptake of siRNA molecules is to use a fluorescently tagged protein. Plasmid tagged with enhanced green fluorescent protein (pEGFP) was transfected into cells using a range of conditions, and successful uptake was measured by counting the number of cells expressing GFP under a fluorescent imaging filter on a microscope.

Initial results using the manufacturer's protocol for each transfection reagent, showed that PEI MAX® was a poor transfection reagent for both Ca9.22 and DOK cells (**Figure 5.1**). In Ca9.22 cells, there was no significant difference in cytotoxicity of PEI MAX® or Lipofectamine™ 2000, with cell viability remaining above 60% in both cases (**Figure 5.1A (i)**). However, Lipofectamine™ 2000 was significantly more efficient at transfecting Ca9.22 cells with pEGFP, with 53% of viable cells expressing GFP (i.e., successfully transfected with GFP) compared to only 7% when using PEI MAX® (**Figure 5.1A (i)**). In DOK cells, Lipofectamine™ 2000 was significantly more cytotoxic compared to PEI MAX®, with cell viability of 59% compared to 91%, respectively (**Figure 5.1A (ii)**). Despite the difference in cytotoxicity of the two transfection reagents, neither was effective at transfecting DOK cells with pEGFP. The percentage of cells transfected to express pEGFP was 9% with Lipofectamine™ 2000 and 6% with PEI MAX® (**Figure 5.1A (ii)**).

To improve transfection efficiency, several ratios of transfection reagent:pEGFP were examined. In Ca9.22 cells, since Lipofectamine™ 2000 was significantly more efficient, the effect of increasing ratios of Lipofectamine™ 2000:pEGFP on cytotoxicity and efficiency were examined (**Figure 5.2**). Increasing the volume of Lipofectamine™ 2000 was significantly more cytotoxic to Ca9.22 cells. It

was found that a ratio of 2:1 was significantly more efficient than 1:1, but any ratio higher than this did not increase transfection efficiency (Figure 5.2).

Similarly, in DOK cells, increasing the volume of Lipofectamine™ 2000 was significantly more cytotoxic compared to lower volumes and compared to PEI MAX® (Figure 5.3). The efficiency of transfection was not significantly improved with either reagent by adjusting ratios of transfection reagent:pEGFP. Transfection rates for DOK cells remained low, but overall Lipofectamine™ 2000 provided slightly better live:transfected ratios of cells (Figure 5.3).

In conclusion, Ca9.22 cells were more readily transfected compared to DOK cells. Lipofectamine™ 2000 proved a more efficient transfection reagent for both cell lines, albeit with minimal success in DOK cells for pEGFP. Based on these experiments, Lipofectamine™ 2000 was used for optimising transfection of cells with siRNA targeting the protein of interest, ALDH1A1.

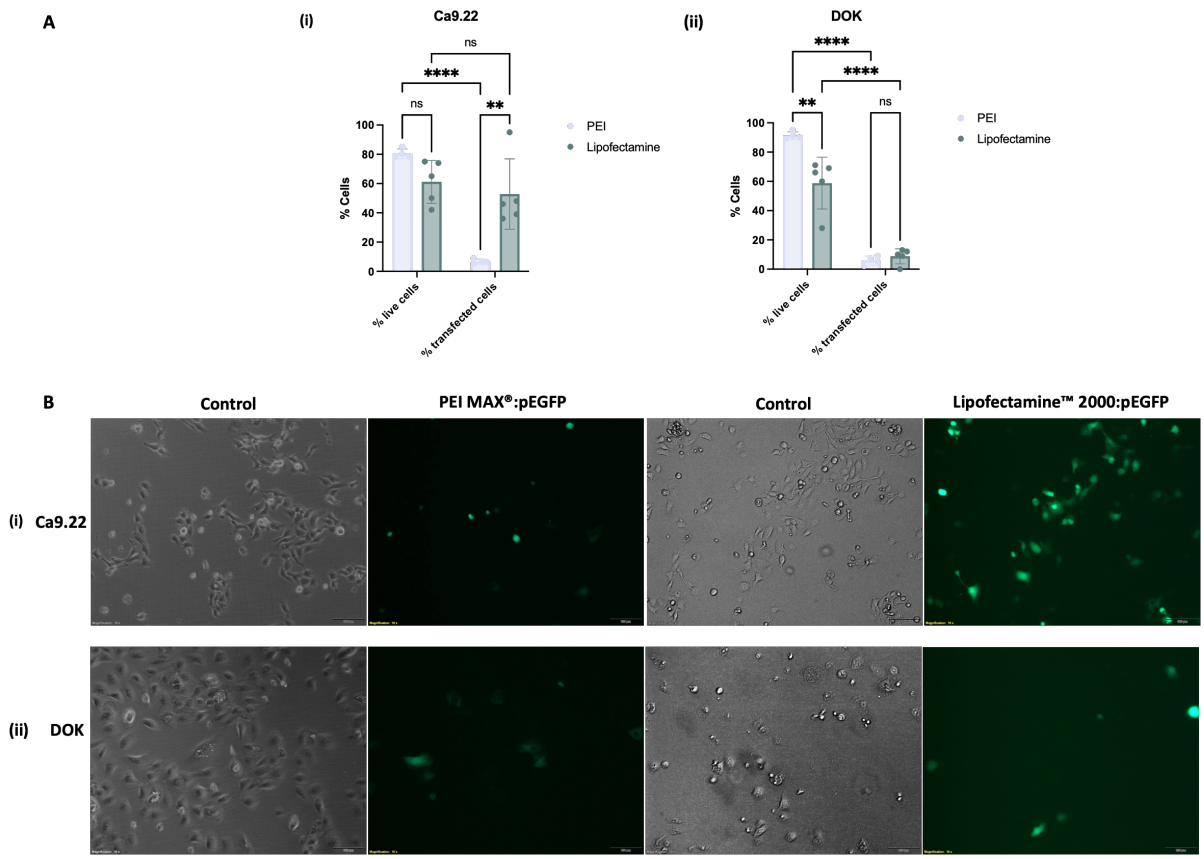
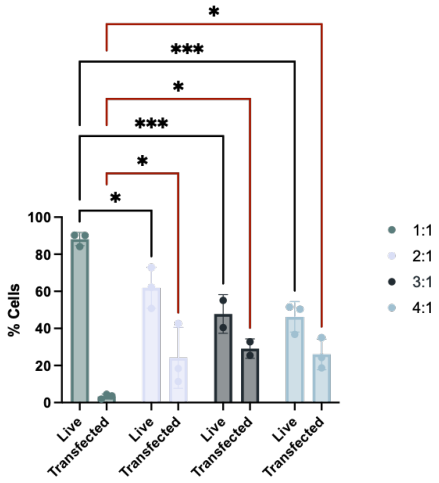
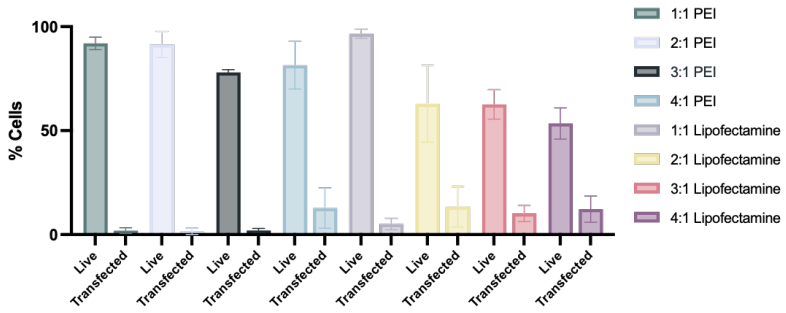


Figure 5.1. (A) (i) Lipofectamine™ 2000 was significantly more efficient than PEI MAX® at transfecting Ca9.22 cells with pEGFP. (ii) Transfection efficiency was low with both Lipofectamine™ 2000 and PEI MAX® for pEGFP in DOK cells. (B) Representative images of Lipofectamine™ 2000 vs PEI MAX transfection of pEGFP into (i) Ca9.22 and (ii) DOK cells. Cells were transfected with pEGFP (2μg) using either Lipofectamine™ 2000 or PEI MAX® in Opti-MEM™ media for 4 h before replacing with full serum media. The transfection efficiency (expression of GFP) was evaluated 24 h post transfection by imaging on an Olympus IX81 microscope with a GFP filter. Cells were counted using ImageJ software and percentage live vs transfected cells plotted. Data shown n=2, mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns = not significant.



Ratio Lipofectamine: pEGFP	% Live cells	% Transfected cells
1:1	88	4
2:1	62	24
3:1	48	29
4:1	46	19

Figure 5.2. A ratio of 2:1 Lipofectamine™ 2000:pEGFP was the least cytotoxic and the most efficient for transfection of Ca9.22 cells. Black significance lines compare the percentage of live cells and red significance lines compare the percentage of transfected cells between ratios of Lipofectamine:pEGFP. Cells were transfected with pEGFP (2μg) using Lipofectamine™ 2000 in increasing volumes in Opti-MEM™ media for 4 h before replacing with full serum media. The transfection efficiency (expression of GFP) was evaluated 24 h post transfection by viewing on an Olympus IX81 microscope and imaging with a GFP filter. Cells were counted using ImageJ software. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001, ns = not significant.



Ratio PEI:pEGFP	% Live cells	% Transfected cells	Ratio Lipofectamine: pEGFP	% Live cells	% Transfected cells
1:1	92	1	**1:1**	97	6
2:1	91	3	**2:1**	63	9
3:1	78	1	**3:1**	63	11
4:1	81	13	**4:1**	53	5

Figure 5.3. DOK cells were not readily transfected with pEGFP using either Lipofectamine™ 2000 or PEI MAX®. Overall, Lipofectamine™ 2000 provided a better ratio of live:transfected cells, but transfection efficiency did not exceed 11%. Cells were transfected with pEGFP (2μg) using either Lipofectamine™ 2000 or PEI MAX® in increasing volumes in Opti-MEM™ media for 4 h before replacing with full serum media. The transfection efficiency was evaluated as described above.

5.4 siRNA-MEDIATED KNOCKDOWN OF ALDH1A1 IN DOK CELLS

Using conditions indicated by pEGFP transfection experiments, DOK cells were transfected using Lipofectamine™ 2000 and siRNA molecules specific to ALDH1A1, using GAPDH as a positive control and non-specific siRNA as a negative control. Cells were evaluated 72 h post transfection for protein expression via Western blot analysis and flow cytometry, and assayed for ALDH enzyme activity. Despite low uptake of pEGFP in previous transfection experiments, ALDH1A1 was successfully knocked down by 48% according to densitometric analysis of Western blot (*** $P < 0.0001$, **Figure 5.4A**). This correlated to a 63% reduction in enzyme activity (** $P < 0.01$, **Figure 5.4C**). Despite this, flow cytometry using a live cell dye for ALDH1A1 showed no significant difference in ALDH1A1 expression (**Figure 5.4B**). Previously, chronic ethanol reduced ALDH1A1 protein expression in DOK cells according to flow cytometry, ALDH activity assay and IF microscopy (**Chapter 3**) but siRNA knockdown decreased ALDH1A1 expression to a much greater extent (**Table 5.1**). The impact of this disparity will be considered when comparing effects on cell behaviours in the discussion.

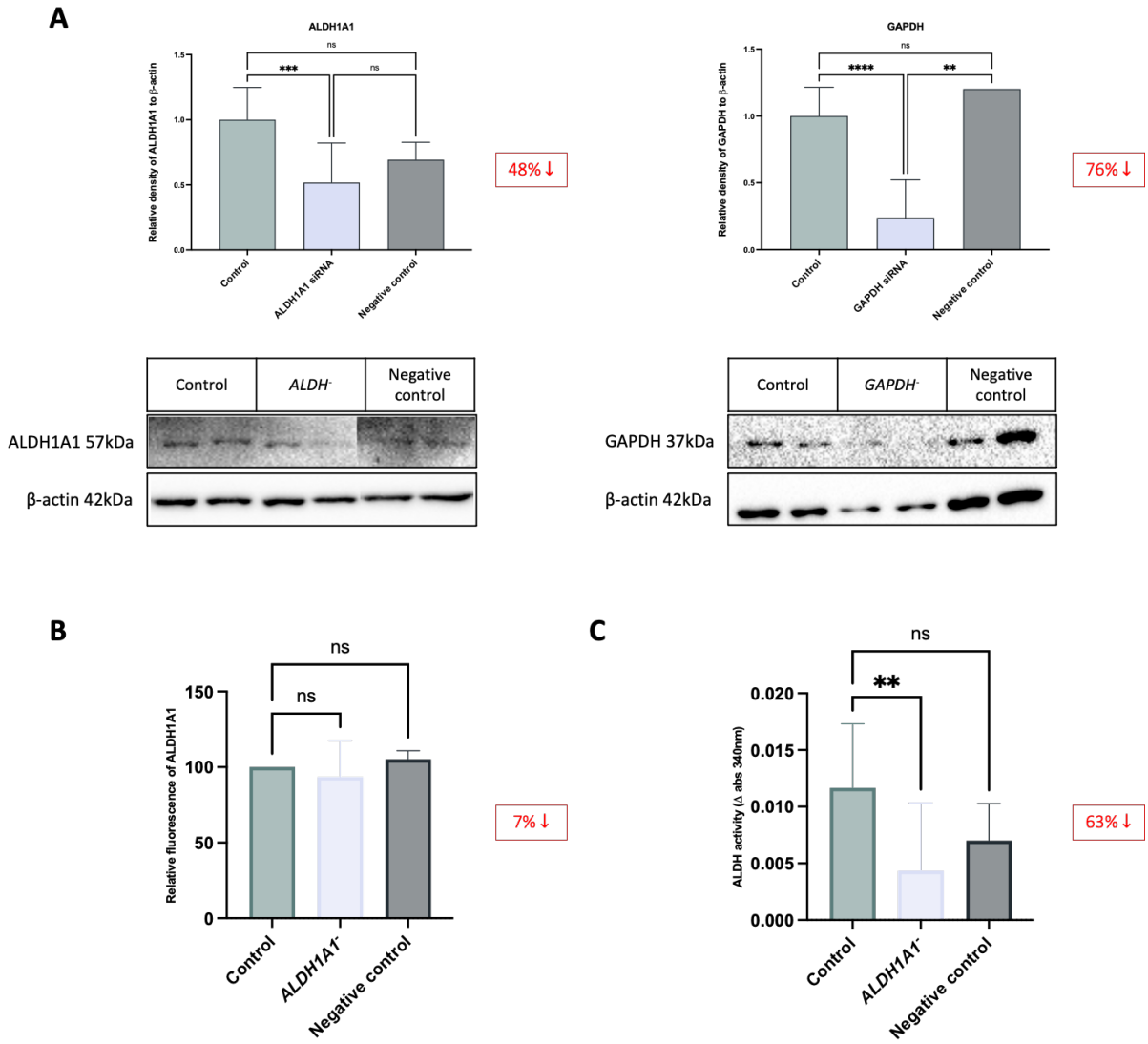


Figure 5.4. (A) siRNA achieved 48% and 76% knockdown of ALDH1A1 and GAPDH respectively in DOK cells as determined by Western blot. DOK cells were transfected using specifically targeted siRNA molecules and Lipofectamine™ 2000. Western blot and densitometric analysis using ImageJ software was carried out, with β-actin as a loading control. Percentage differences between control and knockdown were calculated from the graphs. Western blot for ALDH1A1 are composite images of samples prepared and run at the same time but loaded in a different order. Samples were blotted in duplicate. Images shown are representative of n=6. **(B) siRNA did not significantly affect ALDH1A1 protein expression in DOK cells according to flow cytometry**. Cells were analysed using a live cell dye specific for ALDH1A1, as previously described. Data shown n=4, mean ± SD. **(C) Knockdown of ALDH1A1 resulted in a 63% reduction in ALDH enzyme activity in DOK cells.** ALDH activity assay was measured as previously described. Data shown n=3, mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns = not significant.

Table 5.1. Comparison of the effect of chronic ethanol exposure and siRNA of ALDH1A1 in DOK cells. Significance is shown with respect to the control. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001, ns = not significant.

DOK	Treatment					
	Chronic ethanol exposure			siRNA knockdown of ALDH1A1		
Analysis	% change from control	Significance	Figure	% change from control	Significance	Figure
ALDH1A1 expression via flow cytometry	43% ↓	*	3.9B	7% ↓	ns	5.4B
ALDH1A1 expression via Western blot	45% ↑	ns	3.9D	48% ↓	***	5.4A
ALDH activity assay	76% ↓	ns	3.5D	63% ↓	**	5.4C

5.5 KNOCKDOWN OF ALDH1A1 AND GAPDH IN DOK CELLS SIGNIFICANTLY AFFECTED RESPONSES TO ACETALDEHYDE

Following knockdown of ALDH1A1 (*ALDH*$^-$), DOK cells were treated with ethanol (0.5-5% v/v) or acetaldehyde (50-900μM) for 24 h and viability and proliferation assessed using AlamarBlue assay. Knockdown of GAPDH (*GAPH*$^-$) and a scrambled siRNA molecule ('negative control') were used as controls. No significant differences were detected in the viability of non-transfected cells, *ALDH*$^-$, *GAPDH*$^-$ and the negative control cells when treated with ethanol (**Figure 5.5A**). However, when treated with acetaldehyde up to ~350μM, non-transfected DOK cells and negative control cells increased in proliferation and overall viability (**Figure 5.5B**). The same effect was not observed in *ALDH*$^-$ and *GAPDH*$^-$ cells. Lack of ALDH1A1 expression in *ALDH*$^-$ cells, and knockdown of GAPDH, the sixth enzymatic step in glycolysis, prevented acetaldehyde-induced proliferation.

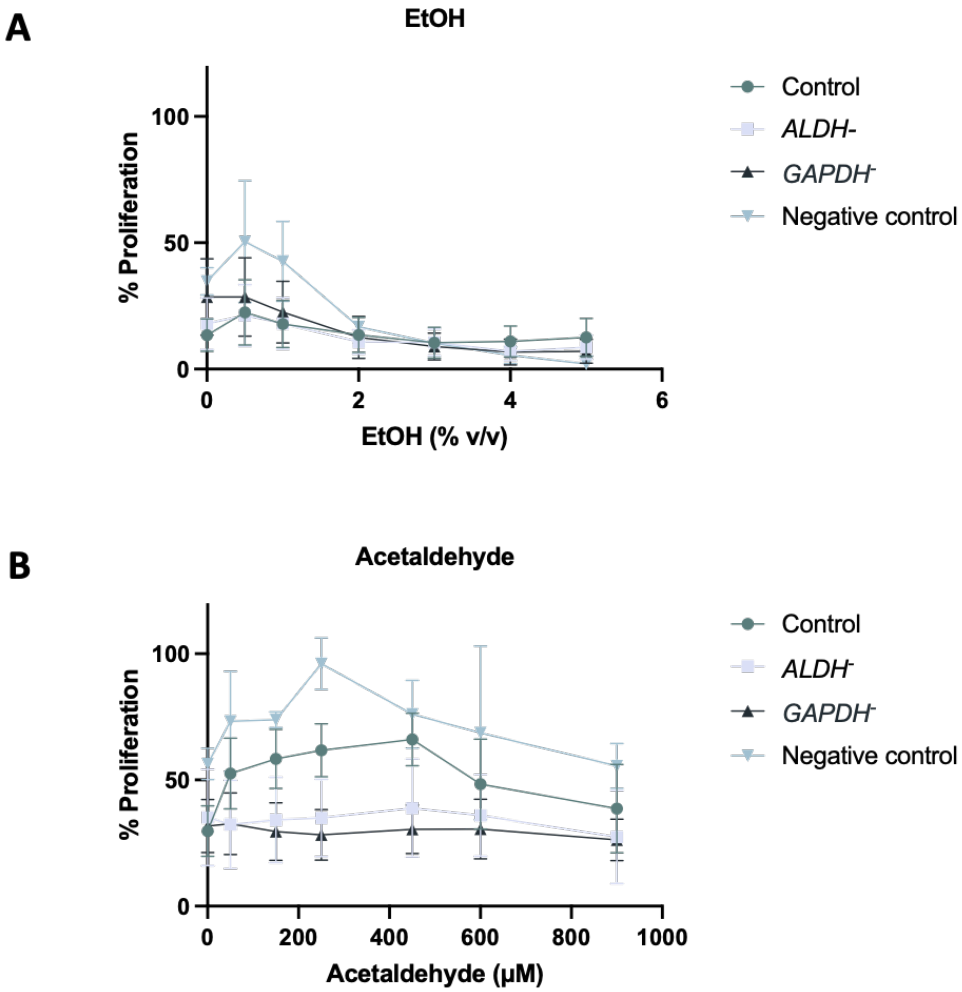


Figure 5.5. (A) Knockdown of ALDH1A1 did not affect DOK cell responses to ethanol treatment. (B) Knockdown of both ALDH1A1 and GAPDH significantly decreased proliferation of DOK cells in the presence of acetaldehyde compared to controls. Cells were transfected with siRNA using Lipofectamine™ 2000 to knockdown ALDH1A1 and GAPDH, as previously described. Cells were treated with either ethanol (0.5-5% v/v) or acetaldehyde (50-900µM) for 24 h. 4 h prior to endpoint, AlamarBlue reagent was added (10% v/v). Absorbance was read on a Spectra MAX Plus Microplate reader at wavelengths 570nm/600nm. Data shown n=3-4, mean ± SD.

5.6 KNOCKDOWN OF ALDH1A1 DID NOT AFFECT TRANSFORMATION OF DYSPLASTIC DOK CELLS

Chronic ethanol treatment was previously shown to increase the rate of wound closure in scratch assays, increase anchorage-independent growth and increase invasive capacities in DOK cells (**Chapter 3, Figure 3.11, 3.14, 3.16**). To examine if chronic ethanol induced transformation in DOK cells via ALDH1A1 expression and activity, *ALDH*$^{-}$ DOK cells were subjected to the same assays. Both control and siRNA-treated cells were either untreated or treated with ethanol (1% v/v) for 24 h. In direct contrast to chronic ethanol treatment of DOK cells, *ALDH*$^{-}$ cells demonstrated a significantly reduced rate of wound closure in scratch assays (**** $P < 0.0001$, **Figure 5.6**). Ethanol (1% v/v, 24 h) significantly increased wound closure rate in control cells, however, the addition of ethanol in *ALDH*$^{-}$ cells had no such effect. Overall, *ALDH*$^{-}$ DOK cells proliferated significantly less compared to control cells. Therefore, chronic ethanol did not induce increased proliferation and wound closure in DOK cells via reduced expression of ALDH1A1.

When *ALDH*$^{-}$ cells were tested for anchorage-independent growth, no significant differences were noted between control and siRNA-treated cells (**Figure 5.7**). The previous observation that chronic ethanol significantly increased anchorage-independent growth is therefore not attributable to reduction of ALDH1A1 expression. The addition of acute ethanol (1% v/v, 24 h) also had no effect on anchorage-independent growth in control or siRNA-treated cells. Similarly, when the invasive capacities of *ALDH*$^{-}$ cells were investigated, no significant differences were noted between control and siRNA-treated cells (**Figure 5.8**). The ability of both acute and chronic ethanol exposure to increase invasion of DOK cells is therefore also not attributable to reduction of ALDH1A1 expression as demonstrated by siRNA.

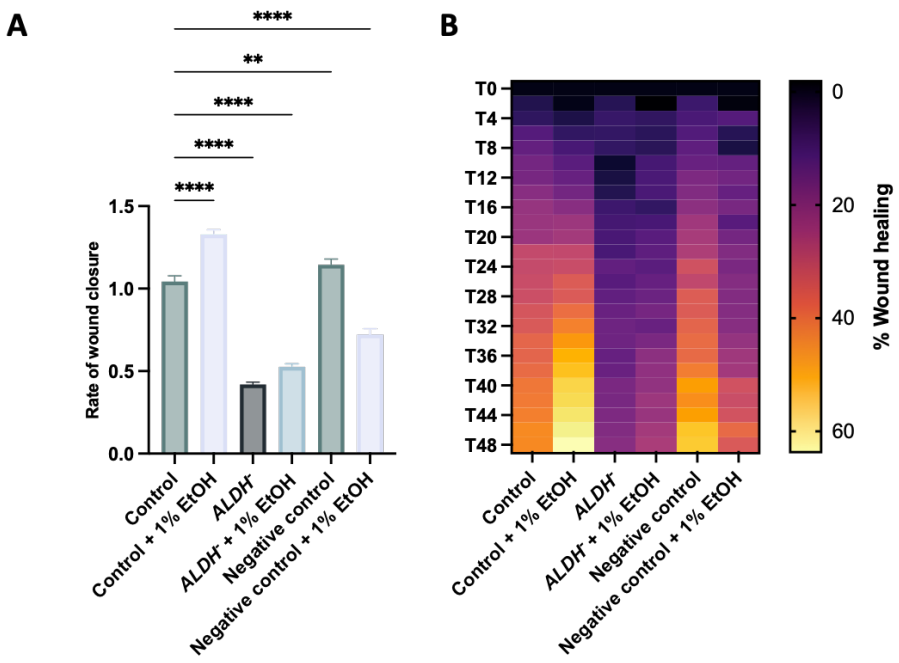


Figure 5.6. Knockdown of ALDH1A1 in DOK cells significantly decreased the rate of wound closure in scratch assays. (B) Heatmap showing the rate of wound closure of DOK cells. Cells were transfected with siRNA using Lipofectamine™ 2000 to knockdown ALDH1A1 as before, prior to seeding around a 3D printed mould to create a zone of exclusion in the monolayer. The area of the monolayer 'wound' was quantified and the rate of closure calculated. Data shown n=3, mean ± SD. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001, ns = not significant.

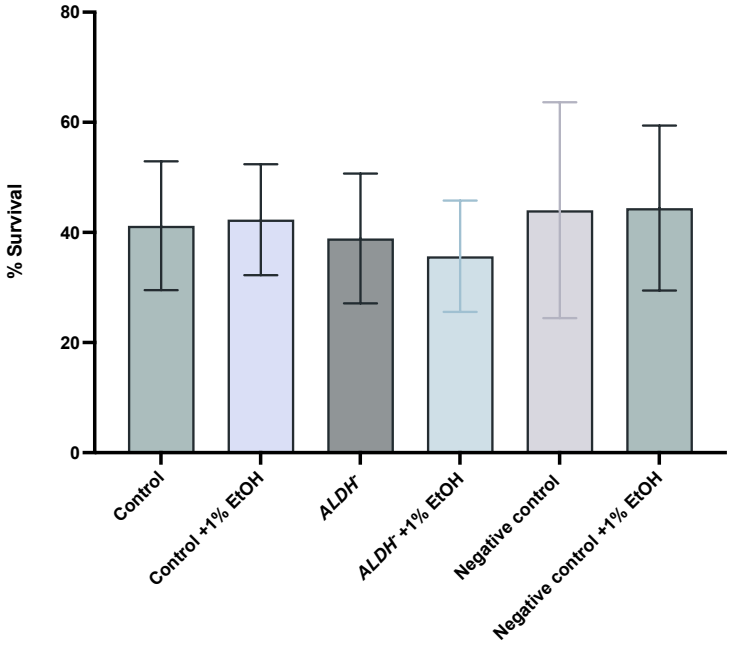


Figure 5.7. Knockdown of ALDH1A1 in DOK cells did not affect anchorage-independent growth. Cells were transfected with siRNA using Lipofectamine™ 2000 to knockdown ALDH1A1 as before. Tissue culture plates were coated with p-HEMA to remove adherent coating before seeding cells. Viability was measured using AlamarBlue assay as previously described. Data shown n=3, mean ± SD.

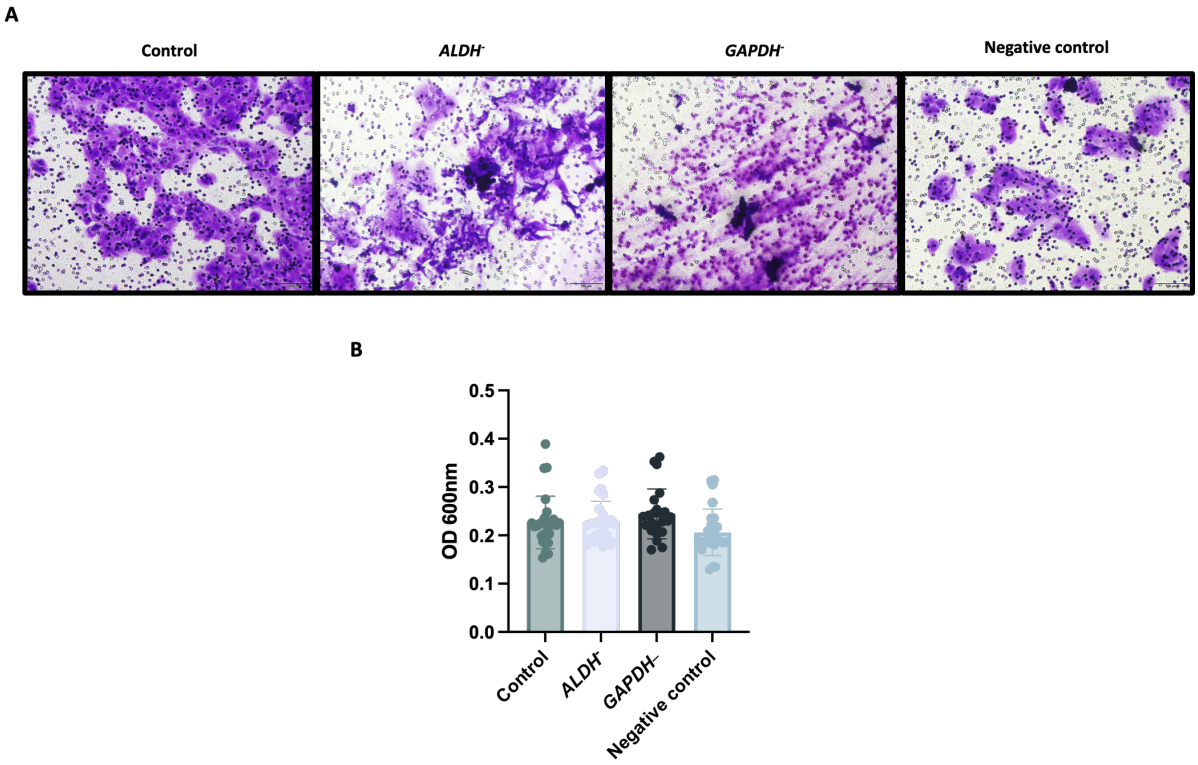


Figure 5.8. Knockdown of ALDH1A1 in DOK cells did not affect invasive characteristics of cells. (A) Representative images of crystal violet stained cells which invaded through ECM-coated inserts. (B) No significant differences in invasion capacities were observed in DOK cells following siRNA treatment. Transwell® inserts (8.0µM pore size) were coated with ECM gel and cells were grown on the inserts in serum-free media. Media with 10% serum was placed in the well below to create a serum-gradient. After 24 h cells were fixed with ethanol, stained with crystal violet, and imaged using an Olympus IX81 phase-contrast microscope. Crystal violet dye was eluted using 10% acetic acid and the absorbance read on a Spectra MAX Plus Microplate reader at 600nm. Data shown n=3, mean ± SD.

5.7 siRNA-MEDIATED KNOCKDOWN OF ALDH1A1 IN CA9.22 CELLS

Despite the initial success transfecting Ca9.22 cells with pEGFP, knockdown of ALDH1A1 in Ca9.22 cells required further optimisation. To ensure uptake of siRNA, the same sequence siRNA molecules were designed and tagged with a Cyanine5 (Cy5) molecule at the 5' end for fluorescent detection (**Figure 5.9**). Using this Cy5-tagged siRNA at a higher concentration compared to that used for DOK cells (siRNA 100mM for Ca9.22 cells and 50nM for DOK cells), it was possible to achieve 85% knockdown of ALDH1A1 expression as detected via Western blot, but only 33% knockdown of positive control GAPDH (** $P < 0.01$ and ns, respectively, **Figure 5.10A**). Knockdown of ALDH1A1 in Ca9.22 cells as determined by flow cytometry showed a 27% reduction in protein expression and this was non-significant (**Figure 5.10B**). ALDH enzyme activity was reduced by 59% (* $P < 0.05$, **Figure 5.10C**). Chronic ethanol exposure and siRNA knockdown had opposing effects on ALDH1A1 expression as summarised in **Table 5.2**.

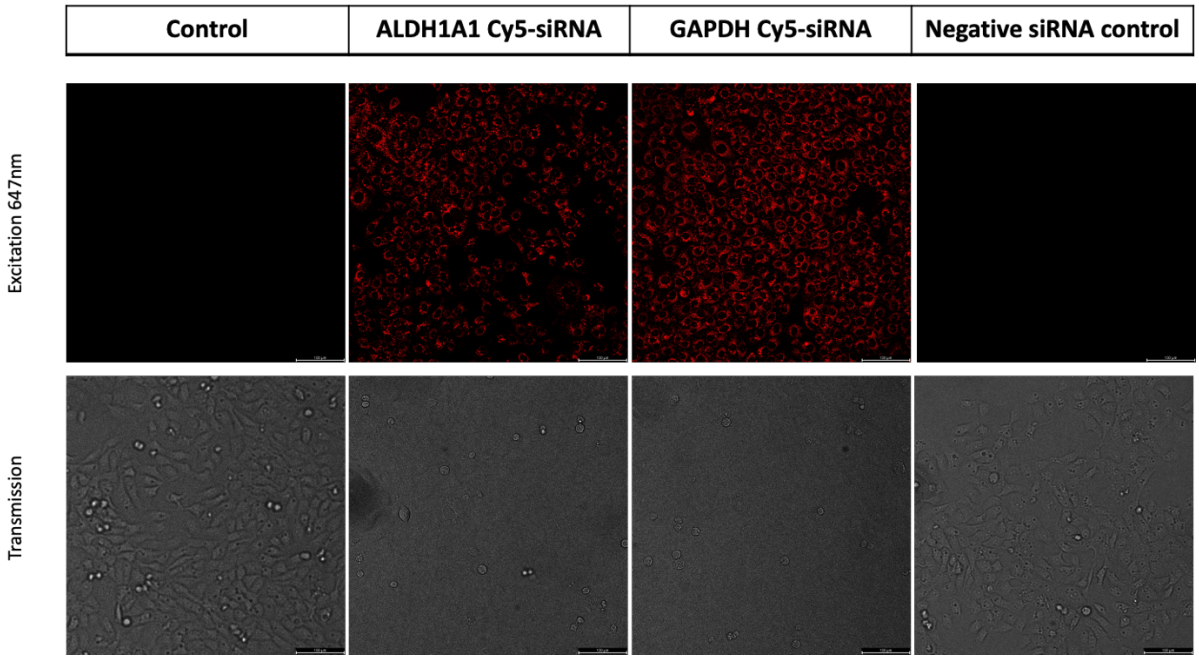


Figure 5.9. Ca9.22 cells successfully transfected with Cy5-tagged siRNA using Lipofectamine™ 2000. To validate uptake of siRNA molecules, Cy5-tagged (red) versions of ALDH1A1 and GAPDH siRNA molecules were used. Cells were seeded onto Ibidi® chambered polymer coverslips and transfected in situ. Images were taken on a Leica SP8 confocal microscope at excitation 647nm 24 h post transfection.

