Life History Trade-offs associated with Evolution of Cancer

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A Thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy.

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Declaration

I, Catherine Mary Worsley, declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other university.

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Signature

On this day the 14th of July 2023 in Johannesburg

Dedication

This thesis is dedicated to my parents Alan and Tina Worsley, and to my grandparents who are no longer here - with love and gratitude for your support through this journey.

This work is also dedicated to those who have suffered from this dreaded disease. Your courage is inspiring.

“Darwin’s theory of evolution explains how lower lifeforms can evolve into higher ones, which in turn makes it entirely reasonable that a human should evolve into an orangutan (while remaining a librarian, since there is no higher life form than a librarian).”

— Sir Terry Pratchett, The Science of Discworld

Publications and Presentations arising from this Study

The work presented in this thesis has been published in the following peer-reviewed manuscripts and has been presented at the conferences as listed.

Publications

1. **Worsley CM**, Mayne ES, Veale RB. “Clone Wars: The Evolution of Therapeutic Resistance in Cancer”. *Evolution, Medicine, and Public Health*, 2016(1): 180-1
2. **Worsley CM**, Veale RB, Mayne ES. “The Acidic Tumour Microenvironment: Manipulating the Immune Response to Elicit Escape”. *Human Immunology*, 2022; 83: 399-408
3. **Worsley CM**, Veale RB, Mayne ES. Inducing apoptosis using chemical treatment and acidic pH, and detecting it using the Annexin V flow cytometric assay. *PLoS One*, 2022: 17(6): e0270599
4. **Worsley CM**, Veale RB, Mayne ES. “The effect of acute acid exposure on immunomodulatory protein secretion, cell survival, and cell cycle progression in tumour cell lines”. *Cytokine*; 2023, 162: 156118

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Presentations

1. **Worsley CM.** “The role of the tumour microenvironment in immune evasion”. Oral presentation at the 9th South African Immunology Society (SAIS) Conference 2022, Muldersdrift, South Africa.
2. **Worsley CM**, Mayne ES. “Acidic extracellular microenvironments assist tumour cells in immunological escape.” Poster presented at the 6th South African Immunology Society (SAIS) Conference 2017, Cape Town, South Africa.
3. **Worsley CM**, Durand PM. “Trade-off genes in germ-soma differentiation in Volvocine algae – a model for cancer evolution.” Oral presentation at the Phycological Society of South Africa (PSSA) Conference, Johannesburg, South Africa, Jan 2011.

Abstract

The evolution of multicellularity requires cooperation between single cells to form new multicellular individuals. Changes in levels of selection occur during this process, with selection at the multicellular level overriding that at the single cell level. For a multicellular individual to function, somatic mutations and selection must be under tight regulation. Nevertheless, mutations and selective environmental pressures can select for cells with fitness advantages relative to normal cells, resulting in cancer. Therapeutic drugs and radiation are forms of artificial selection that can drive the development and selection of cell populations that are resistant to treatment.

Cancer occurs because of the failure of multicellular systems to suppress somatic evolution. This somatic evolution results in tumour cells with a wide range of phenotypes with either fast (proliferating) or slow (quiescent) life history strategies. Evolutionary theory provides a framework for understanding what drives the formation of these phenotypes and the ecological niche that supports them, and helps in predicting tumour progression and response to therapy.

The key hypothesis of this study was that selective pressures in the tumour microenvironment drive trade-offs between tumour cell survival, proliferation, and apoptosis. An extensive literature review was conducted to identify key selective pressures affecting tumour progression. Low extracellular pH was identified as a component of the tumour microenvironment that affects life history trade-offs, and particularly drives escape from immune-mediated destruction. A protocol was then developed to expose cancer cells to low pH in cell culture. Breast carcinoma and oesophageal squamous cell carcinoma cell lines were selected for these experiments based on the prevalence of these cancers and because of their different anatomical locations. Exposure to low pH induced different levels of apoptosis in each cell line. This also affected cell cycle progression and the secretion of growth factors and immunomodulatory cytokines. The oesophageal cell line, WHCO6, adapted to moderate acidity levels with some cells undergoing apoptosis. Factors released by

these cells supported the growth and survival of related cells. In contrast, in the breast carcinoma MCF-7 cell line, low pH induced high rates of apoptosis, and factors released by dying cells stimulated death in related cells.

This study highlights that different life history strategies are employed by different cancer types. It also shows the importance of the tumour microenvironment, and acidity in particular, in driving tumour cell adaptation and survival. This study also identifies apoptosis as a pro-tumorigenic driver of cancer progression which has important therapeutic implications.

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I also wish to acknowledge the editors and reviewers of the published articles for their critique and efforts in ensuring that the final versions were acceptable for publication.

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List of Abbreviations

3-D	3-dimensional
5-PL	5 prime logistic
7-AAD	7-amino actinomycin D
AC	adenocarcinoma
ACE	acetazolamide
ACE-NP	acetazolamide nanoparticle
AiA	apoptosis-induced apoptosis
AiP	apoptosis-induced proliferation
AKT	a serine/threonine-specific protein kinase
ANOVA	analysis of variance
APC	allophycocyanin (reference to fluorophore)
APC	antigen presenting cell (reference to cell type)
Apo-EV	apoptotic-derived extracellular vesicle
ATP	adenosine triphosphate
BCL-2	B-cell lymphoma-2
BRCA	BReast CAncer gene
BrdU	bromodeoxyuridine
Breg	regulatory B cell
CA	carbonic anhydrase
CA II	carbonic anhydrase II
CA IX	carbonic anhydrase 9
CA XII	carbonic anhydrase 12

Ca^{2+}	calcium ions
CAF	cancer-associated fibroblast
CCL	chemokine (C-C motif) ligand
CCL2	chemokine (C-C motif) ligand 2
CCL7	chemokine (C-C motif) ligand 7
CCL8	chemokine (C-C motif) ligand 8
CCL22	chemokine (C-C motif) ligand 22
CD	cluster of differentiation
CI	confidence interval
CK18	cytokeratin 18
CM	conditioned media
CML	chronic myeloid leukaemia
CO_2	carbon dioxide
CSF-1	colony-stimulating factor 1
CST	cytometer setup and tracking
CTL	cytotoxic T-lymphocyte
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
CXCL	chemokine (C-X-C motif) ligand
CXCL1	chemokine (C-X-C motif) ligand 1
CXCL8	chemokine (C-X-C motif) ligand 8
CXCL12	chemokine (C-X-C motif) ligand 12
CXCL13	chemokine (C-X-C motif) ligand 13
CXCL16	chemokine (C-X-C motif) ligand 16
DAMP	damage-associated molecular patterns

DC	dendritic cell
°C	degrees Celsius
DMEM	Dulbecco’s Modified Eagle’s Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EBV	Epstein Barr virus
EC	endothelial cell
ECM	extracellular matrix
EDTA	ethylenediaminetetra-acetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transition
EPO	erythropoietin
ER	oestrogen receptor
ERK	extracellular signal-regulated kinase
EV	extracellular vesicle
FAK	focal adhesion kinase
FasL	Fas-ligand
FCM	flow cytometric method
FCS	foetal calf serum
FGF	fibroblast growth factor
FGF2	fibroblast growth factor 2
FGFR	fibroblast growth factor receptor
FITC	fluorescein isothiocyanate

FoxP3	Forkhead box protein P3
FRC	Faculty Research Committee
FSC	forward scatter
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
G-CSF	granulocyte colony-stimulating factor
GDF-15	growth/differentiation factor 15
GF	growth factor
GLUT	glucose transporter
GLUT-1	glucose transporter-1
GLUT-3	glucose transporter-3
GM-CSF	granulocyte-macrophage colony-stimulating factor
GRO-α	growth-regulated oncogene-alpha
GTPase	guanosine triphosphatase
H^+	hydrogen ion
H^+ ATPase	hydrogen adenosine triphosphatase
HBV	Hepatitis B virus
HCl	hydrochloric acid
HER2	human epidermal growth factor receptor-2
HIF	hypoxia-inducible factor
HIF-α	hypoxia-inducible factor alpha
HIF-1	hypoxia-inducible factor-1
HLA	human leukocyte antigen
HNSCC	head and neck squamous cell carcinoma
HPV	human papillomavirus

hr	hour
HRE	hypoxia response element
HREC	Human Research Ethics Committee
ICAM-1	intercellular cell adhesion molecule 1
IDO	indoleamine 2,3-dioxygenase
IFN-γ	interferon gamma
IL	interleukin
IL-1	interleukin-1
IL-1α	interleukin-1 alpha
IL-1β	interleukin-1 beta
IL-10	interleukin-10
IL-12	interleukin-12
IL-13	interleukin-13
IL-15	interleukin-15
IL-17	interleukin-17
IL-18	interleukin-18
IL-2	interleukin-2
IL-23	interleukin-23
IL-35	interleukin-35
IL-4	interleukin-4
IL-5	interleukin-5
IL-6	interleukin-6
IL-8	interleukin-8
iNOS	inducible nitric oxide synthase

IP-10	IFN-γ inducible protein-10
JNK	c-Jun N-terminal kinase
K	potassium
K^+ ATPase	potassium adenosine triphosphatase
LD	lipid droplet
LFA-1	lymphocyte function-associated antigen-1
LPC	lysophosphatidylcholine
MCP-1	monocyte chemoattractant protein-1
MCP-2	monocyte chemoattractant protein-2
MCP-3	monocyte chemoattractant protein-3
MCT	monocarboxylate transporter
MCT-1	monocarboxylate transporter 1
MCT-4	monocarboxylate transporter 4
MDSC	myeloid-derived suppressor cell
MHC	major histocompatibility complex
µl	microliter
MAPK	mitogen-activated protein kinase
MIG	monokine induced by gamma interferon
min	minute
MIP-1α	macrophage inflammatory protein-1 alpha
MIP-1β	macrophage inflammatory protein-1 beta
ml	millilitre
mM	micromolar
MMP	matrix metalloproteinase

MMP-8	matrix metalloproteinase 8
MMP-9	matrix metalloproteinase 9
MOMP	mitochondrial outer membrane permeabilization
MPO	myeloperoxidase
MV	microvesicle
µl	microliter
Na	sodium
NAD^+	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide plus hydrogen
NE	neutrophil elastase
NET	neutrophil extracellular trap
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor kappa B
NHE	sodium-hydrogen exchanger
NHE1	sodium-hydrogen exchanger 1
NHLS	National Health Laboratory Service
NK	natural killer
NKG2D	natural killer group 2D
NO	nitric oxide
NP	nanoparticle
NRF	National Research Foundation
OSCC	oesophageal squamous cell carcinoma
PBS	phosphate buffered saline
PCD	programmed cell death

PCM	post conditioned media
PD-1	programmed cell death protein-1
PDGF	platelet derived growth factor
PD-L1	programmed death ligand-1
PD-L2	programmed death ligand-2
PerCP-Cy5.5	peridinin-chlorophyll-protein complex conjugate-cyanine 5.5
pg	picogram
PGE2	prostaglandin E2
pHe	extracellular pH
pHi	intracellular pH
PI	propidium iodide
PI3K	phosphatidylinositol 3-kinase
PMT	photomultiplier tube
PPARα	peroxisome proliferator-activated receptor alpha
PPI	proton pump inhibitor
PR	progesterone receptor
PS	phosphatidylserine
ROS	reactive oxygen species
rpm	revolutions per minute
S1P	sphingosine-1-phosphate
SA-PE	streptavidin-phycoerythrin
SCC	squamous cell carcinoma
SDF-1α	stromal-derived factor 1 alpha

sICAM-1	soluble intracellular cell adhesion molecule-1
SSC	side scatter
STAT5	signal transducer and activator of transcription 5
STS	staurosporine
sVCAM-1	soluble vascular cell adhesion molecule-1
TAM	tumour associated macrophage
TCR	T cell receptor
TE	trypsin/ethylenediaminetetraacetic acid
TEM	transmission electron microscope
TGF-β	transforming growth factor beta
TGF-β1	transforming growth factor beta 1
Th	T helper
Th1	T helper 1
Th2	T helper 2
Th17	T helper 17
TIL	tumour infiltrating lymphocyte
TLR	Toll-like receptor
TME	tumour microenvironment
TNBC	triple-negative breast cancer
TNF-α	tumour necrosis factor alpha
TNFR1	tumour necrosis factor receptor 1
TRAIL/Apo-2L	TNF-related apoptosis-inducing ligand
Treg	regulatory T cell
TUNEL	Terminal dUTP Nick-End Labelling

UTP	uridine triphosphate
UV	ultraviolet
V-ATPase	vacuolar-type H^+ adenosine triphosphatase
VCAM-1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor
VGSC	voltage gated sodium channels
VSMC	vascular smooth muscle cell

List of Symbols

α	alpha
β	beta
γ	gamma
κ	kappa
μ	mu
ζ	zeta

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Chapter 1: Introduction

Cancer is a significant cause of morbidity and mortality in humans with an estimated 19.3 million new cases and almost 10 million deaths reported in 2020 [1]. Incidence varies with age, gender, and geographical distribution [1]. In additional to genetic susceptibility, cancer occurrence is influenced by several risk factors including smoking, alcohol consumption, diet, obesity and physical inactivity, as well as exposure to chemicals and ultraviolet (UV) irradiation [1], chronic inflammation [2], and infection with viruses such as Hepatitis B (HBV) [3], human papillomavirus (HPV) [4], and Epstein-Barr virus (EBV) [5].

Significant developments in cancer treatment have improved both the quality of life and life expectancy for cancer patients although this is not consistent in all cancer types or in specific patient populations. The global cancer burden is estimated to reach 28.4 million cases by 2040, a 47% increase from 2020 [1]. Cancer incidence is increasing rapidly in sub-Saharan Africa [1], with over 108 000 new cases and 56 000 deaths reported in South Africa in 2020 [6]. Low survival rates can be attributed to several factors including late-stage presentation and weak health infrastructure [7]. Female breast cancer is the most diagnosed cancer globally, followed by lung, colorectal, prostate, and stomach cancers and in South Africa, female breast cancer has the highest incidence and causes the third highest number of deaths [1, 6]. Other cancers with high incidence rates during 2020 include prostate, cervix uteri, lung, and Kaposi sarcoma, while those with the highest mortality included lung, cervix uteri, prostate, and oesophageal cancers [6] (Fig. 1).

Cancer cells adapt to different conditions and create environments that support their growth which may result in resistance to both immunity and therapy. Tumours are dynamic areas of evolution. Within a neoplasm, an abundance of phenotypically distinct cells compete for space and resources, avoid immune destruction, and cooperate to metastasize to other sites [8]. Looking at cancer through the lens of

evolution helps to understand its development, progression, and resistance to treatment [9].

