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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.

Author contribution statement

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

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, such as alcohol [19]. HICA has been shown to induce increased cell migration, MMP activity and oncometabolite production

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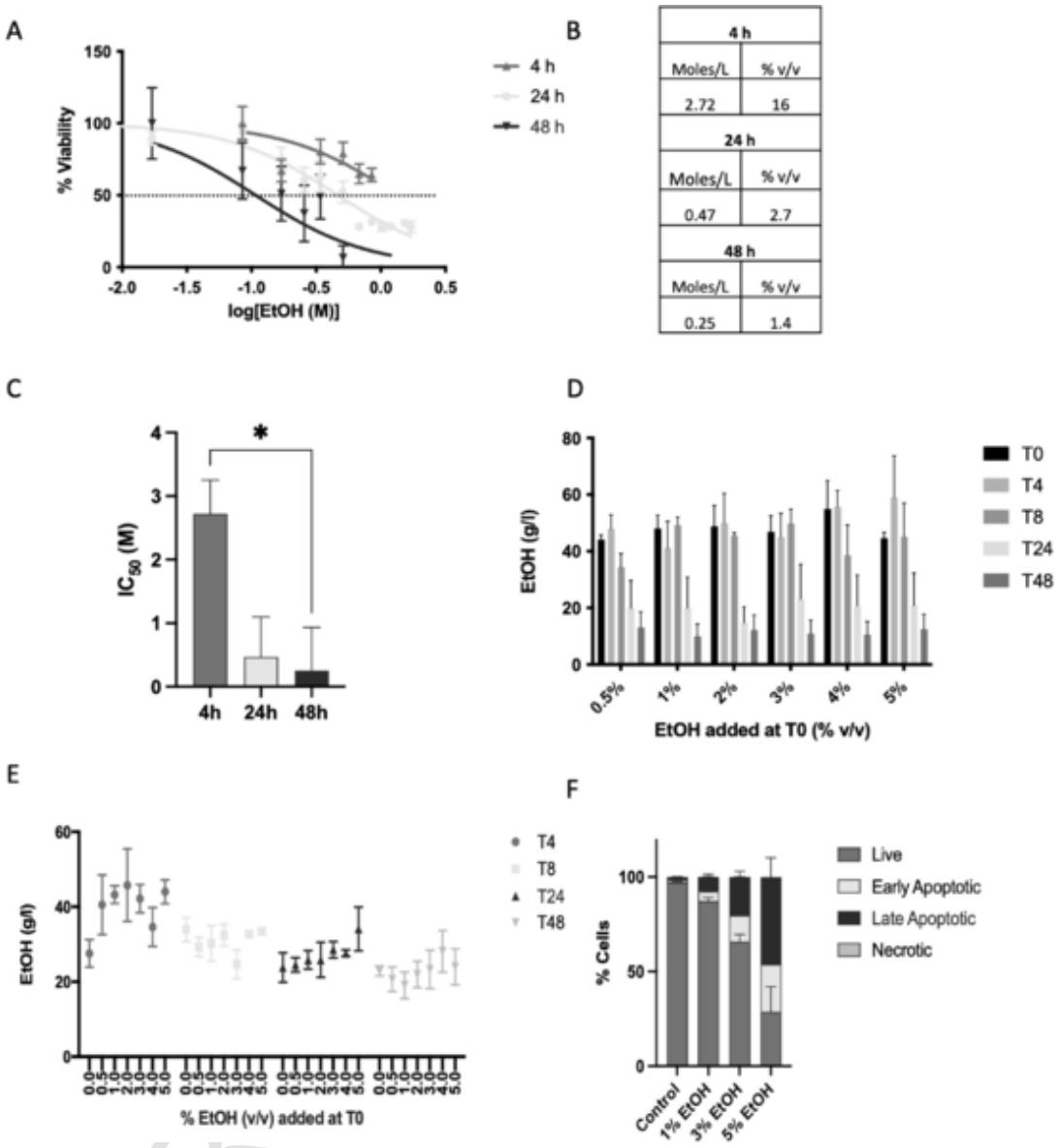

Fig. 1. (A) Ca9-22 cells were treated with ethanol (1–5% v/v) and viability tested from 4 to 48 h. **(B)** IC_{50} values for ethanol treatments were extrapolated from graphs in **(A)**. **(C)** A significant decrease in IC_{50} was seen from 4 h to 48 h. **(D)** Ethanol was assayed from cell culture 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 ≥ 3% v/v over 24 h.