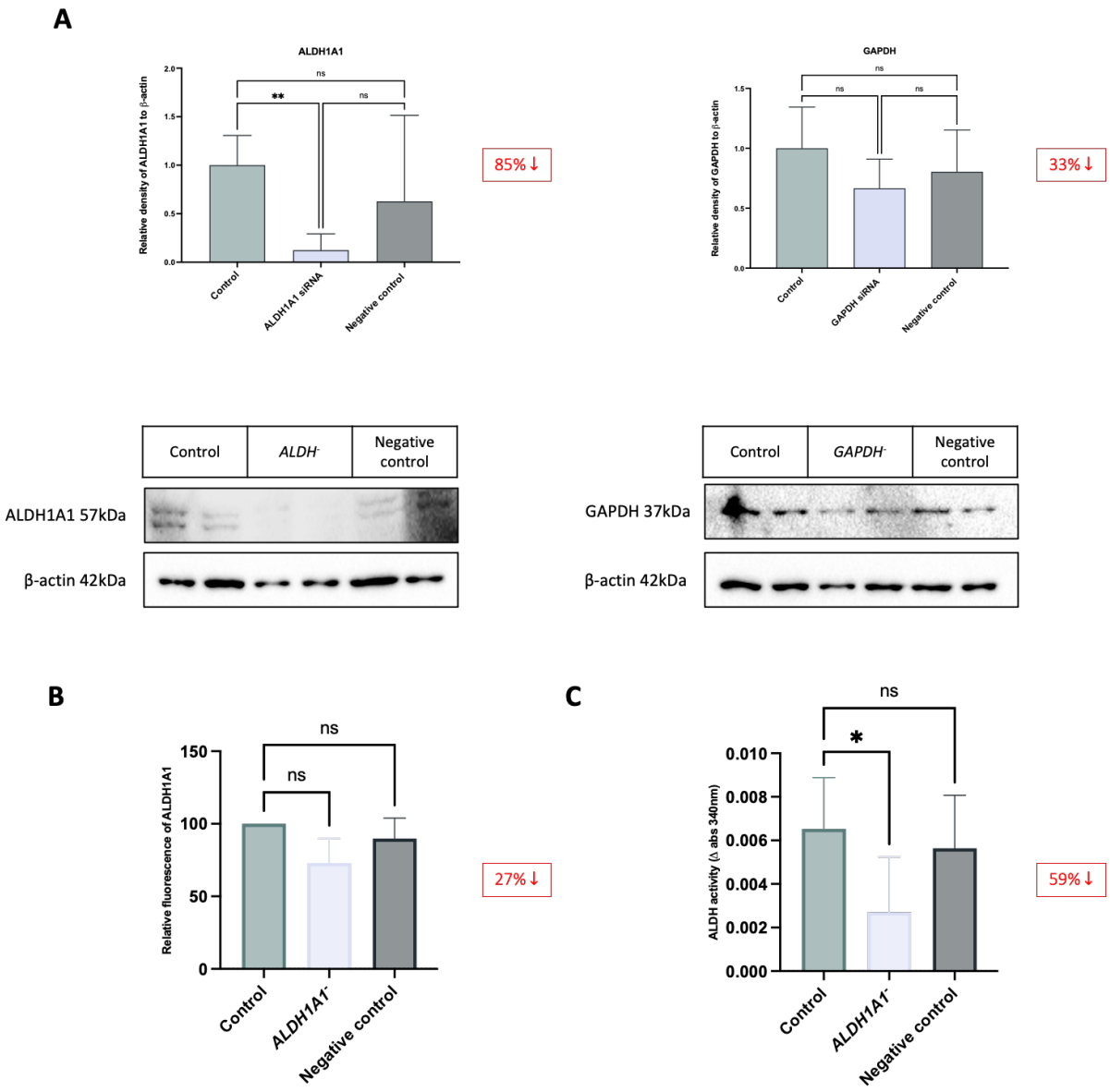


Figure 5.10. (A) siRNA achieved 85% and 33% knockdown of ALDH1A1 and GAPDH respectively in Ca9.22 cells as determined by Western blot. Ca9.22 cells were transfected using Cy5-tagged siRNA molecules and Lipofectamine™ 2000. Western blot and densitometric analysis using ImageJ software was carried out, with β-actin as a loading control. Percentage differences between control and knockdown were calculated from the graphs. Some non-specific binding is visible in the blot image of ALDH1A1 however, the bottom band corresponds to the expected molecular weight of 57kDa for ALDH1A1. Samples were blotted in duplicate. Images shown are representative of n=4. **(B) Knockdown of ALDH1A1 resulted in a 27% knockdown of protein expression in Ca9.22 cells according to flow cytometry.** Cells were analysed using a live cell dye specific for ALDH1A1, as previously described. Data shown n=3, mean ± SD. **(C) Knockdown of ALDH1A1 resulted in a 59% reduction in ALDH enzyme activity in Ca9.22 cells.** ALDH activity assay was measured as previously described. Data shown n=3, mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns = not significant.

Table 5.2. Comparison of the effect of chronic ethanol exposure and siRNA of ALDH1A1 in Ca9.22 cells. Significance is shown with respect to the control. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001, ns = not significant.

Ca9.22	Treatment					
	Chronic ethanol exposure			siRNA knockdown of ALDH1A1		
Analysis	% change from control	Significance	Figure	% change from control	Significance	Figure
ALDH1A1 expression via flow cytometry	25% ↑	**	3.9A	27% ↓	ns	5.10B
ALDH1A1 expression via Western blot	83% ↑	ns	3.9C	85% ↓	**	5.10A
ALDH activity assay	90% ↓	ns	3.5D	59% ↓	*	5.10C

5.8 KNOCKDOWN OF ALDH1A1 IN CA9.22 CELLS SIGNIFICANTLY AFFECTED RESPONSES TO ACETALDEHYDE

ALDH^{-} Ca9.22 cells were treated with ethanol (0.5-5% v/v) and acetaldehyde (50-900μM) for 24 h and viability and proliferation assessed using AlamarBlue assay. No statistically significant difference was noted between the viability of non-transfected cells, *ALDH*$^{-}$*, GAPDH*$^{-}$ and the negative control cells when treated with ethanol (**Figure 5.11A**). When treated with acetaldehyde, less then 20% of *ALDH*$^{-}$ cells were viable, at any concentration used (**Figure 5.11B**). No significant differences were noted between controls when treated with acetaldehyde. Knockdown of ALDH1A1 ablated Ca9.22 cell's ability to tolerate acetaldehyde exposure.

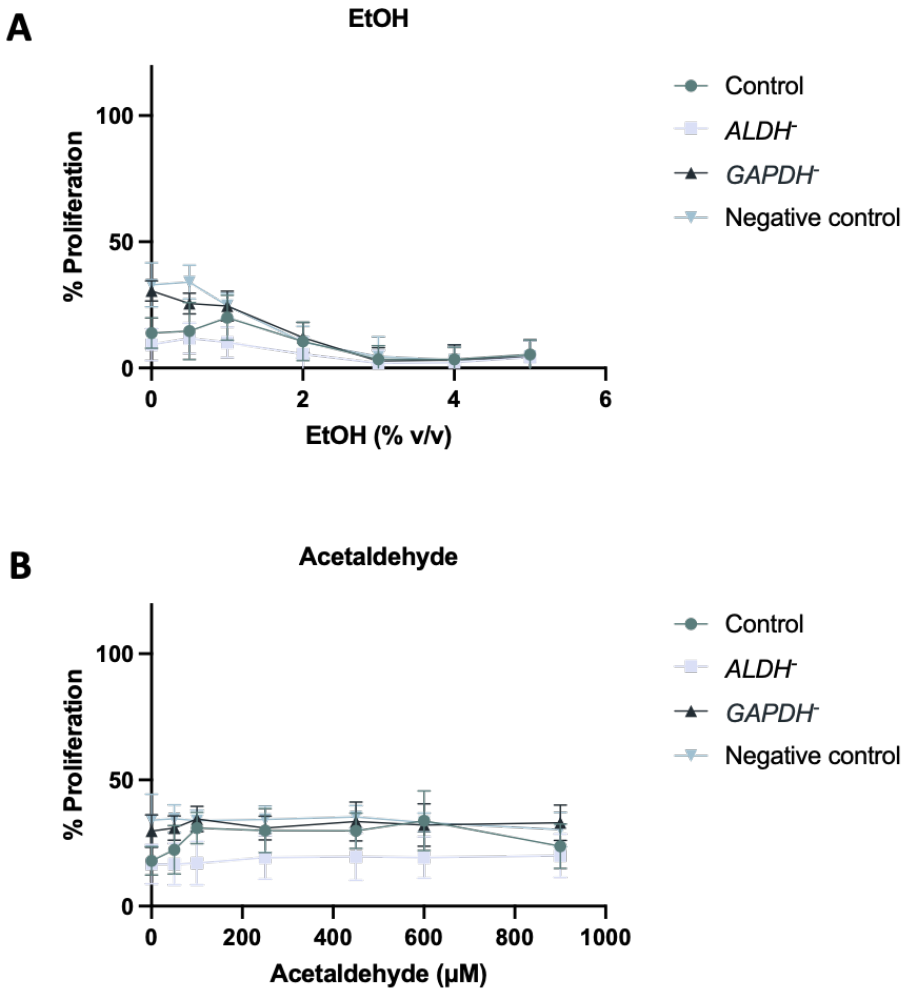


Figure 5.11. (A) Knockdown of ALDH1A1 did not affect Ca9.22 cell responses to ethanol treatment. (B) Knockdown of ALDH1A1 significantly decreased proliferation of Ca9.22 cells in the presence of acetaldehyde compared to controls. Cells were transfected with siRNA using Lipofectamine™ 2000 to knockdown ALDH1A1 as previously described. Cells were treated with either ethanol (0.5-5% v/v) or acetaldehyde (50-900µM) for 24 h. 4 h prior to endpoint, AlamarBlue reagent was added (10% v/v). Absorbance was read on a Spectra MAX Plus Microplate reader at wavelengths 570nm/600nm. Data shown n=3-4, mean ± SD.

5.9 KNOCKDOWN OF ALDH1A1 DID NOT PROMOTE METASTATIC PROCESSES IN CA9.22 CELLS

Chronic ethanol exposure was shown to increase the expression of ALDH1A1, increase the rate of wound closure in scratch assays, increase invasive capacities and decrease expression of E-cadherin in Ca9.22 cells (**Chapter 3**, **Figure 3.9, 3.11, 3.15, 3.16**). *ALDH*$^{-}$ Ca9.22 cells were subjected to the same assays to test if these effects were downstream of ALDH1A1 expression. Both control and siRNA-treated cells were either untreated or treated with ethanol (1% v/v) for 24 h. It was observed that *ALDH*$^{-}$ Ca9.22 cells had significantly reduced rates of wound closure in scratch assays (**** $P < 0.0001$, **Figure 5.12**). This observed effect was the opposite to chronic ethanol exposure. However, the addition of ethanol (1% v/v, 24 h) to *ALDH*$^{-}$ Ca9.22 cells significantly increased the rate of wound closure (** $P < 0.01$). Since acute ethanol exposure was capable of rescuing decreased proliferation and wound closure of ALDH1A1 knockdown cells, the effects of ethanol are therefore not attributable to ALDH1A1 modulation in Ca9.22 cells.

When *ALDH*$^{-}$ Ca9.22 cells were examined for anchorage-independent growth, no significant differences were noted between control cells and siRNA-treated cells (**Figure 5.13**). This was not surprising, as neither acute nor chronic ethanol was previously shown to affect anchorage-independent growth in Ca9.22 cells (**Figure 3.14**). Similarly, when the invasive capacities of *ALDH*$^{-}$ Ca9.22 cells were investigated, no significant differences were noted between control and siRNA-treated cells (**Figure 5.14**). While both acute and chronic ethanol exposure were previously shown to significantly increase invasion of Ca9.22 cells (**Chapter 3, Figure 3.16**), this effect is not attributable to modulation of ALDH1A1 expression. The positive siRNA control of *GAPDH*$^{-}$ demonstrated a slightly lower ability to invade through the ECM, while this was non-statistically significant, it was possibly due to decreased glycolytic activity and proliferation as a result of GAPDH knockdown (**Figure 5.14**).

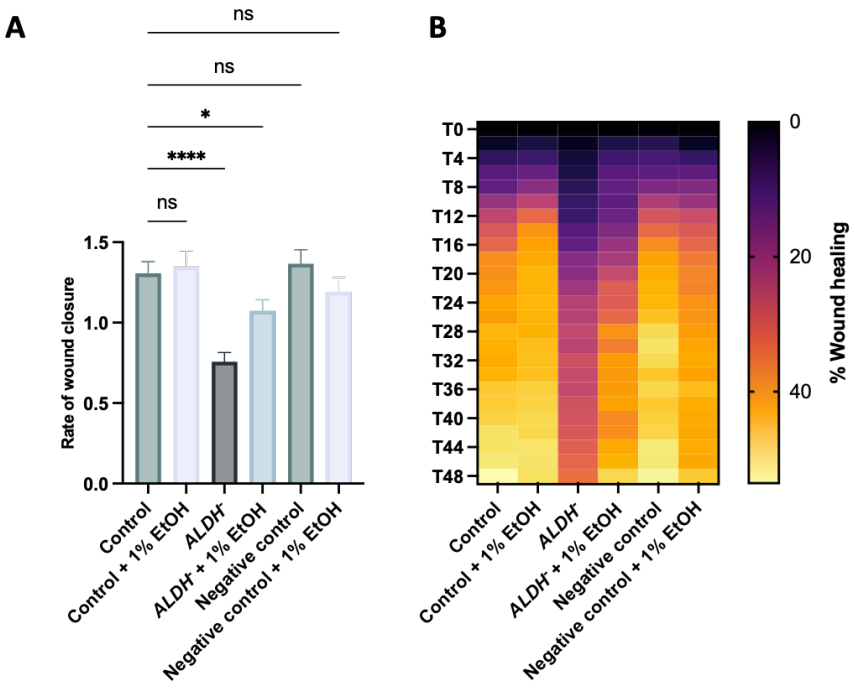


Figure 5.12. (A) Knockdown of ALDH1A1 in Ca9.22 cells significantly reduced the rate of wound closure in scratch assays. (B) Heatmap showing the rate of wound closure of Ca9.22 cells. Cells were transfected with siRNA using Lipofectamine™ 2000 to knockdown ALDH1A1 prior to seeding around a 3D printed mould to create a zone of exclusion in the monolayer. The area of the monolayer 'wound' was quantified and the rate of closure calculated. Data shown n = 3, mean ± SD. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001, ns = not significant.

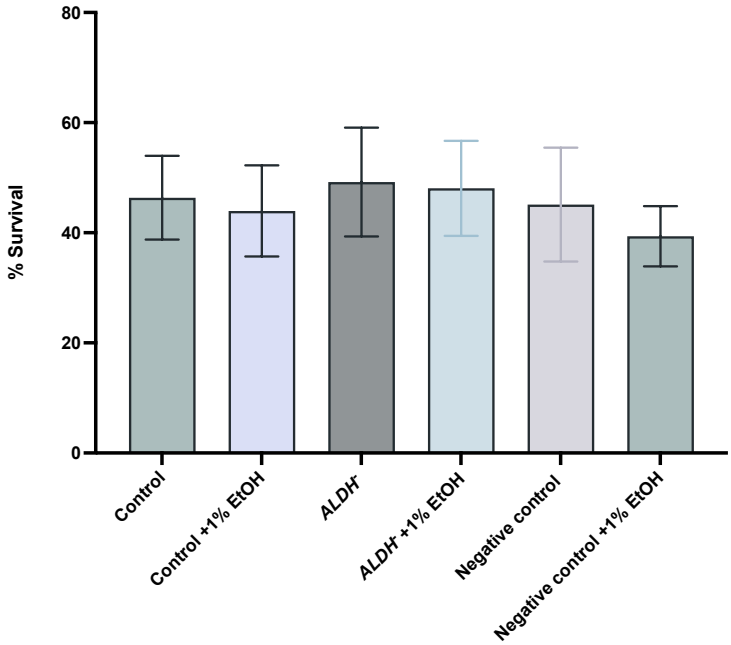


Figure 5.13 Knockdown of ALDH1A1 in Ca9.22 cells did not affect anchorage-independent growth. Cells were transfected with siRNA using Lipofectamine™ 2000 to knockdown ALDH1A1 as previously described. Tissue culture plates were coated with p-HEMA to remove adherent coating before seeding cells. Viability was measured using AlamarBlue assay as previously described. Data shown n=4, mean ± SD.

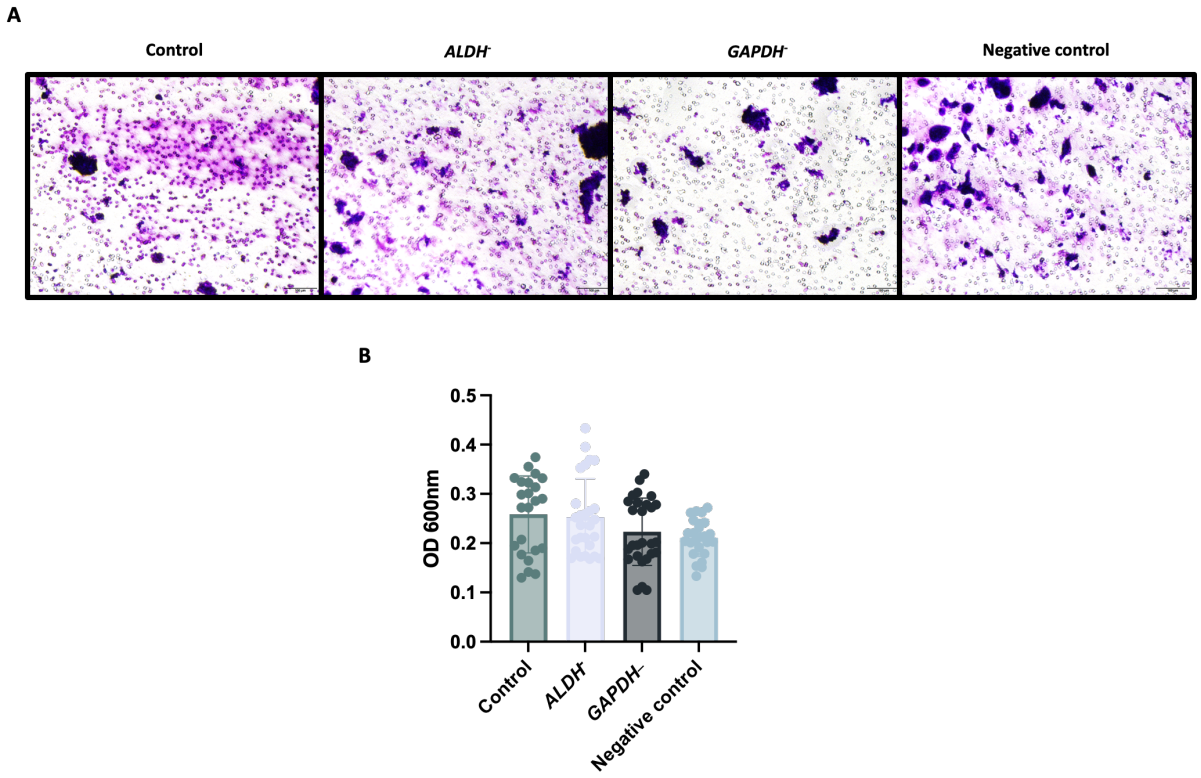


Figure 5.14. Knockdown of ALDH1A1 in Ca9.22 cells did not affect invasive characteristics of cells. (A) Representative images of crystal violet stained cells that invaded through ECM-coated inserts. (B) No significant changes in invasion capacities were observed in Ca9.22 cells following siRNA treatment. Transwell® inserts (8.0µM pore size) were coated with ECM gel and cells were grown on the inserts in serum-free media. Media with 10% serum was placed in the well below to create a serum-gradient. After 24 h cells were fixed with ethanol, stained with crystal violet, and imaged using an Olympus IX81 phase-contrast microscope. Crystal violet dye was eluted using 10% acetic acid and the absorbance read on a Spectra MAX Plus Microplate reader at 600nm. Data shown n=3, mean ± SD.

5.10 Discussion

Validation of successful siRNA of ALDH1A1 and GAPDH in Ca9.22 and DOK cells was tested utilising flow cytometry, Western blot analysis and ALDH activity assay. These experiments all rely on different detection methods and by using multiple assays it increases the information about the efficacy of the knockdown. There are advantages and disadvantages to each method of validation. Similarly, the existence of multiple ALDH isoforms with similar sequence homology is a major confounding factor in the investigation of their role in cancer development and progression. The extent of knockdown varied between detection methods, as summarised in **Table 5.1** and **Table 5.2**. There are a few possible explanations for these discrepancies. The use of acetaldehyde as a substrate for the ALDH activity assay eliminates the ability of certain isoforms to contribute to detected activity (**Table 1.3**) and following knockdown of ALDH1A1 it is very likely that other isoforms compensate for its absence. Propionaldehyde has been shown to be a more selective substrate for ALDH1A1, so the variation of substrates may yield different results (241, 252). The activity assay gives an overall view of the ability of the cells to metabolise acetaldehyde, but a limiting factor to the information it provides is the use of cell lysates as opposed to whole cells. The capacity of the cells to metabolise acetaldehyde *in vitro* may vary.

The flow cytometry protocol used Green-AIDeSense™ live cell dye, which was developed as a specific fluorescent probe for the ALDH1A1 isoform (252). While the authors provide a thorough examination of the specificity of the probe, they concede it may potentially have unaccounted for cross-reactivity. The molecule is weakly fluorescent until activated, so some non-specific staining may contribute to fluorescent signalling. To counteract this, the dye comes with a matching non-responsive control dye to compensate for non-specific fluorescence (data not shown). The dye was screened against a panel of purified ALDH proteins, K562 human chronic myeloid leukaemia cell line, a CSC cell line and mouse models. However, when compared to the data presented in this

thesis, despite the extensive work done by Anorma et al., there is some contrasting data presented in their paper that may suggest it is not specific to ALDH1A1 (252). This may explain that despite apparent successful knockdown of ALDH1A1 via Western blot and decreased ALDH activity in both Ca9.22 and DOK cell lines, the fluorescence intensity of the ALDH1A1 dye was not significantly lower in knockdown samples when tested using flow cytometry (**Table 5.1, 5.2**). For example, the paper by Anorma et al. uses both DSF and DEAB as 'specific ALDH1A1 inhibitors' to demonstrate specificity of their probe. However, it was previously discussed how DSF is an inhibitor of CYP2E1 and several other ALDH isoforms, as well as acting via a metabolite formed *in vivo* and not via inhibition of ALDH directly (77, 216, 229, 231, 233). Equally, DEAB has also been shown to be a broad spectrum inhibitor of ALDH isoforms, and was an ineffective inhibitor of ALDH activity in the oral cell lines used here, despite both cell lines possessing ALDH1A1 (**Chapter 4**) (241, 242, 248). The authors comment that their attempts to co-crystallise AIDeSense™ dye and ALDH1A1 proteins were unsuccessful, however this structure may be of major importance in ascertaining how and on what the dye is operating on within cell systems. It therefore cannot be assumed that this cell dye is exclusively activated by ALDH1A1 in the cell lines used here. Finally, Western blot utilises an antibody specific to ALDH1A1, a method broadly used across the literature, but is more time-consuming, requires more optimisation and relies on the quality of the antibodies used (253).

An interesting finding of this study was that chronic ethanol exposure and ALDH1A1 knockdown had opposite effects on DOK cells response to acetaldehyde. The previously observed increase in proliferation in chronic ethanol treated cells that were exposed to acetaldehyde suggested a mechanism for ethanol to increase utilisation of acetaldehyde in proliferation pathways (**Figure 3.6**). The knockdown of both ALDH1A1 and GAPDH inhibited this increase in proliferation in the presence of acetaldehyde (**Figure 5.5**). The production of acetate from ethanol metabolism may be a source of energetic fuel via conversion into acetyl-CoA. Acetaldehyde is converted to acetate via ALDH enzymes, then into acetyl-CoA via acetyl-CoA synthetases. Although this is not a common

physiological pathway, cancer cells can use this pathway to proliferate despite unfavourable growth conditions (155, 156, 254). DOK cells may also possess this capacity or may acquire this ability as malignant transformation progresses. The effects of ethanol on membrane permeability may also increase the bioavailability of acetaldehyde and acetate. The data from siRNA experiments further consolidated this theory that acetaldehyde is being shuttled into glycolytic pathways, as theoretically ALDH1A1 knockdown would reduce its conversion into acetate, and GAPDH knockdown would impede glycolysis overall.

Furthermore, knockdown of ALDH1A1 in DOK cells significantly impaired rate of wound closure, and this effect could not be reversed by addition of ethanol, unlike in Ca9.22 cells (**Figure 5.6, 5.12**). The inability of additional ethanol to restore wound closure rates to normal rates further suggests indirect mechanisms of ethanol-induced carcinogenesis, potentially via acetaldehyde, as its production would be impaired by ALDH1A1 knockdown. Evidently ALDH1A1 is important for the proliferation of DOK cells in general - this effect has been shown previously in primary oral keratinocytes and *ex vivo* oral mucosal tissues, where ALDH1A1 knockdown suppressed proliferation and also led to the development of a more normal differentiation pattern of cells (82). Although addition of acetaldehyde did not previously affect wound closure rates in DOK cells (**Figure 3.12**), it may affect wound closure rates in either chronic ethanol treated, ALDH1A1 or GAPDH knockdown DOK cells. Of note, acetaldehyde production in the oral cavity is not limited to ADH activity of the oral mucosa. As previously reviewed by O'Grady et al., microbial species within the oral cavity have been shown to produce up to 200μM acetaldehyde following ethanol exposure *in vitro* (157). Oral microbiota composition may also be influenced by alcohol consumption, with salivary acetaldehyde being generally higher in drinkers (56, 255-257). Consumption of alcohol may be contributing not only to the increased production of acetaldehyde but also potentiating acetaldehyde-induced transformation of dysplasia.

Despite successful knockdown of ALDH1A1 in Ca9.22 cells (**Table 5.2**), the control for siRNA transfection of GAPDH could not be reduced in expression at any concentration of siRNA tested. The collection of cell lysates for Western blot included both live and dead cells, so the lack of apparent GAPDH knockdown was not because it was lethal to the cells. Since Ca9.22 cells are a cancer cell line, they are likely more metabolically active and highly glycolytic compared to other cell types (258, 259). This reliance on glycolysis might confer a high expression level of glycolytic proteins such as GAPDH, impeding substantial protein knockdown in this cell line.

Proliferation and viability of *ALDH*$^{-}$ Ca9.22 cells was not significantly lower in the presence of ethanol compared to control cells, unlike when treated with acetaldehyde, where the viability of the cells was <20% at any given concentration (**Figure 5.11**). Ca9.22 cells were previously shown to experience relatively high levels of cell death in the presence of acetaldehyde (**Figure 3.3**), and further knockdown of ALDH1A1 enhanced this effect. This data suggests that while ALDH1A1 expression contributes heavily to the metabolism and clearance of acetaldehyde in Ca9.22 cells, cell death via ethanol exposure is not due to subsequent acetaldehyde accumulation. This agrees with previous data showing that expected levels of *in vitro* acetaldehyde following ethanol treatment did not confer the same effects on Ca9.22 cells in scratch or anchorage-independent growth assays (**Figure 3.12, 3.13, 3.14**). It is of note that the cancer cell line did not seem to experience acetaldehyde-induced proliferation as seen in the dysplastic cell line, suggesting a specific role for acetaldehyde in the transformation of dysplasia.

In both Ca9.22 and DOK cells, ALDH1A1 knockdown had no effect whatsoever on anchorage-independent growth or invasion capacities of cells (**Figure 5.7, 5.8, 5.13, 5.14**). Therefore, the previously observed ethanol-induced anchorage-independent growth and invasion in DOK cells and increase in wound closure rates and invasion of Ca9.22 cells were independent of ALDH1A1 expression. Ethanol is not promoting these transformative events or metastatic processes via

ALDH1A1 modulation. Moreover, addition of ethanol to *ALDH*$^{-}$ Ca9.22 cells in scratch assays significantly increased the rate of wound closure, therefore ethanol exposure is evidently acting via mechanisms independent of ALDH1A1 expression. It has been shown multiple times throughout the literature that ALDH expression changes from normal oral mucosa, through dysplasia to OC (175-177). The data presented here suggests that these changes to ALDH expression are likely a secondary feature, and not the primary mechanism of transformation of dysplasia or metastasis of OC.

It is possible that ethanol exerts its effects via other ALDH isoforms not investigated here, or via physical mechanisms such as altering membrane integrity. Ethanol has been shown to greatly affect membrane integrity, modulating permeability in a time-dependent manner (39, 40, 45). This is mainly thought to contribute to carcinogenesis via increased uptake of carcinogens or increased invasion into cells by bacterial species (41, 43). However, these possibilities are excluded due to the nature of the cell culture models used here. Cell samples from alcoholic patients in Brazil showed that when compared to non-drinkers, cytologic changes including an abnormal nucleus:cytoplasm ratio was also observed, indicating carcinogenic changes in the oral mucosa, although the mechanisms were unclear (260). This increase in cell nuclei size and reduction in cytoplasm area has been observed in both rats fed chronic ethanol or acetaldehyde and in patients with oral lesions with a high risk of developing into OC (261-263). Ethanol has also been shown to promote proliferation of mouse intestinal epithelial cells via increased expression of cyclin proteins, proliferation markers such as Ki-67 and target genes of Wnt signalling (264). Ethanol may be acting via indirect effects on cell cytology such as nucleus:cytoplasm ratio or on proliferation markers in the OC cell lines used here.

A possible mechanism for ethanol-induced transformation and metastasis is via the production of NADH. The sudden overproduction of NADH as a by-product of ethanol metabolism may interfere

with glycolysis and oxidative phosphorylation (OXPHOS) in cells. The reactions catalysed by both ADH and ALDH enzymes produce NADH, and therefore it would be unlikely that the knockdown of ALDH1A1 alone would significantly decrease production of NADH from exposure to ethanol. Although NADH is re-oxidized in the electron transport chain (ETC) and therefore becomes an energy source of ATP, surplus NADH has also been shown to decrease the rate of the tricarboxylic acid (TCA) cycle, impair glycolysis and inhibit gluconeogenesis (265, 266). This is paradoxical to the Warburg hypothesis that cancer cells favour glycolysis and have dysfunctional mitochondria – in fact, cancer cells have been found to have well-preserved mitochondrial function or even enhanced mitochondrial OXPHOS compared to normal cells (266). As indicated in DOK cells, the production of acetate from ethanol metabolism may be a source of energetic fuel via conversion into acetyl-CoA. The TCA cycle uses acetyl-CoA to generate ATP and NADH, further feeding into the ETC to ultimately produce ATP through OXPHOS. In theory, acetyl-CoA production could compensate for energy deficits caused directly or indirectly by ethanol, while the changes in expression of ALDH enzymes might also prevent surplus production of NADH, but rather maintain a level that can be used by the cells as metabolic fuel.

Acetate utilisation will depend on its availability to the cells and the expression of acetate-capturing enzymes, such as mitochondrial localised acetyl-CoA synthetase (ACSS1). A confounding factor to this theory is that ALDH1A1 and ALDH2 are the main isoforms of ALDH posited to be responsible for breakdown of acetaldehyde into acetate. Both Ca9.22 and DOK cells lack the expression of the ALDH2 isoforms (**Figure 3.7**), so in ALDH1A1 knockdown cells it would be expected that ethanol-induced proliferation is entirely inhibited. The expression levels of ACSS enzymes in both oral cell lines may provide further insights into the role of acetate in observed ethanol-induced proliferation and warrants further study.

ALDH1A1 has key roles in RA signalling, which is essential in cell proliferation, cancer stemness and angiogenesis. ALDH1A1 has been shown to have a role in the progression of both breast and bladder cancer specifically via RA receptors and signalling pathways (267, 268). It has been reported that the role of ALDH1A2 (an isoform closely related to ALDH1A1, Figure 1.2) in the pathogenesis of HNCs is critically linked to its involvement in RA signalling (125). ALDH1A2 catalyses an irreversible step in the synthesis of RA, and its low expression in HNCs impaired RA signalling, promoting malignant progression (125). Ethanol has been shown to inhibit RA synthesis during fetal alcohol syndrome, and in general disrupt retinoid homeostasis in later life (269, 270). Although the interactions between ethanol and retinoids are still under investigation, the relationship of ethanol, ALDH and RA signalling is an aspect not explored in this thesis that may augment our understanding of ethanol's contribution to oral carcinogenesis.

While the expression of ALDH1A1 is associated with increased histological grade of lesions and the presence of metastasis (83, 89, 176), based on the overall evidence presented in this thesis it is more likely that its expression is secondary to these cell characteristics and processes, and not a driving factor. Nevertheless, ALDH enzymes clearly have important roles in cellular pathways associated with transformation, metastasis, and prognosis of OC. Moreover, ALDH enzymes remain to be potentially valuable prognostic markers. The expression of ALDH1A1 has also been shown in tandem with other biomarkers, such as CSC markers CD44 or PD-L1, to be a prognostic marker for HNC patients (225, 226, 271). The molecular mechanisms of ethanol-induced carcinogenesis require further research to be fully understood.

Chapter 6: Ethanol Modulates *Candida albicans*-Induced Oral Carcinogenesis

6.1 Introduction

Inflammation has been recognised as a fundamental factor in the neoplastic process (272). Pathogen recognition receptors (PRRs) found on oral epithelial cells have the capacity to respond to the presence of bacterial components and influence inflammation in the oral mucosa (273). Habits such as alcohol consumption can have immunosuppressive effects, affecting the sensitivity of PRRs to bacterial ligands depending on the length of exposure time. For example, acute alcohol exposure in monocytes induced a decreased response to bacterial ligands such as LPS, whereas chronic alcohol exposure increased sensitivity, increasing the production of TNF-α in response (274). Simultaneously, alcohol users tend to have worse overall oral health, potentially leading to overgrowth of pathogenic bacteria and subsequently facilitating further inflammation (256, 275-277).

The oral microbiome is host to over 700 identified species, with whole genome sequences of over 400 other taxa not yet identified (278). There is evidence of bacteria having a direct causal relationship with some cancers, such as the colonisation of the human stomach by *Helicobacter pylori* and its role in gastric cancer (279), and *Salmonella typhi* colonisation in the development of gallbladder cancer (280). Recent research into the role of the oral microbiome in OC development has not yet led to any definitive relationship, but the emergence of some interesting patterns. The microbiome of the 'normal' healthy oral cavity consists of the same species as oral cavities of patients with PMDs, dysplasia and established tumours. However, polymicrobial dysbiosis, as opposed to the infection and colonisation by a single pathogen, is posited to be a main contributor to oral diseases. The 'healthy' microbiome consists of an abundance of aerobic bacteria, such as

Streptococci, Staphylococcus and *Neisseria* species (278, 281). The proportion of aerobic species has been shown to decrease linearly from healthy controls, patients with dysplasia and patients with OC (281-288). There have been a multitude of oral microbiome sequencing studies, using 16sRNA sequencing techniques, that have identified a functionally inflammatory bacteriome in relation to its composition, but no single species demonstrated a significant risk association with OC development (285, 289). However, the presence of bacterial species in the tumour microenvironment cannot be taken as causative for OC – rather, the development of OC may foster an environment for the bacteria/fungi to thrive thereafter. Alcohol consumption has been shown to alter the composition of the oral microbiome, which could foster the transformation from normal microbiome composition to disease state composition (290, 291), creating an environment to enhance OC progression. The exact mechanisms through which alcohol and the oral microbiome affect OC progression are not clear.

A ubiquitous species in the oral cavity is *Candida albicans*. It exists as a polymorphic fungus that is part of the human commensal flora. However, it is an opportunistic pathogen, with the potential to become invasive and pathogenic when there is a disturbance in the balance of flora or a debilitation in the host. It has been reported that the presence of yeast in the oral cavity has no statistical significance on mortality rate of cancer patients (292, 293). However, a significant positive correlation is reported between oral cancer occurrence and oral yeast colonisation, with alcohol having an additive effect on risk association (294). Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) is a rare disease with a definitive link between *Candida* and OC. It is caused by a recessive mutation in the immune system that is characterised by chronic candidiasis, and the development of OC is a distinct complication of APECED (295-297).

The innate response of oral epithelial cells to pathogenic *C. albicans* is modulated through NF-κB and MAPK, whereby epithelia are quiescent during low fungal burden but react to damage-inducing

hyphae. Activation of NF-κB and MAPK is initially due to recognition of fungal cell wall structures, such as β-glucan, which remain intact in heat-inactivated *C. albicans* (HICA) (298, 299). The second phase of response is dependent on filamentous forms of *C. albicans* (298, 299). Inflammation and inhibition of apoptosis induced by *C. albicans* has been postulated to be a major cause of malignant transformation (300, 301), as well as acting as a co-carcinogen with other risk factors, such as alcohol (302). The co-culture of primary oral leukoplakia keratinocytes with *C. albicans* showed an increase in inflammatory cytokines, decreased rates of apoptosis and overall contributed to malignant transformation (300). HICA has been shown to induce increased cell migration, MMP activity and oncometabolite production of OC cells in the same way as live *C. albicans*, albeit to a lesser extent (303). HICA is therefore a valid and efficient tool for studying the effects of *C. albicans*-associated oral carcinogenesis.

C. albicans also possess ADH enzymes that are not only capable of producing carcinogenic levels of acetaldehyde, but are immunogenic to oral cells (160, 304, 305). The production of acetaldehyde by *C. albicans* has a clear correlation with other fungal virulence factors – *C. albicans* isolates from OC patients have significantly increased abilities to form a biofilm, produce hydrolytic enzymes and evoke host immune responses compared to controls (305). Similarly, enhanced biofilm formation, invasion and damage of host tissues and larger amounts of acetaldehyde production were observed when genes encoding ADH enzymes in *C. albicans* were genetically modified (306). *C. albicans* therefore may operate via multiple modalities to contribute to progression of OC, aided or enhanced by alcohol consumption.