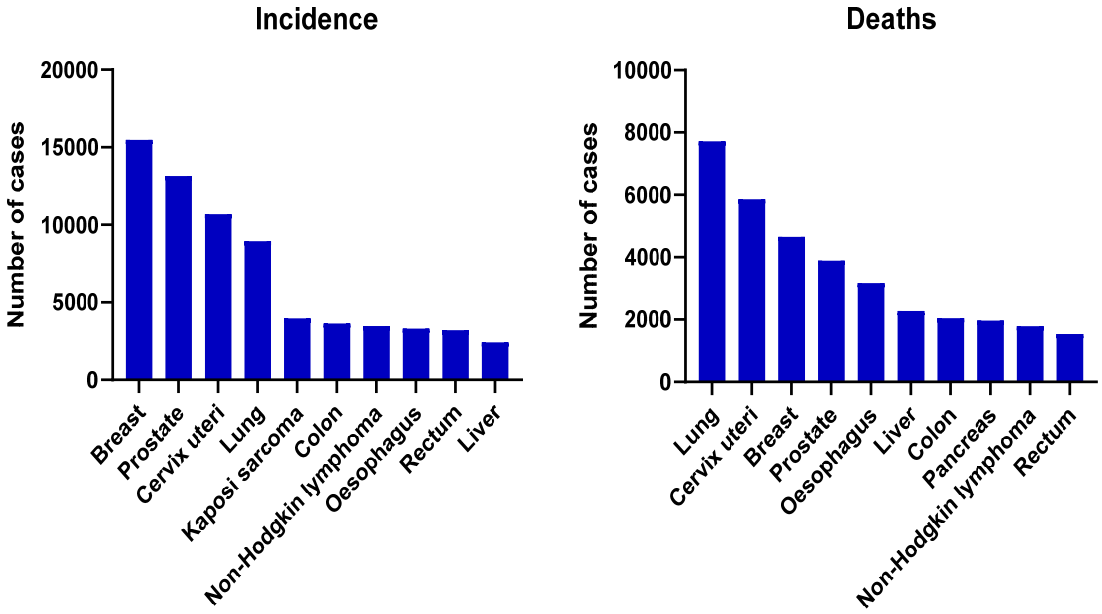

Figure 1. Cancer Statistics in South Africa in 2020.

This illustration represents data obtained from the 2020 GLOBOCAN report detailing the top 10 cancers in South Africa in terms of incidence and number of deaths. (Data source for Figure 1 - [6]).

1.1 Cancer as an Evolutionary Process

Cancer development is a Darwinian process driven by stepwise, somatic cell mutations with sequential subpopulation selection [10, 11]. These subclones are genetically and epigenetically heterogeneous with variable cell survival and replication [8, 12, 13]. Adaptations that are beneficial to a neoplastic clone are usually damaging to the host. These adaptations have variable effects on cancer cell fitness, causing mutant subclones to expand or contract within the tumour because of natural selection [8].

Cancer is closely linked to the transition to multicellularity which has evolved independently in separate lineages from all domains of life (Archaea, Eubacteria, and Eukaryota) [14, 15]. Increases in biological complexity and diversity depend on the evolution of cooperative and altruistic behaviours among single cells, with selection at the multicellular level overriding selection at the single cell level [14, 15]. Multicellular fitness is enhanced by cooperative behaviours including the ability of cells to adhere to and communicate with one another, establish internal transport systems, restrict uncontrolled proliferation and cell division, suppress mutations, remove damaged or mutated cells, and to recognize self from non-self [14, 16, 17]. The breakdown of these cooperative behaviours results in single cells increasing their own fitness at the expense of group fitness [18, 19]. In this context, cancer cells are selfish or cheater cells whose fitness is dependent on the failure of multicellular systems to suppress them [16, 20]. It is these breakdowns in cooperative processes that are linked to the so-called hallmarks of cancer.

1.2 The Hallmarks of Cancer

Hanahan and Weinberg (2000) described six key properties that affect cancer cell fitness including angiogenesis, unlimited replication potential, production of growth signals, insensitivity to anti-growth signals, evading apoptosis, and the ability to invade tissue and metastasize to different sites (Fig. 2) [21]. The reprogramming of energy metabolism and the ability to evade immune-mediated destruction were later included [22], and emerging hallmarks such as dysregulation of differentiation have since been proposed by others [23]. Normal cells within the tumour microenvironment (TME) also contribute to the acquisition of these capabilities [22].

Tumour cells promote angiogenesis, the formation of new blood vessels, by secreting vascular endothelial growth factor (VEGF) [22] (Fig. 2). This ensures that tumour cells have access to sufficient oxygen and nutrients, and waste products are removed. In normal cells, proliferation is controlled by regulating growth-promoting signals that drive entry into and progression through the cell cycle. When these signals are absent, cell cycle progression is halted, preventing uncontrolled proliferation [21]. Telomere

maintenance in tumours allows for unlimited cell replication [21]. Tumour cells also use autocrine signalling to sustain proliferation by producing increasing amounts of growth factors and upregulating cell-surface expression of their corresponding receptors. They also induce surrounding stromal cells to supply growth factors [22]. Normal cells respond to lack of growth signals or deoxyribonucleic acid (DNA) damage by undergoing apoptosis. Neoplastic cells however, often evade apoptosis through mechanisms including inhibition of tumour-suppressor genes and cell cycle checkpoints [21] (Fig. 2).

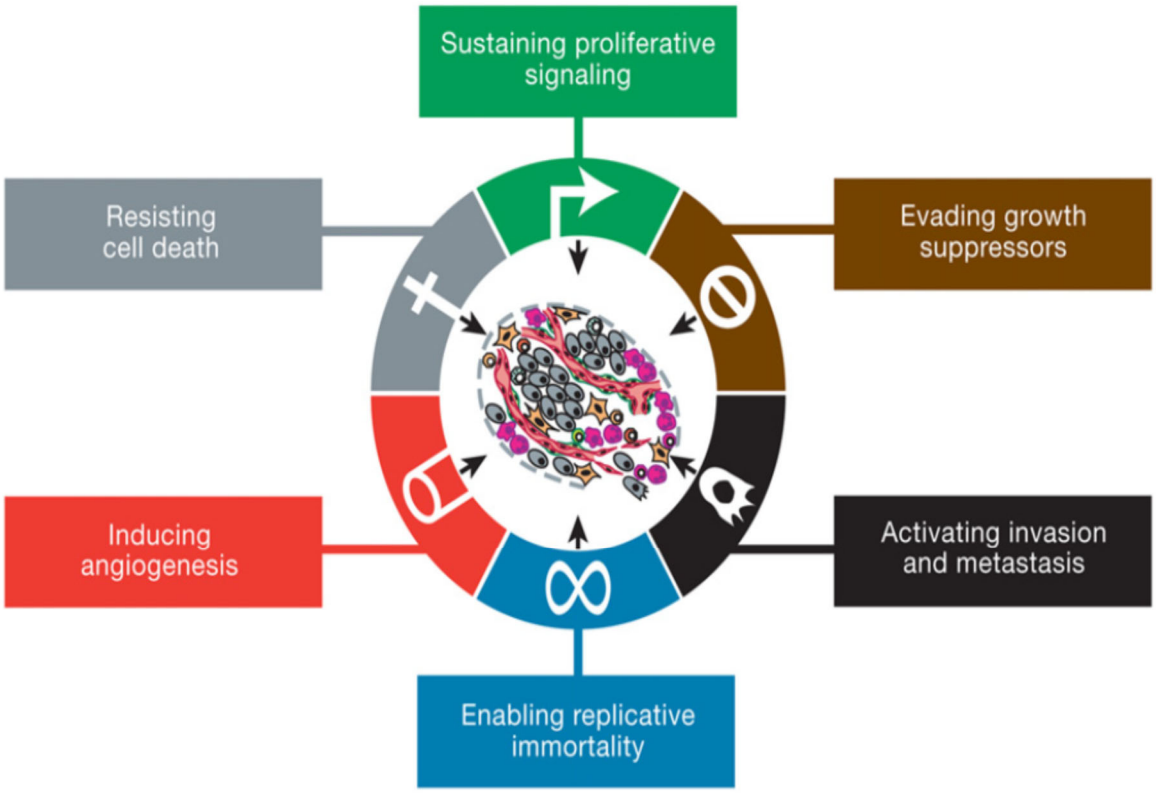

Figure 2. The Hallmarks of Cancer [i]

Hanahan and Weinberg (2000) proposed six hallmarks essential to cancer development which include angiogenesis, resisting apoptosis, sustained proliferation, replicative immortality, evading growth suppression, and invasion and metastasis.

[i] Permission to use this figure from Hanahan and Weinberg (2011) [22] was obtained from the publisher though Elsevier Copyright Clearance Center's RightsLink® - see Appendix C

Continuous proliferation and dysregulated apoptosis causes the tumour to grow, placing constraints on available space and resources. Tumour cells may then secrete proteins such as matrix metalloproteinases (MMPs) to remodel the extracellular matrix (ECM), allowing cells to invade surrounding tissue and escape the constrained environment [21]. Neoplastic cells also interact with neighbouring endothelial cells which they recruit to provide support for invasion and intravasation into the blood or lymph vessels to move to distant sites where they can found new tumour colonies [21] (Fig. 2). Metastasizing cancer cells encounter challenging environments as they migrate which drives adaptation so that they can effectively colonize new sites [21].

1.3 Genetic Instability and Cell Plasticity in Tumours

Cancer cells may respond to environmental stress with or without genomic alteration. Heritable variation within a population is necessary for evolution, and tumours contain heterogeneic cell populations with vast differences in cytogenetic, genetic, and epigenetic capabilities, with the degree of variability linked to malignancy [8]. The genetic machinery meant to suppress cancer is imperfect, which is due in part to antagonistic pleiotropy where genes and pathways that increase survival in certain circumstances have deleterious effects in others [24]. The p53 protein, for example, halts the cell cycle in response to low level DNA damage thereby preventing cancer, however, early apoptosis in stem cells can cause premature aging which lowers reproductive success and fitness [25]. Similarly, processes that support normal tissue repair and wound healing are hijacked to support tumour growth [26].

Genetic instability in cancer cells occurs because of DNA damage, chromosomal changes (losses, gains, or translocations), and mutations in genes that promote tumour progression directly [27, 28]. This instability is evident in both intra- and inter-tumoral heterogeneity, and results in subsequent mutations in daughter cells that undergo clonal expansion [28]. Additionally, this heterogeneity affects treatment decisions where a single biopsy sample may not represent the entire clonal population present in a tumour [29]. Too much genetic instability should be detrimental to a cell, yet tumour cells evolve several strategies to promote growth and survival and to

prevent apoptosis, including the suppression of the anti-apoptotic B-cell lymphoma-2 (BCL-2) family of proteins [30].

Cell plasticity occurs because of dynamic and reversible epigenetic and transcriptional changes that allow cells to adapt rapidly to sudden changes in environmental conditions [31]. Natural selection may favour the phenotype that has the greatest chance of increasing its fitness in a particular environment [24]. Cell plasticity allows for the de-differentiation of cells by epithelial-mesenchymal transition (EMT) with increased migratory and invasive potential, and resistance to apoptosis [31, 32]. Cell plasticity allows for escape from therapeutic intervention with the emergence of drug-tolerant or drug-resistant cell phenotypes [33]. There may be selection for cells that can switch phenotypes to adapt to different environmental pressures including inflammatory, hypoxic, or acidic microenvironments.

While cancers are typically viewed as diseases caused by uncontrolled cell proliferation, they also have to develop survival strategies to persist in challenging environments which may be hypoxic, acidic, and contain destructive immune cells [24]. Somatic cell evolution during cancer progression results in cells of varied phenotypes [24]. Evolutionary life history theory proposes that there are several trade-offs that drive the evolution of these phenotypes.

1.4 Life History Theory and its Relevance in Cancer

Life history theory explains how evolution selects organisms, populations, and species to achieve reproductive success in response to environmental constraints [34]. Variation in life history traits is caused by trade-offs in allocation of resources to competing life functions including maintenance (survival), growth, and reproduction [35, 36]. Organisms cannot maximize all fitness components simultaneously and need to prioritize resource use i.e. investment in one trait occurs at the expense of investment in a competing trait [35]. Trade-offs occur when resources (such as nutrients, energy, time, and space) are limited or where selective pressures are applied to organisms in

an environment [37], and are evident in all species subject to natural selection. As the costs and benefits of different life history trade-offs are dependent on individual characteristics like genetic variation and phenotypic plasticity in response to environmental pressures, strategies can vary across individuals within and between different populations [35]. Natural selection supports mechanisms that enable organisms to modify life history strategies to cope with the challenges faced throughout their lifetime [35].

The combination and coordination of trade-off decisions made by an organism determines its overall life history strategy. Life history strategies lie on a slow-fast continuum depending on the coordination of these trade-offs, and have been documented in a diverse range of species [35]. In response to environmental challenges, organisms that adopt fast life histories invest highly in current over future reproduction which comes at a cost to long-term survival [38]. This is typically seen in mammals such as mice that have small bodies, short life-spans, rapid sexual maturation, and large and frequent litters [24]. In contrast, organisms with slower life history strategies such as elephants invest more in growth, long-term survival, delayed sexual maturation, and fewer but high-quality offspring [24].

Tumour cells are subject to natural selection and can develop either fast or slow life history strategies, depending on trade-offs and environmental conditions. Like other organisms, tumour cells cannot maximize all fitness components at once, with competition for resources between maintenance (survival), cell growth, and proliferation (reproduction) [39]. Fast life history strategies may be adopted because of competition for resources and include rapid and uncontrolled cell proliferation, many but low-quality cells (as a results of bypassing cell cycle checkpoints and suppression of DNA repair mechanisms), and rapid cell turnover (apoptosis). Switching to glycolytic metabolism and invading surrounding tissues are also evident in fast life history strategies in tumours [24]. In contrast, slow life history strategies in resource-limited but stable environments may include cell maintenance (increased DNA repair, autophagy), low levels of proliferation, longer cell survival (evading immune

destruction, suppression of apoptosis), and oxidative phosphorylation [24, 40]. Many of these strategies overlap with the hallmarks of cancer.

As tumours are heterogeneous, it is likely that cells with slow (or quiescent) and fast (rapidly proliferating) phenotypes co-exist. For instance, there may be strong competition driving cell proliferation in hypoxic interior regions of the tumour, whereas at the edge of the tumour where resource availability fluctuates, cell populations with different traits could co-exist [24]. The selection for life history strategies can change over time as cells encounter fluctuations in resource availability, and adapt to overcome environmental challenges and to develop resistance to therapy [41].

1.5 Trade-offs Drive Cancer Progression

Cancer cells must be able to adapt to survive in environments within tumours that are hypoxic, acidic, or contain predatory immune cells [24]. Tumour cell fitness and proliferation is influenced by competition or cooperation with other cancer clones or normal cells within the microenvironment, and trade-offs help drive the evolution of cell types with higher fitness [8, 19]. Evolution may initially select for highly proliferative clones with fast life histories, yet as the tumour develops over time may select for less proliferation, invasion, and metastasis, and more treatment resistance [8]. In a challenging microenvironment with limited resources, cells that express survival genes may be selected at the expense of those expressing growth promoting genes, and cells in hypoxic or acidic regions may survive by invading surrounding tissue to find environments that better sustain growth at the cost of decreased proliferative activity [37]. Other key tasks also compete, and more than two tasks could trade-off simultaneously. These include tasks such as cell division, growth and energy production, lipogenesis, immune interactions, and invasion and metastasis [24, 37].

Both proliferative and apoptotic cells are part of a dynamic environment, which they can alter to promote further cancer development. The TME is the primary regulator that controls the proliferation and apoptosis of different cell types. Apoptosis in

subpopulations in the TME direct several responses which can be directly linked to immune evasion and drug resistance. Understanding the TME could help identify new avenues for the development of successful treatment.