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

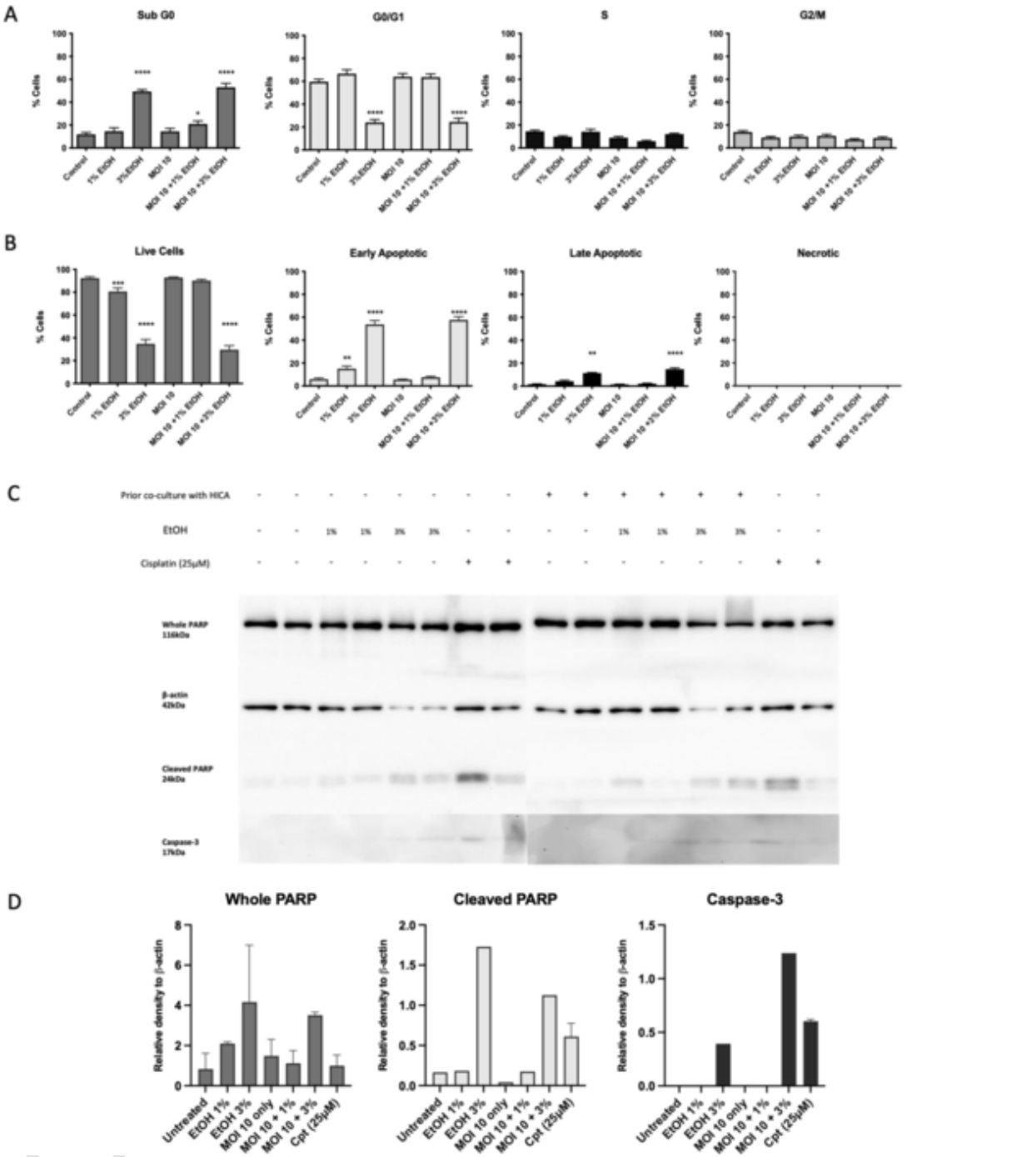

Fig. 2. Ca9-22 cells co-cultured with HICA (MOI 10) for 24 h before treatment with EtOH (1% and 3% v/v) for a further 24 h. **(A)** Ethanol ≥ 3% v/v increased the percentage of cells in the Sub G0 phase and decreased cells in the G0/G1 phase. Prior co-culture with HICA did not influence cell cycle irrespective 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.