6.2 Aims

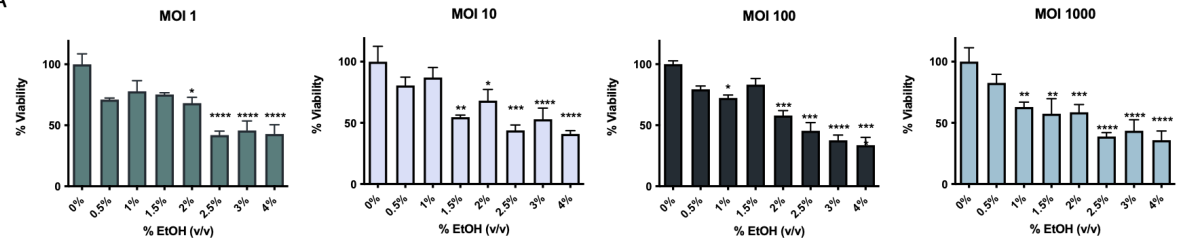
- Characterise *in vitro* co-culture models of oral squamous cell carcinoma cell line Ca9.22 with ethanol and heat-inactivated *C. albicans.*

- Determine the effect of ethanol and heat-inactivated *C. albicans* in combination on cell death, inflammation, and ROS production in Ca9.22 cells.
- Determine the influence of ethanol and heat-inactivated *C. albicans* in combination on metastatic characteristics of Ca9.22 cells.

6.3 Establishing a co-culture model

In order to establish an *in vitro* model of ethanol and HICA co-culture, Ca9.22 cells were co-cultured with HICA at several ratios (MOI of 1, 10, 100, 1000) for 24 h before addition of ethanol (1-4% v/v) for a further 24 h and cell viability was assessed using the AlamarBlue assay. As the MOI was increased, the concentration of ethanol required to significantly affect cell viability decreased (**Figure 6.1A**). At an MOI of 1, cell viability was not significantly affected until ethanol concentration was ≥2%, at an MOI of 10, cell viability was significantly decreased at ethanol concentrations ≥1.5%, and at an MOI of 100 and of 1000, cell viability was significantly decreased at ethanol concentrations ≥1% (**Figure 6.1B**). It was determined that an MOI of 10 would be used for ensuing experiments as it allowed the broadest range of ethanol concentrations to be used while maintaining cell viability ≥40% (**Figure 6.1A**).

A



B

EtOH (% v/v)	MOI 1	MOI 10	MOI 100	MOI 1000
0.5	ns	ns	ns	ns
1	ns	ns	*	**
1.5	ns	**	ns	**
2	*	*	***	***
2.5	****	***	***	****
3	****	****	****	****
4	****	****	****	****

Figure 6.1. (A) As MOI of heat-inactivated *C. albicans* co-culture increased, the concentration of ethanol that significantly affected viability of Ca9.22 cells decreased. Cells were co-cultured with several MOI of HICA (1, 10, 100, 1000) for 24 h, then ethanol was added (1-4% v/v) for a further 24 h. AlamarBlue reagent was added (10% v/v) 4 h prior to endpoint. Absorbance was read on a Spectra MAX Microplate reader at 570/600nm. **(B) Table summarising the effect of several MOIs of HICA and ethanol concentration on cell viability.** Significance of MOI ratio to ethanol concentration on cell viability is shown with respect to co-culture with HICA alone. Data shown n=2 ± SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns = not significant.

6.4 Co-culture with HICA prior to ethanol treatment did not influence cell proliferation, cell cycle distribution or cell death

Ca9.22 cells were co-cultured with heat inactivated *C. albicans* (HICA) at an MOI of 10 for 24 h before treatment with ethanol (1% or 3% v/v) to determine if prior exposure to HICA would influence cell proliferation and death. Ethanol concentrations ≥3% increased cell cycle arrest but prior co-culture with HICA did not exacerbate this effect (**Figure 6.2A**). Similarly, ethanol increased cell death via apoptosis, as previously observed, but the effect of prior co-culture with HICA had no significance on cell death either alone or in combination (**Figure 6.2B**).

To confirm that cell death was occurring via apoptosis, Western blot analysis was used to detect protein markers poly(ADP-ribose) polymerase (PARP), cleaved PARP and cleaved caspase-3. Caspase-3 is an early apoptotic marker and is responsible for the cleavage of PARP – a hallmark event of apoptosis. Activation of caspase-3 requires proteolytic processing of its inactive zymogen into activated fragments – p17 (detected here) and p12. An increase in cleaved PARP was observed following ethanol treatment (1% and 3% v/v), while cleaved caspase-3 was only detectable at ethanol 3% v/v (**Figure 6.2C**). Densitometric analysis demonstrated that prior co-culture with HICA did not significantly affect the levels of these apoptotic proteins detected compared to ethanol alone (**Figure 6.2D**).

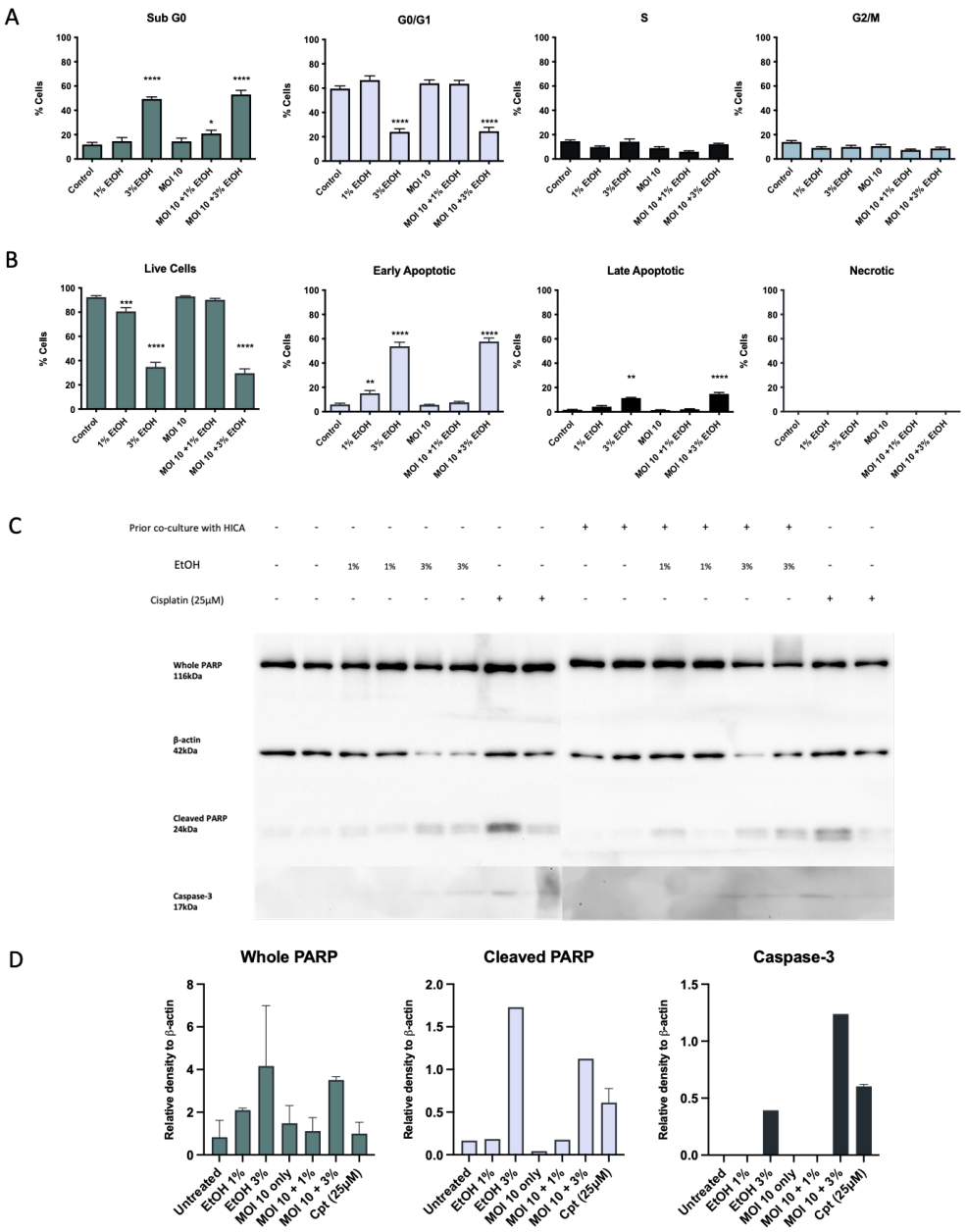


Figure 6.2. (A) Ethanol ≥3% v/v increased cell cycle arrest, prior co-culture with HICA did not influence cell cycle distribution irrespective of ethanol concentration. Ca9.22 cells were co-cultured with HICA (MOI 10) for 24 h before treatment with ethanol (1% and 3% v/v) for a further 24 h. Cells were fixed and stained with PI before analysis on a FACs Canto flow cytometer at 488nm excitation. Significance is shown with respect to the control. **(B) Ethanol induced apoptosis as measured by flow cytometry, prior co-culture with HICA did not influence this effect.** Cells were treated as before then stained with Annexin V and PI prior to analysis on a BD Accuri C6 flow cytometer at 488nm excitation. Significance is shown with respect to the control. **(C) Ethanol increased cleaved PARP and cleaved caspase-3 in Ca9.22 cells, prior co-culture with HICA did not influence this effect.** Western blot was used to analyse expression of apoptosis-related proteins. Cells were treated with a known apoptosis inducer (Cisplatin 25μM) for 24 h as a positive control. Samples were blotted in duplicate, and data shown is a composite image representative of an experiment done in triplicate. **(D) Densitometric analysis of apoptosis-related proteins.** Relative density is shown in relation to loading control β-actin. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns = not significant.

6.5 Ethanol treatment prior to co-culture with HICA increased cell cycle arrest and cell death

To determine if prior treatment with ethanol would influence the effect of HICA on cell proliferation and death, cells were treated in reverse to the previous conditions as in **Section 6.4**. Cells were treated with ethanol (1 or 3% v/v) for 24 h before co-culturing with HICA at an MOI of 10 for a further 24 h. In contrast to previous results, cells co-cultured with HICA *following* ethanol treatment showed a significant increase in Sub G0 cells and decreased cells in the G0/G1 phase compared to ethanol alone (**Figure 6.3A**), indicating cell cycle arrest.

While ethanol alone significantly increased apoptotic cells at both concentrations used, at 3% v/v ethanol, the addition of HICA significantly increased early apoptotic cells and decreased live cells compared to ethanol treatment alone. The same effect was not observed at a lower concentration of ethanol (1% v/v). Therefore, prior treatment with higher concentrations of ethanol sensitised Ca9.22 cells to the effects of co-culture with HICA (**Figure 6.3B**).

Despite apoptotic cells detected in flow cytometry analysis, when Western blot was used to detect apoptotic proteins in ethanol pre-treated cells, no detectable levels of either cleaved PARP or cleaved caspase-3 were observed under any conditions (**Figure 6.3C, 6.3D**). While co-culture with HICA following ethanol exposure does appear to increase cell cycle arrest and early apoptotic events, this was not detectable via caspase-3 or PARP cleavage.

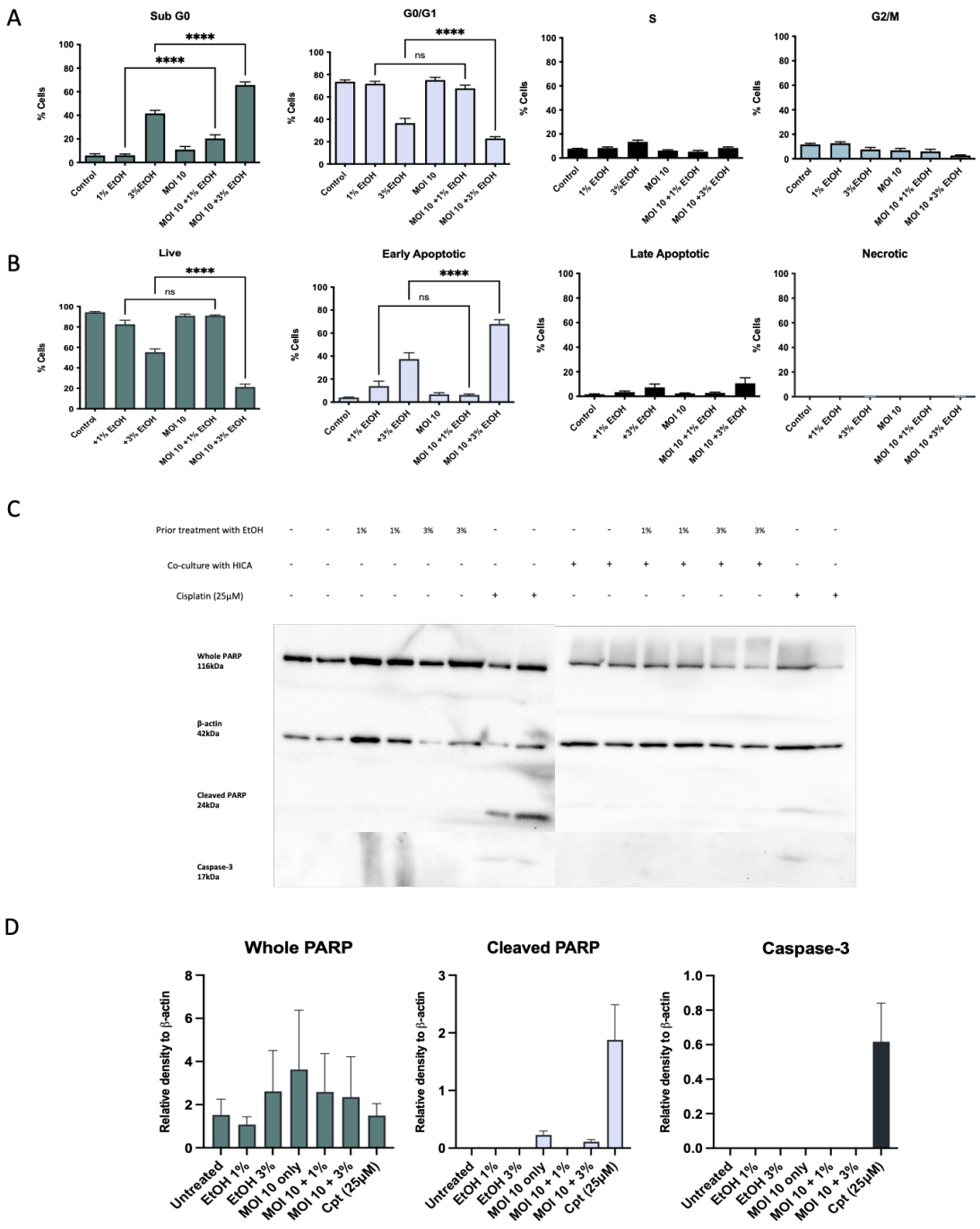


Figure 6.3. (A) Ethanol significantly increased cell cycle arrest, and addition of HICA exacerbated this effect. Ca9.22 cells were treated with ethanol (1 and 3% v/v) for 24 h before co-culture with HICA (MOI 10) for a further 24 h. Cells were fixed and stained with PI before analysis on a FACs Canto flow cytometer at 488nm excitation. **(B) Ethanol induced apoptosis as measured by flow cytometry, and the addition of HICA significantly increased the number of apoptotic cells.** Cells were treated as before then stained with Annexin V and PI prior to analysis on a BD Accuri C6 flow cytometer at 488nm excitation. **(C) Cleaved PARP and cleaved caspase-3 were not detected via Western blot analysis in cells treated with ethanol prior to HICA co-culture.** Western blot analysis was carried out as previously described. Samples were blotted in duplicate, and data shown is a composite image representative of an experiment done in triplicate. **(D) Densitometric analysis of apoptosis-related proteins.** Relative density is shown with relation to loading control β-actin. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns = not significant.

6.6 Ethanol and HICA did not affect ROS Production in Ca9.22 cells

To determine if co-culture or ethanol treatment would affect ROS production of Ca9.22 cells, the production of ROS species was assessed using the DCF-DA assay. Under the same conditions as previous experiments, with either prior co-culture with HICA or prior ethanol treatment, ROS production was not affected in Ca9.22 cells (**Figure 6.4A (i), 6.4A (ii)**).

ROS production is transient; the molecules are short-lived and high doses can lead to cell death via apoptosis. Therefore, shorter timepoints for ROS production were also assessed. At no timepoint tested (20, 30, 60 min) did ethanol or HICA affect ROS production via DCF-DA assay in Ca9.22 cells (**Figure 6.4B**). DCF-DA has wide recognition of total intracellular radicals, so to consolidate these results an extracellular Amplex Red assay was also used to measure production of H_2O_2. Similarly, no differences in ROS production were observed in this assay at any timepoint used (**Figure 6.4C**).

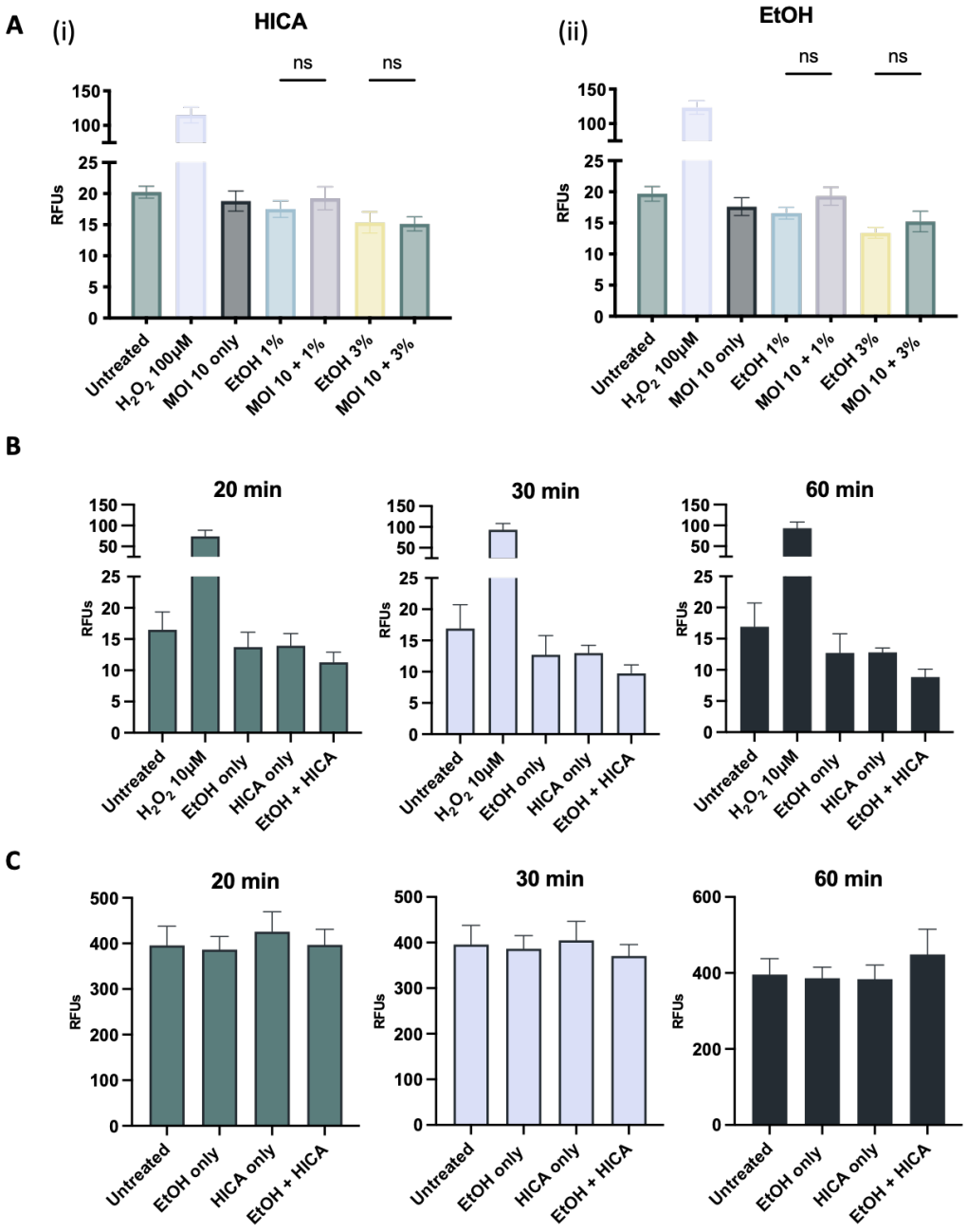


Figure 6.4. Neither ethanol nor HICA in any combination affected ROS production of Ca9.22 cells. (A) (i) Ca9.22 cells were co-cultured with HICA for 24 h before being treated with ethanol (1% and 3% v/v) for a further 24 h or **(ii)** cells were treated with ethanol prior to co-culture with HICA. Cells were stained with DCF-DA and fluorescence read on a Spectra MAX Plus Microplate reader at excitation 490nm and emission 529nm. Data shown n=3, mean ± SEM, ns = not significant. **(B)** Cells were treated with ethanol (1% v/v) for 24 h before co-culture with HICA at an MOI of 10 for 20, 30 and 60 min. ROS production was measured via DCF-DA assay as before. Data shown n=4, mean ± SEM. **(C)** Cells were treated as before and extracellular ROS measured using Amplex Red assay. Cells were washed in Krebs buffer before adding Amplex Red and HRP. The plate was incubated in the dark at 37°C for 1 h before reading on a Spectra MAX Plus Microplate plate reader at excitation 550nm and emission 585nm. Relative fluorescent units (RFUs) were expressed as concentration of H_2O_2 based on a standard curve. Data shown n=4, mean ± SEM.

6.7 Ethanol delayed and dampened immune responses of Ca9.22 cells to HICA

The canonical pathway of NF-κB activation induces transcription of genes regulating several inflammatory pathways. NF-κB exists as a dimer bound to regulatory IκB proteins until a stimulus triggers their ubiquitination and degradation. This obligatory step in NF-κB activation was measured via Western blot analysis for degradation of IκB-α to measure inflammatory responses of Ca9.22 cells to both ethanol and HICA. Ca9.22 cells were treated with bacterial LPS as a positive control (200ng/ml), where degradation of IκB-α occurred in the first 10 min and gradually recovered over 60 min. Co-culture with HICA elicited a similar response to LPS (**Figure 6.5A**). However, when Ca9.22 cells were treated with ethanol (1% v/v) for 24 h prior to HICA exposure, the degradation of IκB-α was a much shorter-lived and weaker response. The degradation of IκB-α in ethanol pre-treated cells only reached the same magnitude as LPS and HICA at the 20-30 min timepoint (**Figure 6.5B**). At 60 min, the recovery of IκB-α was significantly higher in cells treated with ethanol compared to HICA alone, demonstrating a shortened duration of immune response from Ca9.22 cells elicited by HICA (**Figure 6.5C**).

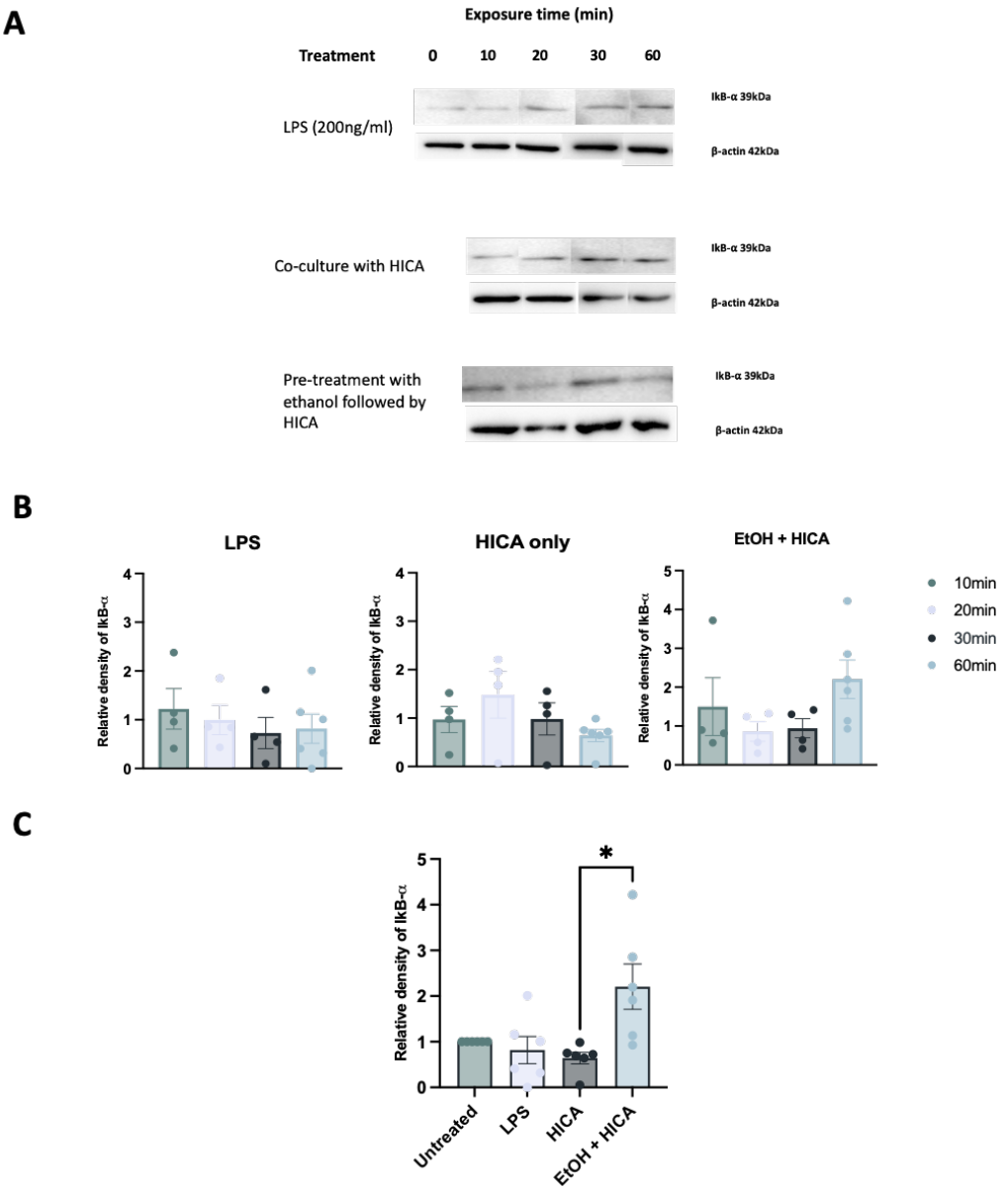


Figure 6.5. (A) Ethanol delays Iκ-B degradation following HICA exposure. Cells were treated with either LPS (200ng/ml), co-cultured with HICA alone, or treated with ethanol (1% v/v) for 24 h prior to co-culture with HICA. Western blot was used to analyse expression of IκB-α after 10, 20, 30 and 60 min. Data shown is a composite image representative of an experiment done four times. **(B) Densitometric analysis of Iκ-B degradation. (C) Ethanol shortens the duration of Iκ-B degradation.** At 60 min following exposure to HICA, cells pre-treated with ethanol had a significant difference in expression levels of IκB-α detected. Relative density of Iκ-B is shown in relation to the loading control β-actin and normalised to the untreated control. Data shown n=4 ± SD. * $P < 0.05$.

6.8 Ethanol increased TIMP-2 expression and decreased MMP-2 secretion in Ca9.22 cells

The activation of NF-κB leads to production of several pro-inflammatory cytokines produced by a variety of cell types in the oral cavity, including gingiva epithelia. A multi-protein cytokine array was used to analyse expression of 20 human cytokines (**Figure 6.6A**). No significant differences were detected in the levels of cytokines typically associated with *C. albicans* infection (IFN-γ, IL-1α, IL-1β, IL-6, IL-7 and IL-8) following exposure to either HICA or ethanol (**Figure 6.6B**). The expression of IL-10 was downregulated following exposure to LPS, HICA, ethanol, and ethanol in combination with HICA but with no significant difference between treatments detected. Significantly higher expression of tissue inhibitor of metalloproteinase 2 (TIMP-2) was observed in cells treated with ethanol, with or without HICA (**Figure 6.6B**).

TIMP proteins are key regulators of matrix metalloproteinases (MMPs), enzymes important in ECM degradation and cell surface biology. The corresponding MMP-2 regulated by TIMP-2 was analysed for expression using an ELISA kit. The expression of MMP-2 by Ca9.22 cells was lower than the recommended minimal detectable dose of the kit. However, when cells were treated with ethanol, with or without HICA, MMP-2 secretion was completely ablated. This finding was consistent with ethanol significantly increasing TIMP-2 expression, as they are inversely correlated. Cells treated with HICA demonstrated increased MMP-2 secretion, but this effect was suppressed by ethanol treatment (**Figure 6.6C**).

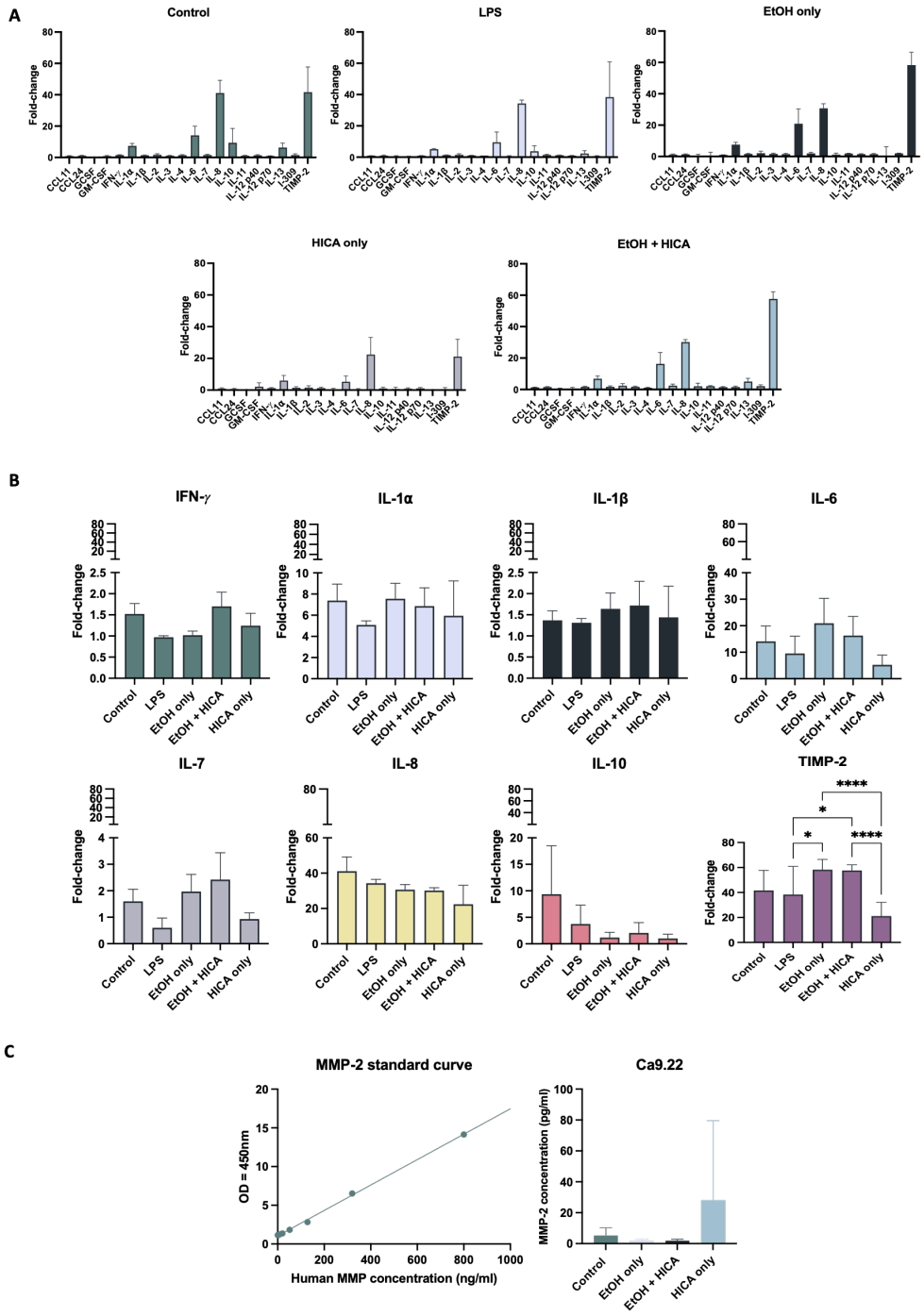


Figure 6.6. (A) Multi-protein cytokine array was used to analyse expression of 20 human cytokines. Cells were treated with ethanol (1% v/v) for 24 h before co-culture with HICA at an MOI of 10 for a further 24 h. Human Inflammation Array C1 (RayBiotech®) was used to detect cytokines according to the manufacturer's protocol. Signal was detected using a Chemi-Luminescent gel documentation system (Bio-Rad), using ImageLab software. **(B) TIMP-2 expression was significantly increased in cells treated with ethanol.** Cytokines of interest in the inflammatory pathways activated by *C. albicans* are shown in the figure. Anti-inflammatory cytokine IL-10 was downregulated in Ca9.22 cells following addition of LPS, HICA or ethanol + HICA. Data shown n=3 ± SEM. (**C**) **Ethanol prevents MMP-2 secretion following co-culture with HICA.** Human MMP-2 ELISA kit (RayBiotech®) was used according to the manufacturer's protocol. Data shown n=2 ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns = not significant.

6.9 Ethanol and HICA increased rates of wound closure and anchorage-independent growth in Ca9.22 cells

Scratch assays were used to evaluate proliferation and migratory capacities of Ca9.22 cells. Co-culture with HICA (MOI 20), ethanol alone and the combination of ethanol with HICA (MOI 10 or 20) increased the rate of wound closure in Ca9.22 cells (**Figure 6.7A, 6.7B**). The rate of wound closure was not significantly different between any combinations of ethanol and HICA. Neither HICA nor ethanol alone affected anchorage-independent growth of Ca9.22 cells, but the combination of both significantly increased the capacity of the cells to survive without an anchor (** $P < 0.01$, **Figure 6.7C**). This contrasts with the scratch assays, as ethanol and HICA appear to cooperate to increase anchorage-independent growth of Ca9.22 cells.

Protein expression of E-cadherin was analysed using Western blot to determine if changes corresponding to EMT-like processes had occurred. Despite increased rates of wound closure in scratch assays and increased capacity for anchorage-independent growth, the expression of E-cadherin, which is typically regulated in accordance with migration of cells, remained unchanged in Ca9.22 cells treated with ethanol, HICA or the combination of both (**Figure 6.7D, 6.7E**).

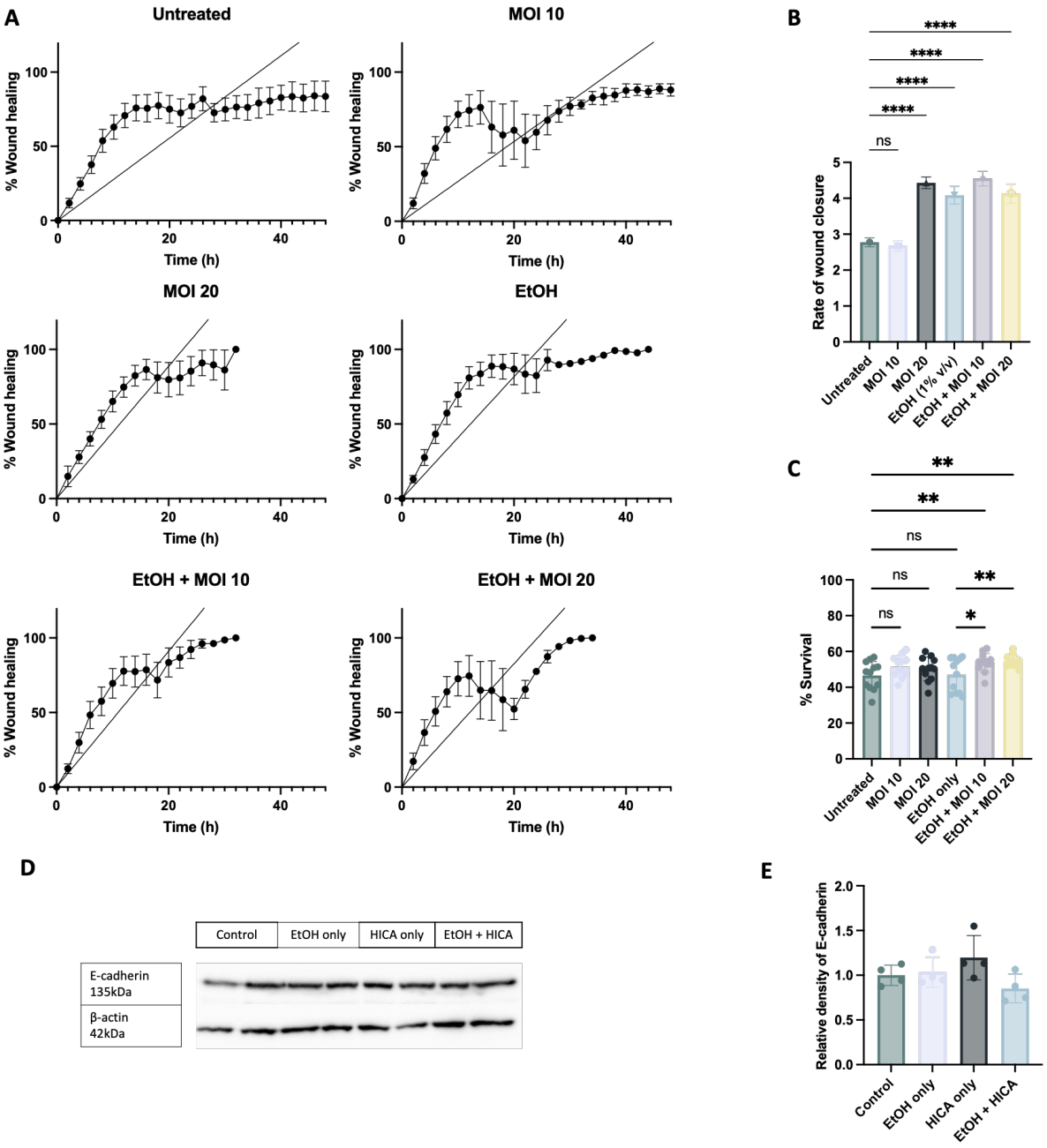


Figure 6.7. (A)(B) Ethanol and HICA significantly increased rate of wound closure in scratch assays. Scratch assays were carried out as previously described. The rate of wound closure was obtained from the graphs in **(A)** and plotted in **(B).** The combination of ethanol and HICA was not significant compared to ethanol or HICA alone. Data shown n=4 ± SEM. **(C) Ethanol and HICA in combination significantly increased anchorage-independent growth of Ca9.22 cells.** Anchorage-independent growth assays were carried out as previously described. Data shown n=4 ± SD. **(D) E-cadherin expression was not affected by ethanol, HICA or ethanol and HICA in combination. (E) Densitometry analysis of E-cadherin expression**. Relative density of E-cadherin is shown in relation to the loading control β-actin and normalized to the untreated control. Samples were blotted in duplicate. Data shown n=4, mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns = not significant.