1.6 The Tumour Microenvironment

A cancer cell-centric view where tumorigenesis was thought to be exclusively driven by multiple genetic alterations has dominated cancer research [42]. Recently this view has shifted to tumour evolution being shaped by the surrounding microenvironment which imposes a number of selective pressures [43]. Cancer heterogeneity occurs at different levels both inter– and intra-tumorally, between different lesions, and between patients [42]. Intra-tumoral heterogeneity is caused by genetic and epigenetic modifications, as well as acquired characteristics driven by the TME [42]. As there is a high degree of variability between cancer cells, it is likely that phenotypic traits, such as invasion and metastasis, are not only caused by genetic alterations but are driven by components of the TME [44]. This is supported in experiments where a healthy microenvironment repressed cancer development by promoting immune surveillance and maintenance of healthy normal tissue surrounding the tumour [45].

The TME is an ecosystem created by the cancer cells themselves and involves both tumour and host components [46]. It imposes different selective pressures on neoplastic cells including changes in access to blood flow providing oxygen and nutrients, differences in ECM components, and altered contact with both stromal and immune cells [43, 47-49] (Fig. 3). The bi-directional communication between these components and tumour cells drives cell adaptation, survival, and migration to other tissues [50]. As these components have an impact on clinical outcome, they provide potential novel therapeutic targets [9].

Interactions between different TME elements promote the development of localized and specialized niches which impact on tumour evolution. Uncontrolled cancer cell proliferation and limited blood supply causes oxygen deprivation and hypoxia (Fig. 3).

Cancer cells adapt to this environment by expressing the hypoxia inducible factor-1 (HIF-1) which in turn promotes expression of angiogenic factors such as VEGF; however, the vessels formed are often sub-optimal with reduced oxygen and nutrient transport and poor removal of metabolic waste [46, 50, 51]. Rapidly dividing tumour cells have high metabolic rates [52] and under hypoxic conditions, anaerobic glycolysis is required to supply intracellular energy needs with release of H^+ ions and lactic acid into the TME [49, 51] (Fig. 3 and 4). Oncogenes can drive aerobic glycolysis even under oxygenic conditions – known as the Warburg effect [53]. This persistent glycolysis under normoxic conditions may confer a selective advantage.

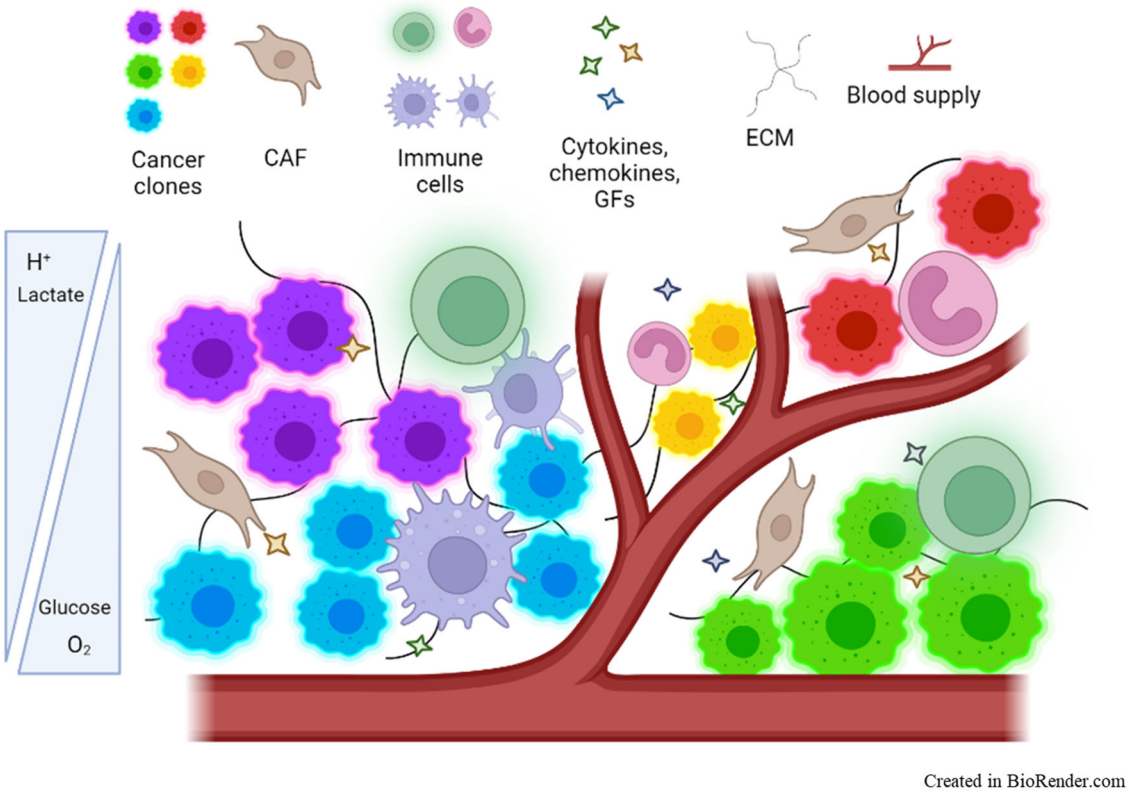

Figure 3. Components of the tumour microenvironment.
Different components in the TME including stromal cells such as endothelial cells (ECs) and cancer-associated fibroblasts (CAFs), immune cells, ECM and secreted components such as cytokine and growth factors (GFs). Hypoxic conditions drive glycolytic metabolism and the subsequent decrease in extracellular pH.

The release of lactic acid and H^+ ions cause both the intracellular pH (pHi) and extracellular pH (pHe) of the tumour cell to decrease [51]. Cells need to maintain an alkaline pHi (between 7.0 - 7.4) to maintain cellular processes including cell adhesion, and proliferation [49, 51]. In tumour cells, the hypoxia inducible factor (HIF) transcription factors regulate pHi by promoting the expression of glucose transporters and net acid extruders responsible for the removal of lactate and hydrogen (H^+) ions from the cell [50] (Fig. 4). This stabilizes the pHi while increasing extracellular acidity. The pHe in solid tumours is much more acidic than in normal tissues and may decrease with increasing tumour size [52].

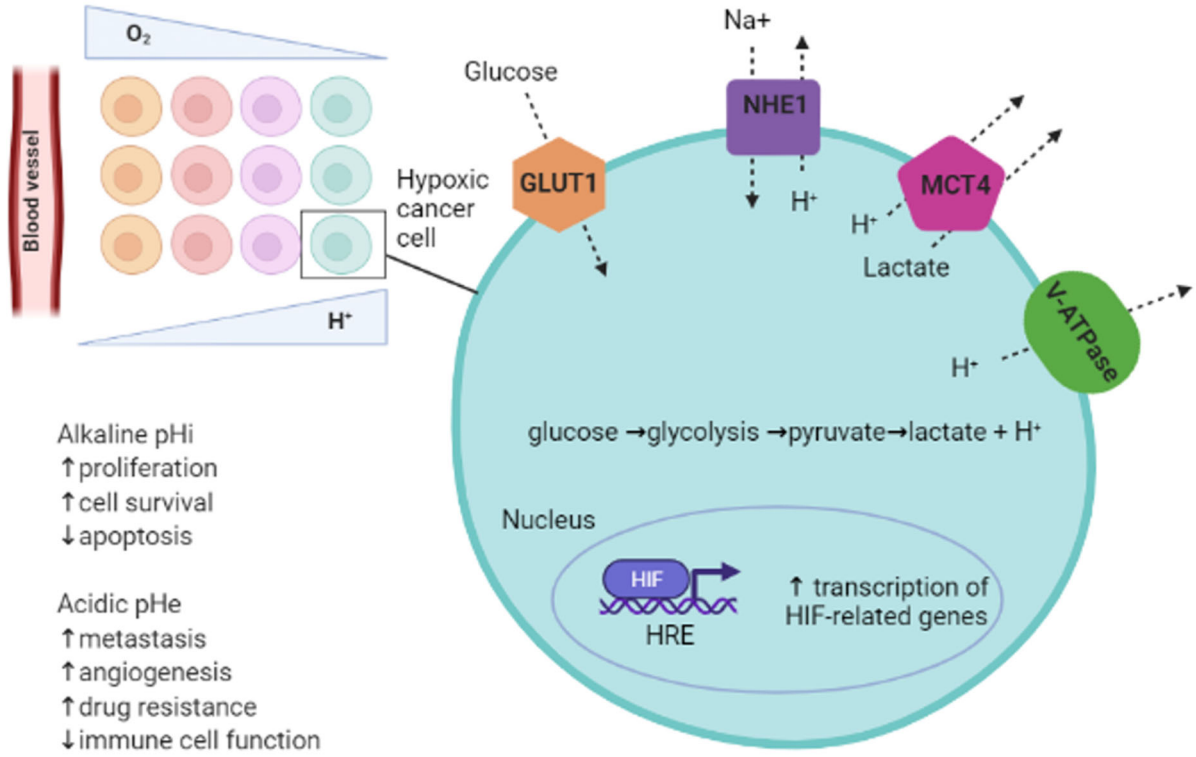

Figure 4. The HIF family regulate lactate and hydrogen ion removal. [ii] HIF-1 promotes the expression of glucose transporter-1 (GLUT-1), and glucose transporter-3 (GLUT-3) that increase glycolysis, and the expression of net acid extruders. These are responsible for regulating pHi by transporting H^+ and lactate out of the cell [50].

[ii] Figure used with permission from Worsley *et al. Hum Immunol* 2022 [50]. See Appendix C for proof of permission.

Initially the acidic TME may trigger apoptosis in sensitive cancer cells [54, 55]. Persistent acidification however drives cancer cell adaptation resulting in aggressive tumours that are difficult to treat [46, 56]. Cells with both increased glycolysis and resistance to extracellular acidosis have a fitness advantage and can create increasingly toxic conditions for surrounding non-adapted cells [56]. Different regions within a tumour can also have different levels of acidity depending on oxygen availability and rates of glycolysis with pockets of extremely low pH scattered across the tumour mass [57]. External factors may also contribute to high levels of acidity. The oesophagus, for example, is continuously exposed to bile acids, gastric fluids, and even food and drink of low pH [58, 59]. This chronic acidosis then drives an inflammatory phenotype and that contributes to the activation of oncogenes, redox pressure, release of exosomes, immune cell inhibition, invasion, metastasis, and resistance to treatment [60, 61].

The low pH of the TME can cause selective stress, causing host stromal, immune cells, and even non-adapted cancer cells to die [62]. In heterogeneous tumours, some cell populations are sensitive to acid-induced apoptosis, while other tumour cells that are adapted to acidity become invasive and metastatic [44]. Programmed cell death (PCD) plays a crucial role in modulating the immunosuppressive TME and impacts on response to therapy [63].

1.7 Programmed Cell Death as an Adaptive Process

Dying cells release signals that profoundly affect their local microenvironment, including mitogens that stimulate proliferation and survival, and death signals that promote cell death [64]. Accidental cell death or necrosis causes the release of signals that are harmful to other cells and tissues [65], whereas PCD is ordered and promotes wound healing and cell survival [66, 67]. Programmed cell death is important in many processes including embryonic development, the destruction and removal of aged, damaged, or malignant cells, and in maintaining tissue homeostasis [64]. Several distinct mechanisms of PCD are reported including autophagy, necroptosis, pyroptosis, ferroptosis. Autophagy typically occurs in the absence of nutrients and growth factors, and involves the degradation of cytoplasmic components and

organelles by autophagosomes [64]. Necroptosis is caused by the ligation of tumour necrosis factor receptor 1 (TNFR1) when caspases are inhibited, and shares morphological features such as membrane swelling with necrosis [64]. Pyroptosis is an inflammatory form of cell death that results in the release of interleukin-1 (IL-1) and -18 (IL-18) and may be tumour-promoting [68], while ferroptosis is an intracellular iron-dependent form of cell death that plays a role in tumour suppression [69].

Apoptosis is the most commonly studied form of PCD, and usually involves caspase activation to induce death [70]. In normal tissues, cells undergo apoptosis in response to DNA damage or the removal of survival factors [71]. Apoptosis is genetically controlled and involves mechanisms such as DNA fragmentation and the exposure of phosphatidylserine (PS) on the cell membrane [72, 73]. Caspases are activated by two distinct but connected pathways: the extrinsic pathway involves interactions between activated death receptors and their ligands (e.g., Fas and Fas ligand (FasL)), while the intrinsic pathway relies on mitochondrial outer membrane permeabilization (MOMP) [70, 74]. To ensure adequate cell clearance, apoptotic cells release ‘find-me’ and ‘eat-me’ signals. ‘Find-me’ signals such as lysophosphatidylcholine (LPC), sphingosine-1-phosphate (S1P), and nucleotides adenosine triphosphate (ATP) and uridine triphosphate (UTP) recruit phagocytic cells, whereas ‘eat-me’ signals such as PS induce phagocytosis [64, 74].

The role of apoptosis in cancer is important as resistance to apoptosis is a hallmark of cancer cells, and consequently, the efficacy of cancer treatments largely depends on activating apoptotic pathways [74]. Research has focused not only on drugs that induce massive tumour cell death, but on inhibiting the underlying processes that help tumour cells evade apoptosis. In some tumours however, cancer cells may have low levels of resistance and may be more susceptible to apoptosis than normal tissues [75]. High levels of apoptosis correlate with worse prognosis in some cancer types including breast, bladder, colorectal cancers, and other squamous cell carcinomas [74, 76, 77]. This indicates that dying tumour cells may promote tumour survival overall.

In multicellular organisms, PCD is an adaptive process that contributes to the development and functionality of the organism [78]. In this context, altruistic cell death is seen as a form of cooperation that is beneficial at a higher multicellular level but is costly at the lower single cell level [78, 79]. While the benefit of apoptosis is evident in multicellular individuals, PCD-like processes have been reported in many unicellular lineages including bacteria, ciliates, chlorophytes, and fungi [80-83]. As PCD is typically viewed as a cooperative behaviour, this raises questions around how cell death could be beneficial in a unicellular context.

The main benefit of apoptosis in unicells is in a kin selection model. Here, apoptosis is an individual-level adaptation that benefits kin and thus the related population overall [78]. This model has been tested in the unicellular alga *Chlamydomonas reinhardtii*, where PCD was induced by heat treatment, and was compared to death caused by non-PCD (necrotic) mechanisms (sonication). Cultures treated with PCD supernatant grew better than controls did, while populations treated with non-PCD supernatant experienced mass death due to the release of chlorophyll and reactive oxygen species (ROS) [84]. Further experiments in *C.reinhardtti* revealed that PCD only benefitted other cells of the same species, while having harmful effects on unrelated species [72]. Similar results have been shown in ciliates, yeast, and bacteria. Taken together, these data suggest that stress-induced PCD could be a trait selected for in order to provide a competitive advantage to kin, while actively inhibiting competitor populations [78]. These experiments may help in understanding the unicellular-like effects induced by PCD in some tumour types.