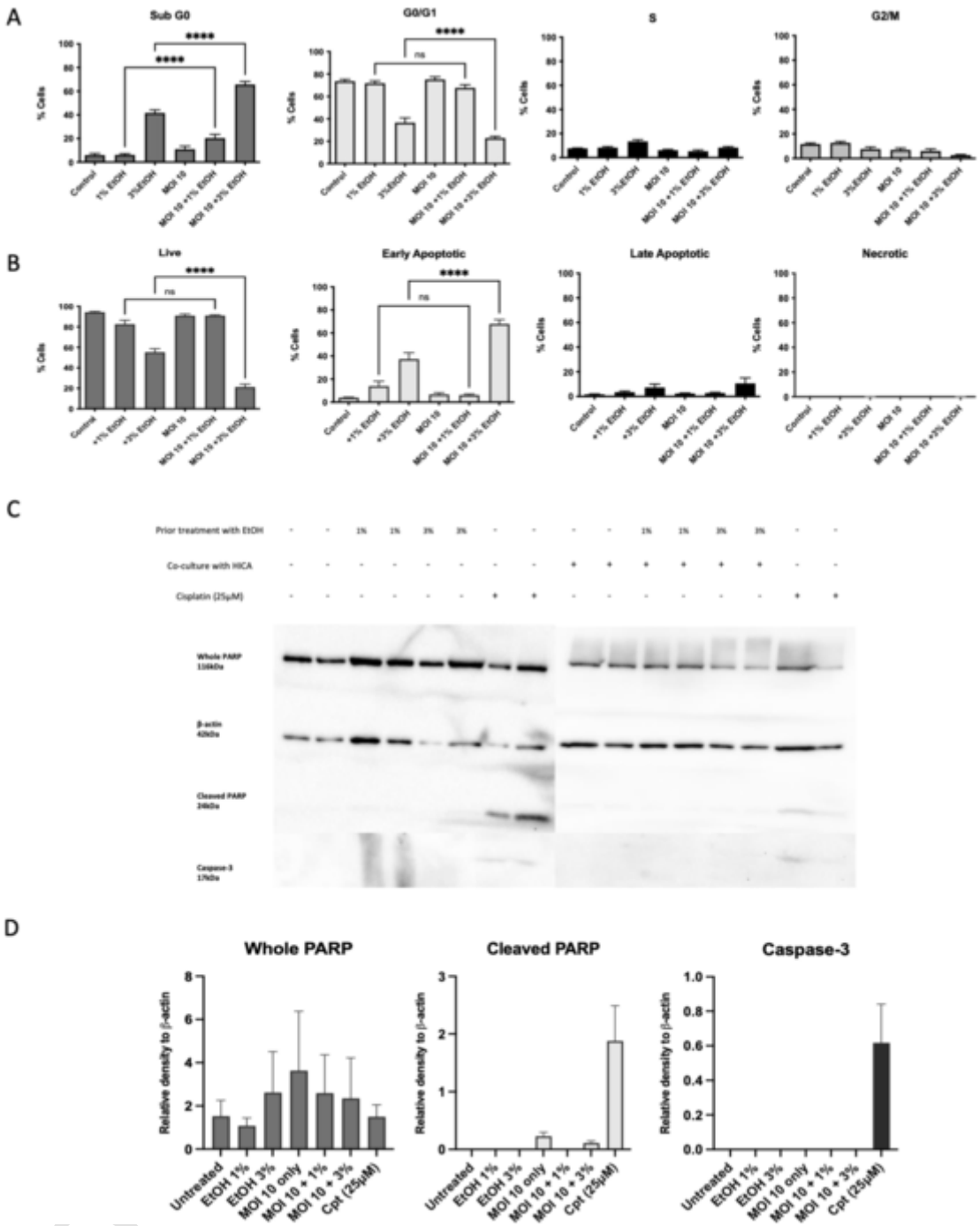

Fig. 3. Ca9-22 cells were treated with EtOH (1% and 3% v/v) for 24 h before co-culturing with HICA (MOI 10) for a further 24 h. **(A)** Ethanol (3% v/v) significantly increased cells in the Sub G0 and decreased cells in G0/G1 phase, with addition of HICA significantly increasing the shift in distribution. **(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.

(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.