6.10 Discussion

The oral cavity is a complex environment with different cell types, tissues, structures, and microbial species. The exchanges and interactions of these factors are highly intricate, so the use of simplistic co-culture methods allow for a rudimentary analysis of cell-cell interactions. Interpretation of data from co-culture models must take into consideration the simplicity of the model in the broader context of *in vivo* scenarios. For example, the data presented here used HICA, which excluded the possibility of a fully mounted immune response without live invasive hyphae (298, 299), biofilm formation (307, 308) and enhanced invasion or adherence of microbes aided by other species residing in the oral cavity (309). Given that host cell responses can vary greatly even between hyphal and yeast cell forms (310), a broad analysis of the influence of ethanol on cellular responses to *C. albicans* was the main aim of this study.

Some factors to consider in optimisation of co-culture models include seeding density of cells, MOI of heat-inactivated microbes and analysis methods, particularly when using two eukaryotic species. *C. albicans* inhabits the oral, vaginal, and gastrointestinal mucosa of healthy individuals as a harmless commensal. It is difficult to ascertain a precise oral carriage rate that is 'normal', as it depends on the age and health of the population being studied. The mean carriage rate cited for healthy individuals ranged from 1.9-62.3%, according to Cannon et al. (311) and 2-71.3% in a more recent study by Al-Amad et al. (312). To optimize a co-culture model of ethanol and HICA with Ca9.22 cells, several concentrations of ethanol and MOIs were tested, and the viability of cells assessed. As the MOI of HICA was increased, the sensitivity of Ca9.22 cells to ethanol treatment increased – i.e., decreased viability was observed. Previous studies have used MOI ratios of 5:1 to elicit ROS production from oral cells (313, 314). An MOI of 10 was also used on two OSCC cell lines to demonstrate the ability of HICA to promote oral carcinogenesis (303). In this study, at an MOI of

10 ≥40% of Ca9.22 cells were still viable at ethanol concentrations up to 3% v/v; hence these upper limits were chosen for further analysis.

Alcohol consumption is known to affect the composition of microbiomes in the body, which may increase the colonisation of the oral cavity by *Candida*. Heavy drinkers and individuals with dysplasia in the oral cavity experience a higher oral carriage of *Candida* (315-317). When Ca9.22 cells were co-cultured with HICA prior to ethanol treatment (a representation of an oral cavity colonised by *Candida* that is exposed to alcohol), the rate of cell death was not enhanced (**Figure 6.2**), most likely due to the lack of a concerted immune response. The *in vitro* conditions used did not include the required immune cells and the invasive hyphae of live *C. albicans* which would adversely affect the cell line utilised in this study. However, when the cells were treated with ethanol prior to co-culture with HICA (replicating increased *Candida* colonisation following alcohol consumption, as seen in heavy drinkers), Ca9.22 cells underwent a significant increase in apoptotic cell death (**Figure 6.3**). These results suggest that a high burden of *Candida* in the oral cavity may render oral cells more sensitive to cell death or damage via alcohol consumption. Despite the increase in cells staining positive for early apoptosis according to flow cytometry, the detection of cleaved caspase-3 (an early apoptotic protein) was not observed in ethanol pre-treated cells (**Figure 6.3**). While caspase-independent death is possible, it would not stain positively for Annexin V (318). It is more likely that the levels of caspase-3 at the timepoints used here were low and therefore difficult to detect. Further cell death markers including other caspase proteins or TUNEL assays could be used to confirm apoptotic cell death.

It was observed that neither ethanol, HICA or a combination of both affected ROS production by Ca9.22 cells (**Figure 6.4**). It was demonstrated previously that ethanol had no influence on ROS production in oral cell lines (**Chapter 3**, **Section 3.2**), and this was hypothesised to be due to a) the timepoints used, b) lack of CYP2E1 enzymes and c) high endogenous levels of ROS production by

cancer cells, leading to negligible changes. However, alcohol consumption and exposure to *C. albicans* are exogenous sources of ROS, which is posited to be a major factor in oral carcinogenesis (319, 320). ROS production has also been shown to play a role in periodontitis in increasing the risk for the development of OSCC (321). *C. albicans* can induce ROS production in cells either indirectly via production of acetaldehyde from endogenous ADH enzymes or via inflammation. Since the yeast cells were heat-inactivated, the ADH activity would be attenuated. HICA did not induce ROS production in Ca9.22 cells, in contrast with previous studies where isolated cell wall components of *Candida* as well as whole HICA stimulated ROS production in oral keratinocytes and phagocytes (313, 314). The use of isolated cell wall components at higher concentrations may induce a more robust ROS production in Ca9.22 cells. ROS production in response to *C. albicans* is evidently both cell-type and morphotype of *Candida*-dependent (310).

A significant change in immune signalling was observed in ethanol pre-treated Ca9.22 cells exposed to HICA (**Figure 6.5, 6.6**). Typically, *C. albicans* activates STAT-3 and NF-κB signalling and increases downstream inflammatory cytokines (IL-6, IL-1β and TNF-α), which are considered to be salivary-based biomarkers for detection of OSCC (322-325). Both *C. albicans*-induced ROS production and inflammatory NF-κB signalling are implicated in malignant processes such as hyperproliferation of cells, angiogenesis and metastasis (322). Co-culture of primary OLP keratinocytes with *C. albicans* (MOI 2:1) showed increased inflammation via NF-κB and inhibition of apoptosis, both of which are markers of transformation (300, 301). NF-κB activation can therefore be anti-apoptotic, promoting cellular growth and malignancy in tumours.

Conversely, the effect of ethanol on immune responses is dependent on dose, exposure time and cell type both *in vitro* and *in vivo* (274, 326, 327). Chronic alcohol consumption has disparate effects on circulating immune cells, depending on the health status of the individual and immune cell population being studied. Overall, alcohol consumption leads to hyper-inflammation with

decreased early immune responses, but in some advanced diseases immunosuppression is observed (328). Immunosuppressed individuals have been shown to have a higher risk for development of OC in general (329). There is limited molecular level evidence clarifying the role of alcohol and inflammation in OC. The expression of TLRs is enhanced in OSCC tissues and is associated with invasion and metastasis, and chronic alcohol consumption has also been shown to be associated with increased TLR9 expression in OSCC tumours correlating with lower overall survival (330). Alcohol consumption also significantly increased populations of cytotoxic $CD8^+$ T cells in OC, but with lower levels infiltrating tumours compared to non-drinkers (330, 331). Despite a clear causal relationship between alcohol consumption and HNCs, the specific molecular mechanisms are yet to be defined.

Ethanol was immunomodulatory in Ca9.22 cells, whereby activation of NF-κB by HICA was both delayed and dampened, but anti-inflammatory cytokine IL-10 secretion was decreased (**Figure 6.5, 6.6**). Maximal activation of NF-κB following exposure to HICA was slower in cells treated with ethanol, and recovery of the inhibitory complex Iκ-Bα to control levels was more rapid compared to cells without ethanol pre-treatment. It is worth noting that while LPS led to a decrease in IκB-α in Ca9.22 cells, it was not significant with respect to the control. LPS concentrations used on oral cells for NF-κB activation and cytokine induction range from 1-20μg/ml (332, 333), compared to 200ng/ml which was employed here. Higher concentrations of 5μg/ml did not yield significant results in Ca9.22 cells (data not shown), indicating that Ca9.22 cells require powerful stimuli to evoke an immune response. Ca9.22 cells have been shown to possess the necessary TLRs and are capable of cytokine production (334, 335). It is possible that LPS from alternative species of bacteria found in the oral cavity, such as *P. gingivalis*, may have elicited a stronger inflammatory response from Ca9.22 cells (335). At the same time, oral epithelial cells have been shown to be 'desensitised' to microbial ligands whereby stimulation with purified cell wall components activates NF-κB and MAPK/c-JUN signalling but does not activate cytokine production (336). However, exposure of

Ca9.22 cells to ethanol, HICA or a combination did not significantly affect the production of IFN-γ, IL-1α, IL-1β, IL-6, IL-7, or IL-8. It was also observed that anti-inflammatory cytokine IL-10 was downregulated following exposure to all stimulants and ethanol did not influence this effect, despite delaying NF-κB signalling.

Nevertheless, since NF-κB signalling is typically anti-apoptotic, attenuated signalling following ethanol exposure may be contributing to the increased levels of apoptosis in cells exposed to both ethanol and HICA. It has been established that chronic alcohol consumption severely alters the function of the NF-κB signalling pathway via multiple modalities. These direct or indirect effects of ethanol on NF-κB include epigenetic changes, changes to regulatory proteins, induction of metabolic shifts and ROS production in pathologies such as advanced liver disease, lung cancer, HCC and potentially breast cancer (220, 337-339). Interestingly, increased NF-κB signalling has been shown to be associated with overall worse survival rates in HNSCC, as well as being higher in metastatic clinical specimens and mouse models (340, 341). NF-κB plays an important role in the effectiveness of chemotherapeutic treatment of HNCs, which therefore may be impaired by alcohol consumption (342, 343). While evidence links ethanol and NF-κB in other diseases, their connection in OC is yet to be shown. Ethanol may be affecting NF-κB by modulating the sensitivity of TLRs or MAPK/STAT-3 signalling (49, 339). The scope of NF-κB signalling includes immune cells and parenchymal cells, controlling multiple cell signalling pathways including regulation of oncogenic proteins. It would be pertinent to investigate the effect of chronic ethanol exposure on NF-κB signalling in Ca9.22 cells, as this may provide radical results compared to acute ethanol exposure.

Of note, the expression of TIMP-2 by Ca9.22 cells was significantly increased by ethanol (**Figure 6.6B**). A high expression score of TIMPs/MMPs has been shown to be associated with LNM status, invasion and metastasis of HNCs (344). Levels of MMP expression have been shown to increase

from normal tissue through dysplasia to oral carcinomas (345). HICA was shown to increase MMP-2 secretion, whereas ethanol decreased MMP-2 secretion to below detectable levels. Although high expression of MMPs is associated with overall worse progression of OC, there are some controversial reports that correlate high TIMP expression with unfavourable prognosis in oesophageal cancer, despite TIMP proteins being MMP regulators (346, 347).

The effect of ethanol on TIMP expression appears to be tissue-dependent, and the mechanisms are unclear. For example, exposure of cardiac fibroblasts to ethanol increased expression of TIMP-1 and -2, however, rats that were fed ethanol showed a decrease in these same TIMPs and a reduction in MMP activity (348, 349). Low-dose ethanol exposure in rats was also shown to upregulate TIMP-1, decreasing pro-inflammatory cytokines (350). In contrast to the data presented here, ethanol has been shown to induce MMP-2 activation in breast cancer, driven by ROS production (339). There is evidence that TIMP-2 acts as both an inhibitor of MMPs and an activator of pro-MMPs, potentially explaining the discrepancies in the relationship between TIMP/MMP and malignant phenotype that is observed both *in vitro* and *in vivo* (351). *Candida* has also been shown to affect TIMP expression and MMP activity in human cells. Exposure of oral cell line HSC-2 to *C. albicans* increased secreted MMP activity (303). In a model of human oral mucosa, infection with *C. albicans* increased MMP-9 secretion with a parallel decrease in secretion of TIMP-2 (352). The results of this study suggest that acute ethanol exposure may have protective effects against increased MMP expression induced by *C. albicans*, however this may change depending on exposure time of ethanol.

Scratch assays and anchorage-independent growth assays were employed to investigate metastatic characteristics of Ca9.22 cells. Interestingly, both ethanol and HICA caused an increase in the rate of scratch closure in Ca9.22 cells (Figure 6.7A, 6.7B). This effect was not additive, and there were no significant differences between treatments. The mechanisms by which ethanol may increase

proliferation and migration of Ca9.22 cells in scratch assays were previously discussed, e.g., via by-products of ethanol metabolism or the use of acetate as bioenergetic fuel (Chapter 5, Section 5.10). The increase in wound closure rate following exposure to HICA is possibly due to induced metabolic reprogramming of Ca9.22 cells. Both heat-inactivated and live *C. albicans* have been shown to induce metabolic reprogramming of monocytes and macrophages, via increased expression of genes associated with glycolysis and glutaminolysis (353, 354). HICA has been demonstrated to increase wound closure rates in scratch assays, increase MMP activity and oncometabolite production of two alternative OC cell lines not used here (303). Despite increased proliferation and migration in scratch assays, proliferation of these OC cell lines measured by bromodeoxyuridine assays was not increased, so observed changes were hypothesised to be due to altered glycolytic and other metabolic processes, namely increased GAPDH activity. In the context of data presented in the previous chapter where GAPDH knockdown of Ca9.22 cells could not be achieved via siRNA (Chapter 5), the propensity of cancer cells for increased GAPDH activity may be enhanced by *C. albicans* infection, supporting cancer growth and metastasis (355). Of note, live *C. albicans* had significantly greater effects on these same pathways in OC cell lines (303). The effects of acute vs chronic ethanol exposure and live vs heat-inactivated *C. albicans* should be further examined to investigate the molecular pathways at play.

Unlike in the scratch assays, in anchorage-independent growth assays it was observed that only a combination of ethanol and HICA caused significant anchorage-independent growth (Figure 6.7C). This is in agreement with previous data, where neither acute nor chronic ethanol stimulated anchorage-independent growth in Ca9.22 cells (Figure 3.14). However, the additive effect of ethanol and HICA is likely due to modulation of other MMPs not examined here. Multiple MMPs have been associated with anchorage-independent growth and metastasis of OC (356-358). MMP-2 and MMP-9 in particular are strongly associated with metastasis and prognosis of oral cancers (359, 360). The observed decrease in MMP-2 secretion caused by ethanol (Figure 6.6C) by may be

rendered futile by combined modulation of other MMPs in the presence of both ethanol and HICA. Further investigation of multiple MMP isoforms would be beneficial.

To evaluate EMT processes, the expression levels of E-cadherin were analysed in Ca9.22 cells exposed to ethanol and HICA. Despite the changes to scratch closure rates and anchorage-independent growth, expression of E-cadherin was unchanged by ethanol or HICA in Ca9.22 cells at the timepoints used (Figure 6.7D, 6.7E). This would suggest that early EMT processes are not occurring under these conditions. This is likely an artefact of using HICA over live *C. albicans*, as live infections in mouse models have been shown to induce EMT progression, as detected by E-cadherin and vimentin staining (303). Likewise, modulation of E-cadherin expression in epithelial cells can vary throughout *C. albicans* infection, increasing invasion and enhancing other *Candida* virulence factors (309, 361).

The findings of this study suggest that acute ethanol exposure reduced the risk of *C. albicans*-associated oral carcinogenesis by increasing cell death via apoptosis, delaying NF-κB signalling and increasing TIMP-2 with a concurrent decrease in MMP-2 secretion. However, when considered in the context of the literature, it is possible that longer exposure time may abrogate these effects and actually enhance *C. albicans* associated oral carcinogenesis. As suggested by the increase in Ca9.22 wound-closure rates in scratch assays and increase in anchorage-independent growth, the combinatory effect of alcohol consumption and *C. albicans* colonisation may overall contribute to OC progression. Another factor to consider is the conversion of ethanol into carcinogenic acetaldehyde by *C. albicans* ADH enzymes; any potential risk reduction with low-exposure levels of ethanol may also be counteracted by *in vivo* acetaldehyde production. The influence of this effect would be dependent on the composition of the individual's oral microbiome, i.e., the proportion of species with high ADH-activity within the oral cavity, which can be altered by alcohol consumption. Further research to determine how alcohol consumption habits affect the response of the oral

cavity to commensal microorganisms may provide valuable insights into the molecular mechanisms of oral carcinogenesis.

Chapter 7: General Discussion and Conclusions

7.1 Alcohol and oral cancer

Alcohol has long been established as a major risk factor for the development of oral cancer, but the exact mechanisms by which it influences oral carcinogenesis remain unclear. Part of the difficulty in investigating clear causal relationships between alcohol consumption and OC are the other lifestyle risk factors that typically coincide with alcohol consumption, for example tobacco consumption and overall poor oral health associated with alcoholism (7, 256). Despite efforts to improve early detection methods and therapeutic approaches, the mortality rate of OC remains high with an average five-year survival rate of 68.5%, according to data from 2013-2019 from the National Cancer Institute (362) . Most oral tumours are detected at a late stage, worsening prognosis (21, 22, 38). Treatment modalities of OC include drastic surgical removal of the tumour, radiation, chemotherapy, or regimes combining these methods. Even successful treatment of OC can have a severe impact on patients' quality of life, with impaired physiological functions such as chewing and swallowing, changes to cosmetic appearance and detrimental effects on psychological well-being (363, 364). Likewise, the recurrence and relapse of OC patients is quite common (365). Therefore, there is some urgency to understand how common risk factors contribute to OC to assist in prevention, earlier detection, treatment, and improved prognostic outcomes of patients with OC.

Alcohol has been postulated to contribute to carcinogenesis via multiple pathways including direct DNA damage, inflammation, carcinogenic metabolites, acting as a co-carcinogen, altering hormone regulation and metabolic reprogramming of cells and tumours (38, 228, 366). The ALDH enzymes involved in the metabolism of ethanol have also been recognized as major players in several types of cancers, including HNCs (79, 106, 367). They are fundamental CSC markers with unique substrate

specificities. They also have various roles in tumour growth, metastasis, prognosis, therapeutic resistance, and immune escape, as reviewed throughout this thesis (79, 94). Additionally, alcohol has been shown to affect composition of the oral microbiome, potentially fostering a tumour-promoting environment via interaction with oral epithelia and oral microbial species (157). The overall aim of this research was to establish the pathways by which alcohol consumption promotes malignant transformation and progression of OC, using oral cell lines, *in vitro* ethanol exposure and commensal oral microbiota as a model of these processes.

7.2 KEY FINDINGS OF THIS THESIS

The ADH and ALDH profiles of three oral cell lines were successfully characterised and utilised as models of OC progression and metastasis (**Chapter 3**). The main cell lines used were a dysplastic cell line, DOK, and gingival squamous cell carcinoma cell line, Ca9.22. These cell lines originate from the tongue and the gingiva of the oral cavity, respectively, and possessed unique ALDH expression profiles. Both cell lines lacked the ALDH2 polymorphism, which when mutated has been clearly established in the literature as a risk factor for development of HNCs, the risk for which is multiplied in individuals who consume alcohol (368). Therefore, the ALDH profile of these cell lines provided an opportunity to study the processes by which alcohol consumption contributes to transformation of dysplasia and metastatic characteristics of established OC. When these models of OC were exposed to chronic ethanol, i.e., representative of heavy or long-term alcohol consumption, it was shown to overall promote malignant transformation of dysplastic DOK cells and metastatic processes in cancerous Ca9.22 cells (**Chapter 3**). The data in this thesis also demonstrated the novel finding that chronic ethanol exposure modulated ALDH enzyme expression and activity in Ca9.22 and DOK cell lines, in a cell-line dependent manner. While these findings are consistent across the literature in other cancers, cell-types and in animal models, the specific ALDH isoforms to cancer type vary. The critical role of ALDH1A1 in CSC identification, chemoresistance, metabolic signalling

and prognosis of many cancers prompted further investigation into its involvement in ethanol-induced carcinogenesis.

An aim of this thesis was to verify if ethanol was contributing to transformative and metastatic characteristics of OC cell lines via ALDH1A1 modulation. It was clearly demonstrated that ethanol does not act via modulation of ALDH1A1 to contribute to these processes, but more likely via indirect mechanisms, such as augmenting metabolic pathways, physical alteration of the cell or influencing inflammatory signalling. In Chapter 3, the modulation of ALDH enzymes by chronic ethanol exposure in oral cancer cells suggested a relationship between the observed variables of ALDH expression and activity, and subsequent cell behaviours. However, in Chapter 5, the use of siRNA showed that while ALDH1A1 plays a role in the proliferation and motility of cells in scratch assays, it had no impact on transformation or metastatic characteristics of oral cell lines via anchorage-independent growth or invasion assays. While ALDH1A1 expression did not influence transformation or metastasis of the cell lines used, it did impact on overall cell metabolism in DOK cells. The effect of ALDH1A1 knockdown on acetaldehyde metabolism further consolidated the hypothesis that ethanol contributes to OC development and progression via indirect metabolic reprogramming.

This effect was also observed following successful establishment of an *in vitro* co-culture model of OC cell line Ca9.22 with HICA in Chapter 6. The synergistic effects of ethanol and HICA on anchorage-independent growth and wound closure rates of Ca9.22 cells suggested ethanol is promoting metastasis via modulation of MMP proteins as well as inducing metabolic reprogramming. This phenomenon has been shown independently, but not in combination, in other cell lines (303, 353). In Chapter 6, it was determined that ethanol plays a role in immune signalling modulation in a time-dependent manner. The indirect impact of ethanol also extends to its modulation of the oral microenvironment, with the potential to critically alter acetaldehyde

production *in vivo* (78, 255, 257). Therefore, the data presented in this thesis provides compelling evidence that acetaldehyde exposure following alcohol consumption may be the driving factor in transformation and metastasis of OC.

An unexpected finding of this thesis was that the commonly used ALDH inhibitor, DEAB, was not effective in Ca9.22 and DOK cells despite possessing isoforms that the drug is postulated to target (Chapter 4). The data presented in Chapter 4 highlighted how research on the impact of ALDH1A1 in carcinogenesis, or other isoforms for that matter, is impeded by the lack of robust and reliable detection methods for both activity and expression of ALDH isoforms. Further research is also needed to clarify the mechanism of action of ALDH inhibitors, to aid in the research of ALDH isoforms. This may potentially be achieved via crystallography of drug:enzyme interactions. The data in Chapter 4 exemplified the misinformation that has persisted throughout the literature that drugs such as DEAB or DSF (although not tested in the cell lines used here) are specific inhibitors to ALDH1A1. Despite studies clearly demonstrating the broad specificity of DEAB for multiple ALDH isoforms (241, 242), it continues to be used as a specific inhibitor for ALDH1A1, confounding interpretation of data and leading to obsolete conclusions. Further observations to this were made in Chapter 5, whereby detection of ALDH expression and activity across multiple experimental techniques (enzyme activity assay, flow cytometry and Western blot analysis) were not consistent. Based on the epidemiological research showing strong correlations of ALDH2 polymorphisms with development of HNCs, multiple studies linking ALDH1A1 expression with CSCs, and strong associations of several ALDH isoforms in conjunction with other proteins as prognostic markers of OC, the available methods for ALDH detection and identification fall short (106, 115, 118, 186, 225). This thesis presented novel findings of the effect of ethanol in malignant transformation and metastasis, but also highlighted the requirement for the development of more specific and sensitive assays for ALDH identification and inhibition for use in both research and potentially clinical settings.

7.3 FUTURE DIRECTIONS

There are several experimental approaches that could be used to further investigate the hypothesis that ethanol is acting via metabolic reprogramming of cells. Changes in oxygen consumption rate or extracellular acidification rate, to investigate mitochondrial respiration and glycolysis in the presence or absence of chronic ethanol, could be monitored using the Seahorse XF Cell Mito Stress Test assays (369). Likewise, if acetaldehyde consumption could be measured and compared between untreated and chronic ethanol treated cells, this may clarify the effect ethanol is having on DOK cells' acetaldehyde metabolism. Current detection methods for acetaldehyde both *in vitro* and *in vivo* are typically focused on quantifying its *production* via high performance liquid chromatography (HPLC) or mass spectrometry (370-372). However, the use of stable carbon isotopes (^{13}C) has been used to follow the incorporation of acetaldehyde into DNA adducts in lymphoblastoid cell lines, lung fibroblasts and the brain and lungs of rat models (373-375). It has been noted that aldehydes are generally poor targets for ^{13}C labelled studies, so labelled pyruvate and lactate have been used to indirectly measure ALDH2 activity via NADH concentration in rat liver (376). Studies on *E. coli* strains also noted that acetyl coA, a product of acetaldehyde metabolism, could not be reliably measured using ^{13}C labels, so ^{13}C labelled intermediary compounds such as glycolaldehyde were used instead (377). If these methodologies could be employed to measure conversion of acetaldehydes or their incorporation into oral cell metabolic pathways, it could provide novel insights into ethanol's impact on acetaldehyde metabolism.

This study utilised dysplastic and oral carcinoma cell lines. Comparison of the effects observed here on normal oral cells would be of great interest, particularly to follow the change in expression of ALDH isoforms from normal cells, through dysplasia to OC. This change in ALDH expression throughout OC is observed in the literature from patient cohorts, although with mixed results for specific isoforms (84, 85, 88, 345, 378). To clarify this, the conditions for ethanol exposure would

need to be optimised for normal oral cell lines, as typically normal or primary cells are not as robust as cancer cell lines. Therefore, they may not tolerate the same concentrations of ethanol, or the same lengths of exposure times. Investigation into the effects of even longer time points e.g., up to 3 months in OC cell lines (165), could also highlight further changes to the cells as a result of chronic ethanol exposure. At the same time, while changes to ALDH protein expression were observed in both Ca9.22 and DOK cell lines, this did not correlate to changes to mRNA expression (Chapter 3). This disparity was potentially due to the effects of ethanol on global protein synthesis rates (183). To test this hypothesis, puromycin assays could be used to measure global protein synthesis rates of the cell lines and how these change over time with ethanol exposure. Ethanol has been shown to decrease global protein synthesis rates of muscles in both rat and mouse-models in a time-dependent manner (379, 380) and this effect may explain the observed discrepancies. In addition to normal cell lines for comparison, the use of organoids or 3D cell-culture systems to mimic more complex interactions and physiological functions of the oral cavity in ethanol-induced oral carcinogenesis should be considered.

Further translational research could include the use of pharmacokinetic prediction models based on ALDH profiles. These models are typically used for drugs but have been combined with whole-body human genome scale models to investigate the impact of ALDH enzymes on alcohol consumption pharmacokinetics and pharmacodynamics. These prediction models are intended to give personalised predictions for individuals with genetic variations that may lead to adverse reactions to alcohol consumption (381). If a definitive ALDH risk profile for ethanol-induced carcinogenesis could be determined, this may have major implications for early detection and treatment of OC. For example, pharmacologic agents have been tested that *enhance* ALDH3A1 activity to compensate for loss of ALDH2 with genetic mutations; highlighting the multiple potential roles for ALDH isoforms in OC (382).

7.4 IMPLICATIONS FOR POLICY AND PRACTICE

Oral cancer induced by alcohol consumption is, in principle, avoidable; however, with its social and cultural significance it is more rational to focus on harm reduction and prevention efforts. The research presented here provides compelling arguments for the use of genetic ALDH variations and microbiome sequencing as predictive markers for OC development, or as a framework to provide more targeted screening programmes. There is emerging evidence that risk-based OC screening may be an effective model for the greatest cancer mortality reduction (383). This may be via testing subsets of the population at higher risk, or via model-predicted risk for OC (383-385). Population screening has been proven to be very effective in reducing mortality in breast, cervical and colorectal cancers. As of 2020, 25 EU countries had introduced National Cancer Control programmes for breast cancer, 22 countries for cervical cancer, and 20 countries for colorectal cancer (386, 387). According to The World Health Organisation, oral cavity cancers meet many of the criteria that justify screening (385, 388, 389). To avoid unnecessary testing and improve feasibility and affordability, identification of those at higher risk of developing OC is essential. This would avoid inefficient allocation of resources for low-risk individuals and avoid unnecessary burden and distress to patients.

A randomised controlled trial targeting the general population of Kerala, India, demonstrated that screening specifically alcohol and/or tobacco users for OC risk resulted in a significant reduction in OC-related deaths compared to those in the control group (390). A case-control study in Cuba, carried out opportunistically on patients reporting for dental problems, aimed to estimate the degree of protection that could be conferred by screening programmes (391). This study indicated a 33% reduction in the incidence of stage III and IV OC, and this degree of protection increased to 59% in those who had two or more follow-up screening examinations (391). Similar population-based screening for OC was carried out in Taiwan, where more than 2 million individuals were

screened for OC based on oral habits of cigarette smoking and/or betel quid chewing (392). Of the population tested, 8033 OC cases were detected and a 26% reduction in mortality rate was observed (392). An oral screening programme was conducted in Japan on 19,056 individuals and showed a higher positive rate of leukoplakia or OC in those with tobacco and alcohol consumption habits (393). The results of these trials further support the need to develop more selective screening programmes based on those at higher risk.

The success of these screening programmes was achieved via oral visual inspection, which has inherent variability due to human error, and lacks the specificity and sensitivity achievable with other methods. The sensitivity of oral visual inspections to detect lesions is approximately ~60% (390). Several techniques exist to supplement clinical examination with the aim to improve early diagnosis, such as toluidine blue staining oral exfoliative cytology (394), as well as the development of automated screening processes using artificial intelligence (395). Since the incidence of OC is highest in low- to middle-income countries, the improvement of screening using oral visual inspection alone would be favourable (396), but combining this method with selective based screening might be more effective. The aforementioned ALDH2 polymorphism is an example of genetic profiling information that could prove useful in detecting cancer before it is symptomatic (397). Individuals with this polymorphism and/or the presence of dysplasia may be at a significantly higher risk for ethanol-induced transformation. The same polymorphism is also associated with increased risk for colon cancer, especially in alcoholics, as well as an increased risk for nausea following chemotherapy treatment, substantiating the benefits of screening for such genetic mutations (398). The lack of specific and sensitive OC screening methods could therefore be improved by more targeted screening for those at higher risk for developing OC, i.e., alcohol consumers, and supplemented by ALDH genetic testing or microbiome sequencing.

Genetic testing is available for several types of cancer, including but not limited to breast, ovarian, colon, thyroid, and prostate cancer. OC is not considered to be a hereditary cancer, therefore does not qualify for genetic testing based on usual criteria (399, 400). The use of non-invasive, next-generation sequencing for oral screening of high-risk individuals has been suggested, with a focus on genetic markers such as *TP53* and *NOTCH1* (401). They achieved a high level of sensitivity using DNA extracted from brushed cells, with significantly enriched levels of mutations detected in patients with oral dysplasia (401). While this study looked at patients with pre-existing oral leukoplakia and other PMDs, these approaches may be applied to healthy individuals for early detection of asymptomatic lesions. Saliva sampling offers a non-invasive approach that could theoretically be used to look at ALDH enzymes, alongside microbiome composition and the detection of premalignant lesions - if a more definitive risk profile of these categories for OC risk could be determined (402). An important consideration in genetic testing is the psychological, legal and ethical implications of patients being provided with information on their genetic cancer susceptibility (403). It would need to be determined if these kinds of test are mandatory for higher-risk groups, who has access to the results and how that may engender genetic discrimination; for example, if the information would be disseminated to third parties, such as health insurance companies (404). This further highlights the need for consolidation of the mechanisms and risk factors for oral carcinogenesis before these strategies could be implemented into policy and practice.

Alongside a strong argument for more targeted and enhanced screening programmes, the research presented here also suggests that individuals found to be at higher risk of developing OC should be targeted for more effective harm reduction campaigns pertaining to alcohol consumption. Ethanol enhances progression and metastasis of OC via multiple modalities, including interfering with oral microbiome interactions. It is well documented that alcohol consumption induces a modified microbiome composition in otherwise healthy individuals and in those with alcohol use disorders,

with the potential to introduce more pathogenic bacteria and their oncometabolites, such as acetaldehyde, into the oral cavity (290, 405-407). Multiple 16s RNA sequencing projects have shown that the variations to microbiome composition are dynamic and dependent on the population studied, with one study even citing diurnal changes to oral microbiome composition in individuals with alcoholism (408). Importantly, these changes can be rectified by cessation of alcohol consumption (405). Intervention methods that have proven useful in reducing alcohol use include cognitive behavioural therapy, social network approaches and pharmacological interventions (409). The awareness of cancer risks associated with alcohol use has been reported to be relatively low (410), but if patients were provided with information on their relative risks for alcohol-related OC based on either microbiome sequencing or even ALDH genetic profiling, this may improve sustained reduction in alcohol use. Likewise, if microbiome sequencing efforts could be used to identify dysbiosis and/or harmful oral microbiome compositions, other preventative measures could be implicated, such as prophylactic probiotics (411, 412). This approach is made more attractive by the existing usefulness of oral microbiome sequencing as a colorectal cancer biomarker, and a prospective risk association with the development of both pancreatic and lung cancer (413-415).

7.5 LIMITATIONS

The data presented in this thesis is not without its limitations. Firstly, the use of cell lines is inherently limited in its ability to replicate human pathophysiology. Cell culture models lack the complexity of *in vivo* interactions of the oral cavity, hence future studies should ideally utilise normal oral cell lines, 3D-organoids and patient samples for comparisons. Likewise, this thesis focused on the detection of a limited number of ALDH isoforms, and the siRNA-mediated knockdown of only one isoform, ALDH1A1. However, the preserved sequence homology and structure of the ALDH isoforms confers multiple overlapping roles that warrant further research. As previously discussed, the revelation that detection methods for ALDH proteins proved

inadequate also limited the research and the conclusions that could be drawn from the data presented here. In the investigation of the impact of ethanol on OC cells and oral microbiome interactions, the use of heat-inactivated over live *C. albicans* was a major limiting factor. This was due to lack of available laboratory facilities to culture live *C. albicans*. Even with the use of live co-culture models, complex physiological interactions of oral microbial species are difficult to faithfully replicate *in vitro*.

7.6 Conclusions

In conclusion, ethanol contributes to transformative and metastatic properties of oral dysplasia and oral cancer respectively, via multiple modalities. This was demonstrated via scratch assays, anchorage-independent growth assays, invasion assays and changes to EMT-related protein expression. It was a novel finding of this thesis that ALDH1A1 modulation is not a driving factor in ethanol-induced oral carcinogenesis. Nevertheless, ALDH1A1 was demonstrated to play a key role in cellular pathways such as proliferation and metabolism. Ethanol-induced OC progression includes changes to expression levels of ALDH isoforms that may be of use clinically in the future.

It was shown in this thesis how ethanol contributes to oral carcinogenesis via indirect mechanisms, such as altering cell metabolism, interfering with inflammatory signalling and disturbing interactions with oral microbiome species. The exposure of dysplastic cells to ethanol significantly impacted acetaldehyde metabolism, and this may be a key factor in transformation. Exposure of the oral cavity to acetaldehyde not only increases the risk for potentially carcinogenic DNA mutations caused by acetaldehyde, but it was also shown that it may increase the capacity of cells to use acetaldehyde for increased proliferation and cell growth. Ethanol exposure was immunosuppressive to oral cancer cells following short-term exposure, suggesting protective effects against *C. albicans* induced carcinogenesis. However, this effect may be rendered insignificant in the context of increased acetaldehyde production and utilisation triggered by alcohol consumption. Likewise, it was concluded that both ethanol and *C. albicans*, and potentially

other microbial species not explored here, work together to modulate metabolic pathways in oral cells. This interplay of ethanol and the oral microbiome is heavily influenced by length of exposure time and likely varies between microbial species.

Although ethanol was shown to be acting independently of ALDH modulation, understanding the role of ALDH isoforms in oral carcinogenesis remains to have huge potential to expedite the discovery of earlier detection markers, improve earlier diagnosis of OC as well as having potentially significant clinical implications for treatment and overall survival. The roles of ALDH isoforms in cell proliferation and link to acetaldehyde metabolism may be utilised in future preventative and diagnostic methods and warrants further research. Furthermore, this thesis highlighted the need for more robust and reliable ALDH detection methods as well as inhibitors fit for use in research settings, with potential clinical significance. In summary, the data presented here provided novel insights into the mechanisms behind ethanol-induced oral carcinogenesis, strengthened the argument for the use of microbiome sequencing and selective genetic screening for OC prevention, and provides a framework for further research questions about the effects of ethanol in oral carcinogenesis.