As apoptotic cells are rapidly cleared in vertebrates, it was previously thought that they had limited signalling capabilities. Apoptotic cells are, however, rich sources of signals that affect their environment. In the context of cancer, apoptosis exerts pleiotropic effects with the release of mitogens that promote proliferation and death signals that cause additional apoptosis [64]. There is growing evidence that PCD occurs in cancer cells and impacts other cells in immediate and distant environments while contributing to carcinogenesis (Table 1). This counters the established view of apoptosis being a mechanism of preventing and treating cancer, with many studies describing the

oncogenic effects of apoptosis failure [85]. This is important as oncogenes, environmental conditions (e.g., hypoxia or acidosis), and treatments such as chemotherapy or radiation work by causing cell death.

The c-Myc oncoprotein, for instance, induces p53 activity and PCD in some cancer cells, thereby removing competition for cells that then proliferate extensively. This process is known as apoptosis-induced proliferation (AiP) [85, 86]. Caspases contribute to tumorigenesis by promoting inflammation and the release of mitogens that stimulate AiP [64, 87]. Apoptotic cells secrete prostaglandin E2 (PGE2) which promotes the proliferation of surrounding tumour cells, blocks immune cell activation, and stimulates the growth of tumours after chemotherapy [70, 85] (Table 1). Apoptotic cells secrete various molecules to attract and activate phagocytic cells, and to polarize tumour-associated macrophages (TAMs) towards a pro-oncogenic state [85]. Other secreted factors include those that support tumour growth, stimulate angiogenesis, and enhance drug resistance [70]. Apoptotic cells can also exert long-range effects on distant environments by exporting apoptotic-derived extracellular vesicles (Apo-EVs) and PS-charged particles. Together these transport oncogenes, PS, and metabolites that promote tumour survival, proliferation, and migration [70] (Table 1).

Table 1. Pro-oncogenic effects of apoptotic cells on immediate and distant environments

Local environment (short-range)	Distant sites (long-range)
Tumour cell proliferation	Metastasis
Immune silencing – cytotoxic T-lymphocytes (CTLs) and natural killer (NK) cells	Release of Apo-EVs
Anti-inflammatory – myeloid-derived suppressor cells (MDSCs), TAMs	Immune silencing - CTLs and NK cells
Angiogenesis	Anti-inflammatory - MDSCs, TAMs
Altered TME	Drug resistance
Matrix remodelling	Aggressiveness
Drug resistance	Drug resistance

1.8 PCD and the Acidic TME Affect Immune Surveillance

Tumour cells can influence immune components in the TME. Robust anti-tumour immune response influence patient morbidity and mortality and exert selective pressure on subclones within the tumour. Although tumour cells secrete several molecules that block infiltration by proinflammatory anti-tumour leukocytes and recruit anti-inflammatory cell types to the tumour, the exact pathophysiology of the pro-tumorigenic microenvironment is still unclear [50]. It is likely though that conditions within the TME, including relative hypoxia and acidity, may play a role. The immune system plays a paradoxical role in cancer development with different immune cells either preventing or promoting tumour growth [42].

Immune cells secrete many substances that are cytotoxic to tumour cells such as the cytokines interleukin-1 (IL-1), interleukin-15 (IL-15), interleukin-17 (IL-17), interleukin-

23 (IL-23), tumour necrosis factor-α (TNF-α), and interferon-γ (IFN-γ), as well as ROS, perforin, and granzyme B [46]. The presence of CTLs and NK cells is key to tumour cell clearance. While acute inflammation creates a hostile environment for tumour growth, persistent inflammation and hypoxic and acidic conditions drive the formation of an immunosuppressive niche [46].

While CTLs and NK cells can infiltrate the tumour periphery, they may not be able to penetrate to the hypoxic and acid interior of the tumour. In contrast, MDSCs, TAMs, and regulatory T and B lymphocytes (Tregs and Bregs, respectively) are actively recruited to the TME [46, 88]. Apoptosis promotes tumorigenesis by recruiting and activating TAMs to clear dying cells at the tumour site [85]. TAM accumulation is closely linked to angiogenesis and improved tumour growth in many cancers [74]. TAMs, MDSCs and other suppressor cells release pro-tumour cytokines and growth factors that promote tumorigenesis [88].

There is evidence to suggest that all mammalian cells have the innate capacity to react to apoptosis of neighbouring cells. Experiments where fibroblasts and epithelial cells were in direct contact with apoptotic cells showed that they recognized the apoptotic cells, with corresponding mitogen-activated protein kinase (MAPK) signalling and ablation of proinflammatory cytokine secretion in these cell types [89]. Neighbouring homotypic cells other than professional phagocytes are also able to remove dying cells *in vivo* [90]. Essentially, this innate recognition of apoptotic cells by non-immune cells reveals inherent anti-inflammatory mechanisms that suppresses conventional inflammatory responses triggered via Toll-like and other innate immune receptors [89]. The ubiquity of this apoptotic cell recognition across cell types suggests this may be an evolutionarily conserved feature [89]. Furthermore, apoptotic immunosuppression may be exploited in tumorigenesis [90].

The pleiotropic responses to apoptosis in tumours may be influenced by different cellular and molecular components of the TME. Together these responses may have a profound effect on trade-offs that influence proliferation, survival, or further apoptosis

within the tumour. Understanding this complex network could help predict whether a tumour is benign, malignant, regressing, or responding to treatment.

1.9 Treatment Influences Cancer Life History Strategies and the Development of Resistance

Cancer causes morbidity and mortality by affecting essential organ functions both in the host organ and following metastasis to different tissues, as well as through a number of para-neoplastic effects [9]. Current treatment models follow the Hippocratic view that extreme treatments are needed to cure severe disease [91]. These include surgery, chemotherapy, radiation, hormone treatments, immunotherapy, and even stem cell transplants with the goal of completely eradicating the tumour burden to cure the disease [46, 91]. Newer anti-tumour drugs and immunotherapy especially have become more tumour-specific, although mainstream interventions like chemotherapy and radiation continue to have significant off-target effects and toxicity prompting efforts to improve specificity of treatments.

Therapeutic interventions generally fall into one of two groups: those that target proliferating tumour cells to reduce tumour size, and those that try to correct dysregulated apoptotic processes. Both target tumour cells directly. The balance between proliferation and apoptosis is crucial in determining whether a tumour grows or regresses [85]. In an ecological context, chemotherapy or radiation are agents of artificial selection that can influence the life history characteristics of tumours and may select for resistant clones [11, 24, 33]. Standard high-dose chemotherapy may kill off rapidly proliferating cells, leaving behind cells with slow life history characteristics such as efflux pumps, or improved DNA repair, increased drug metabolism, and blocking of apoptotic pathways that promotes their survival [24, 92]. These advantages may only be temporary, as any surviving cells with a fast life history strategy will claim the advantage of space and available resources after the demise of sensitive clones [24, 33].

As tumours occur within an ecological niche, treatment should focus on targeting components of the TME. In order to treat cancer, investigations into tumour evolution are needed to understand tumour pathophysiology. The main hypothesis explored in this study is that selective pressures in the TME drive trade-offs between tumour cell survival, proliferation, and apoptosis. A key determinant is the acidic microenvironment which affects the composition of the TME, as well as tumour life history traits of survival, death, proliferation, and immune evasion. These traits in turn affect the TME in a reciprocal manner. Understanding these complex dynamics may be useful to modifying therapeutic approaches to efficiently control cancer.

1.10 Study Design and Aims

1.10.1 Selection of Cancer Cell Lines

Breast cancer is the most common cancer in South Africa [1], while oesophageal squamous cell carcinoma (OSCC) is the most common cancer in South African men [93]. Here, cancer is often only detected at advanced disease stage, with patients having poor prognosis and low 5-year survival rates [7]. This study was conducted using OSCC and breast carcinoma cell lines. Ethical clearance was obtained from the Human Research Ethics Committee (HREC) of the Faculty of Health Science, University of the Witwatersrand (approval number M120205) (Appendix A).

1.10.2 Aims and Objectives

This study aimed to investigate how acidity acts as a selective pressure impacting on tumour life history traits including proliferation, survival, and apoptosis, and by measuring the effect of low pH on tumour cell production of immunomodulatory factors.

This was achieved by the following objectives:

1. Identify how evolution drives the emergence of resistant cancer clones (Chapter 2)
2. To review relevant literature published on the impact that the acidic tumour microenvironment has on driving immune evasion (Chapter 3)
3. To develop a method to replicate the acidic extracellular pH in cancer cell culture and use it to induce PCD (Chapter 4)
4. To assess the humoral effects of acidic pH on the breast and oesophageal cancer selected cell lines treated with acidic pH (Chapter 5)
5. Assess the effects of acute acid exposure on apoptosis (Chapters 4 and 5), cell cycle progression, and cell survival (Chapter 5)
6. To measure the effects of apoptosis on life history traits in related cell cultures (Chapter 5)

Chapter 2: Clone Wars: The Evolution of Therapeutic Resistance in Cancer

Publication: Worsley CM, Mayne ES, Veale RB. “Clone Wars: The Evolution of Therapeutic Resistance in Cancer”. *Evolution, Medicine, and Public Health*, 2016(1): 180-1

This project considers the Darwinian evolution of cancer. To provide context, a commentary was written to highlight that chemotherapy often fails because of the development of resistant clonal cell populations. Cancer cells acquire mutations that affect their fitness, with some cell populations adapting to become resistant to treatment while less fit ones succumb and die. By removing competition for resources, chemotherapy promotes the survival of resistant cells that then proliferate rapidly causing tumour growth and metastasis. Here, the need to better understand the evolutionary principles of cancer evolution and the development of resistance is highlighted in order to improve therapeutic strategies. This commentary crystalizes the hypothesis of this project that life history trade-offs drive tumour evolution and resistance to treatment.

Contribution of the candidate: Conceptualization, design, and writing of the paper.

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CLINICAL BRIEF

Clone wars: the evolution of therapeutic resistance in cancer

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THE FAILURE OF CHEMOTHERAPY

Cancer chemotherapy kills some tumour cells, leaving behind resistant clones with less competition for space and resources. These clones, groups of cells that share common ancestry, proliferate without restriction causing disease relapse.

Tumours contain a host of cancer clones that are genetically and epigenetically different from one another [1]. These clones follow a Darwinian process of somatic selection where they compete for space and resources within their microenvironments [2]. Cancer cells acquire mutations over time that affect their fitness, giving some clones a survival advantage over others [3].

Chemotherapy itself is a selective pressure that influences tumour heterogeneity and clonal evolution. Most cancer deaths are caused by clones that are therapeutically resistant [3]. The evolution of resistant clones occurs rapidly, resulting in the appearance of new clones that are not susceptible to conventional therapy. Evolutionary principles describe the process of clonal evolution and aid in formulating novel strategies for disease management and prognosis [4].

EVOLUTIONARY PERSPECTIVES

An understanding of tumour heterogeneity and clone fitness is key to developing better treatment options. Intra-tumoural genetic variability and instability affect the process of somatic evolution [4].

Variations in the tumour microenvironment, including nutrient availability and blood supply access, drive clone evolution in both the presence and absence of chemotherapeutic drugs [3, 5]. The more genetic or environmental variation there is, the greater the likelihood that some clones will develop a survival advantage over others [4].

In some instances, resistant clones can cooperate with one another in ways that promote their survival [6], leading to faster cancer progression or increased aggressiveness. When chemo-sensitive cells are killed, space and resources become abundantly available to resistant clones, which then proliferate without inhibition by neighbouring cells. Understanding clonal evolution and the pressures that select for resistant clones can inform the development of new therapeutic approaches.

FUTURE IMPLICATIONS

With the advent of molecular data and computational frameworks, alternate strategies are being investigated to manipulate the microenvironment to control and contain tumours [5, 7]. Genetic profiling of tumour progression over time allows analysis of DNA methylation patterns and base pair mutations of clones, linking the evolution of clones to specific genetic events [8]. Single cell analysis helps in understanding tumour progression and intra-tumoural heterogeneity [9], and next-generation sequencing uncovers genetic complexities in individual clones [3, 10].

Mathematical and computational modelling provides frameworks for determining vital mutations and microenvironmental changes, bridging the gap between laboratory data and clinical information [7, 9, 11].

The strategies being investigated include using cell competition to control resistant clones, manipulating blood and nutrient supply, and using drugs that contain tumour growth instead of killing cells [7, 8, 11]. Many of these approaches are being tested in animal models, with the hope that some of these interventions will make their way into clinical practice.

One promising approach is to utilize tradeoffs by manipulating clones to compromise themselves by becoming dependent on mutations that protect them from one drug, but that make them susceptible to other drugs [12].

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Chapter 3: The Acidic Tumour Microenvironment: Manipulating the Immune Response to Elicit Escape

Publication: Worsley CM, Veale RB, Mayne ES. “The Acidic Tumour Microenvironment: Manipulating the Immune Response to Elicit Escape”. *Human Immunology*, 2022; 83: 399-408

The evolution of cancer clones that can adapt to different environmental conditions is a barrier to successful control and treatment. Cancer evolution is largely driven by trade-offs in the tumour microenvironment. A comprehensive literature review was conducted to identify key selective pressures affecting tumour progression. From this low extracellular pH was identified as a TME component that affects life history trade-offs, and the immune response in particular. The immune system is integrally involved in the TME and can either control cancer growth or can be manipulated to promote tumorigenesis. Tumour cells themselves influence the immune composition of the TME largely through extracellular acidity driven by tumour processes such as glycolysis. Tumour acidity can vary within the tumour, as well as between tumour types and anatomical locations. This study investigates drivers of tumour acidity and the effects of low pH on different immune components to drive immune escape. These factors ultimately affect tumour life history strategies, cancer progression, and treatment options.

Contribution of the candidate: Conceptualization, extensive literature search, article design, design of artwork, writing, and editing of the paper.

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Review

The acidic tumour microenvironment: Manipulating the immune response to elicit escape

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ABSTRACT

The success of cancer treatment relies on the composition of the tumour microenvironment which is comprised of tumour cells, blood vessels, stromal cells, immune cells, and extracellular matrix components. Barriers to effective cancer treatment need to be overcome, and the acidic microenvironment of the tumour provides a key target for treatment. This review intends to provide an overview of the effects that low extracellular pH has on components of the tumour microenvironment and how they contribute to immune escape. Further, potential therapeutic targets will be discussed.