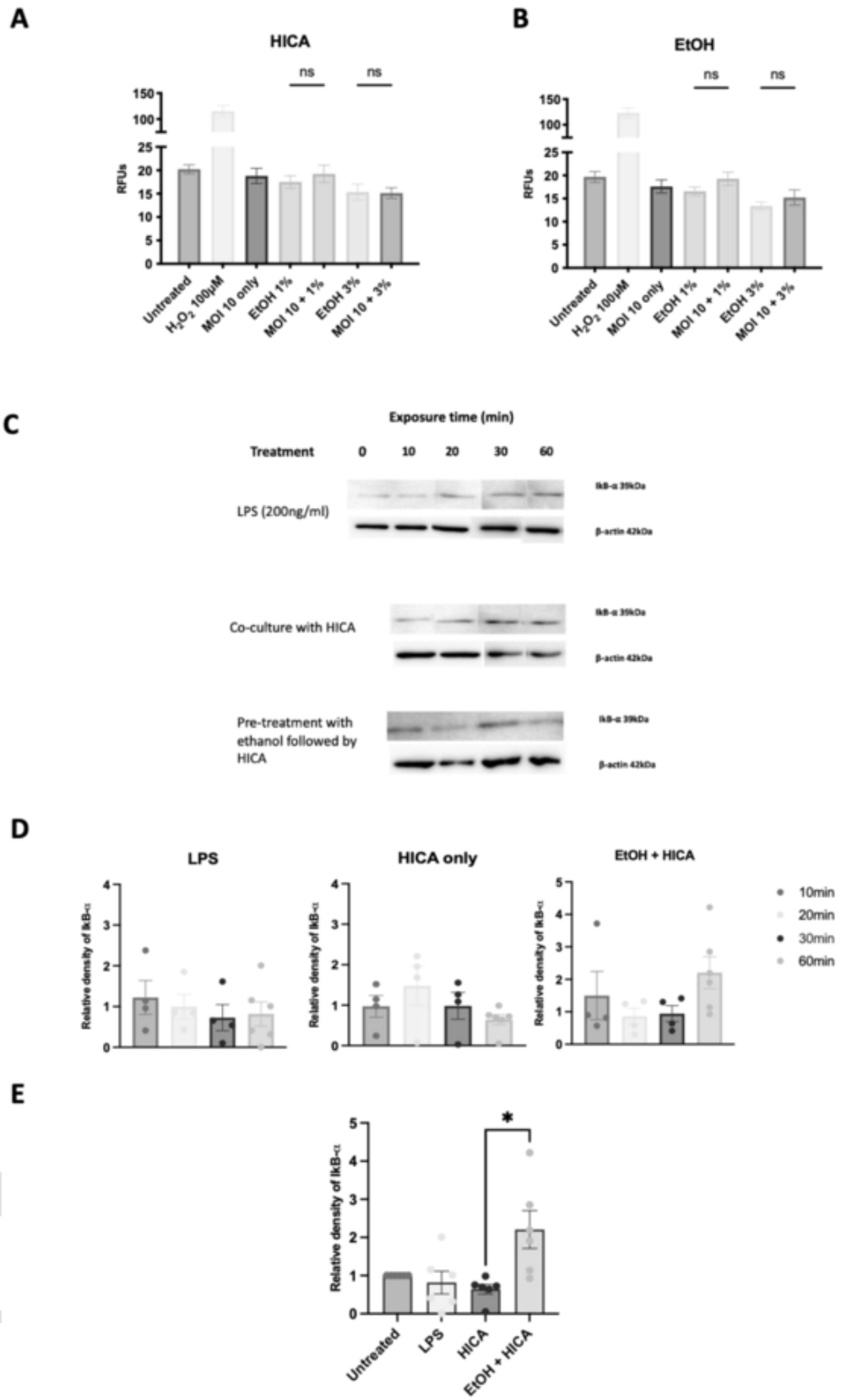

Fig. 4. (A) Ca9-22 cells were co-cultured with HICA 24 h before being treated with EtOH (1% and 3% v/v) for a further 24 h or the reverse, **(B)** whereby they were treated with EtOH prior to co-culture. Cells were then stained with DCF-DA before reading fluorescence on a Spectra MAX Plus Microplate 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 com-

Fig. 4.—*continued*
posite 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.