Supplementary Figures

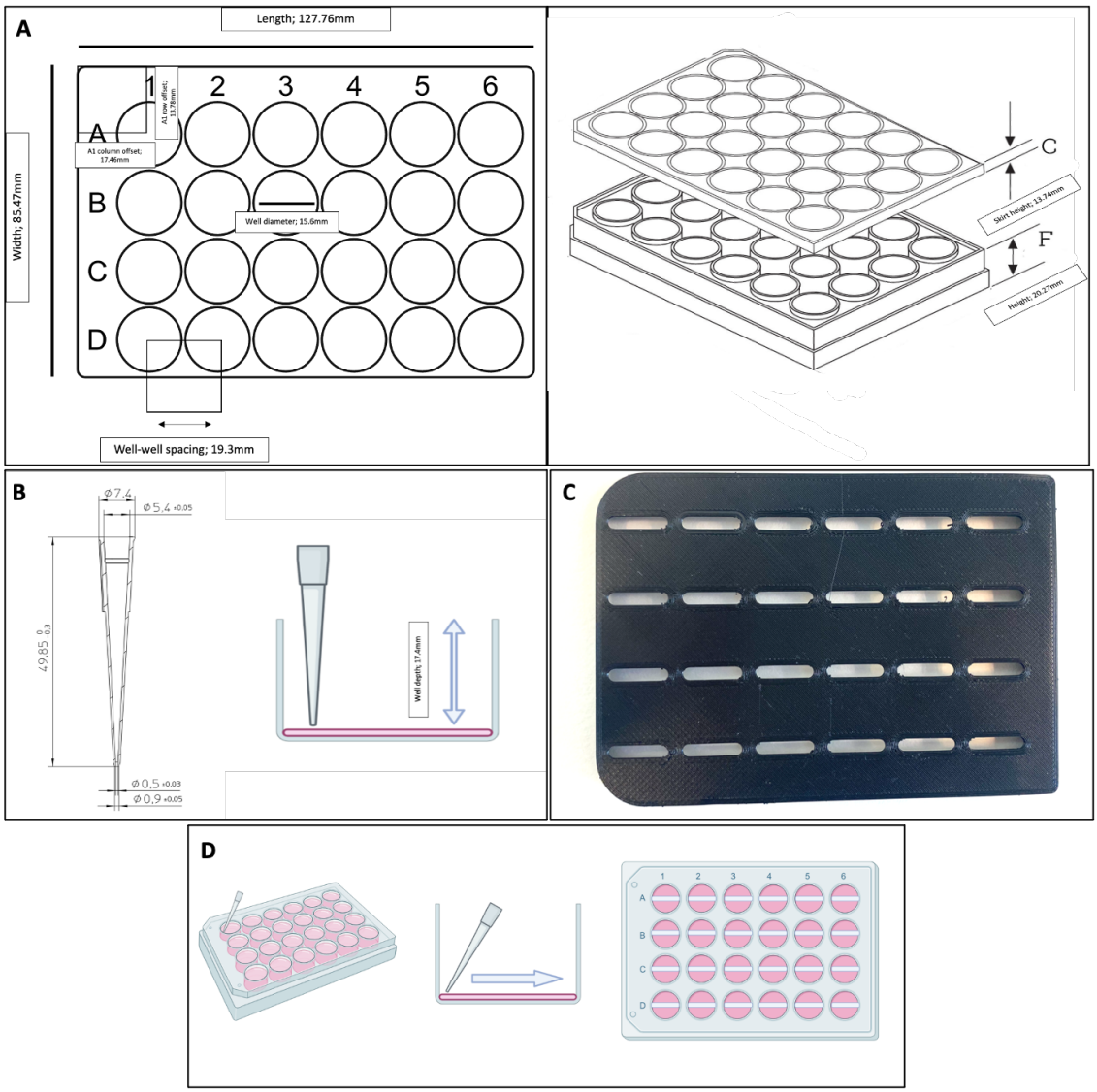


Figure A. (A) The dimensions of Corning™ Costar™ Flat Bottom Cell Culture Plates 24-well (Cat no; 3526) and (B) standard p200 pipette tips. (C) These dimensions were used to design a prototype of a 3D printed plate lid with holes cut over each well, (D) to create even gaps in the cell monolayer. Different size holes were tested to reduce variability in scratch size by limiting the amount of movement, angle and pressure that could be applied to pipette tips when creating the scratch in the monolayer.

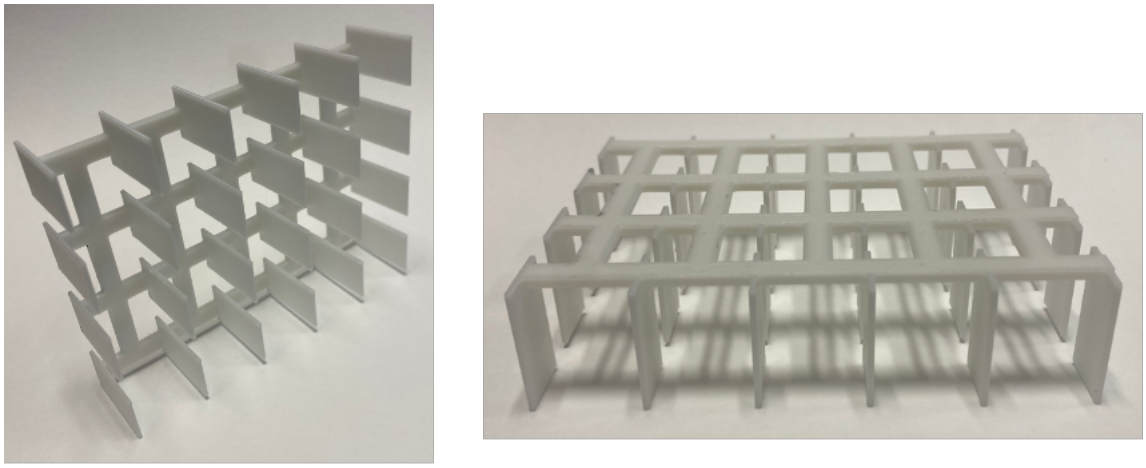


Figure B. 3D printed cell exclusion moulds. Moulds were made to measure for Corning™ Costar™ Flat Bottom Cell Culture Plates 24-well (Cat no; 3526). The mould was sterilised with 70% ethanol and held in place with rubber bands during seeding of cells to eliminate movement and create a consistent surface pressure. Use of the mould also ensured that gaps in the cell monolayer were consistently within the field of view on the live cell imaging system, Incucyte™ S3.

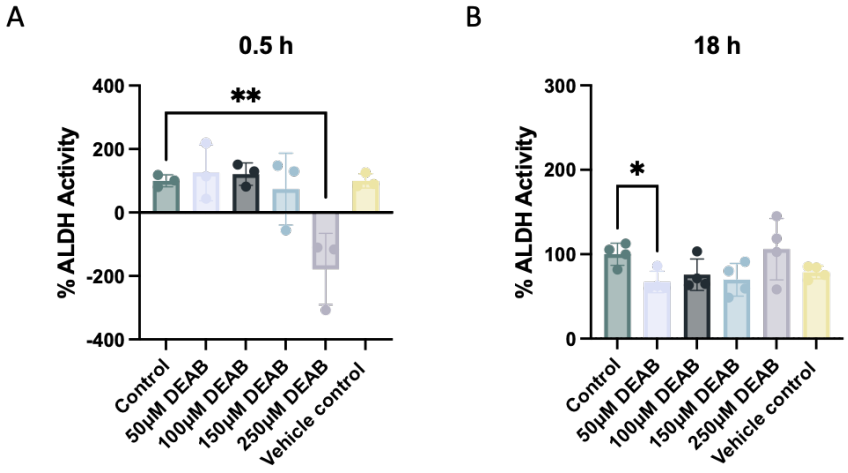


Figure C. DEAB inhibited ALDH activity in HepG2 cells. Cells were treated with DEAB (50-250μM, 0.5 or18 h) before lysing in assay buffer (0.1M sodium phosphate, 1% Triton-X-100, protease inhibitor cocktail (Roche), pH 8). ALDH activity was determined by adding reaction mix containing assay buffer as previously described, 10mM acetaldehyde and 2.5mM NAD. Absorbance was read on a Spectra MAX Microplate reader at 300/340/360nm every 3 min at 30°C to follow the production of NADH. Data shown n=3-4, mean ± SD. These experiments were carried out by undergraduate student Claire Enaholo, 2022, under the supervision of Isabel O'Grady.

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Appendix 1

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Review

The interplay of the oral microbiome and alcohol consumption in oral squamous cell carcinomas

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ABSTRACT

Oral cancer (OC) is among the top twenty occurring cancers in the world, with a mortality rate of 50%. A shift to a functionally inflammatory or a 'disease state' oral microbiome composition has been observed amongst patients with premalignant disorders and OC, with evidence suggesting alcohol could be exacerbating the inflammatory influence of the oral microorganisms. Alcohol dehydrogenase (ADH, EC 1.1.1.1) converts alcohol into a known carcinogenic metabolite, acetaldehyde and while ADH levels in oral mucosa are low, several oral commensal species possess ADH and could produce genotoxic levels of acetaldehyde. With a direct association between oral microbiome status, alcohol and poor oral health status combining to induce chronic inflammation with increased acetaldehyde levels – this leads to a tumour promoting environment. This new disease state increases the production of reactive oxygen species (ROS), while impairing anti-oxidant systems thus activating the redox signalling required for the promotion and survival of tumours. This review aims to highlight the evidence linking these processes in the progression of oral cancer.

Introduction

Head and neck cancers (HNCs) are a group of cancers affecting the mouth, nose, throat, larynx, sinuses or salivary glands. Oral squamous cell carcinomas (OSCCs), originating in the squamous cells lining the mucosal surfaces, account for 90% of all HNCs [1]. The most common sites for OSCC formation are the floor of the mouth and the lateral/ventral surfaces of the tongue [2], however they can also affect the lips, gingiva, buccal mucosa and hard palate. GLOBOCAN estimated a total of 354,864 new cases of lip and oral cavity cancer in 2018, with a mortality rate of at least 50%. The incidence rate is 4.3 cases per 100,000 people in the world irrespective of age or gender [3] (Fig. 1). The high mortality rate associated with OSCC is largely due to the asymptomatic presentation in the early stages of the disease. Late clinical detection therefore lends itself to poor prognosis. The frontline therapy option for treatment is surgery. Radiation therapy and chemoradiotherapy are used as adjuvant therapies to shrink tumours prior to surgery. In recent years the field of research into OSCC has tilted towards the discovery of new biomarkers. In order to elucidate these markers, the mechanisms by which risk factors contribute to OSCC need to be fully understood.

Traditional risk factors of oral cancer (OC) such as alcohol consumption and tobacco smoking, increase the risk of developing cancer substantially when consumed concurrently [4]. More recently, the oral microbiome has been implicated in the progression of OC, due to changes in its basal composition and its influence on inflammatory responses in the mouth throughout OSCC development [5]. A link between alcohol consumption and the oral microbiome has been identified in the progression of oral cancer chiefly through the expression of the enzyme alcohol dehydrogenase (ADH) by certain bacterial species [6–8]. However, the exact relationship of alcohol and the oral microbiome has not yet been determined. It has been suggested that alcohol influences the inflammatory effect of the oral microbiota, or facilitates enhanced pathogenicity of commensal microorganisms [6,9–11]. Furthermore that there is a potential interaction between alcohol and the oral microbiome, leading to a malignant transformative event. This review will look at the effects of the oral microbiome and alcohol consumption both independently and synergistically in the progression of oral cancer.

Pre-malignant disorders & OSCC

There are many premalignant oral diseases (premalignant disorders, PMDs) implicated in the development of OSCC alongside well-documented risk factors, such as alcohol consumption, human papillomavirus (HPV), tobacco smoking and betel quid chewing [12–15]. Oral

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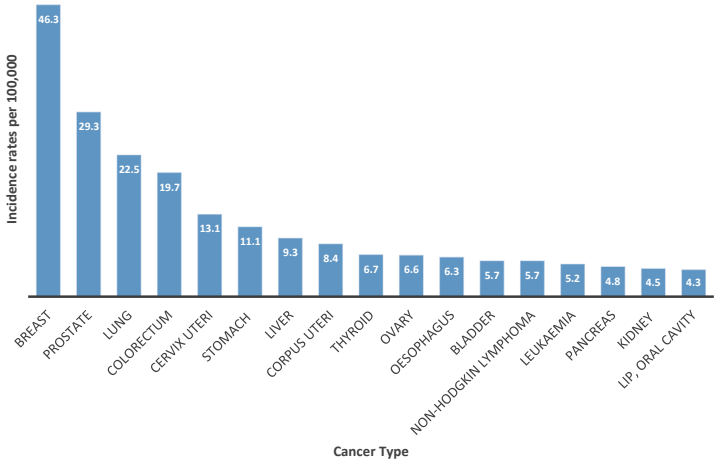

Fig. 1. Worldwide incidence rates of top 20 cancers in 2018 according to GLOBOCAN. Oesophageal cancer is at number 11 of the most commonly occurring cancers. At number 17 in the list of top 20 cancers, oral cavity cancer has an incidence rate of 4.3 cases per 100,000 people worldwide (3).

diseases such as oral lichen planus (OLP), periodontitis and oral submucous fibrosis (OSF) have been associated with the development of OC. Patients with OLP have a similar dysbiotic status of bacterial community in the mucosa compared to those with OSCC [16], and it has been demonstrated that OLP patients are 5 times as likely to develop OSCC [17]. Chronic periodontitis (an inflammatory disease of the gums) and OSCC share several common risk factors, however no causal relationship has been defined [5]. OC resulting from OSF has been defined as a clinico-pathologically distinct entity in comparison to OSCC that develops in its absence [18]. There is controversy in the literature as to whether or not the presence of a PMD confers a better or worse prognosis to patients who develop OSCC. There is also an entirely different incident rate of HPV-associated OC - it mainly contributes to the development of oropharyngeal cancers [19]. In addition to this, patients presenting with HPV-associated OC lack traditional risk factors [15,20]. For this reason, this review will focus on non-HPV associated OC only.

Overview of the oral microbiome in OSCC

The oral microbiome is host to over 700 identified common oral species, with only 70% cultivatable in the lab. There are whole genome sequences of over 400 other taxa not yet identified [21]. In recent years, there has been increasing interest in the oral microbiome with regard to its contribution to tumour development. There is evidence in other cancers of bacteria having a direct causal relationship with carcinogenesis, such as the colonization of the human stomach by *Helicobacter pylori* and its role in gastric cancer [22] and *Salmonella typhi* colonisation in the development of gallbladder cancer [23]. However, no distinct relationship between the oral microbiome and OSCC has been yet identified. The relationship between bacteria and cancer is complex, and depends on several factors such as host susceptibility and genetics, in addition to environmental factors such as diet, smoking and alcohol consumption (reviewed by Mager et al.) [24]. It is known that alcohol consumption (along with other exogenous factors), can alter the composition of the oral microbiome [9,10,25]. In oral diseases, general dysbiosis is posited to be the main contributor to pathogenesis, as opposed to the infection and colonization by a single pathogen. Bacteriomes and mycobiomes identified in patients with PMDs, surfaces of OSCC tumours, deep-tissue tumour specimens and OSCC biofilms consist of the same species that would be found in a healthy mouth. The healthy microbiome commonly consists of viridians *Streptococci, Staphylococcus* and *Neisseria* species, alongside approximately 51 other aerobes [21,26].

An overall trend exists in the literature as to the composition of a "disease state oral microbiome". The general consensus is that the proportion of anaerobes increases with oral cancer, whereas healthy oral microbiota constitutes of mostly aerobic bacteria. In PMDs, oral biofilms and in oral cancer tissues, the proportion of aerobic viridian *Streptococci*, such as *S. mitis* or *S. salivarius*, decreases. The proportion of anaerobes tends to increase relative to healthy controls. This trend is found as a linear gradient whereby the proportion of anaerobic bacteria increases as the proportion of aerobic bacteria decreases from healthy controls, to patients with a PMD and to patients with oral cancer [11,26–32]. The same associations are seen in periodontitis, where *Prevotella, Fusobacterium, Alloprevotella* and *Veillonella* bacterial species are considered periodontopathogenic, along with *Porphyromonas gingivalis* [32]. *Capnocytophaga,* a putative periodontopathogenic bacteria, is specifically associated with high recurrence rates of oral cancer, where no other taxa were found to have the same association [32].

Investigation of the healthy microbiome status of streptococci species has reported that *S. mitis* and *S. oralis* improve the rate of oral epithelial wound healing, and mitigate the inhibitory effects on wound healing seen by periodontopathogenic bacteria such as *P. gingivalis* [33]. In conjunction with this, a study undertaken in China investigated *Streptococci*-reactive cytotoxic $CD8^+$ T-cell responses in OSCC patients that underwent tumour resection. This work found that OSCC patients had more $CD8^+$ T-cells for all *Streptococci* species compared to healthy controls, and at advanced disease stages the frequency of *S. anginosus*-reactive $CD8^+$ T-cells increased. In patients that did not present tumour recurrence over a 24-month period follow-up, *S. salivarius*-reactive and *S. mitus*-reactive $CD8^+$ T-cells were significantly higher than those that did present with recurrent tumours. The frequency of *Streptococcus*-reactive $CD8^+$ T cells was positively correlated with prognosis, suggesting a role for *Streptococci* in stimulating an anti-tumour immune response [34].

It has been reported that the presence of yeast in the oral cavity has no statistical significance on mortality rate of cancer patients [35,36]. However, a significant positive correlation is reported between oral cancer and both the presence and severity of oral yeast colonisation. In

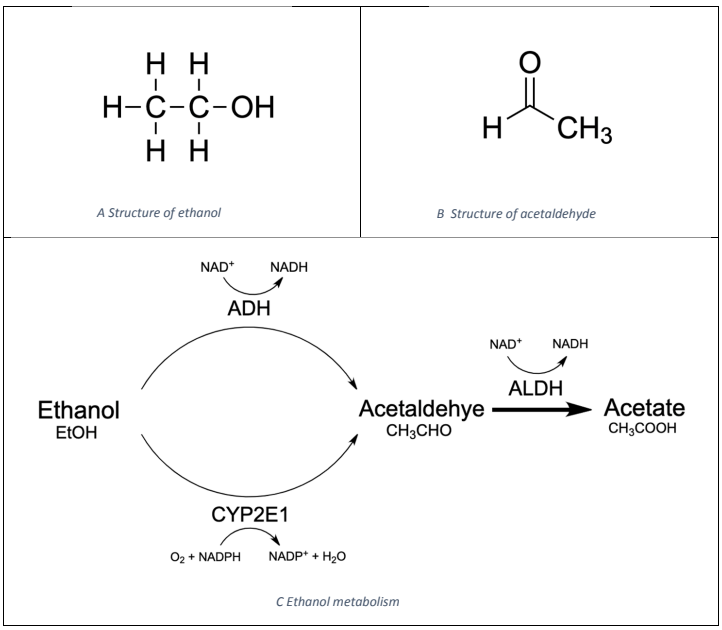

Fig. 2. (A) **Structure of ethanol**. (B) **Structure of acetaldehyde.** The carcinogenic metabolite product of ethanol oxidation by alcohol dehydrogenase. (C) **Ethanol metabolism.** Oxidative pathways of ethanol - alcohol dehydrogenase (ADH) catalyses the metabolism of ethanol into acetaldehyde. Acetaldehyde is then metabolised by acetaldehyde dehydrogenase (ALDH) into acetate. Ethanol metabolism contributes indirectly to free radical formation via production of NADH molecules. Cytochrome P450 2E1 (CYP2E1) is part of the microsomal ethanol oxidising system and metabolises ethanol principally in the liver.

a study comparing OC patients with healthy patients, adjusted for confounding factors such as tobacco/alcohol consumption, HPV infections and periodontal disease, it was shown that the presence of *Candida albicans* was a significant risk marker in OC [37]. Importantly, both presence of *C. albicans* and frequent alcohol consumption was shown to have more than an additive effect on risk association [37].

Despite the trends of multiple 16sRNA sequencing projects, no definitive pathogenic role has been determined for oral commensals in OSCC. There is not yet sufficient evidence to implicate specific bacterial species in the etiology of OSCC. Only one study has produced experimental evidence of bacteria promoting malignant transformation – using a mouse model of chronic periodontitis, administration of oral carcinogen 4-nitroquinoline-1-oxide (4NQO) and co-infection with *P. gingivalis* and *F. nucleatum* significantly enhanced OSCC progression [38]. Rather, it is hypothesised that general polymicrobial dysbiosis may alter the oral environment, leading to inflammation and malignant transformation. The association of the oral microbiome with HNSCCs was examined over a 4-year study. This study showed no significant risk associated with specific bacterial species or oral microbiome composition in the patients who developed HNSCC [39]. A study conducted by Al-hebshi *et al* investigated the functional potential of the bacteriome in conjunction with the composition. They found that the profile of bacteria found in OSCC held similarities to those found in periodontitis, suggesting that the bacteriome associated with OSCC could be functionally described as 'inflammatory' [11]. Both OSCC and periodontitis are associated with poor oral health, as will be discussed in a later section. Further functional studies on the bacteriome may prove more useful with regard to the role of bacteria in OSCC. It is likely there are other confounding factors such as smoking or drinking alcohol, exacerbating the influence of the microbiome on disease progression. The presence or dysbiosis of bacterial species alone in the oral cavity cannot be taken as causative or even contributory to the development of oral cancer. Rather, the tumour microenvironment might create an environment for opportunistic bacteria or fungi to thrive thereafter. The question remains; are the bacteria simply thriving in the tumour microenvironment or are they cancer promoting?

The influence of alcohol metabolism on oral cancer

Alcohol can greatly affect the integrity of mucosa via protein-alcohol interactions, hydrogen bonding and subsequent changes in membrane properties. Short-term exposure of oral mucosa to EtOH has been shown to cause increased permeability of the epithelial barrier [40]. The local permeabilizing effects of alcohol could provide enhanced penetration of carcinogens or enhanced invasion of bacteria across the oral epithelia. This effect is seen in the concurrent consumption of alcohol and tobacco, where alcohol acts as a solvent for penetration of carcinogens in cigarette smoke [41]. Although short-term or acute alcohol exposure increases membrane fluidity, chronic alcohol exposure has been shown to result in production of fatty acid ethyl esters, changing membrane make-up and ultimately leading to increased rigidity of the membrane [42]. Changes to membrane characteristics not only have the potential to render the cell more vulnerable to carcinogens and bacterial infections, but it could also alter intracellular signalling and subsequently affect the inflammatory response. Increased rigidity of cell membranes may delay dissociation of lipid rafts, which are crucial for many immune signalling processes. This could lead to prolonged signalling and an increase in pro-inflammatory mediators [43].

Chronic alcohol consumption has also been shown to increase basal cell nuclei size, reduce epithelia thickness and increase the number of cells in the S-phase of the cell cycle in oral epithelia [44]. Increased nucleo:cytoplasmic ratio is seen in oral lesions associated with risk of developing OSCC such as leukoplakia and oral lichen planus (OLP), as well as in established squamous cell carcinomas [45]. The increase in nuclei size and number of cells in S-phase could indicate increased DNA synthesis, which would imply a higher degree of vulnerability to mutations, or potentially a halt in cell cycle progression following the intra

S-phase checkpoint for DNA damage.

Acetaldehyde – The carcinogenic metabolite of alcohol metabolism

Alcohol is metabolised by alcohol dehydrogenase (ADH) into its first metabolite product, acetaldehyde (Fig. 2B). Acetaldehyde is then cleared via the enzyme acetaldehyde dehydrogenase (ALDH). This oxidative pathway results in acetate, which can be excreted by the body. While alcohol consumption has been indicated as a risk factor in a number of cancers, it was acetaldehyde that was declared a carcinogen by the International Agency for Research on Cancer (IARC) in 1999 and confirmed as a Group 1 carcinogen to humans in 2009 [46]. Both ADH and ALDH are expressed in oral mucosa, however ALDH activity is lower than that of ADH. This could potentially cause a build-up of cytotoxic acetaldehyde [47].

Acetaldehyde is a genotoxic carcinogen, capable of causing point mutations, chromosomal alterations and forming DNA adducts [48]. Acetaldehyde has also been shown to inhibit O-6-methylguanine-DNA methyltransferase (MGMT), an enzyme required for repair of DNA adducts from alkylating agents, in a dose-dependent manner [49]. The carbonyl carbon group of acetaldehyde can act as an electrophile to interact with DNA directly and the end product is an ethyl-adduct. The most common adduct is N^2-ethyldeoxyguanosine [50]. Both O^6- or N^2-adducts can interfere with the fidelity of DNA replication, leading to mutations [51]. In Rhesus monkeys chronically exposed to alcohol (4% w/v in water) over a period of 12 months, levels of acetaldehyde-derived N^2-ethyldeoxyguanosine were found to be increased in oral mucosa DNA [52]. It has been suggested that quantification of N^2-ethyldeoxyguanosine could be a potential biomarker in alcohol-related carcinogenesis, however further characterisation is needed [53]. Overall, the effects of ethanol metabolism result in an increased ratio of NADH:NAD^+ and the potential for the first metabolite, acetaldehyde, to form adducts or other genotoxic products (Fig. 2C).

Alcohol dehydrogenase & acetaldehyde dehydrogenase polymorphisms

Dr. Yokoyama and colleagues in Japan have extensively studied the common polymorphism in the ALDH gene, in which the mutant enzyme is inactive. The mutant gene contains a single nucleotide polymorphism (SNP) at position 487, where a lysine substitutes a glutamate, and is dominant over the wild-type allele [54]. The homozygous or heterozygous mutant alleles (ALDH2*2/2*2 and ALDH2*1/2*2) can cause severe acetaldehydemia and flushing responses, as a result of acetaldehyde not being metabolised into acetate at an appropriate rate. Subsequently, inactive ALDH polymorphisms and therefore decreased clearance of acetaldehyde showed a strong association with the risk of oesophageal and HNCs. This risk was increased multiplicatively in patients with both an inactive ALDH polymorphism and the more active ADH2*1/2*1 allele [55–58]. These studies highlight the role of acetaldehyde from alcohol consumption as a potent carcinogen and risk factor for OC.

Bacterial alcohol dehydrogenases

Consumed alcohol passes transiently through the mouth before reaching the liver or other organs. However, acetaldehyde concentrations of up to 450 μM have been found present in saliva following alcohol consumption [7]. Concentrations of acetaldehyde greater than 100 μM have been shown to induce mutagenic adducts [59]. The highest production of acetaldehyde per gram of tissue occurs in the colonic mucosa and comparatively, the activity of ADH in oral mucosa is low [47,60]. When patients with normal ALDH activity were treated with 4-methylpyrazole (a competitive inhibitor of human ADH) no significant changes were seen in salivary or blood acetaldehyde levels. This suggested that acetaldehyde production in the oral cavity is of microbial origin [61]. This lead to numerous studies investigating the ADH activities of specific oral bacteria and yeasts and their contribution to salivary acetaldehyde levels.

Neisseria mucosa alcohol dehydrogenase

Neisseria mucosa is a Gram-negative, diplococcus aerobic bacteria. The capacity of *N. mucosa* to produce acetaldehyde is 100-fold higher than other genera of bacteria studied in the mouth, as well as producing significantly more NADH [8,62]. Typically, *Neisseira* species are considered non-pathogenic, although extremely rare and severe cases of endocarditis resulting from infection with *N. mucosa* have been reported [63]. The local production of acetaldehyde by *N. mucosa* in the oral cavity was hypothesised to contribute to cancer promotion; however, a recent paper reported an inverse correlation between the abundance of *Neisseria* and the capacity of the oral microbial community to produce acetaldehyde [6]. Furthermore, the presence of oral *N. mucosa* has been shown to be decreased in smokers, indicating a potential protective role for *N. mucosa* in the oral microbiome [8,25]. *Neisseria* species are generally associated with good oral health [64].

Streptococci alcohol dehydrogenase

Streptococci of normal oral flora, including *S. salivarius, S. intermedius, S. gordonii* and *S. mitis* all have significant ADH-enzyme activity, and produce high amounts of acetaldehyde when incubated with ethanol [65]. No functional ALDH enzyme has been found in *Streptococci* species, and of those species investigated, *S. gordonii* was found to be the highest producer of acetaldehyde [66]. Older studies suggest that *S. salivarius* is capable of producing the most acetaldehyde however, a more recent paper reported *S. mitis* to be the most dominant acetaldehyde producer, producing in excess of 50 μM acetaldehyde in the presence of 11 mM ethanol [62,65]. While there is a link between *Streptococci* species and alcohol consumption via acetaldehyde production, the influence of *Streptococci* in tumour promotion is partly mitigated by the fact that it is largely associated with the healthy microbiome, and reported to be decreased in several oral diseases, PMDs and in OC itself [9].

Candida albicans alcohol dehydrogenase

Candida also possess the ability to metabolise ethanol into its carcinogenic metabolite acetaldehyde. In patients with PMDs such as OLP, oral lichenoid lesion and oral leukoplakia, where colonisation by *Candida* is common, the acetaldehyde production of yeasts was found to be producing potentially carcinogenic levels of acetaldehyde (> 100 μM). Yeast species isolated from smokers and drinkers produced more acetaldehyde than control groups [67]. The contribution of oral yeasts to acetaldehyde production in the oral cavity was first investigated in 1999. Yeast was found in 78% of high-acetaldehyde producing saliva samples, with *C. albicans* being the main species isolated. The rate of acetaldehyde production was 73.1 nmol acetaldehyde/10^6 colony-forming units. They also found that heavy smokers or drinkers correlated with a higher yeast burden [68]. Genomic databases show seven putative genes for ADH in *C. albicans*, but only three are thought to encode functional proteins (CaADH1-3) [69]. Bakri *et al* investigated mRNA of these genes. They expressed each recombinant protein in *Saccharomyces cerevisiae* and found ethanol-utilising ADH activity above the normal level of endogenous alcohol metabolism. In *S. cerevisiae* where the endogenous acetaldehyde producing gene (ScAdh2p) was deleted, acetaldehyde production was detected and it was determined that CaADH1 is the largest contributor to ethanol metabolism in *C. albicans* [69]. However these experiments failed to address the ethanol metabolising capabilities of clinical isolates, as only lab strains were used. It is also worth noting that the yeast were grown in glucose excess, which does not mimic *in vivo* salivary glucose concentrations and may have influenced the results.

Candida albicans ADH in the progression of oral cancer in APECED patients

Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) is a rare monogenic disease, caused by an autosomal recessive mutation in the autoimmune regulator (AIRE) gene. The disease is characterised by chronic mucocutaneous candidiasis. OSCC has been recognised as a distinct complication of APECED, often developing at the site of fungal lesions, and potentially leading to death [70–72]. Therefore *Candida* has a significant bearing on the progression of oral cancer in APECED.

C. albicans isolates from APECED patients have been shown to produce high levels of acetaldehyde *in vitro* [73]. The expression of the ADH gene, CaADH1, was investigated in *C. albicans* isolated from APECED patients. A negative correlation was found between the expression of ADH1 and Candida drug resistant 1/2 (CDR) genes conferring azole (anti-fungal drugs) resistance. In samples where CDR1/2 were highly expressed, meaning the isolate was resistant to anti-fungal fluconazole treatments, ADH1 was expressed at low levels [74]. These findings suggest that the more pathogenic *C. albicans* has down-regulated expression of ADH1 - decreasing exposure to carcinogenic acetaldehyde. This highlights the complex role of CaADH genes in *Candida* virulence and progression of oral carcinogenesis.

Candida albicans ADH and other virulence factors

Nieminen *et al.* cultured *Candida*-only biofilms and investigated the effect of a novel anti-fungal, D,L-2-hydroxyisocaproic acid (HICA), on *Candida* virulence. HICA has anti-inflammatory properties, was found to inhibit biofilm formation and reduce the mutagenic potential of *Candida* biofilms. Biomass and metabolic activity of the biofilms was reduced as well as significantly lowered acetaldehyde production compared to controls. However, CaADH1 gene expression in *C. albicans* was upregulated following treatment with HICA [75]. CaADH1 deletion has been shown to enhance the ability of *C. albicans* to form a biofilm and to invade and damage host mucosal tissues, where CaADH1 mutant strains actually produced larger amounts of acetaldehyde [76]. This research demonstrated that CaADH1 protein can also catalyse the production of ethanol in the reversible ethanol-acetaldehyde conversion, subsequently modulating the ability of *C. albicans* to form a biofilm. Alnuaimi et al. was the first to demonstrate a clear relationship between virulence factors and acetaldehyde production capability of *Candida* yeasts and oral cancer. They compared ability to form a biofilm, hydrolytic enzyme production and acetaldehyde production in *Candida* isolates from OC patients and age/gender/denture status-matched non-OC patients. It was found that OC patients had significantly increased biofilm biomass, metabolic activity and acetaldehyde production in comparison with non-OC patient controls [77].

Alcohol induced poor oral health increases the risk for OSCC

Patients presenting with oral cancer typically have poor oral health and dental status, and it has been shown that poor oral health increases the risk of developing oral cancer, potentially by increasing salivary acetaldehyde levels [78–80]. Chronic alcohol users typically have a poor dental score, with increased occurrence of caries and tooth loss. Chronic alcohol users also suffer a lower status of oral mucosal health, alongside higher prevalence of periodontitis and mucosal lesions [81–83]. There is some controversy as to whether the type of alcoholic beverage consumed influences the level of risk for oral cancer. There are studies that report wine, spirits or beer to be of equal risk while others report beer and spirits to be the highest risk [84,85]. There are many limitations when studying the impact of beverage type on risk of cancer development. Data collection on consumption habits is subjective and discrepancies exist in alcohol measurements and ethanol content e.g. units or standard drink measurements between different countries. An important confounding factor is concurrent tobacco consumption. Similarly, the consumption habits of individuals are usually not restricted to one type of alcoholic beverage. Even 'heavy-drinking' relative to 'light-drinking' is ill-defined, particularly in countries where 'binge-drinking' cultures exist. Therefore it remains unclear if any specific types of alcoholic beverage impacts overall oral health more so than others.

The oral microbiome influences oral health status

It has been hypothesised that poor oral hygiene would lead to overgrowth of pathogenic bacteria in the mouth, inducing chronic inflammation and promoting the development of OSCC. A positive association has been shown between periodontopathogenic bacteria and OSCC risk, as well as an association of alcohol use and poor oral hygiene with a higher percentage of oral periodontopathogenic bacteria. Interestingly, the ALDH2 deficient genetic polymorphisms results in a stronger positive association between alcohol and periodontopathogenic bacteria [9]. Hsiao *et al* found that the level of inflammatory cytokines such as IL-1β and IL-1 positively correlated with the percentage of periodontopathogenic bacteria (*Fusobacterium nucleatum, Prevotella intermedia and Prevotella tannerae*) within the literature [9]. Therefore, alterations in the microbiome associated with poor oral health and alcohol use may promote tumour development.

ROS-mediated inflammation in oral cancer

A notable link between alcohol consumption, the oral microbiome, inflammation and oral cancer is the production of reactive oxygen species (ROS). ROS such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\cdot}OH$) are double-edged swords in cancer. They are produced from endogenous and essential reactions, either as signalling molecules or as by-products. The addition of exogenous sources of ROS, from alcohol consumption or bacteria in the oral cavity, can disturb the balance between free radical generation and free radical scavenging.

Despite the role of ROS in normal physiological functions, increased levels can result in oxidative stress, implicated in countless pathologies. Cancer cells produce abundant ROS due to their hyper-proliferative and metabolic states in comparison to normal cells. Increased ROS activates redox signalling required for survival and promotes tumorigenesis, however, cancer cells also maintain an active antioxidant defence system, in order to protect themselves from ROS-induced cell death [86]. This includes enzymes such as superoxide dismutase (SOD) or the glutathione peroxidase (GPx) system [86]. The tumour suppressor gene p53 (Tp53) is capable of up-regulating antioxidant genes, contributing to regulation of redox signalling [87]. Tp53 is a highly mutated gene in numerous cancers, and whole-exome sequencing has shown that OSCC is no exception [88]. p53 mutations have been found in up to 70% of OC patients [89]. Therefore, it can be seen that redox biology and cancer are intrinsically linked, having both a promoting and suppressive effect on cancer progression.

Alcohol metabolism and the production of ROS

Ethanol metabolism has the potential to produce free radicals, depending on a number of influencing factors. In the oxidation of alcohol to acetaldehyde and subsequent oxidation to acetate, both reactions result in the formation of an NADH molecule (Fig. 2C). The change in both cytosolic and mitochondrial NADH/NAD^+ ratio would increase the activity of the electron transport chain (ETC) and contribute to ROS generation via oxidative phosphorylation.