Abbreviations: ACE, acetazolamide; ACE-NP, acetazolamide nanoparticle; AKT, a serine/threonine-specific protein kinase; APC, antigen presenting cell; ATPase, adenosine triphosphatase; Breg, regulatory B cell; CA, carbonic anhydrase; CA II, carbonic anhydrase II; CA IX, carbonic anhydrase 9; CA XII, carbonic anhydrase 12; CAF, cancer-associated fibroblast; CCL, chemokine (C-C motif) ligand; CCL2, chemokine (C-C motif) ligand 2; CCL7, chemokine (C-C motif) ligand 7; CCL8, chemokine (C-C motif) ligand 8; CCL22, chemokine (C-C motif) ligand 22; CD, cluster of differentiation; CO_2, carbon dioxide; CSF-1, colony stimulating factor 1; CTL, cytotoxic T-lymphocyte; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; CXCL, chemokine (C-X-C motif) ligand; CXCL1, chemokine (C-X-C motif) ligand 1; CXCL8, chemokine (C-X-C motif) ligand 8; CXCL12, chemokine (C-X-C motif) ligand 12; CXCL13, chemokine (C-X-C motif) ligand 13; CXCL16, chemokine (C-X-C motif) ligand 16; DAMP, damage-associated molecular patterns; DC, dendritic cell; DNA, deoxyribonucleic acid; EC, endothelial cell; ECM, extracellular matrix; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EMT, epithelial-mesenchymal transition; EPO, erythropoietin; ERK, extracellular signal-regulated kinase; EV, extracellular vesicle; FAK, focal adhesion kinase; FGF, fibroblast growth factor; FGF2, fibroblast growth factor 2; FoxP3, forkhead box protein P3; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GLUT, glucose transporter; GLUT-1, glucose transporter-1; GLUT-3, glucose transporter-3; GTPase, guanosine triphosphatase; H, hydrogen; H^+ ATPase, hydrogen adenosine triphosphatase; HIF, hypoxia-inducible factor; HIF-α, hypoxia-inducible factor alpha; HIF-1, hypoxia-inducible factor-1; HLA, human leukocyte antigen; HNSCC, head and neck squamous cell carcinoma; HRE, hypoxia response element; ICAM-1, intercellular cell adhesion molecule 1; IDO, indoleamine 2,3-dioxygenase; IFN-γ, interferon gamma; IL, interleukin; IL-1α, interleukin-1 alpha; IL-1β, interleukin-1 beta; IL-10, interleukin-10; IL-12, interleukin-12; IL-13, interleukin13; IL-2, interleukin-2; IL-35, interleukin-35; IL-4, interleukin-4; IL-6, interleukin-6; IL-8, interleukin-8; iNOS, inducible nitric oxide synthase; JNK, c-Jun N-terminal kinase; K, potassium; K^+ ATPase, potassium adenosine triphosphatase; LD, lipid droplet; MCT, monocarboxylate transporter; MCT-1, monocarboxylate transporter 1; MCT-4, monocarboxylate transporter 4; MDSC, myeloid-derived suppressor cell; MHC, major histocompatibility complex; MMP, matrix metalloproteinase; MMP-8, matrix metalloproteinase 8; MMP-9, matrix metalloproteinase 9; MPO, myeloperoxidase; MV, microvesicle; Na, sodium; NAD^+, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide plus hydrogen; NE, neutrophil elastase; NET, neutrophil extracellular trap; NFAT, nuclear factor of activated T cells; NF-κB, nuclear factor kappa B; NHE, sodium-hydrogen exchanger; NHE1, sodium-hydrogen exchanger 1; NK, natural killer; NKG2D, natural killer group 2D; NO, nitric oxide; NP, nanoparticle; PD-1, programmed cell death protein-1; PDGF, platelet derived growth factor; PD-L1, programmed death ligand-1; PD-L2, programmed death ligand-2; pHe, extracellular pH; pHi, intracellular pH; PPARα, peroxisome proliferator-activated receptor alpha; PPI, proton pump inhibitor; ROS, reactive oxygen species; STAT5, signal transducer and activator of transcription 5; TAM, tumour associated macrophage; TCR, T cell receptor; TGF-β, transforming growth factor beta; TGF-β1, transforming growth factor beta1; Th, T helper; Th1, T helper 1; Th2, T helper 2; Th17, T helper 17; TIL, tumour infiltrating lymphocyte; TLR, Toll-like receptor; TME, tumour microenvironment; TNF-α, tumour necrosis factor alpha; TRAIL/Apo-2L, TNF-related apoptosis-inducing ligand; Treg, regulatory T cell; V-ATPase, vacuolar-type H^+ adenosine triphosphatase; VCAM-1, vascular cell adhesion molecule 1; VEGF, vascular endothelial growth factor; VGSC, voltage gated sodium channels; α, alpha; β, beta; γ, gamma; κ, kappa; ζ, zeta.

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Contents

1. Introduction

Understanding the effects of low extracellular pH (pHe) on tumour biology is vital to treating and curing cancer. The tumour microenvironment (TME) is the physical niche in which a cell develops into a tumour cell. This environment influences different stages of oncogenesis by inducing angiogenesis, promoting sustained and uncontrolled clonal proliferation, stimulating invasion and metastasis, and by assisting tumour cells in evading checkpoint regulation and avoiding cell death [1].

The TME contains a number of different cell types including fibroblasts, innate and adaptive immune cells, adipocytes, and endothelial cells (ECs), as well as extracellular matrix (ECM) components, signalling molecules, and antibodies [2,3]. These components within the TME, controlled and manipulated by the tumour itself, co-operate in a series of complex interactions to create an environment in which tumour cells adapt, survive, and spread to other tissues. As tumour cells proliferate and tumour size increases, molecular and cellular changes occur in the TME [4].

Tumour cells use a number of strategies to evade the immune response including impaired antigen presentation, the release of immunosuppressive molecules and extracellular vesicles (EVs) (containing multiple humoral factors), and overexpression of checkpoint molecules [5,6]. Checkpoint molecules, such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed-death cell protein 1 (PD-1), are instrumental in preventing T cell activation. The T cell receptor (TCR) binds to major histocompatibility complex (MHC) molecules presenting antigen, and requires a second signal for activation. CD28 on the T cell binds to B7 molecules (CD80/CD86) on antigen-presenting cells (APCs) (e.g. dendritic cells, macrophages) leading to stimulation of the T cell [7]. CTLA-4 is a CD28 homolog that competes with a higher affinity for B7. It inhibits T cell stimulation, and causes decreased interaction of T cells with APCs [7]. PD-1 also regulates T cell activation by binding to its ligands programmed death ligand-1 (PD-L1) or PD-L2. This interaction inhibits T cell proliferation, the production of interferon gamma (IFN-γ), tumour necrosis factor alpha (TNF-α) and interleukin-2 (IL-2), and decreased T cell survival. Many tumour cells express high levels of PD-L1, which is associated with more tumour infiltrating lymphocytes (TILs) and worse prognosis [7].

Evolution of the TME is strongly influenced by tumour cell metabolism which causes hypoxia, glucose and nutrient depletion, waste accumulation, oxidative stress, lactate accumulation, and increased extracellular acidosis [4,8]. This acidic TME is incompatible with normal cell survival, with low pHe promoting cancer development in all stages from dysplasia to malignant disease [8]. Tumour cells adapt to survive acid-mediated toxicity, evade the immune system, and escape therapeutic intervention [9]. These strategies enable the fittest clones to survive in the acidic TME. These factors, combined with the acidic microenvironment can affect both treatment pharmacokinetics and effectiveness. This review focusses on how the acidic TME directly impacts tumour cells, and how it manipulates both stromal and immune cells to drive immune escape. This has implications for targeting specific processes in the acidic TME to improve therapeutic intervention and patient outcomes.

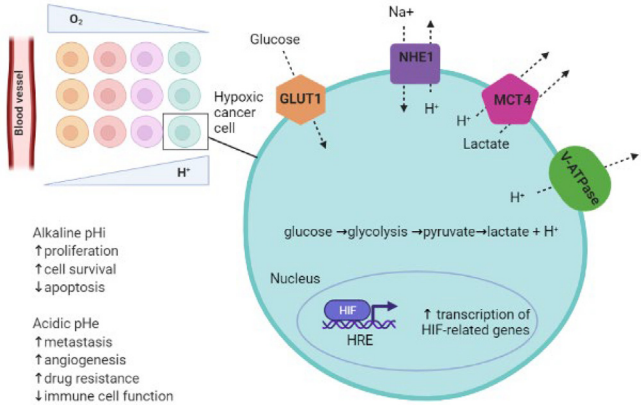

Fig. 1. The HIF family regulate lactate and hydrogen ion removal. As cells are positioned further away from adequate blood supply, oxygen levels decrease to produce a hypoxic environment. HIF-1 promotes the expression of glucose transporters GLUT-1, GLUT-3 which increase glycolysis, and expression of net acid extruders. These are responsible for regulating pHi by transporting H+ and lactate out of the cell. These include monocarboxylate transporters (MCTs), Vacuolar-type H+ -ATPases (V-ATPases), and sodium-hydrogen exchangers (NHEs). The resultant decrease in pHe contributes to metastasis and immune evasion.

1.1. Tumour cell metabolism lowers extracellular pH in the TME

The development of low pHe in tumours is associated with hypoxia. Although tumours promote angiogenesis, the resulting vascular network is often chaotic and inefficient leading to reduced oxygen and nutrient transport, and removal of metabolic waste [10]. Cancer cells have a high demand for glucose, and use anaerobic glycolysis to maintain intracellular energy levels under hypoxic conditions [3,10]. This causes accumulation of H^+ ions and lactic acid in the TME. In aggressive cancers, oncogenes drive aerobic glycolysis even under normoxic conditions, known as the Warburg effect [11].

Glycolysis increases the production of both lactic acid and H^+ ions that cause the intracellular pH (pHi) of the tumour cell to decrease [10]. A slightly alkaline pHi (7.0–7.4) is required to maintain many cellular processes including cell adhesion, proliferation, metabolism, and apoptosis, while intracellular acidification can block cell cycle progression [3,10]. Histone acetylation, required to allow access of chromatin to transcription factors, is negatively affected by decreasing pHi [10,12].

To mitigate the effects of intracellular acidification, the pHi of cells is tightly controlled by the hypoxia-inducible factor (HIF) family of transcription factors which regulate the expression of genes involved in lactate and hydrogen ion removal [10] (Fig. 1). HIF proteins induce the transcription of over 70 genes in tumours via the hypoxia response element (HRE) in their promoter regions, including glucose transporters such as glucose transporter-1 (GLUT-1) and GLUT-3, and net acid extruders such as monocarboxylate transporters (MCTs), hydrogen/potassium adenosine triphosphatases (H^+/K^+ ATPases), vacuolar-type H^+-ATPases (V-ATPases), carbonic anhydrases (CAs), and sodium-hydrogen exchangers (NHEs) [12] (Fig. 1). Maintaining an alkaline pHi during glycolysis results in extrusion of more H^+ ions from the cell with a concomitant decrease in extracellular pH [12].

Lactate extruded by tumour cells lowers the pH of the TME, however, lactate itself also has direct immunosuppressive functions. Increased levels of lactate are sensed by both tumour and immune cells in the TME, and impacts on the behaviour and function of these cells. Lactate efflux from immune cells depends on the concentration gradient between the extracellular and intracellular environment, and is prevented by the abundance of tumour-secreted lactate in the TME. The accumulation of lactate in $CD8^+$ cytotoxic T lymphocytes (CTLs) impairs cytotoxicity by inhibiting nuclear factor of activated T cells (NFAT), causing decreased IFN-γ production. It promotes CTL and natural killer (NK) cell apoptosis by increasing CTLA-4 and PD-L1 expression [13]. T cell polarization is also affected by lactate, with a decrease in anti-tumoural T helper 1 (Th1) cells with concomitant increases in regulatory T cells (Tregs) [14]. Increased lactate causes a reduction of nicotinamide adenine dinucleotide (NAD^+) to NADH, leading to depletion of post-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) glycolytic intermediates and serine which are vital for T cell proliferation [15]. Lactate also impacts on antigen processing and presentation, differentiation and activation of dendritic cells (DCs), and induces a tolerogenic M2 tumour-associated macrophage (TAM) phenotype [13].

1.2. The survival of adaptive cancer clones is promoted by the acidic TME

Normal mammalian cells cannot survive long in a microenvironment with an pHe of less than 7.2 [3]. The pHe of cancer cells is usually between 6.7 and 7.1, but can drop as low as 6.0 [10]. Heterogeneous cell clones within the TME compete for space and resources, providing a selective survival advantage over suboptimal clones. In addition, the presence of the proton extruder channels shown in Fig. 1 provide an adaptive advantage. The acidic TME drives the evolution of cancer cells with more malignant phenotypes adapted to survive the acidic TME [8]. Depending on the tumour type and microenvironmental conditions, cancer cells have to adapt their metabolic strategies [8]. Cells adapted to an acidic environment metabolize glucose more rapidly at low pH, and may adapt alternate strategies in the hypoglycaemic TME including the metabolism of fatty acids [16].

Extracellular acidity also drives epithelial-mesenchymal transition (EMT), induces genetic instability and DNA damage, redistribution of lysosomes, and promotes ECM remodelling and invasiveness [8]. During EMT, dynamic cytoskeletal reorganization is needed to enable morphology change, migration, and invasive capabilities of cancer cells [17]. The acidic pHe regulates cytoskeletal changes by promoting focal adhesion maturation, activating Rho-GTPases, and regulating microfilament reorganization through β1-integrin-activated focal adhesion kinase (FAK) signalling [17]. Reorganization of cytoskeletal proteins such as actin, tubulin, and vimentin, contributes to acidic pHe-triggered morphology change and cell migration [17]. Genetic instability is induced by extracellular acidosis which causes double-stranded DNA breaks through reactive oxygen species (ROS) production, and prevention of damage repair [8]. This allows for the mutation of clones, and selection of clones with survival advantages.

Extracellular acidity promotes autocrine transforming growth factor beta 2 (TGF-β2) signalling in cancer cells, which enhances lipid droplet (LD) formation enabling storage of energy to resist apoptosis [18]. Acidosis-induced TGF-β2 activation promotes EMT as well as fatty acid metabolism [18]. Another advantage stimulated by chronic acidosis is the increase in distribution of lysosomes to the plasma membrane where they protect the plasma membrane against acid-mediated damage [19]. Lysosomes containing cathepsins are also stimulated, increasing ECM remodelling [20].

The acidic pHe shifts tumour cell metabolism. Human breast cancer cells under acute acidic pH exposure show reduced proliferation, G1 cell cycle arrest, and increased cytoplasmic vacuolization [21]. These cells reduce their glucose metabolism, while increasing their metabolism of both glutamine and fatty acids [22,23]. The acidic TME stimulates autophagy in which organelles and proteins are self-digested to maintain metabolism and promote cell survival [21]. Cells that were exposed to low pH for months had restored proliferative ability, however, autophagy markers LC3-II and double-membrane markers were maintained [21]. Thus autophagy may be used as an adaptive strategy to maintain survival in an acidic TME.

Acidity also promotes release of EVs by cancer cells, with increasing numbers of EVs released as the pHe drops from 7.4 to 6.5 [6]. EVs include microvesicles (MVs), exosomes, and apoptotic bodies, which have different origins, properties, and functions [24]. Their main function is cell–cell communication between local and distant cells. EVs can contain different lipids, nucleic acids, and proteins, that are selectively taken up by stromal or tumour cells. They can confer molecules that promote tumour cell survival and metastasis, angiogenesis, and suppression of immune cells [6,24].

While the intrinsic effects of acidity are important for understanding tumour behaviour, acidity has larger influence beyond the tumour itself. Here we describe the relationship between the acidic TME and stromal cells, the influence of the acidic TME in mediating and manipulating immune evasion, and potential therapeutic targets requiring investigation.

Table 1
The effects of acidic pHe on cells in the tumour microenvironment.