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 ≥ 3% increased cell cycle arrest, prior co-culture with HICA did not exacerbate this effect

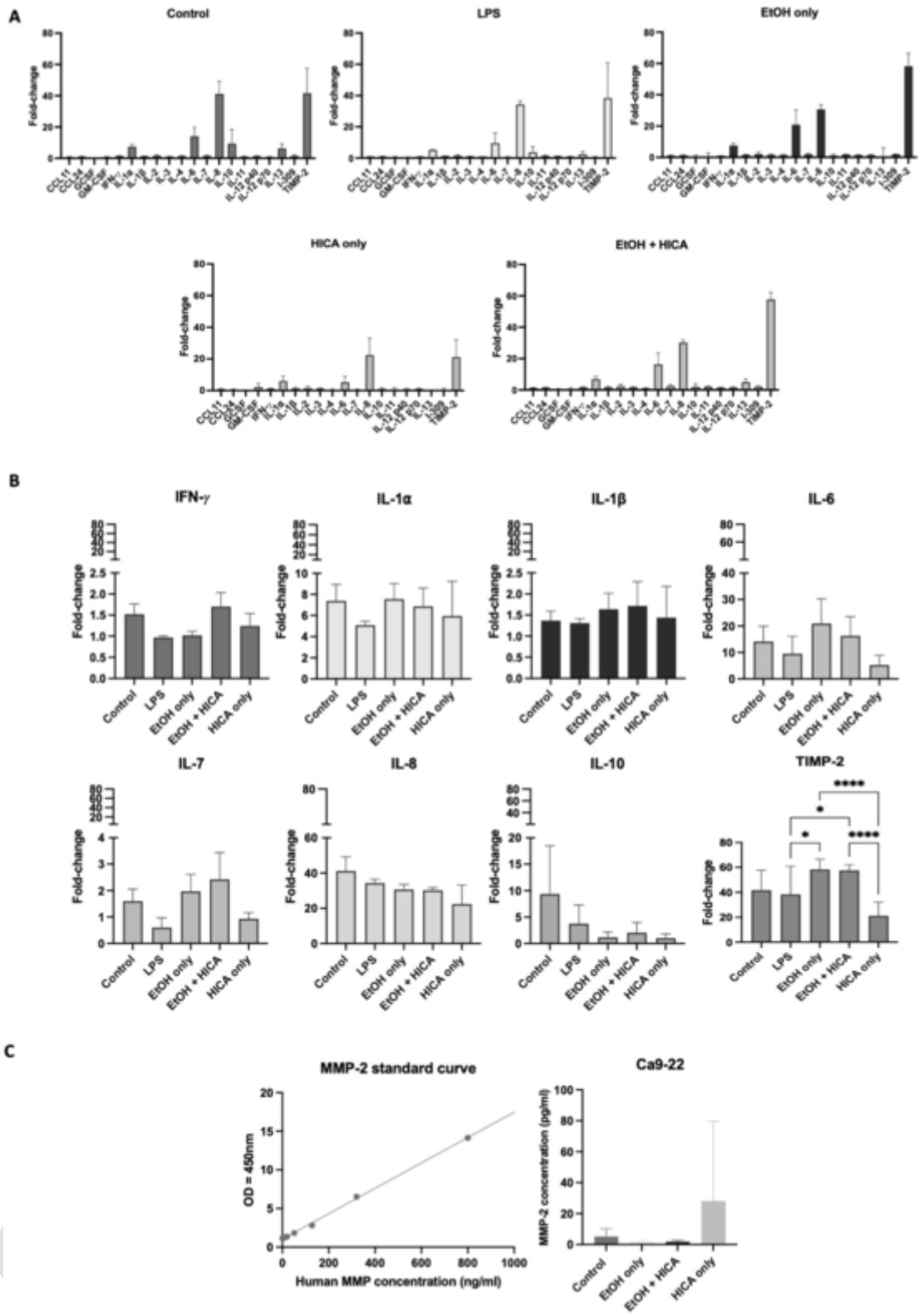

Fig. 5. **(A)** Multi-protein cytokine array was used to analyse expression of 20 human cytokines following treatment of Ca9-22 cells with HICA alone or in combination with EtOH. **(B)** Cytokines of interest in the inflammatory pathways activated by *C. albicans*. No significant differences were observed 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.