Other potential sources of ROS from chronic alcohol consumption would be via aldehyde oxidase (AO), xanthine oxidase (XO) and NADPH oxidase (NOX). AO and XO are both enzymes of the same family, molybdenum hydroxylases. AO can use NADH as a substrate for $O_2^{\cdot-}$ formation [90], whereas both NADH and acetaldehyde are

substrates for XO in the formation of several by-product ROS species [91]. NOX can mediate direct ROS-release that results in respiratory burst, a process seen typically in phagocytic cells, although it is also expressed in other tissues. Alcohol has been shown to upregulate NOX expression and activity in both human neurons and in lung tissue [92,93]. While the same effect has not yet been demonstrated in oral mucosa, NOX activity and superoxide production are found to be increased in OSCC [94]. In periodontal pathologies, increased ROS generation has been postulated to be caused by oral pathogens that enhance NOX function [95].

Alcohol metabolism impairs anti-oxidant systems

Alcohol metabolism can also contribute to ROS generation by impairing the body's natural anti-oxidant system. The GPx system utilises glutathione to scavenge ROS, acting as a defence system against oxidative stress. Acetaldehyde can bind to glutathione molecules and reduce scavenging glutathione levels, subsequently increasing susceptibility to oxidative damage [96]. Furthermore, glutathione can reduce NOX expression and prevent DNA adducts forming by conjugating to 4-hydroxynoneal – an aldehyde that forms DNA lesions [97,98]. If glutathione is sequestered by acetaldehyde, ethanol-mediated ROS production would increase, as would the chances of DNA adducts forming.

Periodontopathogenic bacteria increase ROS

ROS have been shown to play a role in periodontitis, associated with increased risk for OSCC [99]. In a Japanese study, serum reactive oxygen metabolites were found to have a positive correlation with serum antibody titres to putative periodontopathogenic bacteria, including *P. gingivalis* [100]. A NOX homolog exists in bacteria that plays a role in ROS generation [101]. The NOX gene possesses NADH oxidase activity that is important in yeasts for biofilm formation in the oral cavity, likely by using ROS as signalling molecules [102,103].

Inflammatory response to bacterial pathogens

The tumour microenvironment is largely influenced by inflammatory cells, and inflammation has been recognised as a fundamental factor in the neoplastic process [104]. It is a commonly accepted hypothesis that ROS can trigger inflammation, and that inflammation itself can induce the generation of ROS. Hyperactive inflammation through ROS can arise from the induction of nuclear transcription factor NF-κB, which has been demonstrated in OSCC cells [105]. In some cancers, NF-κB is over-active or loses feedback regulation in order to aid tumorigenesis [106]. NF-κB has been shown to be involved in invasion and metastasis in HNCs as well as in OSCC specifically [107,108]. ROS and a pro-inflammatory environment can interfere with DNA methylation. By altering DNA methyl transferase activity, tumour suppressor genes can be silenced via CpG methylation whereas oncogenes could be up-regulated from *reduced* methylation [109], further contributing to the development of cancer.

A pro-inflammatory environment can also produce ROS – typically in response to pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs). Innate immune cells, such as macrophages or neutrophils, as well as oral epithelial cells, have pathogen recognition receptors (PRR) that detect PAMPs and DAMPs [110]. The NF-κB signalling pathway (Fig. 3) is activated upon ligand binding to PRRs. PRRs found on oral epithelial cells have been shown to be capable of responding to bacterial components and influencing inflammation in the oral mucosa [110]. Alcohol can have immunosuppressive affects following consumption, affecting the sensitivity of TLRs to bacterial ligands. Acute alcohol exposure in monocytes has been shown to induce a decreased response to bacterial ligands such as LPS, however chronic alcohol exposure has been shown to increase sensitivity to LPS, increasing the production of TNF-α in response [111].

Stimulation of NF-κB via PAMPs leads to inflammation and recruitment of inflammatory cells, as well as enhanced proliferation, cell survival and can even aid in invasion and metastasis in cancerous cells. Phagocytic cells also release ROS as they engulf pathogens [112]. The response of these phagocytes, such as neutrophils, can be modified by periodontopathogenic bacteria – affecting ROS and neutrophil extracellular traps (NETs) release [113]. As the bacteria and fungi that exist in the mouth are commensal organisms, the oral cavity is not in a constant state of inflammation. However, polymicrobial dysbiosis in combination with alcohol consumption has potential to influence the inflammatory response. Abnormal responses to commensal oral bacteria could lead to bacteria-induced ROS and NET release, influencing an inflammatory environment and contributing to the development of OC.

Inflammatory response to Candida albicans - alcohol dehydrogenase is a multi-functional enzyme

Soluble ADH from *C. albicans* was cloned, purified and tested for immunogenicity. The multi-functional or 'moonlighting' protein ADH was evaluated for its effect on the differentiation of a monocytic cell line, THP-1. THP-1 cells are typically induced to differentiate into a simplified macrophage-model by phorbol 12-myristate-13-acetate (PMA). It was found that ADH was able to induce differentiation of the THP-1 cells in the same way as the positive PMA control. This differentiation was carried out through the ERK1/2 pathway and resulted in increased adhesion, phagocytosis and killing of *C. albicans*. The differentiated cells also showed an increase in the production of inflammatory cytokines IL-1β and TNF-α. Monocyte to macrophage differentiation is an important step in the onset of immune responses, hence ADH is not only involved in ethanol metabolism but may also illicit a host immune response against fungal invasion [114]. A limitation of this study is the use of soluble ADH. *In vivo* ADH is a cell-wall related protein that can be expressed in the cytoplasm, therefore the use of soluble ADH only cannot clarify its role and interactions with host immune systems.

Although *C. albicans* is a commensal member of the oral mycobiome, it is capable of triggering an immune response. Candida hyphae trigger lysis and DAMP signalling in oral epithelial cells, leading to the production of cytokines and chemokines. This is typically initiated by the recognition of β-glucan on the fungal cell wall that are exposed during hyphal transition. This leads to the production of IL-6 and IL-23, eventually leading to TH17 cell differentiation. *C. albicans* secrete pore-forming peptide candidalysin upon invasion of epithelial tissue and the cytokines released following this secretion are also associated with TH17 recruitment. Subsequently it was discovered that candidalysin can prompt the release of cytokines driving the TH17 response in conjunction with IL-17. This feed-forward loop of amplified inflammation does not require T-cell receptor activation or typical fungal pattern recognition but rather is regulated by the virulence factor candidalysin upon tissue damage by *C. albicans* [115]. The inflammatory cytokines induced by *C. albicans* are similar to those considered to be salivary-based biomarkers in OSCC. Culturing oral epithelial cells with *C. albicans* results in increased production of IL-6, IL-1β, TNF-α, which have been shown to be increased in salivary samples from OSCC patients when compared to dentally compromised patients and to controls [115–117].

C. albicans is a commensal organism but also an opportunistic pathogen. The host immune system must be able to distinguish between these forms of the yeast. Oral epithelial cells have an innate response to pathogenic *C. albicans* via NF-κB and MAPK, however this response is dose dependent meaning the epithelia can remain quiescent during low fungal burden but react specifically to damage-inducing hyphae. The activation of NF-κB and the first MAPK phase is independent of the morphological form and is due to recognition of fungal cell wall structures such as chitin, mannans and β-glucans. The second MAPK

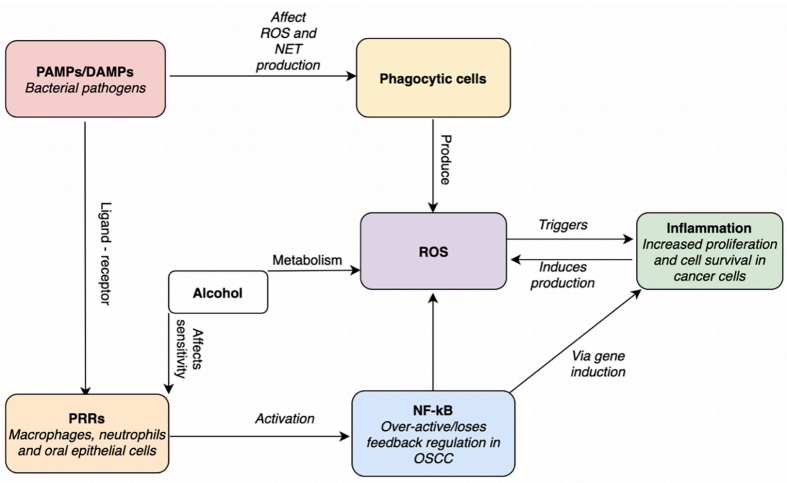

Fig. 3. Inflammation, reactive oxygen species, alcohol and bacterial pathogens. NF-κB regulates inflammation by directly increasing the gene production of inflammatory cytokines. It also up-regulates cell cycle regulators and anti-apoptotic factors in favour of cell survival. Inflammation, engulfing phagocytic cells and alcohol metabolism can increase the production of reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\cdot}OH$). The ROS production from phagocytic cells can be affected by specific PAMPs/DAMPs. The release of neutrophil extracellular traps (NETs) to engulf pathogens by phagocytic cells can be stimulated or suppressed by certain microorganisms [113]. Activation of PRRs by PAMPs/DAMPs stimulate NF-κB and the sensitivity of PRRs can be affected by alcohol exposure.

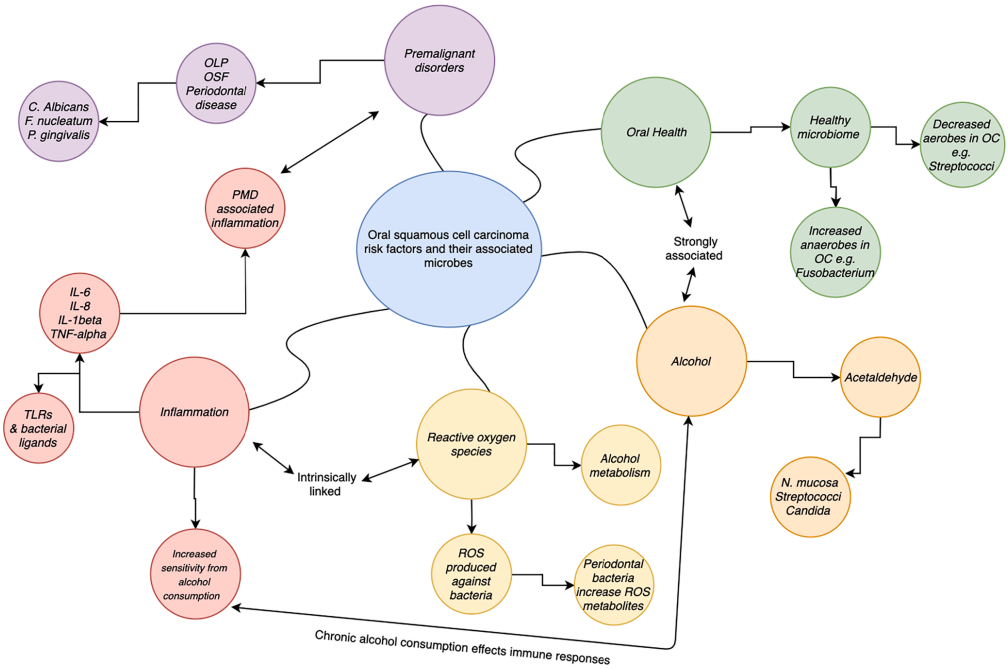

Fig. 4. The links between oral microbiome, alcohol consumption, alcohol dehydrogenases, ROS production and inflammation. The disease state microbiome associated with OSCC and PMDs can be influenced by alcohol consumption. This inflammatory composition includes microbes that can contribute to genotoxic levels of acetaldehyde via possession of ADH (*N. mucosa, Streptococci, Candida*). In addition, OSCC and PMD microbiomes are accompanied by increased ROS production and inflammation. Both ROS production and inflammation can be mediated by alcohol consumption. It is hypothesised that the concurrent influence of alcohol consumption and disease state microbiome on inflammation and ROS contributes to the development and progression of OSCC.

phase response is contact dependent and activated via cell surface moieties specific to filamentous forms of *C. albicans* [118]. In oral leukoplakia, an increased susceptibility to *C. albicans* infection is seen in oral leukoplakia-associated fibroblasts (LAFs). This is due to decreased secretion of anti-fungal chemokine CX3CL1, via the ERK/MAPK pathway. Co-culture of LAFs and *C. albicans* increased subsequent

Table 1
Summary of main findings on oral microbes impact in the oral cavity with respect to OSCC.

Genus	Domain	Species	Possess ADH	Acetaldehyde production levels (μM)	Trend in disease state	Immune response	Main Findings
Neisseria	Bacteria (Gram-negative)	*N. mucosa*	Yes	50–270 μM [8,62,129]	Decreased in OSCC, associated with good oral health [6,64]	N/A	• Produces significant levels of NADH (117.3 ± 3.7 nmol/min/mg protein [8], contributing to ROS production
Streptococcus	Bacteria (Gram-positive)	*S. salivarius* *S. intermedius* *S. gordonii* *S. mitis* *S. oralis*	Yes	~135 μM [65] ~50–60 μM [65] ~20 μM [62,66] ~11 μM-50 μM [62,65,129] ~5 μM [65]	Decreased in PMDs and OSCC [11,26–32]	Hypothesised to stimulate an anti-tumour response [34]	• Proportion of *Streptococci* species decreases in OC and in PMDs [11,26–32] • *S. mitis* and *S. salivarius* reactive $CD8^+$ T-cells positively correlate with prognosis for OC patients [34] • High acetaldehyde producers with no functional ALDH enzyme [66] • *S. mitis* and *S. oralis* mitigate the inhibitory effects on wound healing caused by bacteria such as *P. gingivalis (33)*.
Candida	Fungi	*C. albicans*	Yes	~215 μM [67]	Significant positive correlation between oral cancer and presence/severity of oral yeast colonisation [37]	Evidence for increased production of IL-6, IL-1β, TNF-α, and IL-23 [111–114]	• High yeast burden seen in drinkers [68] and OSCC patients [37] • Ability to evoke immune response through external components as well as soluble ADH [114–117] • Correlation between virulence factors and acetaldehyde production in OC patients [77] • Inflammation caused by *C. albicans* might cause malignant transformation from OLP to OSCC [120] • Significant bearing on progression of OC in patients with APECED [70–72]
Porphyromonas	Bacteria (Gram-negative)	*P. gingivalis*	Putatively [131]	Not documented [62,130]	Increased in OSCC [32]	Increased IL-1β, does not induce Il-6/-8 and decreases TNF-α [124,126]	• Increase ROS generation, potentially via enhanced NOX function [95,100,101,125,127] • Induce pro-MMP expression and cleavage, promoting carcinoma invasion and metastasis [123] • Modify host cytokine response – no secondary response. Subverts protective inflammation [124]
Fusobacterium	Bacteria (Gram-negative)	*F. nucleatum*	Putatively [131]	Not documented [130]	Increased in OSCC [32]	Increase IL-1β, IL-1, IL-6, IL-8 [124,128]	• Increases ROS generation [95,100] • Evidence of promoting malignant transformation in conjunction with *P. gingivalis*, in a mouse model of chronic periodontitis, alongside administration of 4NQO [38]

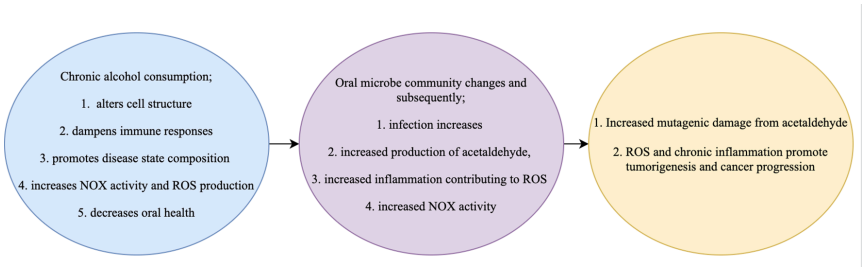

Fig. 5. Potential links between oral microbiome, alcohol consumption, alcohol dehydrogenases, ROS production and inflammation. Alcohol consumption promotes the disease state microbiome associated with OSCC. The effect of this inflammatory composition is heightened by the modulation of the immune response and cell structure by alcohol. Microbes that can contribute to genotoxic levels of acetaldehyde via possession of ADH also increase ROS production and promote inflammation. Both ROS production and inflammation can be mediated by alcohol consumption. The resulting oral environment promotes tumorigenesis via ROS/chronic inflammation and is susceptible to damage from acetaldehyde.

adhesion and invasion of the yeast to an OSCC cell line [119].

Inflammation caused by *C. albicans* has also been postulated to be a cause of malignant transformation from OLP to OSCC. Co-culture of primary OLP keratinocytes at an MOI of 2:1 of candida cells to keratinocytes for 24 h showed increased inflammatory cytokines (IL-10, IL-8, IL-2, TNF-α), increased expression of TLR2, MyD88 and NF-κB, and decreased rates of apoptosis compared to the control [120]. Although it is not clear from this study the mechanisms by which *C. albicans* inhibits apoptosis in OLP keratinocytes, inflammation and inhibition of apoptosis have been shown in OLP patients to be markers of malignant transformation, as reviewed by Tampa et al. [121].

Inflammatory response to periodontal pathogen Porphyromonas gingivalis

While a definitive causal relationship has not yet been defined between periodontitis and OSCC, periodontal pathogens have been identified as an independent risk factor of non-HPV associated OSCC [32]. *P. gingivalis* is a pathobiont – a commensal microorganism that under disrupted homeostatic conditions can deregulate inflammatory responses and cause disease. It is a major etiologic agent in the progression of periodontal disease. *P. gingivalis* possesses several virulence mechanisms, including gingipains – trypsin-like cysteine proteases that are either secreted or membrane-bound. These proteases are arginine- or lysine-specific and play a major role in the etiology of periodontal disease [122]. An important role of gingipains in the virulence of *P. gingivalis* is their ability to induce expression of proenzyme matrix metalloproteinases, and subsequent cleavage to activate them. This effect has been shown in oral mucosa, dendritic cells, monocytes and in OSCC cell lines. Promotion of pro-MMP2/MMP9 expression is via ERK/p38/Nf-κB pathways. Increased expression of MMP9 in particular is associated with increased capacity for degradation of basement membranes and collagen IV, promoting carcinoma cell invasion and migration and ultimately aiding in metastasis. The ability of *P. gingivalis* gingipains to cleave pro-MMP enzymes provides a novel mechanism for progression of OSCC associated with periodontal disease [123].

A unique feature of infection with *P. gingivalis* is the lack of secondary cytokine response. This reduces inflammatory infiltrate and migration of bacteria phagocytosing cells. The production of primary cytokines such as IL-1β but not secondary cytokines such as Il-6/IL-8 is not observed with other periodontal pathogens such as *F. nucleatum*. Lys-gingipains orchestrate this effect by direct degradation of IL-6/IL-8. The same effect is not seen with Arg-specific gingipains. Specific gingipains therefore modify the host cytokine response in order to subvert protective inflammation, and explains the differential expression of cytokines in periodontal pockets [124].

Utispan *et al* report that macrophages subjected to periodontal pathogen *P. gingivalis* exhibit increased NO secretion. NO possesses the ability to promote angiogenesis but also has cytotoxic effects on tumour cells such as suppression of DNA synthesis [125]. Reduction of NO levels could promote *P. gingivalis* survival while induction of NO secretion could promote tumour progression. Utispan et al. also found that stimulation of macrophages with *P. gingivalis* LPS significantly decreased TNF-α production but promoted invasion of HNSCC cell lines compared to a PMA-differentiated control [126]. Furthermore, neutrophils from patients with localized aggressive periodontal disease produce higher levels of ROS against P. gingivalis, as compared with those from healthy donors, and release of ROS results in secretion of pro-inflammatory cytokines [127].

Conclusion

The interactions between the oral microbiome and alcohol metabolism in the progression of OC is a complex and enigmatic process (Fig. 4) with numerous genus/species related outcomes possible (Table 1). One of the main links between alcohol consumption and the oral microbiome is the possession of the enzyme ADH by several commensal bacterial species such as *N. mucosa* and *Streptococci* species, as well as fungi *C. albicans* [8,65,67]. Acetaldehyde, the metabolite product of ADH, is carcinogenic and oral microbes have the potential to produce genotoxic levels. Poor oral health status from chronic alcohol consumption is also associated with an increased level of acetaldehyde in the oral cavity [78–83] and poses a risk for the development of oral cancer. Inflammation is another putative link between alcohol consumption and the oral microbiome in the progression of OC. Inflammation can be mediated by the production of ROS via alcohol metabolism or as a result of polymicrobial dysbiosis in the oral cavity. In the disease state oral microbiome, there is a trend for an increased proportion of anaerobes to appear within the composition of microbes. This is observed in several PMDs and periodontal diseases as well as in OSCC itself [11,26–32]. This disease state dysbiosis has been described as functionally inflammatory [11]. Inflammatory responses to microorganisms in the oral cavity could lead to induction of ROS and NET release, further influencing the oral cavity environment. It is also noted that the host immune response is exacerbated by the presence of alcohol, either via local permeabilizing effects of alcohol [40] providing enhanced invasion of bacteria across the oral epithelia, or by increasing sensitivity of TLR ligands [111]. Alcohol has been shown to alter the composition of the oral microbiome [9,10,25], which could foster the transformation from normal oral microbiome composition to disease state composition, creating an environment for malignant transformation to take place via increased ADH activity, inflammation and ROS (Fig. 5). Future studies on the role of the oral microbiome and alcohol consumption in the development and progression of OC will be necessary to elucidate the mechanisms at play.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix 2

The novel therapeutic potential of bovine α-lactalbumin made lethal to tumour cells (BALMET) and oleic acid in oral squamous cell carcinoma (OSCC)

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Background **Since the serendipitous discovery of bovine α-lactalbumin made lethal to tumour cells (BAMLET)/human α-lactalbumin made lethal to tumour cells there has been an increased interest in the ability of the two components, oleic acid and α-lactalbumin, to form anti-cancer complexes. Here we have investigated the in-vitro efficacy of the BAMLET complex in killing oral cancer (OC) cells, determined the active component of the complex and investigated possible biological mechanisms.**

Materials and methods **Two OC cell lines (±p53 mutation) and one dysplastic cell line were used as a model of progressive oral carcinogenesis. We performed cell viability assays with increasing BAMLET concentrations to determine the cytotoxic potential of the complex. We further analysed the individual components to determine their respective cytotoxicities. siRNA knockdown of p53 was used to determine its functional role in mediating sensitivity to BAMLET. Cell death mechanisms were investigated by flow cytometry, confocal microscopy and the lactate dehydrogenase assay.**

Results **Our results show that BAMLET is cytotoxic to the OC and dysplastic cell lines in a time and dose-dependent manner. The cytotoxic component was found to be oleic acid, which, can induce cytotoxicity even when not in complex. Our results indicate that the mechanism of cytotoxicity occurs through multiple simultaneous events including cell cycle arrest, autophagy like processes with a minor involvement of necrosis.**

Conclusion **Deciphering the mechanism of cytotoxicity will aid treatment modalities for OC. This study highlights the potential of BAMLET as a novel therapeutic strategy in oral dysplastic and cancerous cells.** ***European Journal of Cancer Prevention*** **30: 178–187 Copyright © 2020 Wolters Kluwer Health, Inc. All rights reserved.**

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Keywords: apoptosis, autophagy, bovine α-lactalbumin made lethal to tumour cells, necrosis, oleic acid, oral cancer, oral squamous cell carcinoma, α-lactalbumin

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Introduction

Oral cancer (OC), a subset of head and neck squamous cell carcinoma, remains one of the most common cancers worldwide, with over 650 000 new diagnosis and 330 000 deaths annually (Bray *et al.*, 2018). OC is top-ranking in incidence, mortality and low 5-year survival outlook, reported at 50% (Le Campion *et al.*, 2017). In contrast to historical trends, increased diagnosis in females and the younger population has been reported with an 11.3% increase in adults under the age of 45 years (Gupta *et al.*, 2016). Smoking, drinking and human pipillomavirus (HPV) infections are the highest risk factors for developing OC. A significant problem associated with OC is late-stage diagnosis, attributed in part to the asymptomatic nature of the disease, considerably limiting the therapeutic success rate. Surgery remains the gold standard treatment, despite the advances of modern medicine. Chemotherapy or in combination with radiotherapy have shown modest benefits in OC. Thirty-three percent of patients treated with surgery and adjuvant therapy will experience local or regional recurrence or in combination with distant metastasis (Hosni *et al.*, 2017). The median time for recurrences is 7.5 months after treatment and 86% of all recurrences occur within 24 months (Da Silva *et al.*, 2012).

Current therapeutic strategies are linked to numerous undesirable side-effects. Of the most evident, facial reconstruction at physical level followed by psychological distress and pain, significantly decrease the patient's quality of life. At the cellular and molecular level, undesired toxicity to normal cells leads to considerable side effects, yet in other cases, these therapies are infective due to inherent and acquired resistance. Limited efficacy of current treatment strategies highlights the need for novel therapeutic avenues to improve treatment success rates in OC.

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In this study, the novel potential of bovine α-lactalbumin made lethal to tumour cells (BAMLET) as a therapeutic for OC was investigated. BAMLET has been shown to possess selective ability in killing cancerous cells but its antitumorigenic potential has not been demonstrated in OC (Håkansson *et al.*, 1995; Svensson *et al.*, 2000; Hallgren *et al.*, 2006). The BAMLET complex (Fig. 1a), contains the protein α-lactalbumin (α-LA) and a monounsaturated fatty acid, oleic acid (OA) (Min *et al.*, 2012; Ho *et al.*, 2017). α-LA is found naturally in the whey portion of milk and forms the second most abundant whey protein after β-lactoglobulin. OA is a non-essential fatty acid (ω-9), predominant in olive oil (70–80%), avocados, almonds, peanuts, sesame oil, pecans, pistachio nuts and animal fat.

In this study, we demonstrate that oral dysplastic and cancerous cell lines are sensitive to BAMLET treatment. We show that sensitivity is cell line dependent and that the fatty acid component, forms the active component of the complex. BAMLET was shown to increase cell cycle arrest and thus decrease cell proliferation in oral cancerous cells. Furthermore, BAMLET and OA increased lysosomal vesicle formation indicating a possible autophagic involvement, while necrosis was only a minor event induced after BAMLET administration. Sensitivity to BAMLET was independent of the functional p53 status of the cell indicating a wide spectrum of patients who would benefit from BAMLET therapy. The results of this study show the potential for BAMLET to become an effective therapeutic for OC. Furthermore, this study supports the consumption of a Mediterranean diet, which may provide a means for lowering the incidence of OC.

Materials and methods

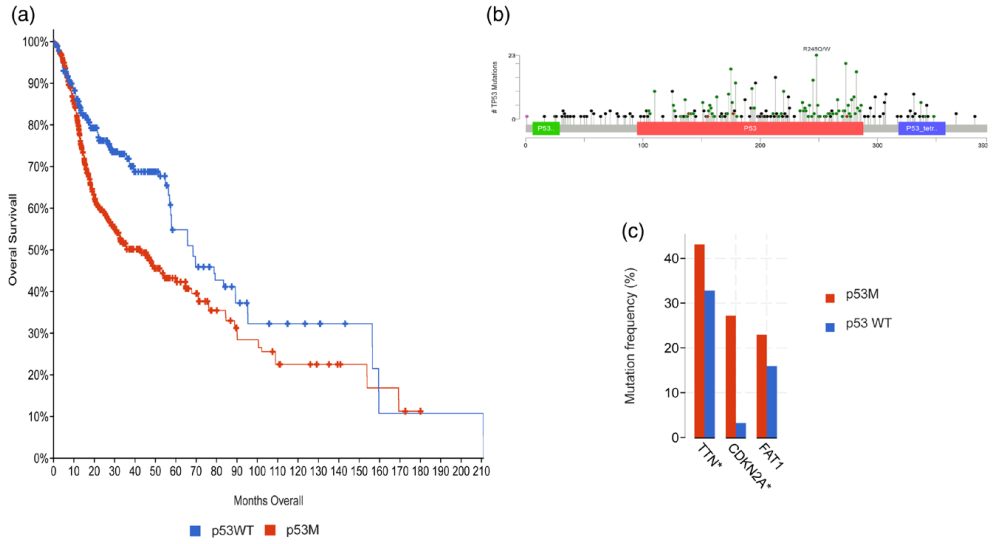
Cell lines and maintenance

Human TR146 and dysplastic oral keratinocyte (DOK) cell lines were obtained from the Health Protection Agency Culture Collection (Salisbury, UK). The TR146 cells were maintained in Dulbecco's Modified Eagle's medium, supplemented with 10% v/v foetal bovine serum (FBS), 10 U/ml penicillin, 0.1 mg/ml streptomycin and 2 mM glutamine. For the DOK cell line, hydrocortisone (5 μg/ml) was also added. The Ca9.22 cell line was obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). The Ca9.22 cells were maintained in minimum essential medium, supplemented with 10% v/v FBS, 10 U penicillin, 0.1 mg/ml streptomycin and 2 mM glutamine.

Complex formation with oleic acid by ion-exchange chromatography

BAMLET was prepared using the chromatographic method previously described by Svensson *et al.* (1999). Briefly, a DEAE column matrix (GE Healthcare HiTrap

Fig. 1



p53 mutations are associated with worse prognosis in OC. (a) Kaplan–Meier plot of overall survival in patients with and without p53 mutations (b) genomic map of the p53 mutation locations and (c) top three genetic mutations found in p53 mutated OC. OC, oral cancer.

DEAE FF, Sigma, Ireland) was preconditioned with OA (200 mg), followed by the addition of the Ca^{2+} depleted α-LA (37 mg). The complex was eluted with 1M NaCl buffer, desalted through dialysis (membrane cut-off 3.5 kDa) against distilled water overnight and lyophilized.

AlexaFluor 647 labelled BAMLET was prepared using the same method with the only modification that OA was mixed with the chromophore, as per manufacturers guidelines, prior to its addition on the column. The concentration of the complex was estimated spectrophotometrically. The molar extinction, $A^{1\%}$ value of 20.1 (for native α-LA) and 22.8 (for α-LA in BAMLET) at 280 nm were used for determining the protein concentration.

Cell viability

Cell viability was determined after BAMLET or OA treatment using the AlamarBlue redox indicator. Fifteen thousand cells were serum-starved overnight (1% FBS), washed with PBS and treated with a range of drug concentrations or vehicle control (sterile distilled water or EtOH), in serum-free media (SFM) or 10% FBS media and incubated for required time. AlamarBlue reagent (10% v/v) was added to each well 4 h before the time points elapsed. Fluorescence was measured using the SPECTRAmax PLUS Microplate Spectrophotometer at 560 nm emission and 590 nm excitation. Cell viability was expressed as percentage of control with all values corrected for blank using Microsoft Excel. Statistical analyses were performed using GraphPad Prism 5 software.

Transient knockdown of p53

p53 knockdown was achieved using the oligonucleotide sequences of small interfering RNAs (siRNAs), using the following sequences: Guide strand 5′-3′ UCACAAUUGUAAUCCCAGCCCCC, UGUCAUCCAAAUACUCCACCCCC, ACUAACCCUUAACUGCAAGCCCCC, UUAUUUCAUUAACCCUCACCCCC and passenger strand: GGGGGCUGGAUUACAAUUGUGA, GGGGGUGGAGUAUUUGGAUGACA, GGGGGCUUGCAGUUAAGGGUUAGU, GGGGGUGAGGGUUAAUGAAAUAA. The nucleotides were pooled to achieve maximal efficiency. Cells were serum-starved overnight (1% FBS). Cells were transfected with transfection solution [the targeting siRNA 20 nM IBONI p53-siRNA pool was mixed with RIBOXX-FECT transfection reagent (3% v/v) in OPTI-MEM and incubated at room temperature for 10 minutes]. Cells were incubated for 24 h and were then subjected to different treatments.

Lactate dehydrogenase assay

Measurement of necrosis by lactate dehydrogenase (LDH) release (Pierce LDH Cytotoxicity Assay Kit, Sigma, Ireland) was performed as per manufactures guidelines. Cells were treated with the drug or vehicle control (water, EtOH and lysis buffer) in SFM or 10% FBS media, and incubated for the required time. Equal amount of medium (from the treated cells) and LDH reaction mixture were transferred to a new plate and incubated for 45 minutes at 37°C in darkness. Absorbance readings at 490 and 690 nm were obtained using the SPECTRAmax Plus Microplate Spectrophotometer. Necrotic cell death was expressed as percentage of maximum control. All values were corrected for blank and LDH activity in media. Statistical analyses were performed using GraphPad Prism 5 software.

Microscopy

Analysis of lysosomal activation

Cells were seeded in a four-compartment Cellview culture dish overnight in serum-starved media (1% FBS). Cells were treated with the drug or control (water or Earls balanced salt solution) for different time periods. After treatment acridine orange (1 μg/ml) was added and the dishes were incubated for 15 minutes at 37°C. The medium was discarded and replaced with fresh medium. Live cells were viewed using the Olympus FV1000 Point Scanning Confocal Microscope, FV10-ASQ Olympus Fluoview Ver.2 software at 20, 40 or 60X oil immersion objective.

Time-lapse microscopy of BAMLET

Cells were seeded in 4 well compartment confocal dishes and serum-starved (1% FBS) overnight. Alexafluor 647 labelled BAMLET (IC_{50}) was added along with Hoechst dye (1 μg/ml) and Propidium Iodide dye (100 μg/μl) to stain the nucleus and dead cells respectively. Images were acquired every 5 minutes for a total of 200 images. Excitation at 405, 488 and 546 nm were provided by near-violet laser diode, argon and helium-neon gas lasers, respectively. The imaging chamber was heated to 37°C, humidified and contained 5% CO_2 to facilitate live-cell imaging.

Flow cytometry

Cells were treated as required followed by trypsinisation and centrifugation as previously described. Cells were washed with PBS (1X) and fixed in ice-cold ethanol (100%) at 4°C overnight. Cells were re-suspended in PBS containing RNAse A (10 μg/μl) and PI (100 μg/μl) and incubated for 30 minutes at 37°C in darkness. Cells were analysed using the FACSCanto II. Maximum excitation of PI bound to DNA was achieved at 536 nm and emission at 617 nm. The blue 488 nm laser was used for optimal excitation of PI fluorescence. Results were analyzed using Flow Jo software (Treestar, Ashland, USA).

Bioinformatic analysis

To analyze p53 gene alternations in OC, we queried the online database www.cbioportal.org. A total of six studies of head and neck squamous cell carcinoma and 1018

patients were included in the search. The relationship of p53 expression and overall survival was investigated and the corresponding Kaplan–Meier plot was extracted. Topological and mutation types as well as top three genes with copy number alteration frequency associated with p53 are also displayed.

Results

Oral cancers associated with p53 mutation show worse prognosis

We first queried the online genomic database cBioportal to determine generic patient characteristics as well as specific genomic alternations in head and neck cancers from six studies including 1018 patients. Details of the studies included, anatomical location and other relevant information pertaining to patients included in this analysis are presented in Supplementary Fig. 1, Supplemental digital content 1, *http://links.lww.com/EJCP/A305*. From the available data, 53.8% were male and 23.6% female; 23.3% and 4% of the reported cases were HPV negative and HPV positive, respectively. In accordance to previously reported results, we found p53 to be mutated in 69% of cases. The mean overall survival of patients with a p53 mutation was 45.93 months compared to 68.43 in p53 WT patients (Fig. 1a). A total of 929 mutations were reported in the p53 gene, with 57.6% being missense mutations, 40% truncating mutations and 2.15% inframe mutations (Fig. 1b). A higher mutational frequency in the TTN (involved in cell plasticity), CDKN2A (involved in cell cycle control) and FAT1 (involved in cellular polarization) genes was also associated with p53 mutated cancers (Fig. 1c). Together these results demonstrate that p53 mutations are frequent in OCs and contribute to worse prognosis.

BAMLET induces cellular cytotoxicity in dysplastic and cancerous oral cell lines

To recapitulate different stages and molecular profiles of OC in our study, we selected a dysplastic cell line derived from the tongue (DOK), a gingival cancerous cell line with a reported p53 mutation (Ca9.22) and an OC cell line derived from the buccal mucosa with a wild-type p53 gene (TR146).

To determine the potential therapeutic role of BAMLET in these cell lines we first carried out cytotoxicity assays. We found that the cytotoxicity of BAMLET is cell type-dependent with IC_{50} similar to the cytotoxicity of BAMLET in other cancerous cell lines as shown by Rammer *et al.* (2010). The IC_{50} values ranged from 0.05 to 0.4 mg/ml, with the dysplastic cell line showing the highest sensitivity followed by the Ca9.22 cell line ($p53^{M}$) and the TR146 cell ($p53^{WT}$) when treated in SFM (Fig. 2).

Studies have previously reported that blood components partially inactivate the BAMLET complex due to the presence of calcium and albumin. Thus, we also investigated the cytotoxicity potential of BAMLET in medium containing full serum to ensure in-vivo translatability. The presence of serum components increased the IC_{50} values by a factor of at least two, an effect that is in accordance with previously reported studies. Interestingly, the dysplastic cell line displayed the highest increase in IC_{50}, by factor of six, when treated in full serum conditions, indicating a possible involvement of α-LA. In summary, these results show that BAMLET is cytotoxic to both dysplastic and cancerous oral cell and the effective concentration is cell type-dependent.