Functional group	Cell type	Effect of acidic TME	References
Stromal cells	Fibroblasts	↑ CCL7 and MMP secretion to enhance cell migration and invasion ↑ CXCL1 secretion to promote tumour cell proliferation ↑ CXCL12 and FGF to promote tumour growth ↑ CXCL16 ↑ erythropoietin (EPO), FGF, and VEGF to promote angiogenesis ↑ TGF-β to suppress T and NK cells ↑ IL-1β, IL-6, IL-8 production ↑ IL-6, TGF-β, IL-1α, EGF, PDGF, FGF2 stimulates activation of CAFs	[1,2,25,26]
	Endothelial cells	↑ expression of VEGF and FGF to promote angiogenesis and cell migration ↑ secretion of IL-6 and CSF-1 ↑ recruitment of innate immune cells to create an immunosuppressive environment ↑ PD-L1 expression to disable T cells ↑ Treg recruitment ↑ polyunsaturated fats to support tumour growth ↓ apoptosis ↓ ICAM-1 and VCAM-1	[28,31]
Phagocytic and antigen-presenting cells	Myeloid-derived suppressor cells (MDSC)	↑ Recruitment to tumour site ↑ MDSC secretion of arginase and iNOS ↑ immunosuppression of NK and T cells	[28,33,35,36]
	Neutrophils	Switch to N2 phenotype ↑ NETs release ↑ release of MMP-8, MMP-9 and NE to promote ECM remodelling ↓ Cytotoxic killing of tumour cells ↑Hydrogen peroxide and MPO release ↑H^+ATPase release to block NK and T cell activity	[44,46,47,49]
	Macrophages	Switch to M2 phenotype ↑ Th2 cytokine release e.g. IL-10 and TGF-β to suppress T cells ↑ iNOS and ROS ↑ Prostaglandin release ↑ release of EGF, VEGF, CXCL8, IL-6 to promote tumour cell survival and angiogenesis ↑ MMP secretion to promote invasion ↑ oncogenic EV release ↓ Antigen presentation	[2,28,39,40,42]
	Dendritic cells	↑ anti-inflammatory cytokines e.g. IL-10 ↓ Maturation and migration to lymph nodes ↓ HLA class II expression and antigen presentation ↓ co-stimulatory molecule expression (CD40, CD80, CD86) ↓ Pro-inflammatory cytokines e.g. IL-12 ↑PD-L1 and CTLA-4	[10,28,38]
Natural Killer cells	Natural Killer cells	↑ Anergy and apoptosis ↓ Cytotoxic activity (perforin and granzyme release) ↓ NKG2D expression and activation ↓ IFN-γ production ↓ Degranulation ↓ metabolic activity Inhibited by TGF-β, IL-10	[33,50–53]
T lymphocytes	$CD8^+$ (cytotoxic) and $CD4^+$ (helper) T cells	↑ CTLA-4 ↑ Anergy and apoptosis ↑iNOS and arginase depletes arginine needed to TCR function ↓ CD3 ζ chain expression ↓ release of cytokines e.g. IL-2, IFN-γ, TNF-α ↓ cytotoxic activity (perforin and granzyme release) ↓ STAT5, ERK, AKT, JNK, c-Jun and p38 phosphorylation ↓ NFAT activation ↓ Metabolism, chemotaxis and proliferation of T cells ↓TCR activation - blocked by IDO converting tryptophan to kynurenine	[11,54,55,58–66]
	Regulatory T cells (Tregs)	↑ numbers of Tregs recruited by IDO, TGF-β, and CCL22 Lactic acid in TME supports Treg metabolism ↑ Immunosuppression of DCs, B Cells, T cells and NK cells through release of TGF-β, IL-10 and IL-35 ↑ VEGF to promote angiogenesis	[2,33,43,68]

Table 1 (*continued*)

Functional group	Cell type	Effect of acidic TME	References
B cells	B lymphocytes	↓ recruitment to the TME Different B cell subsets exert different effects in different cancer types	[69,70,73]
	Regulatory B cells (Bregs)	Tumour metabolites and IDO recruit Bregs to tumour site ↑ IL-10, IL-35 and TGB-β production to inhibit T cells and stimulate FoxP3 expression in Tregs ↑ PD-1, PD-L1 and CTLA-4 expression to block T and NK cells ↑ adenosine production to suppress T cells Impairs Th17 response Suppresses IFN-γ release by CTLs	[69,71,75,77,78,80]

1.3. Stromal cells within the TME promote tumourigenesis

In solid tumours, cancer cells are embedded in stromal connective tissue that is also affected by the acidity of the TME [8]. Numerous bi-directional interactions occur between cancer and stromal cells, and the acidic TME can alter the composition and function of the stromal cells present [8]. Fibroblasts are abundant in the TME and play important roles in tumour growth, survival, proliferation, migration and invasion [25,26]. Fibroblasts are usually inactive, however through interactions with tumour cells and other TME components, they are stimulated to become cancer-associated fibroblasts (CAFs) that promote tumour growth. Although it remains to be seen whether the acidic pHe plays a direct role in the activation of CAFs, the acidic TME promotes tumour cells to release a number of factors which promote their activation including cytokines such as IL-6 and TGF-β, and growth factors such as epidermal growth factor (EGF), platelet derived growth factor (PDGF), and fibroblast growth factor 2 (FGF2) (Table 1) [1]. CAFs secrete matrix proteins and proteolytic enzymes that degrade the ECM, chemokines and growth factors that promote cell growth, proliferation and malignancy, as well as various cytokines that recruit immune cells to the TME (Table 1) [1,25]. CAFs can also contribute to extracellular acidification by secreting carbonic anhydrase 9 (CA IX) into the TME [8]. In head and neck squamous cell carcinomas (HNSCC), CAFs have been shown to have high glucose uptake, generate high levels of lactate, and have increased expression of GLUT-1 and monocarboxylate transporter 4 (MCT-4) [27]. They therefore further contribute to the acidity of the TME.

Endothelial cells line blood vessels in the TME, and are responsible for critical functions such as angiogenesis, transportation of immune cells, and intra- and extravasation of tumour cells during metastasis [28]. They adapt to survive low pHe, maintaining their pHi by extruding lactate via carbonic anhydrase II (CA II) [29]. Tumour cells release EVs that transfer the EGF receptor (EGFR) to ECs, activating the autocrine vascular endothelial growth factor (VEGF) pathway in ECs to promote angiogenesis [30]. Induction of VEGF expression by ECs also downregulate the expression of adhesion markers, such as intercellular cell adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), on blood vessels in the TME. This prevents CTLs from infiltrating the tumour [31]. VEGF also stimulates PD-L1 expression, inhibiting CTLs [31]. Tumour-associated ECs also preferentially attract immunosuppressive Tregs to the TME (Table 1) [31].

1.4. Acidosis in the TME drives immune escape by inhibiting effector cells while promoting immunosuppressive phenotypes

Immune cells comprise a large component of the TME, although their anti-tumour effects are mostly downregulated. The immune cell infiltrate is comprised of innate immune cells (macrophages, neutrophils, dendritic cells, innate lymphoid cells, myeloid-derived suppressor cells, and natural killer cells), as well as adaptive immune cells (T cells and B cells). The presence of effector cells including CD4 or CD8 T cells, and NK cells, is associated with good disease prognosis, while the presence of regulatory immune cells such as monocyte derived suppressor cells (MDSCs), Tregs, and regulatory B cells (Bregs) are linked to poor outcomes. During the early stages of tumour development, cancer cells in the TME stimulate a weak immune response. Over time these cells become resistant to the innate immune response, and go on to undermine and manipulate the adaptive immune response.

1.4.1. Monocyte derived suppressor cells

Monocyte derived suppressor cells comprise a heterogeneous group of immature, poorly differentiated myelomonocytic cells including monocytes, dendritic cells, macrophages, and neutrophils [32]. Tumour exosomes can drive monocyte differentiation to immunosuppressive MDSCs, and tumour cells secrete chemokine (C-X-C) motif ligand 1 (CXCL1) to recruit MDSCs to the TME [28,33]. In the acidic TME, MDSCs are activated by a number of cancer-derived signals including IL-1β, IL-4, IL-6, IL-13, toll-like receptors (TLRs), VEGF, and TGF-β [32,34]. MDSCs upregulate expression of arginase and inducible nitric oxide synthase (iNOS), thereby blocking activation of NK cells and T cells (Table 1) [35,36]. The numbers of circulating and TME-infiltrating MDSCs correlate with tumour progression and poor patient outcomes [37].

1.4.2. Dendritic cells

The acidic pHe inhibits DC maturation, allowing them to become tolerogenic. Tolerogenic DCs have reduced levels of co-stimulatory molecules such as CD80 and CD86, and increased levels of PD-L1 and CTLA-4 [28]. They also show reduced capacity to present antigen to T cells [10]. In mouse models, extracellular acidosis causes damage to DC cytoskeletal structure, with reduced DC migration and altered membrane charges and membrane fluidity of DCs [38]. This may reduce DC migration to lymph nodes and antigen presentation. Treatment of DCs with pH 6.5 medium also impaired DC ability to stimulate T cells [38]. Tolerogenic DCs release immunosuppressive molecules such as indoleamine 2,3-dioxygenase (IDO) and nitric oxide (NO) which suppress T cells, and immunosuppressive cytokines TGF-β and IL-10 which recruit Tregs (Table 1) [28].

1.4.3. Macrophages

Macrophages are actively recruited to tumours in response to growth factors such as VEGF, colony-stimulating factor 1 (CSF-1), and granulocyte–macrophage colony-stimulating factor (GM-CSF), cytokines (IL-6, IL-10 and TGF-β) and chemokines such as chemokine (C–C) motif ligand 2 (CCL2) and CCL8 released by

tumour and stromal cells in the TME [2,28,39]. Lactic acid in the TME drives macrophage polarization towards the M2 phenotype, with the release of anti-inflammatory cytokines, ROS production, and release of prostaglandins [2,40,41]. M2-like TAMs have poor antigen-presenting capabilities as they downregulate human leukocyte antigen (HLA) class II expression, and suppress T cells by releasing IL-10 and TGF-β [40].They release growth factors, cytokines and matrix metalloproteinases (MMPs) which further promote angiogenesis, tumour survival, invasion and metastasis (Table 1) [2,39,40,42]. High numbers of TAMs are found in many tumour types and are correlated with poor prognosis [40]. TAMs also release chemokines such as CCL22, which recruit Tregs to the TME [43].

1.4.4. Neutrophils

Neutrophils migrate to the tumour site in response to damage-associated molecular patterns (DAMPs), chemokines and cytokines, although their role in the TME is controversial. Environmental stimuli can drive neutrophil polarization towards either an anti-tumourigenic N1, or pro-tumourigenic N2 phenotype. Acidic extracellular pH was shown to polarize neutrophils to an N2 phenotype *in vitro* [44]. In murine models, the absence of TGF-β can induce anti-tumourigenic neutrophils (N1), that may recruit and stimulate activation of $CD8^+$ CTLs [45]. As the acidic TME stimulates release of TGF-β by tumour cells and certain immune cells, this may favour the development of the immunosuppressive N2 neutrophils. In addition to polarization, neutrophil function is influenced by acidic extracellular pH. Studies conducted *in vitro* show that acidic pH medium causes neutrophils to upregulate hydrogen peroxide production and myeloperoxidase (MPO) release [46]. Extracellular acidosis also delays neutrophil apoptosis, allowing the neutrophils to sustain an inflammatory response [46]. Neutrophils release neutrophil extracellular traps (NETs) that consist of nuclear or mitochondrial DNA, histones, and proteases [47]. The NETS stimulate the nuclear factor kappa B (NF-κB) pathway, block apoptosis, and stimulate cell proliferation. They promote ECM remodelling through release of proteolytic granules containing neutrophil elastase (NE), MMP-8 and MMP-9, and MPO [47,48]. Neutrophils also further contribute to acidosis in TME by the release of granular H^+ ATPases, which block NK and T cell activity (Table 1) [49].

1.4.5. Natural killer cells

T and B lymphocytes as well as NK cells are present at the tumour site. NK cells are vital for curtailing tumour growth, as they are able to directly kill tumour cells by using death receptor-mediated apoptosis, and by releasing perforin and granzyme [40]. Tumour cells escape NK cytotoxicity by downregulating their surface antigens. Natural killer group 2D (NKG2D) receptors that are vital for NK cell function are blocked by NKG2D ligands on tumour-derived exosomes [50]. NK cells are inhibited in the acidic TME. Acidic pH and lactate accumulation in the TME results in decreased IFN-γ production by NK cells [51]. Within the acidic TME, NK cells are also less efficient at producing pro-inflammatory cytokines, and their function is inhibited by cytokines secreted by tumour cells and other immune cells such as TGF-β and IL-10 [52]. When exposed to tumour-conditioned media of low pH, NK cells undergo apoptosis caused by decreasing intracellular pH and mitochondrial stress [53].

1.4.6. T cells (CD4, CD8 and Treg)

The intratumoural T cell population is heterogeneous and includes naïve, effector, memory, or regulatory T cells. CD8 + CTLs recognize tumour antigens presented on HLA molecules, and then release perforin-containing granules that damage the tumour cell membrane, and granzyme which causes apoptosis by damaging tumour cell DNA [54]. Once the CTL is activated, maturation and clonal expansion occur with the release of immuno-stimulatory cytokines including IL-2, IFN-γ, and TNF-α+ [54]. The release of these factors is important in sustaining anti-tumour immunity, and in developing immunological memory [54].

CTL activation is sustained by the presence of $CD4^+$ T cells, particularly Th1 cells, which provide essential cytokine support. Tumours evade a Th1 response through downregulation of HLA molecules, release of EVs and immunosuppressive cytokines, and loss of cell adhesion molecules such as ICAM-1. The acidic microenvironment however drives T cell differentiation towards Th2, Th17, or Treg polarisation [55]. Th2 and Th17 cells promote angiogenesis and tumour growth, and inhibit Th1 immunity [56]. Some T cells are sensitive to changes in pH and reduced glucose availability and are thus inhibited in the acidic TME [57].

The acidic TME affects T cell metabolism, as elevated glycolysis in cancer cells themselves can limit the availability of glucose to other cells [57,58]. T cells increase their glucose uptake when they encounter a stimulatory antigen in order to proliferate and support effector functions [58]. Owing to poor vasculature within the TME, there is tough competition between cells for nutrients [59]. Although tumour cells can become quiescent in the absence of glucose or switch to fatty acid metabolism, activated T cells cannot survive or expand without glucose [58]. Lactate, released as a waste product by tumour cells, downregulates T cell metabolism and blocks lactate export from T cells [59]. Lactic acidosis impairs TCR-triggered phosphorylation of c-Jun N-terminal kinase (JNK), c-Jun and p38 [60]. Acidosis impairs CTL function, blocks signalling through NFAT, reduces IL-2, IFN-γ, perforin and granzyme production, and increases anergy and apoptosis [58,61–63]. In the presence of lactic acid, CTL motility is reduced and contact with tumour cells is prolonged. This prolonged contact means that the number of tumour cells they interact with is reduced, and this slows down the rate of serial cytotoxic killing, allowing tumour cells to escape death [64].

T cells require both arginine and tryptophan to function. Tumour cells release IDO into the TME which converts tryptophan into kynurenine. This blocks T cell activation and proliferation, and promotes T cell apoptosis. A lack of tryptophan in the TME also causes downregulation of the TCR ζ-chain in cytotoxic T cells, which impairs their function [65]. The acidic TME also contains elevated levels of arginase 1 and (iNOS which deplete arginine [11,65]. Arginine is crucial to T cell function as it is also required for the expression of the TCR ζ-chain [35], and depleting it blocks T cell proliferation, activation, glycolysis, and cytokine production [11,66]. The acidic TME also reduces CTL motility and prolongs cell-CTL contact which reduces the number of CTL-tumour cell interactions and hence tumour cytolysis [64].