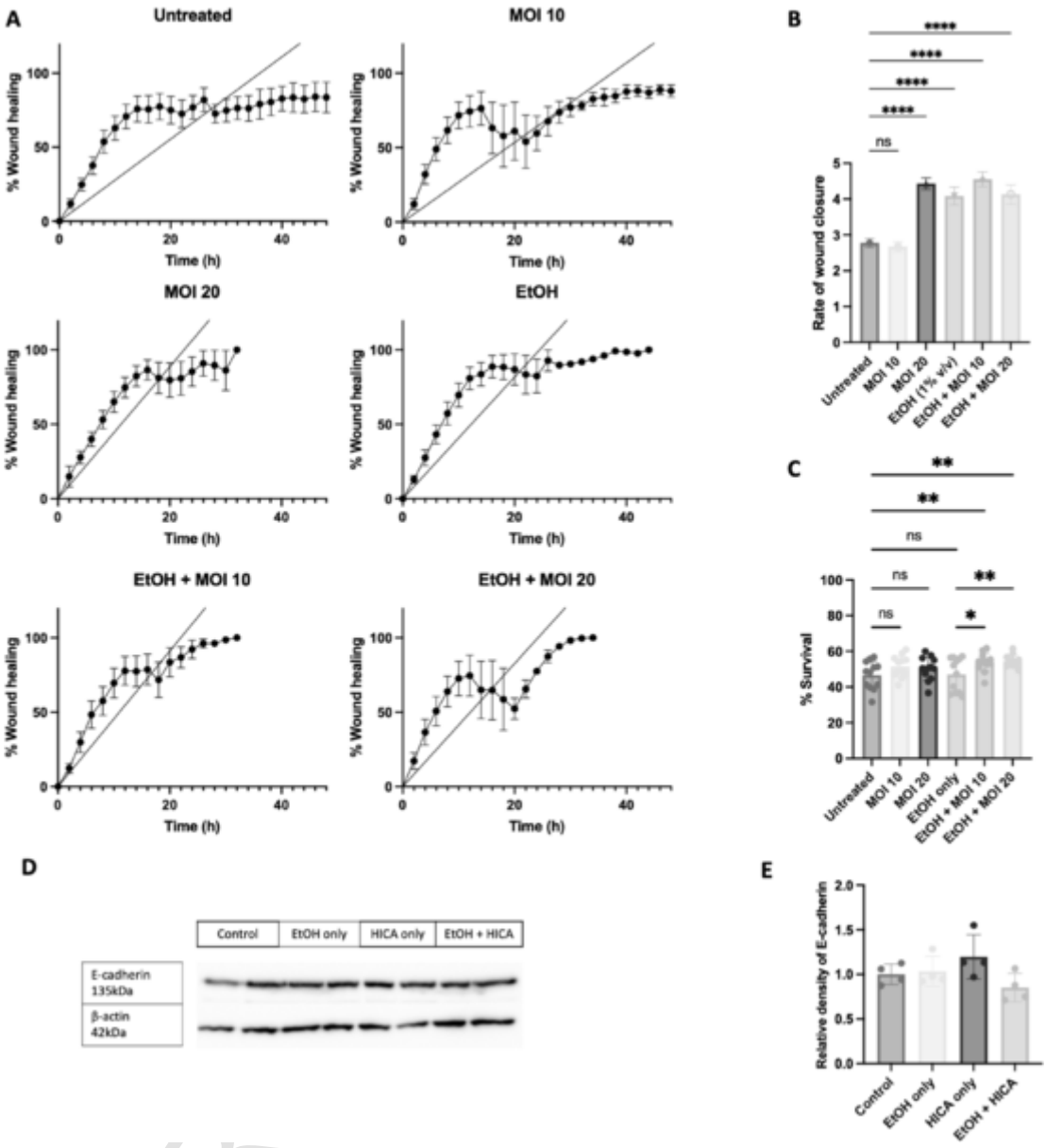

Fig. 6. (A) The effect of EtOH and HICA on Ca9-22 cell rate of wound closure in scratch assays. **(B)** The rate of wound closure obtained from slopes of graph **(A)** were plotted and analysed. Cells treated with a higher MOI of 20 of HICA, EtOH alone or a combination of both proliferated significantly 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.

(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.

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 anchor-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* [27,28]. 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 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 would not stain positively for Annexin V [29]. It is more likely that the levels of caspase-3 at time-points 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 [30,31]. 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 [32,33]. ROS production in response to *C. albicans* is likely both cell-type and morphotype of *Candida*-dependent [34].

C. albicans can activate NF-κB signalling and increase downstream inflammatory cytokines (IL-6, IL-1β and TNF-α) [35]. 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 [13,36].

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 Iκ-Bα 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 [37,38], 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 [39]. 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 expo-

sure 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 [40]. Levels of MMP expression have been shown to increase from normal tissue through dysplasia to oral carcinomas [41]. 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 [42,43].

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 [44,45]. Low-dose ethanol exposure in rats was also shown to upregulate TIMP-1, decreasing pro-inflammatory cytokines [46]. 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* [47]. *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 [48]. 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 [49,50].

Decrease in E-cadherin is observed in EMT processes [20,51–53] 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 [54,55]. 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 [20,56–58]. 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.

Ethical approval

Ethical approval was not required for this article.

Uncited References

[26].

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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