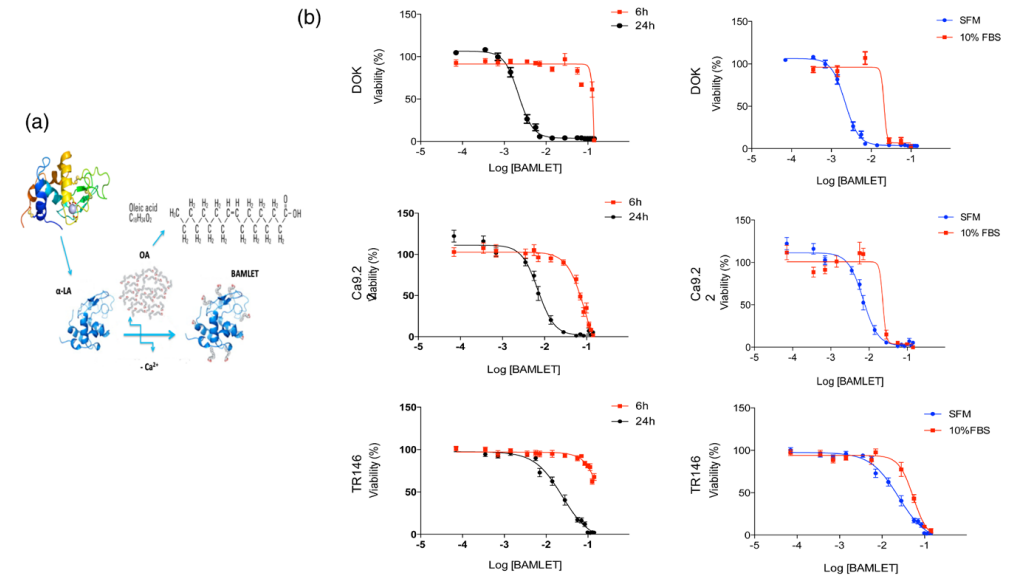
Oleic acid is the active component of the BAMLET complex

Studies investigating the cytotoxic component of BAMLET have yielded diverse results regarding the active component, with most studies showing OA as the principal cytotoxic-inducing agent. We next carried out cytotoxicity assays with OA alone (Fig. 3a) and show that in OC cells, OA is the main active component of the BAMLET complex. The IC_{50} concentration increased by a factor of at least two, with the same sensitivity profile of the cell lines: DOK, Ca9.22 and TR146, demonstrating that OA mediates cell death in the BAMLET complex.

A number of studies describing the tumoricidal action of BAMLET observed little toxicity of native or apo-α-LA against tumour cells. We investigated the effect of α-LA to determine if the differential response in the dysplastic cell line could be attributed to the effect of α-LA. Cytotoxicity results show a differential response in viability in response to α-LA in the cell lines tested. In the cancerous cell lines, Ca9.22 and TR146, our results confirm observations of α-LA showing minimal cytotoxic potential (Fig. 3b). However, in the dysplastic cell line, we see a higher reduction in cell viability in response to α-LA after 24 h treatment. The ability of α-LA to induce cytotoxicity in this cell line may explain the lower sensitivity observed in response to BAMLET treatment. In summary, OA is the principal active component resulting in cellular cytotoxicity in oral dysplastic and cancerous cells. However, the increase in IC_{50} suggests that α-LA plays a significant role in the BAMLET complex, possibly mediating the transport of the complex to the cell membrane and resulting in enhanced cellular delivery.

Since OA alone is less potent than when in complex with the protein as in the BAMLET complex, we hypothesized that α-LA acts as an important carrier molecule, transporting OA to the cell membrane, mechanism referred to as cargo off-loading (Rath *et al.*, 2018). OA is hypothesized to dissociate from the complex once it is in contact with a hydrophobic membrane such as the cell membrane. Similar synergistic effects of both protein and fatty acid, resulting in a vastly different profile of transcriptomics between fatty acid and complex, have been observed in lung carcinoma cells (A549) and T-cell lymphoma cells (Jurkat) (Ho *et al.*, 2013).

Fig. 2



BAMLET induces cytotoxicity in oral dysplastic and cancerous cells (a) Structure of α-lactalbumin, oleic acid and the BAMLET complex; (b) dose-response curves after treatment with a range of BAMLET concentrations for 6 and 24 h in serum-free media (left) or 10% FBS (right). BAMLET exhibited the highest sensitivity towards the dysplastic cell line DOK, followed by the cancerous gingival cell line Ca9.22 and the buccal mucosal cell line TR146. Experiments were carried out at least three times with three replicates per experiment. Results show the mean ± SEM. BAMLET, bovine α-lactalbumin made lethal to tumour cells; DOK, dysplastic oral keratinocyte; FBS, foetal bovine serum.

Live microscopy imaging experiments show that BAMLET likely penetrates into the cells soon after administration (Fig. 3c). Cell death is visible within the first hour and total cellular death is completed within the 18 h visualisation period. Although the exact localization of BAMLET once inside the cell has not been determined multiple studies have shown the ability of BAMLET to cause multiple attacks on cellular organelles including the mitochondria, endoplasmic reticulum, proteasomes and lysosomes illustrating its multi-target capability (Messner *et al.*, 2012).

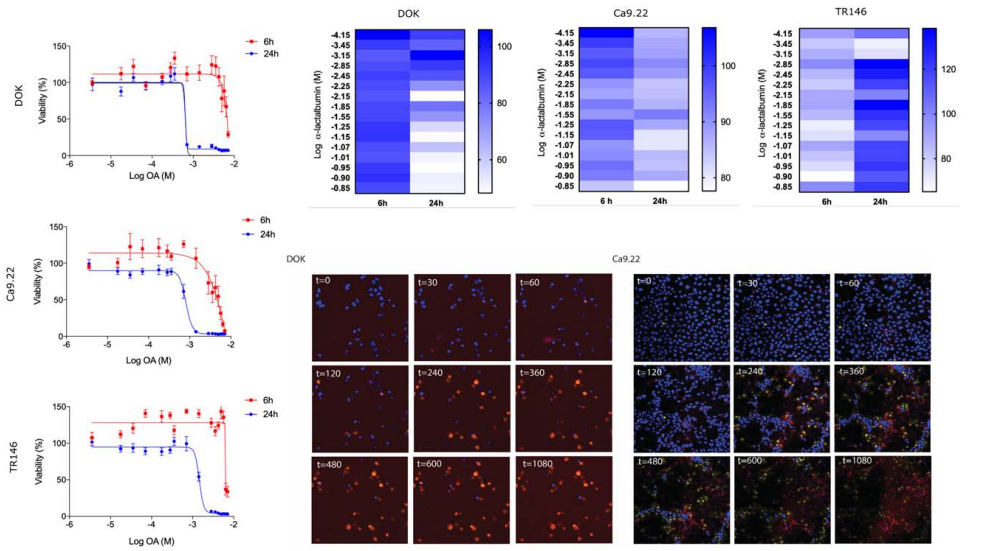
BAMLET induces cell cycle arrest in oral cancer cells

To determine if BAMLET induces changes in the cell cycle of OC cells, cell cycle analysis using flow cytometry was performed. The Ca9.22 cell line (Fig. 4a) showed a significant increase in the number of cells in the pre-G1 peak (~50%), following BAMLET treatment. In contrast, the TR146 cell line showed a differential response with under 20% of cells in the pre-G1 peak and ~40% in the G1 peak. These results show that BAMLET causes a halt in the G1 phase and subsequently causes decreased proliferation. The number of cells in the S and M phases are similar for both cell lines. These results correlate to the cytotoxicity profile of BAMLET/OA in these cell lines. Interestingly, the TR146 cell line shows a slower response in cytotoxicity but a significant halt in the cell cycle. The cell cycle arrest in the G1 phase in the TR146 cell line may be indicative of the functional p53 protein that is characteristic of this cell line in contrast to the Ca9.22 cell line that has a p53 mutation.

BAMLET sensitivity is not affected by functional p53 in oral cancer

The cell cycle is a tightly regulated mechanism with two checkpoints, at G1 point which allows transition into the S phase and at G2/M where cells complete repair mechanism and transition into the mitotic phase. In normal physiological conditions, irreparable DNA damage leads to apoptosis or senescence. p53 mediates part of the response to DNA damage either by stimulating DNA repair or beyond a certain threshold of DNA damage – by initiating apoptosis. Both p53 dependent and independent mechanisms are possible for the induction of apoptosis. To further characterize the effect of BAMLET in OC, the effect of functional p53 on BAMLET cytotoxicity in the TR146 cell line using small interfering RNA was examined.

Fig. 3



OA is the main cytotoxic component of BAMLET. (a) DOK oral dysplastic, CA9.22 and TR146 oral cancerous cells were treated with a range of OA concentrations in serum-free media for 6 or 24 h. Dose-response curves show a similar cytotoxic profile to that of BAMLET, although with higher IC_{50} concentrations. (b) Heat map showing the % inhibition of DOK, Ca9.22 and TR146 cells after α-LA treatment. The highest inhibition in cell proliferation was seen in the DOK cells with approximately a 40% reduction in cell viability. (c) Time-lapse microscopy images showing BAMLET off-loading post-treatment in the DOK and Ca9.22 cell lines. AlexaFlour 647 labelled BAMLET (shown in red), nuclei stained with Hoechst dye (shown in blue) and dead cells stained with propidium iodide (shown in yellow). Cell viability values represent the SEM of the replicate experiments. BAMLET, bovine α-lactalbumin made lethal to tumour cells; DOK, dysplastic oral keratinocyte; OA, oleic acid.

Cytotoxicity experiments demonstrated that the $p53^{WT}$ TR146 cell line displayed the lowest sensitivity to BAMLET treatment and showed a different distribution of cells in different stages of the cell cycle compared to the $p53^{M}$ Ca9.22 cell line. Results shown in Fig. 4b indicate the percent viability from the TR146 cell with and without the p53 knockdown after treatment with BAMLET (IC_{50} concentration) in full serum media (10% FBS). Our experiments show no significant difference in percent toxicity with 52.13 ± 4.2 and 62.72 ± 4.3 in the knockdown versus the functional p53 cells, respectively. These results show that BAMLET sensitivity is p53 independent, and thus a potential therapeutic intervention for both OC subtypes.

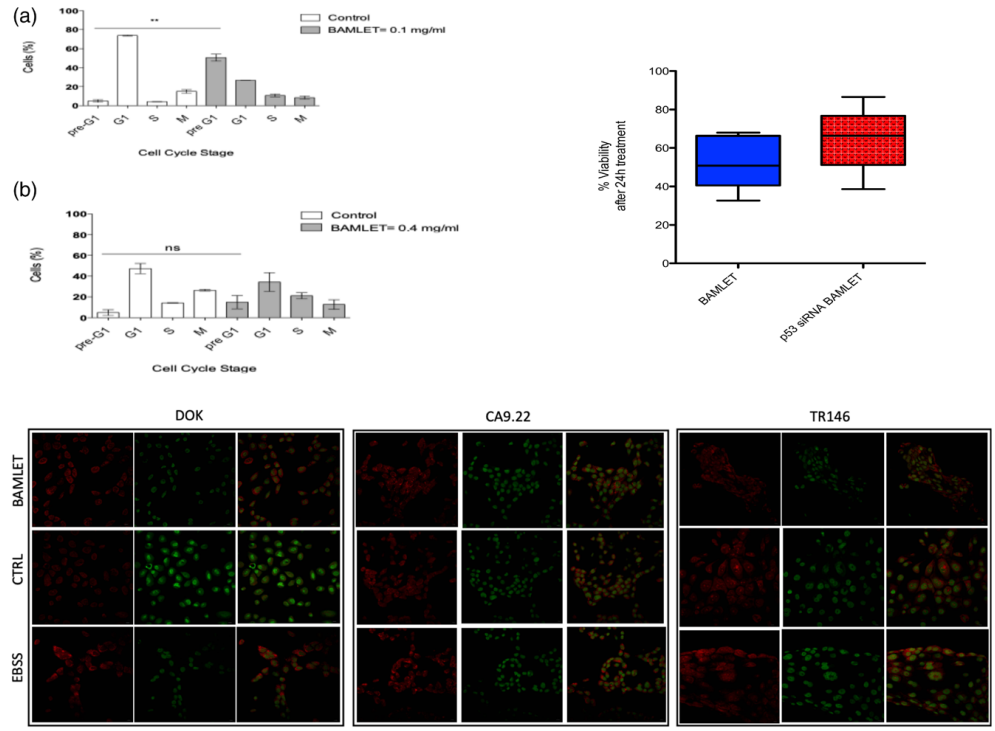
BAMLET increases lysosomal vesicles in dysplastic and oral cancerous cells

Macroautophagy or autophagy is a lysosomal catabolic pathway involved in cellular recycling of macromolecules and organelles. In physiological conditions, autophagy functions as a cytoprotective mechanisms but can be triggered in response to cellular stresses such as starvation, organelle/DNA damage, hypoxia, ER stress and pathogen infection. To investigate a possible autophagic response to BAMLET and OA treatment, the development of acidic vesicular organelles was monitored (Fig. 4c). Compared to the two cancerous cell lines (Ca9.22 and TR146), the DOK dysplastic cell line, showed the highest induction of lysosomal vesicles compared to controls. Treatment with OA alone resulted in a similar response in the induction of autophagic vesicles (data not shown). These results show that BAMLET induces lysosomal formation after BAMLET treatment and the magnitude of the response is cell type dependent. In accordance with our results Jiang *et al.* (2017) demonstrate that OA induces both apoptosis and autophagy in tongue squamous cell carcinoma.

BAMLET induces minimal necrosis in oral dysplastic and cancerous cells

Lastly, we investigated if BAMLET/OA induces necrotic type cell death. Necrosis is considered to occur in a less controlled fashion as a result of trauma or direct toxic injuries. Morphologically, necrosis is characterised by the swelling and rupture of intracellular organelles followed by rupture of the plasma membrane leading to

Fig. 4



BAMLET induces cell cycle arrest in a p53 independent manner. (a) Cell cycle distribution in the Ca9.22 and Tr146 cell lines after treatment with BAMLET for 24 h. Cells were analysed by flow cytometry for the percentage of cells in each stage of the cell cycle preG1/G1, S phase and M phase. (b) Cell viability in TR146 cells after transient p53 or sham control knockdown and treated with BAMLET for 24 h, showing no statistically significant difference suggesting p53 independent cytotoxicity. (c) Staining of acidic vesicles (red) in DOK, Ca9.22 and TR146 cells after BAMLET treatment (IC_{50}) for 24 h. Values represent the mean ± SEM of three independent experiments carried out in triplicate. BAMLET, bovine α-lactalbumin made lethal to tumour cells; DOK, dysplastic oral keratinocyte.

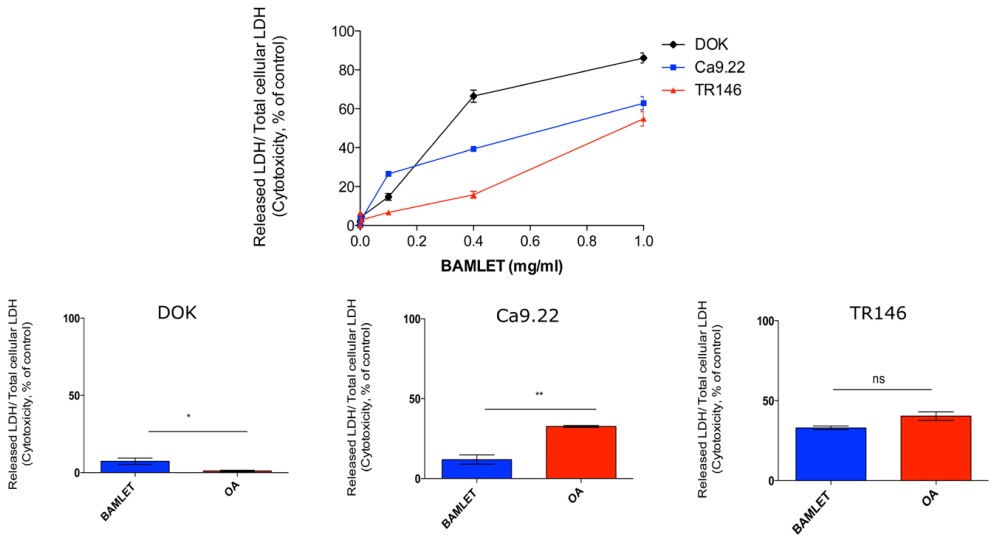
inflammatory responses. Rupturing of the plasma membrane results in the release of the enzyme LDH. The level of LDH released in response to different doses of BAMLET was determined and a linear correlation between BAMLET treatment and LDH release was observed (Fig. 5a). The induction of necrosis appeared to be time-dependent in all cell lines treated with BAMLET (data not shown). Interestingly, the dysplastic cell line showed the lowest induction of necrosis compared to the two cancerous cell lines in response to both BAMLET or OA (Fig. 5b). Taken together these results show that BAMLET and OA can induce an increase in released LDH and indicates that necrosis may be a complementary cytotoxic effect. Future studies are needed to determine if events specific for other forms of necrosis such as necroptosis mediate BAMLET/OA induced cell death.

Discussion

The importance of diet in the prevention of different pathological conditions has been confirmed in multiple studies. The ability of fatty acids to form cytotoxic complexes with soluble transport proteins such as α-LA, under specific conditions, exemplifies the beneficial role in physiological and pathological conditions. This study supports the paradigm of the beneficial effects of OA on the oral cavity, previously reported in a meta-analysis study by Psaltopoulou *et al.* (2011).

BAMLET has shown cytotoxic effects in over 50 cancer cell lines as well as in in-vivo mouse and rat experiments with non-toxic effects to healthy tissue (Rath *et al.*, 2015). Furthermore, phase I/II clinical studies in bladder cancer (NCT03560479) are showing promising clinical translatability. The results shown within

Fig. 5



Effect of BAMLET and OA on necrosis type cell death. (a) BAMLET induces dose-dependent necrosis in the DOK, Ca9.22 and TR146 cell lines. Cells were seeded in reduced medium overnight, followed by treatment with different concentrations of BAMLET in serum-free medium for 24 h. Data expressed as a percentage of released LDH to untreated control. Data shown n = 2 ± SEM. (b) Comparison of percent necrosis after 24 h treatment with BAMLET or OA at their respective IC_{50} concentrations in the DOK, Ca9.22 and TR146. Data shown n = 3 ± SEM and analysed by *t*-tests with ***$P < 0.001$. BAMLET, bovine α-lactalbumin made lethal to tumour cells; DOK, dysplastic oral keratinocyte; LDH, lactate dehydrogenase; OA, oleic acid.

demonstrate that dysplastic and OC may benefit from such a therapeutic approach. We show that the cytotoxic properties of BAMLET and subsequently OA, the active component, are time- and dose-dependent. The IC_{50} for BAMLET ranged from 0.05 to 0.4 mg/ml and 0.1 to 0.8 mg/ml when treated in SFM and full serum medium, respectively. The presence or absence of full serum has an effect on cytotoxicity due to the inherent ability of OA to precipitate with other proteins. OA on its own induces cell death although at higher IC_{50} concentrations.

We show that α-LA does not mediate cell death in the cancerous cell lines tested confirming findings by Fontana *et al.* (2013) and Delgado *et al.* (2015). Contrary, others have shown that α-LA can induce cytotoxicity in a number of cell lines (Hoque *et al.*, 2013) thus suggesting its effect is cell type-dependent. We found a higher sensitivity to α-LA in the dysplastic cell line, effect which may explain the lower sensitivity seen with BAMLET and OA.

Corroborating other studies, these results indicate that α-LA acts as a transport vehicle for the fatty acid to the cell membrane. This is likely to facilitate availability of OA in higher concentrations and subsequently causing increased toxicity. Furthermore, it has been demonstrated that there is a direct correlation between toxicity and the fatty acid concentration in the protein complex (Permyakov *et al.*, 2012).

On the basis of the above knowledge and in accordance with others, we hypothesize that BAMLET mimics the transport of fatty acids in normal physiology but where the effector carrier is albumin and the effect is cellular death rather than supplying energy demand (Van Der Vusse, 2009). It remains to be elucidated if the apparent sensitivity to BAMLET/OA is or it is not related to the energy demands of the respective cells.

Cell cycle analysis revealed accumulation of cells in the pre-G1/G1 peak indicative of decreased cellular proliferation possibly leading to apoptosis. The role of p53 in cell cycle regulation has been well documented and reviewed by Shaw (1996). The phenomena of different sensitivities exhibited by different cell lines with respect to the p53 status of the cell has been previously reported with both positive and negative correlation shown.

We show that the difference in the distribution of the cell cycle was not related to the functional status of p53 protein in the TR146 cell line, compared to the Ca9.22 cell

line that has a mutated p53 gain-of-function (R248W) (Kaneda *et al*., 2006). These results are in line with previously reported studies showing that BAMLET cytotoxicity is independent of p53 (Gustafsson *et al*., 2005; Hallgren *et al*., 2006).

It has been suggested that sensitivity may be influenced by other factors other than p53 mutation such as through the expression of p63 and p73 (members of the same family), as well as the drug and cell type under investigation (Kamiya and Ohshima, 2005). Further investigations are needed to determine cell cycle target proteins mediating BAMLET cytotoxicity. Importantly, these results show that BAMLET could be administered to all OC patients and thus a higher target population would benefit from its effect. This is in contrast to current chemotherapeutics in which p53 plays a significant role in chemoresistance.

The rapid induction of lysosomic vesicles in all cell lines tested is in agreement with previously published results of BAMLET and OA inducing an autophagy type response. We show that necrosis is a minor contributor to cell death in response to BAMLET and OA treatment, and may be related to the environmental changes/events taking place rather than as a direct effect of BAMLET or OA treatment.

BAMLET is an exciting new avenue for OC therapeutics with future animal/clinical studies needed to demonstrate its efficacy in the clinical context. For effective clinical translatability, maximum absorption and reduced complex dissociation should be considered. To achieve significant clinical efficacy, BAMLET may be administered topically with repeated dosing since it has been shown that BAMLET acts on cell membranes. Extended periods of administration of BAMLET are unlikely to result in toxicity to normal tissue as demonstrated by high dosing (of up to 1 mg/ml) in in-vivo animal experiments (Rath *et al*., 2018). Furthermore, the relative ease of large-scale production as well as the low cost of BAMLET production makes it an economically sound alternative.

In conclusion, BAMLET and OA induced cell death in dysplastic and cancerous oral cell lines with cell type-dependent sensitivity. Cytotoxicity was induced through the activation of multiple simultaneous cellular events, independent of the p53 protein. The use of BAMLET alone or combined with other chemotherapeutics to enhance sensitivity of chemotherapy may be a promising approach in OC treatment. Furthermore, these results indicate that a diet high in OA such as the Mediterranean diet may have protective effects on the oral mucosa.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

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Appendix 3

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Original Article

Alcohol consumption modulates *Candida albicans*-induced oral carcinogenesis and progression

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ABSTRACT

Objectives: This study aimed to determine the impact of low levels of alcohol consumption on the interaction of the oral cavity with *Candida albicans*, a species that is commonly found at higher levels in the oral cavities of regular alcohol consumers, patients with pre-malignant diseases, and patients with existing oral cancer (OC).
Methods: The gingival squamous cell carcinoma cell line, Ca9-22, was subjected to low-level ethanol exposure before co-culture with heat-inactivated *C. albicans* (HICA). We performed cell viability assays, measured reactive oxygen species, and used Western blot analysis for cell death markers to examine the effect of ethanol and HICA on cells. Scratch assays and anchorage-independent growth assays were used to determine cell behavioral changes.
Results: The results showed that ethanol in combination with HICA exacerbated cell death and cell cycle disruption, delayed NF-κB signaling, increased TIMP-2 secretion, and subsequently decreased MMP-2 secretion when compared to exposure to HICA alone. Conversely, both ethanol and HICA independently increased proliferation of Ca9-22 cells in scratch assays, and in combination, increased their capacity for anchorage-independent growth.
Conclusion: Low levels of ethanol may provide protective effects against *Candida*-induced inflammatory oral carcinogenesis or OC progression.

1. Introduction

Oral cancer (OC) is the 17th most common cancer worldwide [1]. Early carcinomas tend to go unnoticed due to their asymptomatic nature, and approximately two thirds of OC are diagnosed at stage III and IV [2]. Survival rates of early-stage OC are around 85% whereas advanced stage disease has survival rates of as low as 40% [3].

Alcohol is one of many risk factors associated with OC [4–6]. Alcohol can greatly affect the integrity of oral mucosa - providing enhanced penetration of carcinogens, as seen in the concurrent consumption of alcohol and tobacco, or enhanced invasion of bacteria across the oral epithelia [7–9]. Although short-term alcohol exposure increases membrane fluidity, chronic alcohol exposure has been shown to increase rigidity of the membrane [10]. Changes to membrane characteristics have the potential to alter intracellular signalling, including inflammatory mediators [11].

The tumour microenvironment is largely influenced by inflammatory cells and inflammation is a fundamental factor in neoplastic processes [12]. Alcohol consumption can have immunosuppressive effects, affecting the sensitivity of pathogen recognition receptors (PRRs) to bacterial ligands depending on the length of exposure time. For example, acute alcohol exposure in monocytes induced a decreased response to LPS, whereas chronic alcohol exposure increased sensitivity, increasing the production of TNF-α [13].

Candida albicans is a ubiquitous, polymorphic fungus that is a normal part of the human commensal flora. However, it is an opportunistic pathogen, with the potential to become pathogenic when there is a disturbance in the balance of flora in the host. A significant positive correlation is reported between OC occurrence and oral yeast colonisation, with alcohol increasing risk association [14]. Oral cell's innate response to pathogenic *C. albicans* is modulated through NF-κB and MAPK, and initial immune response is due to recognition of fungal cell wall structures, such as β-glucan, which remain intact in heat-inactivated *C. albicans* (HICA) [15,16]. Inflammation and inhibition of apoptosis induced by *C. albicans* has been postulated to be a major cause of malignant transformation [17,18], as well as acting as a 'co-carcinogen' with other risk factors,

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such as alcohol [19]. HICA has been shown to induce increased cell migration, MMP activity and oncometabolite production of OC cells in the same way as live *C. albicans*, albeit to a lesser extent [20]. HICA is therefore a valid and efficient tool for studying the effects of *C. albicans*-associated oral carcinogenesis.

Alcohol has been shown to alter the composition of the oral microbiome, which could foster the transformation from 'normal' to disease state composition [21,22], facilitating OC progression. The exact mechanisms through which alcohol and the oral microbiome affect OC progression are not clear. The objectives of this study are to characterise *in vitro* co-culture models of OC cell line Ca9-22 with ethanol and HICA and to elucidate the cellular mechanisms underlying the concomitant roles of both in the progression of OC.

2. Materials & methods

All chemicals and reagents were obtained from Merck (Dublin, Ireland), unless otherwise stated.

2.1. Cell culture

Ca9-22 (RRID: CVCL_1102) oral squamous carcinoma cells were obtained from Health Science Research Resources Bank, Osaka, Japan. Cells were maintained in Minimum Essential Medium supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) L-Glutamine (200 mM) and 1% (v/v) penicillin/streptomycin (100 μg/mL).

2.2. Co-culture

C. albicans was cultured in brain heart infusion broth overnight in a shaker (200 RPM, 37 °C). Cultures were centrifuged (14,000 g, 1 min) and the pellet washed in PBS before determining the optical density (OD). At OD_{600nm} of 1, cell count was determined to be 1×10^8 cells/mL of *C. albicans*. Heat-inactivated (1 h, 65 °C) *C. albicans* (HICA) was added to Ca9-22 cells at a multiplicity of infection (MOI) of 10:1 for 24 h before/after treatment with ethanol (1% v/v, 24 h) without changing the media.

2.3. Cell viability

Following required treatment of cells, AlamarBlue reagent was added and incubated for 4 h (10% v/v). Absorbance was read on a Spectra MAX Plus Microplate reader (Molecular Devices, United Kingdom) at 570/600 nm.

2.4. Ethanol measurement

Ethanol concentration was determined using the Megazyme Ethanol Assay Kit™ (Neogen, Scotland, United Kingdom) as per the manufacturer's protocol. Absorbance was read on a Spectra MAX Plus Microplate reader at 340 nm.

2.5. Flow cytometry

For cell cycle analysis, cells were trypsinised, centrifuged (1200 RPM, 5 min), the supernatant discarded, and the pellets washed with PBS. Ice-cold ethanol (2 mL) was added and incubated for 20 min at 4 °C. Following centrifugation (1000 RPM, 5 min), pellets were resuspended in PBS, RNase (10 mg/mL) and propidium iodide (PI) (1 mg/mL). Samples were incubated in the dark (30 min, 37 °C), before excitation using a 488 nm laser on a BD FACs Canto™ II flow cytometer (Cytek Biosciences, Amsterdam, Netherlands). Fluorescence of PI was measured and used to differentiate cells in G_0-G_1, S, and G_2/M phase, as fluorescence intensity correlates to the amount of DNA present in the cell. Histograms were generated using FlowJo software (TreeStar, Version 10.9).

For analysis of apoptotic and necrotic cells, cell pellets were resuspended in ice-cold Annexin V binding buffer (Thermo Fisher Scientific, Dublin, Ireland) and centrifuged (600 g, 5 min at 4 °C). Pellets were resuspended in binding buffer, FITC Annexin V (Thermo Fisher Scientific, Dublin, Ireland) and PI (75 μM). Samples were incubated on ice for 15 min before excitation using a 488 nm laser on a BD Accuri C6 flow cytometer (Cytek Biosciences, Amsterdam, Netherlands). Data compensation was carried out using an unstained sample, an Annexin V-only stained sample, and a PI-only stained sample. Live cells were gated as Annexin V/PI −/−, early apoptotic cells Annexin V/PI +/−, late apoptotic cells Annexin V/PI +/+ and necrotic cells Annexin V/PI −/+ using FlowJo software.

2.6. Western blot analysis

Cells were lysed in RIPA buffer. Equal protein concentrations were combined with Laemmli buffer (Bio-Rad, Kildare, Ireland) (65 °C, 15 min) before separation by 12% SDS-PAGE and transfer to a PVDF membrane. Membranes were blocked in 5% milk powder in TBST then incubated overnight in the following primary antibodies at 4 °C - IkB-α, (Brennan & Co, Dublin, Ireland, 44D4), β-actin (Merck, Dublin, Ireland, A5441), PARP (Abcam, Cambridge, United Kingdom, ab32138), Caspase p17 (Abcam, Cambridge, United Kingdom, ab2302) and E-cadherin (Brennan & Co, Dublin, Ireland, 3195T). Membranes were incubated with secondary antibodies for 2 h at RT (Anti-rabbit 4812S, Anti-mouse 7076S, Brennan & Co, Dublin, Ireland). Antibody-labelled proteins were visualised using Enhanced Chemiluminescence HRP substrate. The signal was detected using a Chemi-Luminescent gel documentation system (Bio-Rad, Kildare, Ireland) using ImageLab software (Version 6).

2.7. Reactive oxygen species measurement

To determine levels of ROS, cells were seeded in a black 96-well plate. Dichlorodihydro fluorescein diacetate (DCF-DA) (Thermo Fisher Scientific, Dublin, Ireland) was added to each well (20 μM) and incubated for 30 min in the dark. Fluorescence was read on a Spectra MAX Plus Microplate plate reader at Ex 490 nm and Em 529 nm.

2.8. Cytokine detection

Inflammatory cytokines were detected using the Human Inflammation Array C1 (RayBiotech®, Generon, Dublin, Ireland) as per the manufacturer's protocol, using cell culture supernatants. Membranes were imaged on a Chemi-Luminescent gel documentation system (Bio-Rad, Kildare, Ireland).

2.9. Scratch assay

A polylactic acid 3D-printed mould was designed and used to create a uniform zone of exclusion in the cell monolayer (Fig. A). Cells were seeded onto a 24-well plate with the mould. At 100% confluency, the mould was removed, cells washed with PBS and treated as required. An IncuCyte™ S3 (Sartorius BioAnalytics, Göttingen, Germany) was used to obtain images of the scratches at 2 h intervals over a 48 h period. Rate of gap closure was measured using ImageJ software (Version 1.53) and GraphPad Prism (Version 10).

2.10. Anchorage-independent growth assay

Tissue culture plates were coated with poly-hydroxyethyl methacrylic acid (p-HEMA) (12 mg/mL) in 95% ethanol and allowed to air dry overnight, before this coating procedure was repeated. Cells were seeded onto the plates, treated as required and incubated for 24 h. 4 h prior to endpoint, AlamarBlue (10% v/v) was added to each well. Fluorescence was read on a Spectra MAX Plus Microplate reader at Ex 530 nm and Em 590 nm.

2.11. Human MMP-2 ELISA

Secretion of matrix metalloproteinase-2 was detected using Human MMP-2 ELISA kit (RayBiotech®, Generon, Dublin, Ireland) as per the manufacturer's protocol. Absorbance was read using a Spectra MAX Plus Microplate reader at 450 nm. The concentration of MMP-2 in each sample was calculated using a standard curve generated on GraphPad Prism (Version 10).

3. Results

3.1. EtOH affects Ca9-22 cell viability in a time and dose dependent manner

Ca9-22 cells were treated with a range of concentrations of EtOH (1–5% v/v) for 4, 24 or 48 h and their viability assessed. In addition, cell culture media was assayed for EtOH at various time points. Cell viability decreased in a time and dose-dependent manner with ethanol exposure (Fig. 1A). IC_{50} values for EtOH treatments significantly decreased from 4 h to 48 h (Fig. 1B and C). When ethanol was added to flasks with cell culture media only, there was a significant loss of ethanol by 24 h compared to initial concentration (****, $P < 0.0001$, Fig. 1D). When ethanol was assayed from culture flasks containing Ca9-22 cells, a similar result was seen (Fig. 1E), confirming that Ca9-22 cells did not contribute significantly to ethanol clearance from media. Ca9-22 cells were stained for apoptotic markers following 24 h ethanol exposure, and the percentage of live cells significantly decreased at concentrations 3–5%, while early and late apoptotic cells also significantly increased. No significant changes were observed at 1% EtOH, therefore this concentration was used for future experiments (Fig. 1F).

3.2. Co-culture with HICA prior to ethanol treatment does not influence cell proliferation, cell cycle or cell death

Ca9-22 cells were co-cultured with heat-inactivated *C. albicans* (HICA) at a multiplicity of infection (MOI) of 10:1 for 24 h before treatment with ethanol. While ethanol concentrations $\geq$ 3% increased cell cycle arrest, prior co-culture with HICA did not exacerbate this effect (Fig. 2A). Similarly, ethanol increased cell death via apoptosis but the effect of prior co-culture with HICA had no significance on cell death, either alone or in combination (Fig. 2B).

An increase in cleaved PARP was observed in the presence of ethanol (1% and 3%), while cleaved caspase-3 was only detectable at ethanol 3% (Fig. 2C). Densitometric analysis demonstrated that prior co-culture with HICA did not significantly affect levels of apoptotic proteins detected compared to ethanol alone (Fig. 2D).

3.3. Ethanol sensitises Ca9-22 cells to HICA, increasing cell cycle arrest and cell death

Cells were treated with EtOH for 24 h before co-culturing with HICA (MOI 10) for a further 24 h. When cells were co-cultured with HICA following ethanol treatment, a significant increase in Sub G0 cells and decrease in G0/G1 phase cells was observed compared to ethanol alone (Fig. 3A). Neither treatment with EtOH, or combination of EtOH with HICA, affected cells in the S or G2/M phase.

EtOH significantly increased the percentage of cells undergoing early apoptosis. At 1% ethanol, the addition of HICA did not affect cellular apoptosis. However, addition of HICA significantly increased early apoptotic cells and decreased live cells compared to 3% ethanol treatment alone. Pre-treatment with higher concentrations of ethanol sensitised Ca9-22 cells to the effects of co-culture with HICA (Fig. 3A and B).

Despite detectable apoptotic cells via flow cytometry, when Western blot was used to detect apoptotic proteins in ethanol pre-treated cells, no detectable levels of either cleaved PARP or cleaved caspase-3 were observed under any conditions (Fig. 3C and D). While co-culture with HICA following ethanol exposure appeared to increase cell cycle arrest and early apoptotic events, this was not detectable via caspase-3 or PARP cleavage.

3.4. Ethanol delays and dampens immune response of Ca9-22 cells

The production of ROS by Ca9-22 cells following ethanol and HICA treatment was assessed using the DCF-DA assay. Under the same conditions as previous experiments, ROS production was not affected in Ca9-22 cells (Fig. 4A and B).