In contrast to its impact on T effector cells, the acidic TME is supportive of Treg presence and activity. Their presence correlates with poor outcomes in many tumour types. Tregs are recruited to the TME by stimuli such as hypoxia, the secretion of factors such as VEGF, HIF-α and IDO by tumours, and by tumour-associated chemokines such as TGF-β and CCL22 [43,67]. Studies in murine models show that unlike T effector cells, Tregs thrive under glucose restriction [68]. Instead of metabolizing glucose, tumour-associated Tregs upregulate pathways that metabolize lactic acid which is abundant in the TME. This increases Treg survival and proliferation, supporting the idea that the acidic TME provides metabolic support for Tregs [68]. Tregs contribute to the immunosuppressive TME by releasing additional TGF-β, IL-10 and IL-35, which suppress DCs, B cells, NK cells, and T cells [2,67] (Table 1). Tregs express granzyme B and perforin, allowing them to directly kill CTLs and NK cells [43]. They also express CTLA-4 and compete with T cells for CD28 binding, and downregulate CD80 and CD86 expression in APCs [43].

1.4.7. B cells and Bregs

The TME recruits B cells and controls the localization of the different B cell subsets [69,70]. Phenotypically distinct B cell subsets play different functional roles in tumour responses, and can be either pro- or anti-tumourigenic [71,72]. The B cell chemoattractant CXCL13, secreted by tumour cells and dendritic cells, recruits B cells to the tumour site [69]. Antigens released by tumour cells trigger a humoral response. These antigens can be expressed as a result of mutations, gene overexpression, or expression of aberrant markers not common to that cell type [72]. Some tumour-associated antigens are recognized by B cells, leading to the production of antibodies that aid in killing tumour cells through activation of the complement pathway and stimulating phagocytosis by macrophages, or by activating NK cells [72]. B cells also stimulate T cell responses such as T cell activation in response to antigen presentation, clonal expansion, and memory T cell formation [69]. In some instances, B cells may also exhibit direct cytotoxic activity, by killing tumour cells via granzyme B or via TNF-related apoptosis-inducing ligand (TRAIL/Apo-2L)-dependent mechanisms [69,73]. The presence of tumour infiltrating B cells is associated with good prognosis in some cancers (e.g. non-small cell lung carcinoma and melanoma) [70,71,74], and contribute to both humoral and cellular immunity [69]. In other cancers, such as head and neck squamous cell carcinomas and ovarian cancers, they have little to no beneficial impact [70]. This may in part be due to the high degree of functional plasticity and the high degree of heterogeneity in B cell subsets [70].

Bregs are a subpopulation of B cells that have immunoregulatory or suppressive properties. Unlike other cell types, there are a number of different Breg phenotypes depending on the tumour type and location [75], leading to the idea that they are not lineage specific but arise as a result of environmental stimuli [76]. Although the acidic TME recruits and promotes the differentiation of other immunosuppressive cells such as MDSCs, the direct effect of the acidic TME on Breg differentiation is not known. Tumour cell metabolites can induce Bregs via the peroxisome proliferator-activated receptor α (PPARα) [77], and IDO released by MDSCs has been shown to induce Breg differentiation *in vitro* [78]. Bregs inhibit other immune cells by releasing immunosuppressive cytokines such as IL-10, IL-35, and TGF-β [71]. Bregs release TGF-β which converts CD4+ T cells into Tregs [79], IL-35 which promotes Treg differentiation and impairs Th17 responses [75], while IL-10 producing Bregs suppress IFN-γ production by CTLs [80]. They also perform suppressive functions through direct contact with immune cells, and by upregulating PD-L1 and CTLA-4 they block T and NK cell responses [69]. Some Bregs subtypes ($CD73^+$) induce adenosine production, which causes T cell suppression [71]. The release of pro-inflammatory cytokines by DCs is also suppressed by Bregs, enabling Bregs to indirectly block Th1 and Th17 differentiation [76] (Table 1). Thus Bregs present in the

Table 2
Proton extruder system targets.

Effector molecular target	Therapeutic treatment	Rationale	References
Vacuolar-ATPase (V-ATPase) proton pump	Proton pump inhibitors (PPIs) e.g. omeprazole	Decrease pHi and prevention of protons being extruded into the TME. Increased T cell infiltration. Decreased cell proliferation Increase effects of chemotherapy	[90,91]
	Archazolid	Inhibits endocytic activation of the Rho-GTPase Rac1 and prevents metastasis. Induces apoptosis. Inhibits proliferation .	[92,93]
	Anti-epileptic drugs e.g. Phenytoin	Reduce tumour growth and metastasis	[94,95]
Sodium-hydrogen exchanger-1 (NHE1)	Amiloride or cariporide	Blocks Na + extrusion and pHi increase. Impairs growth factor-induced DNA synthesis Decreases proliferation	[86,96,97]
Voltage gated sodium channels (VGSCs)	Anti-epileptic drugs partially inhibit VGSCs	Inhibits tumour growth	[96]
	Antibiotics (e.g. clofazimine)	Increase mitochondrial ROS production and stimulate apoptosis	[98]
Monocarboxylate transporters (MCTs)	MCT inhibitor e.g. bioflavonoids	Partially inhibit MCT	[57,86,96]
	Ionidamine	Acts on MCT-1 to reduce pHi and sensitize cells to hyperthermia. Inhibits glycolysis and mitochondrial respiration	[99,100]
	Statins	Partially inhibit MCT-1 and MCT-4	[96,101]
Carbonic anhydrases (CAs)	CA inhibitor e.g. acetazolamide, sulfonamide	Impair growth of primary tumour. Reduces cell proliferation and metastasis. Stimulates T cell function	[86,96,102]
	Coumarins	Inhibits carbonic anhydrase IX and XII Inhibits tumour growth and metastasis	[86,103]
	Monoclonal antibody therapy e.g. Imatinib and nilotinib	Inhibit CA and tyrosine kinase activity.	[104]
	Small molecule inhibitor SLC-0111 (targets CA IX and CA XII)	Decrease in metastatic burden Reduced metastasis Inhibit tumour growth	[102]

TME help facilitate immune escape, and provide a potential therapeutic target for future immunotherapy research.

1.5. The acidic tumour microenvironment is a therapeutic target

Chemotherapy is the leading cancer therapy worldwide, alone or in combination with surgery and/or radiotherapy, but limitations including drug resistance exist, with increased instances of relapse, and increased morbidity and mortality for patients [4].

An acidic microenvironment reduces the effectiveness of anti-tumour immunity [81]. The acidic TME alters drug structure and charge. This reduces their uptake into tumour cells, and affects the delivery and efficacy of anti-cancer drugs, as well as chemotherapy and radiation [9,10]. This has prompted evaluation of the TME as a therapeutic target. Elements of the TME provide promising targets for therapy, including the ECM, immune cells, and stromal cells. Trials targeting tumour-associated leukocytes have had variable and limited success [82–85].

General buffer therapy is a direct approach to target tumour pH. In a murine model, neutralization of tumour acidity by bicarbonate increased susceptibility to checkpoint inhibitors such as anti-CTLA-4 and anti-PD-1, and improved survival [81]. Research into inhibiting proton extruder systems in humans is being explored (summarized in Table 2), but as these systems are redundant, targeting more than one may be necessary for successful treatment. The use of proton pump-inhibitors (PPIs) and other drugs to prevent extrusion of protons by tumour cells look promising to increase pHe. An important caveat is the impact of these interventions on the pHi and normal cellular processes [86].

Other approaches exploit the acidic environment including use of pH-sensitive drug delivery systems to deliver drugs selectively to the tumour [86]. pH-sensitive nano-carriers including peptides, liposomes, and polymeric nanoparticles (NPs) have been used with some success. These systems remain stable and intact at normal physiological pH, but are released in acidic environments [86]. This targeted therapy reduces systemic toxicity and allows more predictable pharmacokinetics [4,86]. In mouse models, pH-responsive acetazolamide nanoparticles (ACE-NPs) disintegrate in acidic solution, resulting in rapid release of acetazolamide (ACE) which inhibits expression of CA IX and increases the alkalinity of the pHe [87].

Some NPs target immune cells within the TME and reduce the effects of hypoxia and acidity. Manganese dioxide NPs reduce hypoxia in cancer spheroids by catalysing degradation of hydrogen peroxide to produce oxygen. This revived NK cell cytotoxic activity [88]. Calcium-assisted NPs promote T cell activity and proliferation and prevent relapse and activity in a breast cancer mouse model [89].

2. Conclusion

Tumour cell metabolism drives an acidic TME, selecting for tumour cells with evolved survival strategies in a hypoxic and hypoglycaemic environment. The acidic TME inhibits immune cell activity and has implications for prognosis and treatment. Despite compelling evidence from *in vitro* and animal studies, few drugs that interfere with tumour pH have reached clinical trials because of redundancy in pH-mediating proteins in humans being targeted. New approaches to therapy should consider the acidic microenvironment. The use of pH-responsive nanoparticles is promising, as these can be used to affect tumour cells directly, impact on the TME, and positively influence the proliferation and activity of immune cells. In combination with other conventional therapies currently used, manipulating the acidic TME may aid in successful treatment of cancer patients.

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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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Chapter 4: Inducing Apoptosis using Chemical Treatment and Acidic pH, and detecting it using the Annexin V Flow Cytometric Assay

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Following a comprehensive review of the acidic tumour microenvironment, an experimental protocol was developed to stimulate low pH in cell culture. Environmental stress, such as low pH, impacts on tumour life histories as it affects tumour cell physiology and survival, and drives cancer evolution and immune escape. This protocol describes the method that was optimized to expose breast carcinoma and OSCC cell lines to acidic pH and utilizing PCD as a readout of exposure. The pH of the medium was adjusted with hydrochloric acid (HCl), and a range of acidity was examined (between pH 3.2 and 7.5). Different stages of the PCD process were detected by the flow cytometric assessment of cells staining with Annexin V and propidium iodide (PI). This protocol provides a robust method for investigating adaptation to low pH, and was then used to measure the impact of low pH and apoptosis on life history trade-offs such as cell survival, proliferation, and immune evasion in Chapter 5. In support of open science, the details of the method developed here and used in further experiments is included in Appendix F and can be accessed online at protocols.io too (dx.doi.org/10/17504/protocols.io.b2dfqa3n).

Contribution of the candidate: Performed experiments, analysed data, conceptualized, designed, wrote, and edited the paper.

PLOS ONE

LAB PROTOCOL

Inducing apoptosis using chemical treatment and acidic pH, and detecting it using the Annexin V flow cytometric assay

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Abstract

Cell death is important in physiology, and can happen as a result of structural damage, or as a sequence of programmed cellular processes known as apoptosis. Pathogenic alterations in apoptosis occur in a number of diseases, including cancer, viral infections, autoimmune diseases, immunodeficiencies, and degenerative conditions. Developing accurate and reproducible laboratory methods for inducing and detecting apoptosis is vital for research into these conditions. A number of methods are employed to detect cell death, including DNA fragmentation, the TUNEL assay, and electron microscopy although each has its limitations. Flow cytometry allows for the distinction between live, early apoptotic, late apoptotic and necrotic cells. In this protocol we successfully induce apoptosis using chemical treatment and treatment with low pH in solid tumour cell lines, and have optimized detection using the Annexin V/PI apoptosis assay.

Introduction

The importance of studying death in cell culture is becoming increasingly apparent. Cell death is a key component of physiology, and can happen either passively as a result of structural damage (necrosis), or actively as a sequence of programmed cellular processes such as apoptosis. Apoptosis or programmed cell death (PCD) is a genetically organized process that occurs when survival and proliferative signals are removed, or when the cell has suffered DNA damage [1]. Cells undergoing PCD are broken down into membrane-bound apoptotic bodies or vesicles which are taken up by neighbouring cells and phagocytes [1]. These apoptotic vesicles aid cell-to-cell communication, and are able to transmit nucleic acid, proteins, and lipids to other cells, aiding in both wound healing and cell survival [2, 3]. In contrast, necrosis is characterized by swelling of the cell and mitochondria, and cell membrane rupture with release of inflammatory cellular contents into the surrounding microenvironment [1]. Aberrations in

PCD are pathogenic in many processes. Reduced PCD is associated with malignancies, persistent viral infections, or autoimmune disease, whereas excessive PCD may cause immunodeficiency or degenerative disease [4].

The role that PCD plays in cancer progression is controversial. Apoptosis can promote cancer, and dying cells can affect their environment by stimulating the proliferation of neighbouring cells, affecting intra-tumoural cell competition, and exerting paracrine effects on the tumour microenvironment (TME) [5]. High levels of apoptosis in some tumours correlate with poor prognosis [5]. Prostaglandin E2 (PGE2) is released by apoptotic cells, and has pleiotropic effects on surrounding cells, including proliferative and immune evasive effects [5]. Other studies however show that cancer cells block apoptosis by preventing p53 signalling, overexpressing antagonists to apoptotic pathways, immune evasion, and inducing apoptosis of lymphocytes via programmed death ligand-1 (PD-L1) [6]. As cell death contributes to a number of adverse processes, understanding how it is regulated may provide insight into disease pathology.

1. Inducing apoptosis in the laboratory

Any environmental agent or condition that stresses the cell can cause cell death. In cell culture systems, the severity and type of cell death can be controlled. Death can be induced by heat [7], ultra-violet (UV) light [7, 8], various chemicals [7, 9], production of reactive oxygen species (ROS) [7, 10, 11], nutrient or growth factor deprivation [10–12], Fas introduction [8], cytokines [11, 13], viruses [12], and acidosis [10, 11]. Cultured cells studied in isolation from their normal physiological surroundings often behave differently than *in vivo* as they are unable to communicate with neighbouring cells, and elements of their microenvironment. Our aim was to develop a robust method of inducing apoptosis in cultured cells that would mimic their natural microenvironment. As the tumour microenvironment is typically more acidic [14], we used culture media of decreasing pH to induce cell death and compared this to cell death induced by chemicals such as dimethyl sulfoxide (DMSO) and stauroporine (STS) which have been well reported [9, 11, 15, 16].

2. Confirming apoptosis in the laboratory

A number of techniques can be used to detect apoptosis. Some physical features of PCD can be observed by microscopy [17]. Cells undergoing PCD have distinct morphological features including cell shrinkage, plasma membrane blebbing, with nuclear condensation and DNA fragmentation [4]. Other techniques to study PCD include observing DNA fragmentation, detecting changes in caspase and mitochondrial activity, or changes in phosphatidylserine (PS) detection. These techniques all have their positive and negative attributes which have been well described elsewhere [12, 17, 18] and are summarized in Table 1.