Activation of NF-κB was measured by monitoring the degradation of the inhibitory molecule IκB-α. Ca9-22 cells were treated with bacterial LPS as a positive control (200 ng/mL), where degradation of IκB-α occurred in the first 10 min and gradually recovered over 60 min. Co-culture with HICA elicited a similar response to LPS, showing low-level activation of NF-κB (Fig. 4C). When Ca9-22 cells were treated with EtOH for 24 h prior to HICA exposure, the degradation of IκB-α was a much shorter-lived and weaker response. The degradation of IκB-α reached the same magnitude as LPS or HICA alone only at 20–30 min (Fig. 4D). At 60 min, the recovery of IκB-α was significantly higher in cells treated with EtOH compared to HICA alone, suggesting that EtOH shortens the duration of immune response of Ca9-22 cells elicited by HICA (Fig. 4E).

3.5. Ethanol increases TIMP-2 expression and decreases MMP-2 secretion from Ca9-22 cells

Using a multi-protein cytokine array, no significant differences were detected in inflammatory cytokines associated with *C. albicans* infection (IFN-γ, IL-1α, IL-1β, IL-6, IL-7 and IL-8) following exposure to HICA or ethanol. The expression of the anti-inflammatory cytokine IL-10 was downregulated following exposure to LPS, HICA, ethanol, and ethanol with HICA. Significantly, Ca9-22 cells treated with ethanol (+/-HICA), showed increased expression of tissue inhibitor of metalloproteinase-2 (TIMP-2) compared to untreated cells (Fig. 5B).

The corresponding matrix metalloproteinase-2 (MMP-2) to TIMP-2 was detected using an ELISA kit. The levels of MMP-2 from Ca9-22 cells were lower than the recommended minimal detectable dose of the kit. However, when cells were treated with ethanol, with or without HICA, MMP-2 secretion was completely ablated. This coincided with the significant increase in TIMP-2 expression as a result of ethanol exposure. HICA alone increased MMP-2 secretion, but this effect was suppressed by ethanol treatment (Fig. 5C).

3.6. Ethanol and HICA increase rate of scratch closure and anchorage-independent growth of Ca9-22 cells

Scratch assays were used to evaluate migratory capacities of Ca9-22 cells. HICA (MOI 20), ethanol alone and a combination increased the rate of wound closure in Ca9-22 cells (Fig. 6A and B), but with no significance between treatments.

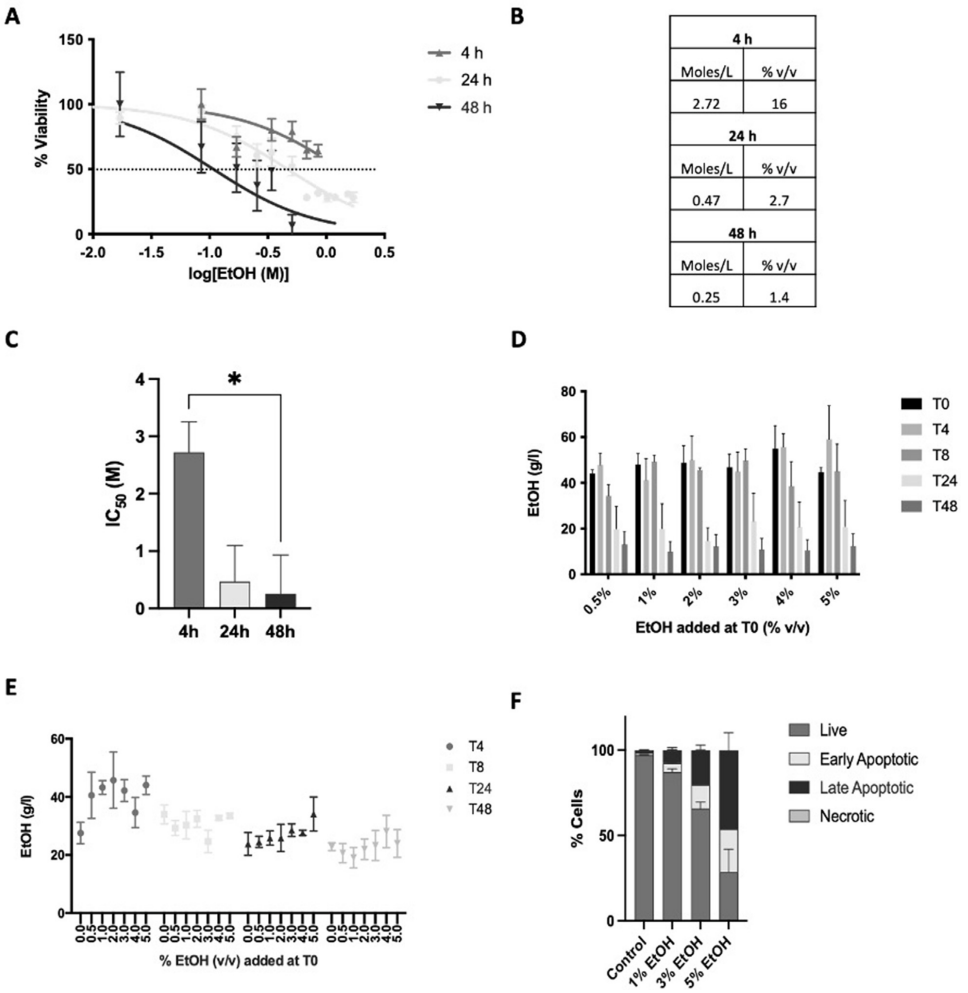

Fig. 1. **(A)** Ca9-22 cells were treated with ethanol (1–5% v/v) and viability tested from 4 to 48 h. **(B)** IC_{50} values for ethanol treatments were extrapolated from graphs in **(A)**. **(C)** A significant decrease in IC_{50} was seen from 4 h to 48 h. **(D)** Ethanol was assayed from cell culture media alone or **(E)** from cultured Ca9-22 cells and a significant decrease in ethanol content was observed in the initial 24 h. **(F)** Ca9-22 cells showed a significant increase in apoptosis at ethanol concentrations $\geq$ 3% v/v over 24 h.

Neither HICA or EtOH alone affected anchorage-independent growth of Ca9-22 cells, but the combination of both significantly increased this capacity of the cells (**P < 0.01, Fig. 6C). In contrast to the scratch assays, EtOH and HICA appeared to work together to increase the capacity of Ca9-22 cells to grow anchorage-free.

The expression of E-cadherin, which is typically regulated in accordance with migration of cells, remained unchanged in Ca9-22 cells treated with ethanol, HICA or in combination (Fig. 6D and E).

4. Discussion

Initial assays established how long cells can be exposed to ethanol *in vitro* (Fig. 1D and E). Some published studies employ protocols to maintain constant alcohol content in cell culture plates via sealed containers and ethanol reservoir chambers [23,24]. However, *in vivo* alcohol exposure is transient as it is ingested and metabolised [25]. Salivary alcohol concentration dissipates 30 min following consumption, so a lower concentration of ethanol with increased exposure time can be taken as representative of regular alcohol consumers.

Alcohol consumption can influence composition of microbiomes in the body; for example, heavy drinkers experience a higher oral carriage of *Candida* [26,27]. When cells were co-cultured with HICA prior to ethanol treatment (a representation of a typical oral cavity colonised by *Candida* that is exposed to alcohol), the rate of cell death was not affected, most likely due to

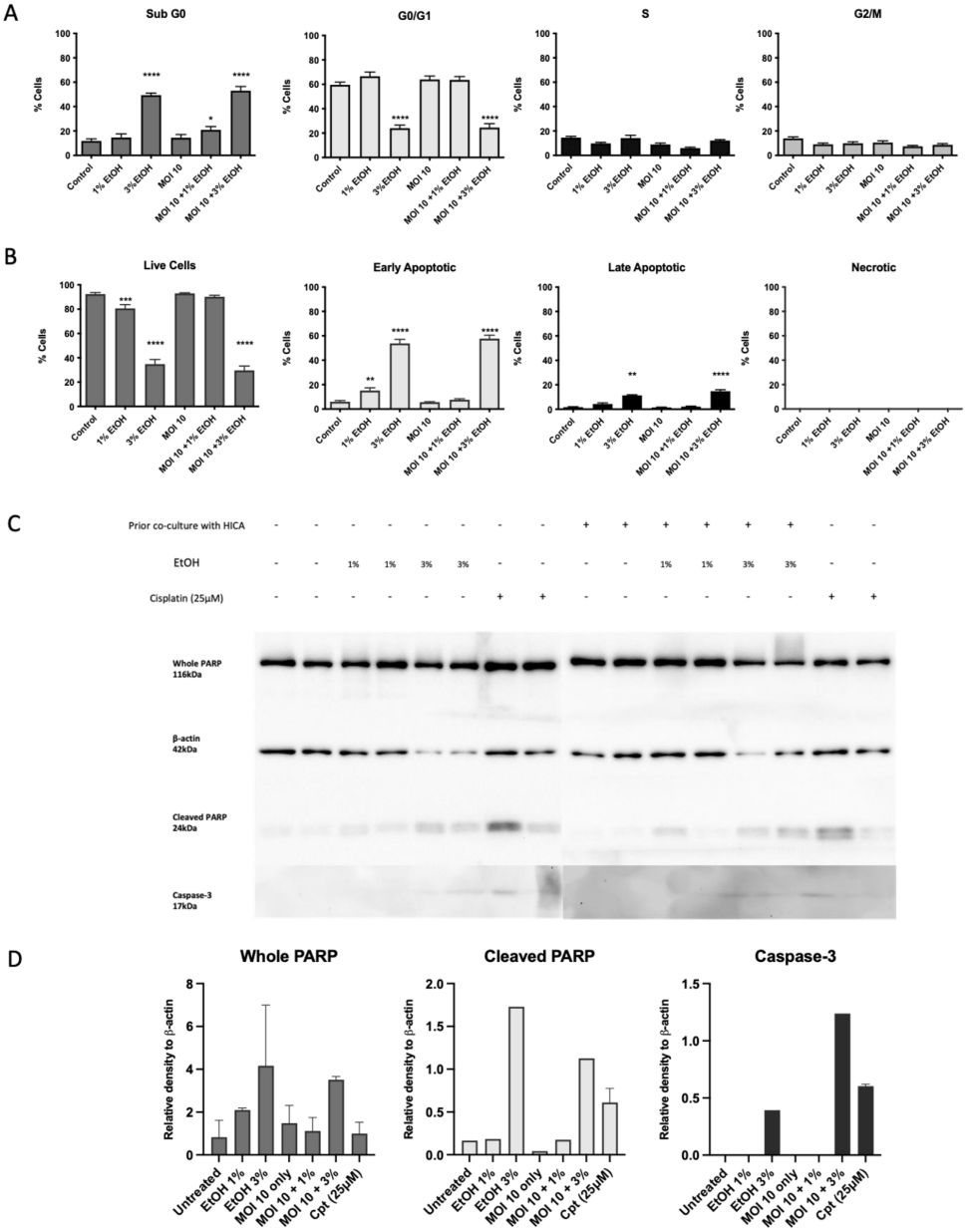

Fig. 2. Ca9-22 cells co-cultured with HICA (MOI 10) for 24 h before treatment with EtOH (1% and 3% v/v) for a further 24 h. **(A)** Ethanol ≥ 3% v/v increased the percentage of cells in the Sub G0 phase and decreased cells in the G0/G1 phase. Prior co-culture with HICA did not influence cell cycle irrespective of ethanol concentration. **(B)**. Ethanol (1% and 3%) increased the rate of cell death by apoptosis. Prior co-culture with HICA did not influence cell death. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns = not significant. Significance shown with respect to untreated control. **(C)** Western blot was used to analyse expression of apoptosis-related proteins. Cells were treated with a known apoptosis inducer (Cisplatin 25 μM) for 24 h as a positive control. Samples were blotted in duplicate, and data shown is a composite image representative of an experiment done in triplicate. Ethanol 1% and 3% increased cleaved PARP and cleaved caspase-3 in Ca9-22 cells; prior co-culture with HICA at MOI 10 did not influence this effect. **(D)** Densitometric analysis of apoptosis-related proteins. Relative density is shown in relation to loading control β-actin.

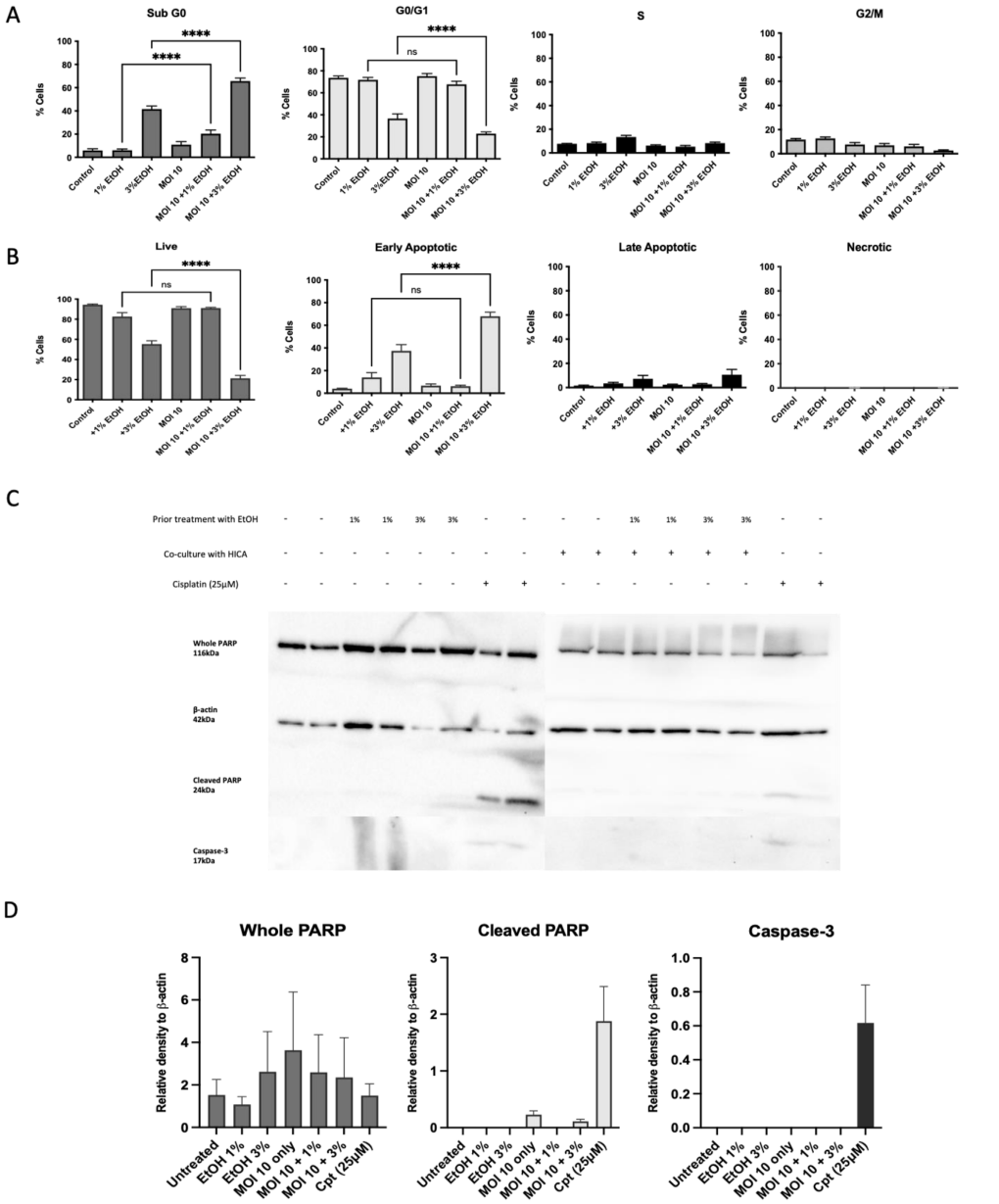

Fig. 3. Ca9-22 cells were treated with EtOH (1% and 3% v/v) for 24 h before co-culturing with HICA (MOI 10) for a further 24 h. **(A)** Ethanol (3% v/v) significantly increased cells in the Sub G0 and decreased cells in G0/G1 phase, with addition of HICA significantly increasing the shift in distribution. **(B)** Both 1% and 3% ethanol decreased live cells and increased early apoptotic cells, with the addition of HICA having no effect at 1% ethanol, but significantly increasing cell death observed at 3% ethanol. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns = not significant. **(C)** Ethanol followed by co-culture with HICA did not influence detection of apoptosis-related proteins by Western blot in Ca9-22 cells. Samples were blotted in duplicate, and data shown is a composite image representative of an experiment done in triplicate. **(D)** Densitometric analysis of apoptosis-related proteins. Relative density is shown with relation to loading control β-actin.

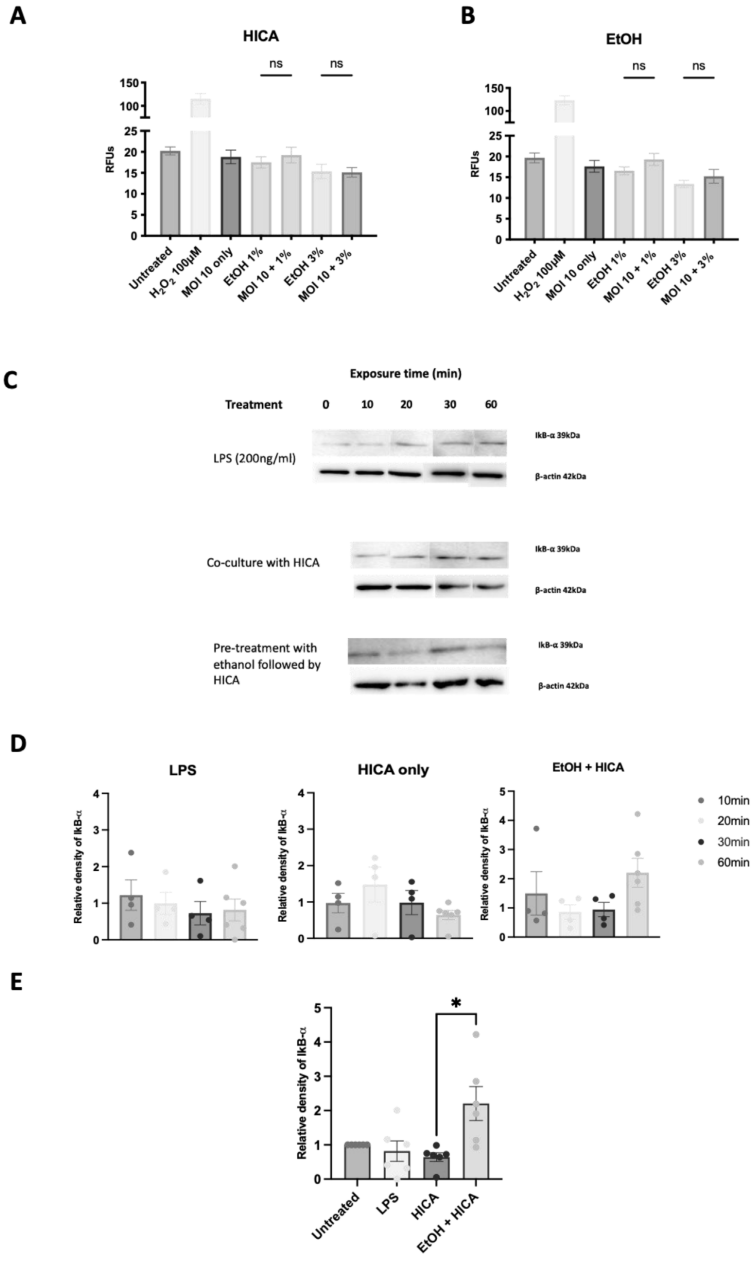

Fig. 4. **(A)** Ca9-22 cells were co-cultured with HICA 24 h before being treated with EtOH (1% and 3% v/v) for a further 24 h or the reverse, **(B)** whereby they were treated with EtOH prior to co-culture. Cells were then stained with DCF-DA before reading fluorescence on a Spectra MAX Plus Microplate reader and expressed as relative fluorescent units (RFUs). Data shown n = 3, mean ± SEM, ns = not significant. **(C)** Western blot was used to analyse expression of IκB-α in Ca9-22 cells co-cultured with HICA either alone or following ethanol exposure. LPS was used as a positive control and β-actin was used as a loading control. Data shown is a composite image representative of an experiment done four times. **(D)** Densitometric analysis of Western blot bands of IκB-α relative to β-actin and normalised to the untreated control. **(E)** At the 60 min timepoint, there was a significant difference in recovery of IκB-α detected in Ca9-22 cells treated with either HICA alone or in combination with EtOH.

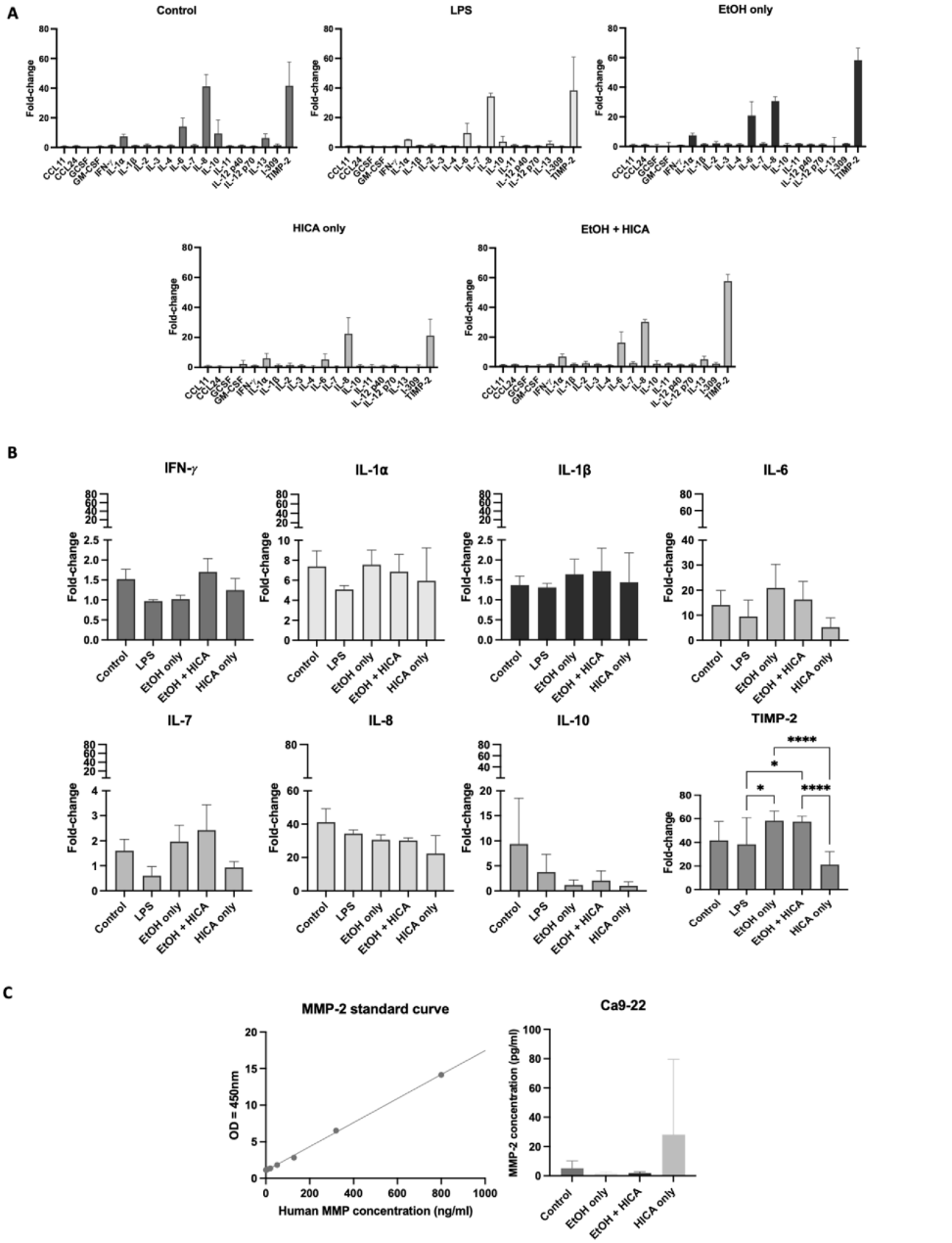

Fig. 5. **(A)** Multi-protein cytokine array was used to analyse expression of 20 human cytokines following treatment of Ca9-22 cells with HICA alone or in combination with EtOH. **(B)** Cytokines of interest in the inflammatory pathways activated by *C. albicans*. No significant differences were observed in inflammatory cytokines IFN-γ, IL-1α, IL-1β, IL-6, IL-7, or IL-8. Anti-inflammatory cytokine IL-10 was downregulated in Ca9-22 cells following addition of LPS, HICA or EtOH + HICA. Ethanol significantly increased expression of TIMP-2 in Ca9-22 cells compared to LPS or HICA only treatment. **(C)** Human MMP-2 ELISA kit was used to determine secretion of MMP-2 from Ca9-22 cells. Ethanol ablated MMP-2 secretion under all conditions whereas HICA alone appeared to increase MMP-2 secretion.

the lack of a concerted immune response given the absence of the required immune cells and subsequent inflammatory mediators which would adversely affect the cell line utilised in this study (Fig. 2). However, when the cells were treated with ethanol prior to co-culture with HICA (replicating increased *Candida* colonisation following alcohol consumption), Ca9-22 cells undergo a significant increase in early apoptosis (Fig. 3). Despite the increase in cells staining positive for early apoptosis, the detection of cleaved caspase-3 (an early apoptotic protein) was not observed in EtOH pre-treated cells. While caspase-independent death is possible, it

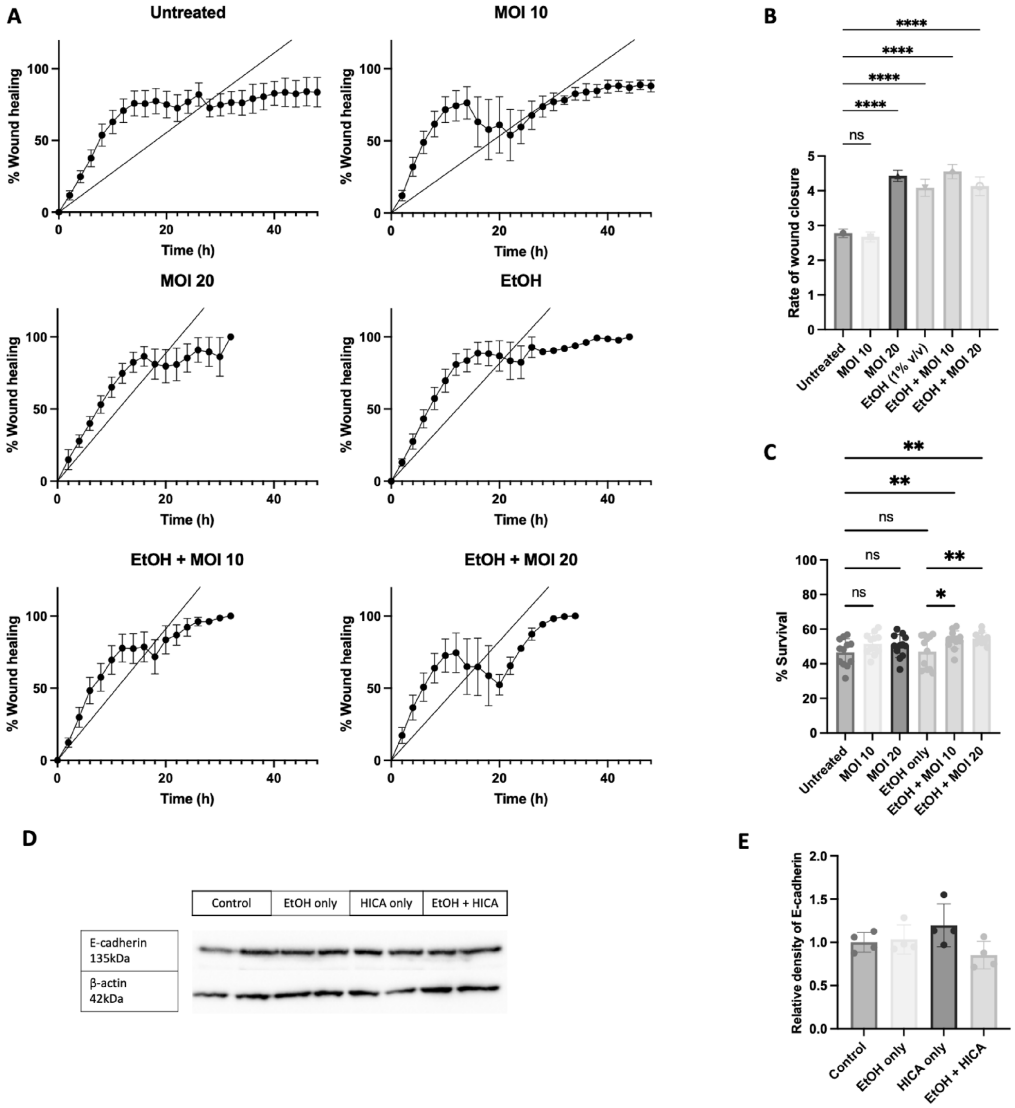

Fig. 6. **(A)** The effect of EtOH and HICA on Ca9-22 cell rate of wound closure in scratch assays. **(B)** The rate of wound closure obtained from slopes of graph **(A)** were plotted and analysed. Cells treated with a higher MOI of 20 of HICA, EtOH alone or a combination of both proliferated significantly faster compared to untreated control. **(C)** The combination of HICA and EtOH significantly increased anchorage-independent growth of Ca9-22 cells, compared to both untreated controls and ethanol alone. **(D)** E-cadherin was analysed using Western blot with β-actin as a loading control. **(E)** Densitometric analysis showed no significant changes to expression of E-cadherin in Ca9-22 cells with either EtOH, HICA or a combination. Data shown n = 4, mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns = not significant.

would not stain positively for Annexin V [28]. It is more likely that the levels of caspase-3 at timepoints used here were low and difficult to detect.

We show that neither ethanol, HICA or a combination of both affected ROS production by Ca9-22 cells (Fig. 4). Alcohol consumption and exposure to *C. albicans* are exogenous sources of ROS, which is posited to be a major factor in oral carcinogenesis [29,30]. Cancer cells generally have higher ROS production compared to normal tissues; therefore, it is possible that this level of ethanol exposure *in vitro* does not affect Ca9-22 cells the same way it would normal oral tissue. *C. albicans* can induce ROS production either indirectly via acetaldehyde from endogenous ADH enzymes or via inflammation. Since the yeast cells are heat-inactivated, the ADH activity would be attenuated. HICA did not induce ROS-production in Ca9-22 cells, in contrast with previous studies where HICA stimulates ROS production in oral keratinocytes and phagocytes [31,32]. ROS production in response to *C. albicans* is likely both cell-type and morphotype of *Candida*-dependent [33].

C. albicans can activate NF-κB signalling and increase downstream inflammatory cytokines (IL-6, IL-1β and TNF-α) [34]. Co-culture of primary oral leukoplakia keratinocytes with *C. albicans* showed increased inflammation via NF-κB and inhibition of apoptosis, both of which are markers of transformation [17,18]. NF-κB activation can therefore be anti-apoptotic, promoting cellular growth and malignancy in tumours. Conversely, the effect of ethanol on immune responses is both exposure-length and cell-type dependent [13,35].

Ethanol was immunosuppressive in Ca9-22 cells, whereby activation of NF-κB by HICA was both delayed and dampened (Fig. 4). Maximal activation of NF-κB was slower in cells treated with ethanol and complete recovery of the inhibitory complex IκBα to control levels was observed 60 min following exposure to HICA. It is worth noting that while LPS led to a decrease in IκB-α in Ca9-22 cells, it was not significant with respect to the control. Previous studies used concentrations ranging from 1 to 20 μg/mL [36,37], compared to 200 ng/mL employed here. Higher concentrations of 5 μg/mL did not yield significant results either (data not shown). Nevertheless, since NF-κB signaling is typically anti-apoptotic, this attenuated signaling following ethanol exposure may be contributing to the increased levels of apoptosis in cells exposed to both ethanol and HICA.

Exposure of Ca9-22 cells to ethanol, HICA or a combination did not significantly affect the production of IFN-γ, IL-1α, IL-1β, IL-6, IL-7, or IL-8 (Fig. 5). Oral epithelial cells have been shown to be 'desensitised' to microbial ligands whereby stimulation with purified cell wall components activates NF-κB and MAPK/c-JUN signaling but does not activate cytokine production [38]. Similarly, for *C. albicans* to mount a complete immune response, hyphae-invasion is required. It was observed that anti-inflammatory cytokine IL-10 was downregulated following exposure to all stimulants and ethanol did not influence this effect, despite its delaying of NF-κB signalling.

The expression of TIMP-2 by Ca9-22 cells was significantly increased by ethanol. A high expression score of TIMPs/MMPs has been shown to be associated with LNM status, invasion and metastasis of carcinomas [39]. Levels of MMP expression have been shown to increase from normal tissue through dysplasia to oral carcinomas [40]. HICA was shown to increase MMP-2 secretion, whereas ethanol decreased MMP-2 secretion to below detectable levels. Although high expression of MMPs is associated with overall worse progression of OC, there are some controversial reports that correlate high TIMP expression with unfavourable prognosis in oesophageal cancer, despite TIMP proteins being MMP regulators [41,42].

Ethanol's effect on TIMP expression appears to be tissue-dependent, and the mechanism is unclear. For example, exposure of cardiac fibroblasts to ethanol increased expression of TIMP-1/-2, however, rats that were fed ethanol showed a decrease in TIMPs and also a reduction in MMP activity [43,44]. Low-dose ethanol exposure in rats was also shown to upregulate TIMP-1, decreasing pro-inflammatory cytokines [45]. There is evidence that TIMP-2 acts as both an inhibitor of MMPs and an activator of pro-MMPs, potentially explaining the discrepancies of the relationship between TIMP/MMP and malignant phenotype that is observed both *in vitro* and *in vivo* [46]. *Candida* has been shown to affect TIMP and MMP expression/activity in human cells. Exposure of oral cell line HSC-2 to *C. albicans* increased MMP activity [20]. In a human oral mucosa model, infection with *C. albicans* increased MMP-9 secretion with a parallel decrease in secretion of TIMP-2 [47]. The results of this study are in line with the literature – ethanol promotes an increase in TIMP-2 and a decrease in MMP-2 in Ca9-22 cells, compared to HICA only.

Scratch assays and anchorage-independent growth assays were employed to investigate migration and motility capacities of Ca9-22 cells. Interestingly, both ethanol and HICA caused an increase in the rate of scratch closure in Ca9-22 cells (Fig. 6). This effect was not additive, and there was no significant differences between treatments, suggesting that ethanol and HICA increased migration of Ca9-22 cells by independent mechanisms. However, it was observed that the combination of ethanol and HICA caused a significant increase in anchorage-independent growth (Fig. 6). This effect is likely due to modulation of several other MMPs not examined here by both ethanol and HICA. A possible explanation for ethanol increasing scratch closure rates but not promoting anchorage-independent growth is the use of acetate by cancer cells as bioenergetic fuel [48,49].

Decrease in E-cadherin is observed in EMT processes [20,50–52] and is indicative of a more metastatic phenotype. Despite the changes to scratch closure rates and anchorage-independent growth, expression of E-cadherin was unchanged by ethanol or HICA in Ca9-22 cells at the timepoints used (Fig. 6). This would suggest that early EMT processes are not occurring under these conditions.

The findings of this study suggest that ethanol reduces the risk of *C. albicans* associated oral carcinogenesis by increasing cell death via apoptosis, delaying NF-κB signalling and increasing TIMP-2 with a concurrent decrease in MMP-2 secretion. However, acetaldehyde exposure following ethanol consumption is posited to be a major player in oral carcinogenesis. It is important to note that oral microbiome species that possess ADH enzymes, including *C. albicans*, have been shown to contribute to potentially genotoxic levels of acetaldehyde in the oral cavity [53,54]. A limitation of this study is the use of HICA – conversion of ethanol to acetaldehyde would be limited to Ca9-22 cells capacity for this reaction and not aided by *C. albicans*. Therefore, any risk reduction effects of ethanol may be counter-acted *in vivo* by acetaldehyde production. This may be dependent on the composition of the individuals oral microbiome, i.e. the proportion of species with high ADH-activity within the oral cavity.

5. Conclusion

In conclusion, it is possible that low levels of alcohol consumption, i.e. acute exposure, has an overall protective effect in the oral cavity against *C. albicans* induced oral carcinogenesis. *Candida* induced oral carcinogenesis is posited to be largely due to increased inflammation in the oral cavity [20,55–57]. However, exposure to ethanol appears to reduce the risk of *C. albicans* associated oral

carcinogenesis by increasing cell death, delaying NF-κB signalling and increasing TIMP-2. Further research to determine how frequent exposure to low levels of alcohol, such as social drinking, affect the response of the oral cavity to commensal microorganisms may help to further elucidate the mechanisms of oral carcinogenesis.

Author contribution statement

Isabel O'Grady: Conceptualization, Methodology, Data collection and analysis, Interpretation, Writing.
Jeff O'Sullivan: Supervision, Review & Editing.

Ethical approval

Ethical approval was not required for this article.

Conflict of interest

The authors do not declare any conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.job.2023.10.002.

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