Flow cytometry has many advantages over other techniques, as it is rapid, and can be performed on relatively simple analysers. The technique is quantitative, and analysis of cell death on individual cells or cell populations can be performed [19]. Cells undergoing PCD can be distinguished from viable cells based on their light scatter properties. In early apoptosis, cell shrinkage occurs while the cell membrane remains intact, causing a decrease in cell size measured by forward scatter (FSC) while cell composition, measured by side scatter (SSC), remains the same. As PCD progresses, changes in both FSC and SSC occur. As the cell membrane becomes more permeable, the cell shrinks causing a decrease in cell size. Nuclear degranulation increases side scatter [4]. During necrosis however, the cytoplasm swells causing an increase in cell size and FSC, while release of intracellular contents causes a decrease in SSC [4]. FSC and SSC alterations alone are however insufficient in distinguishing between apoptotic and necrotic cells.

Table 1. Comparison of apoptosis assays.

Assay	Apoptotic characteristic measured	Benefits	Limitations
DNA laddering	DNA fragmentation by endonucleases	Inexpensive	Only detects late apoptosis
			Needs apoptosis in a large number of cells to be detects
TUNEL (Terminal dUTP Nick-End Labeling)	3'end labelling of DNA fragments	Commercial kits available	High cost
			Fixation and pretreatment affect DNA strand breaks
			Doesn't distinguish between apoptosis and necrosis
Caspase-3 Activity	Measure activity of caspase-3 which is activated in the terminal apoptosis cascade	Quantitative	Integrity of tissue destroyed
			Lots of tissue needed to detect activity
			Can't determine which cell is apoptotic
Mitochondrial assays	Mitochondrial redox status, Ca^{2+} increase, reactive oxygen species (ROS), mitochondrial permeability, mitochondrial depolarization	Assayed in living cells	Difficult to distinguish between apoptosis and necrosis
Viability dyes	Bind to exposed nucleic acids	Fast	Unable to distinguish between apoptosis and necrosis
		Inexpensive	Cannot penetrate thick tissue
Microscopy (TEM or fluorescent)	Morphological changes including membrane blebbing, cell swelling, and nuclear condensation	Structural characteristics of apoptosis observed	Time-consuming
			Limited samples can be analysed
Annexin V assay	Phosphatidylserine exposure on plasma membrane	Detects early apoptosis	Expensive in whole animal studies
		Can be combined with other multiple labels of other proteins or DNA	
		Quantitative	

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Based on the benefits of being able to distinguish between early and late PCD, we used the Annexin V assay performed by flow cytometric methods (FCM). In early PCD, although the plasma membrane is intact, PS begins to be redistributed from the cytoplasmic surface to the exterior of the plasma membrane. As PCD progresses, PS is completely exposed and the plasma membrane becomes more permeable. Annexin V binds to PS and is labelled with a fluorophore that is detected by a flow cytometer [20]. As a number of markers can be used simultaneously in flow cytometry, multiple cell death characteristics in different cell populations can be detected at the same time. FCM also distinguishes between live and dead cells by measuring the uptake of viability dyes such as propidium iodide (PI). The PI intercalates with exposed DNA, and the fluorescence detected indicates how much dye is bound. As the plasma membrane is intact in viable cells and those undergoing early PCD, the PI is excluded. As the plasma membrane becomes more permeable as PCD progresses, more and more dye is able to enter the cell [19]. Viability dyes alone are, however, inadequate to distinguish different stages of PCD. By using Annexin V and PI in one assay, we were able to distinguish between different stages of cell death, including viable, early PCD, late PCD and dead or necrotic cell populations (Fig 1).

Materials and methods

The protocol described in this article is published on protocols.io dx.doi.org/10.17504/protocols.io.b2dfqa3n and is included for printing as S1 File with this article.

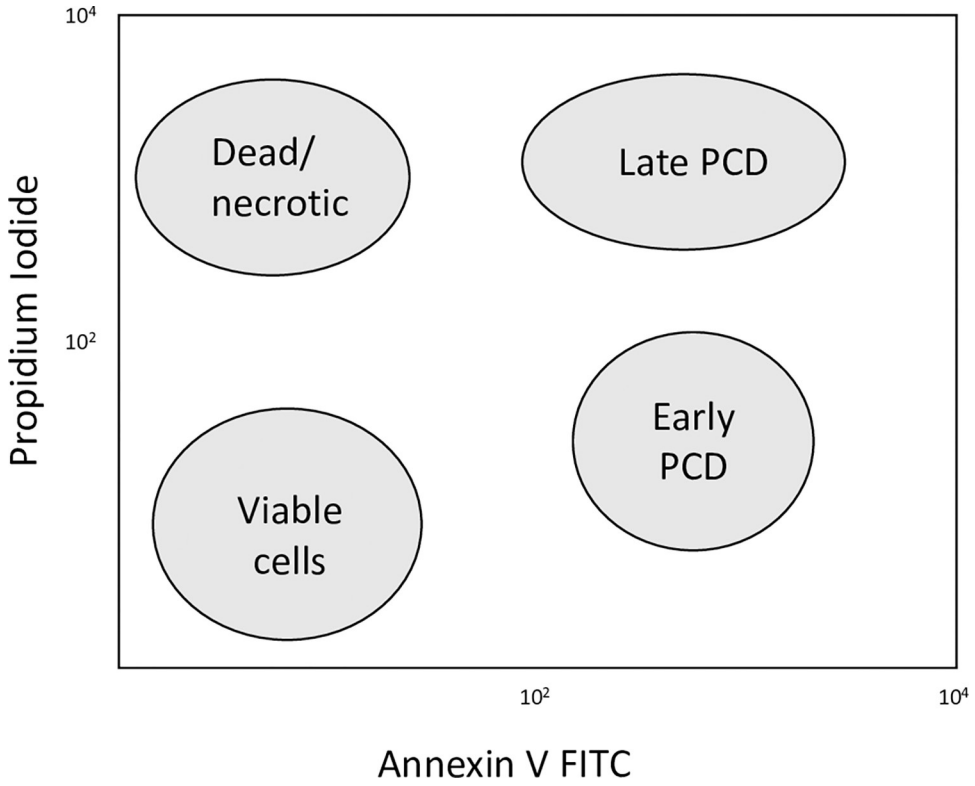

Fig 1. An example of a flow cytometry plot showing stages of cell death. Viable cells do not bind Annexin V or take up PI. Cells in early PCD bind Annexin V but still exclude PI. As the cell membrane becomes more permeable, cells in late PCD bind to Annexin V and PI. Dead or necrotic cells do not bind to Annexin V, but do take up PI.

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This study was conducted on long-established cell lines, and no additional patient consent was needed. This study was approved by Human Research Ethics Committee (HREC) of the University of the Witwatersrand (approval M120205).

Expected results

The assay has been optimised in both breast (MCF-7) and oesophageal squamous cell carcinoma cell lines (SNO). Cells were treated with DMSO and STS, and cell death was measured by flow cytometry. We monitored morphological changes of the cells treated with DMSO and STS under the microscope every 15 minutes. At 30 minutes, no morphological change was observed. At 1 hour, some cells were starting to round up, and at 2.5 hours, some cells had detached and were floating in the medium.

An example of PCD induction in the MCF-7 and SNO cell lines is given in Fig 2. An untreated control should always be included, as the death detected here can be attributed to the harvesting of the cells with enzymatic treatment with TE buffer, and death that may have occurred during centrifugation and assay preparation. Differences in cell death are noticeable between treated and untreated cells, as DMSO and STS exhibited much higher levels of cell death than the untreated control. We observed that cells treated with STS became smaller and less complex as death was induced. STS caused more cell death than DMSO.

In order to determine the effects of pH, SNO cells were treated with media of decreasing pH (pH 7.5, 6.9, 6.5, 3.2) (Fig 3). After 6 hours, we performed the FCM assay, and measured PCD of the different treatments. Cells treated with media of pH 7.5 appeared very much like

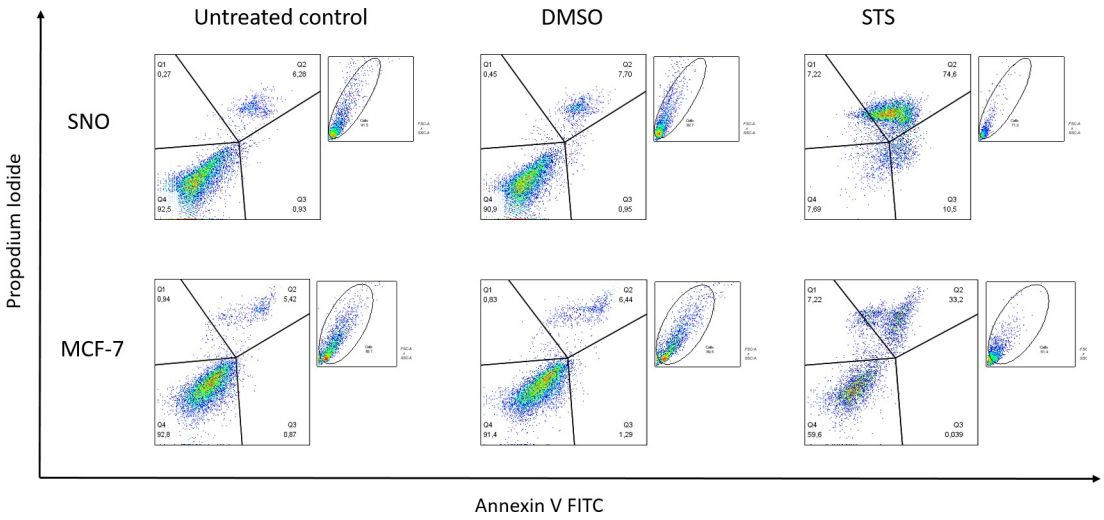

Fig 2. Induction of programmed cell death in an MCF-7 breast carcinoma and SNO oesophageal squamous cell carcinoma cell lines.

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the untreated control. As pH decreased to 6.5 however, more cells were detected in early and particularly late PCD. As the media became more acidic, cells tend to follow a more necrotic form of cell death. We suggest that researchers try the assay with media of varying pH levels to determine the correct pH for their work. Time of treatment also needs to be carefully monitored in order to detect cells still in early PCD.

Conclusion

We successfully optimised induction of cell death in solid tumour cell lines. Lowering pH mimics the acidic TME, and as pH decreases PCD is induced. The flow cytometric Annexin V/PI assay is a useful tool in distinguishing between viable cells, and those in early and late PCD, and can be combined with other markers to characterize PCD in different cell populations. This protocol will assist researchers in studying PCD in a range of tumour cell lines.

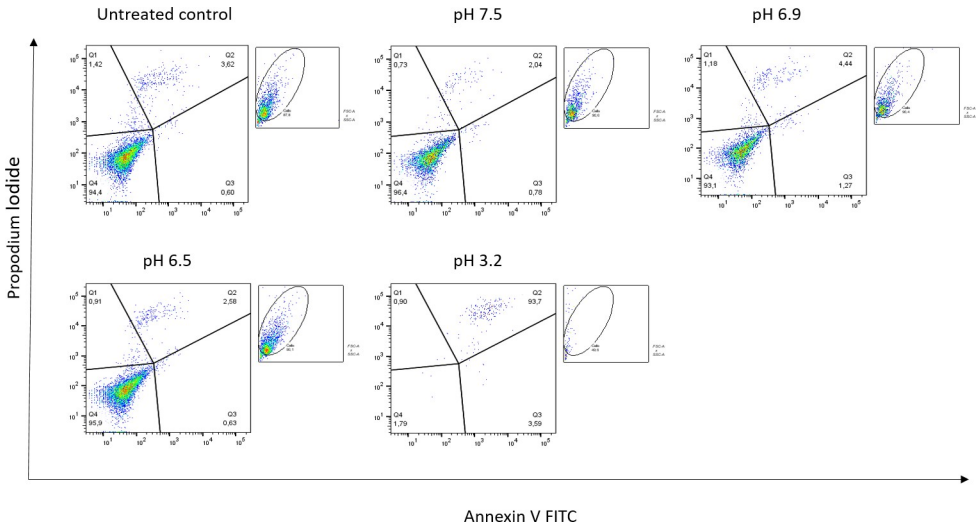

Fig 3. Decreasing pH causes cell death in SNO cell line.

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Supporting information

S1 File. Loaded on protocols.io dx.doi.org/10.17504/protocols.io.b2dfqa3n.
(PDF)

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Chapter 5: The Effect of Acute Acid Exposure on Immunomodulatory Protein Secretion, Cell Survival, and Cell Cycle Progression in Tumour Cell Lines

Publication: Worsley CM, Veale RB, Mayne ES. “The Effect of Acute Acid Exposure on Immunomodulatory Protein Secretion, Cell Survival, and Cell Cycle Progression in Tumour Cell Lines”. *Cytokine*; 2023, 162: 156118*

Utilising the protocol developed in Chapter 4, cell lines from commonly occurring cancers in South Africa were exposed to low pH. Acidity affected the life history traits of survival, proliferation, and immunomodulation to a different extent in each cell line. Moderate acidity caused apoptosis in some populations in both cell lines, but high levels of acidity had a greater effect on viability. The secretion of cytokines and growth factors were reduced, though to a lesser extent in the WHCO6 cell line. Both cell lines were exposed to conditioned media (CM) from these apoptotic cells which promoted viability and proliferation in WHCO6, but caused further apoptosis in MCF-7. Following queries from reviewers, the effect of apoptosis on the cell cultures was examined more closely in additional experiments. Growth factors such as granulocyte colony-stimulating factor (G-CSF) were significantly increased in WHCO6 and likely support the increase in viability and proliferation, while myoglobin and growth/differentiation factor 15 (GDF-15) levels are indicative of the cell damage and death observed in MCF-7. These data highlight the differences in life history strategies adopted by the two cell lines in keeping with their ecological niche. These results support the hypothesis that selective pressure such as low pH and apoptosis drive different trade-offs that affect life history strategies in different tumours. These data support the need to understand evolutionary clonal dynamics in order to improve treatment and control of this disease.

Contribution of the candidate: Performed experiments, analysed data, performed statistical analysis, conceptualized, designed, and wrote the paper.

*Erratum: the following caption was omitted from Figure 3 in the final published article - “Compared to the untreated control (A), DMSO (B) and STS (C) induced significant cell death. Early and late apoptosis was observed in cells incubated with pH 6.5 media for 15 min (D) and 1 hr (E), whereas most cells were in late PCD in cells treated with pH 3.2 media (F and G).” The journal editor has been notified of the omission and a corrigendum has been issued by the journal (Appendix H).

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The effect of acute acid exposure on immunomodulatory protein secretion, cell survival, and cell cycle progression in tumour cell lines

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ABSTRACT

Cancer develops when multiple systems fail to suppress uncontrolled cell proliferation. Breast cancers and oesophageal squamous cell carcinoma (OSCC) are common cancers prone to genetic instability. They typically occur in acidic microenvironments which impacts on cell proliferation, apoptosis, and their influence on surrounding cells to support tumour growth and immune evasion. This study aimed to evaluate the impact of the acidic tumour microenvironment on the production of pro-tumorigenic and immunomodulatory factors in cancer cell lines. Multiple factors that may mediate immune evasion were secreted including IL-6, IL-8, G-CSF, IP-10, GDF-15, Lipocalin-2, sICAM-1, and myoglobin. Others, such as VEGF, FGF, and EGF that are essential for tumour cell survival were also detected. Treatment with moderate acidity did not significantly affect secretion of most proteins, whereas very low pH did. Distinct differences in apoptosis were noted between the cell lines, with WHCO6 being better adapted to survive at moderate acid levels. Conditioned medium from acid-treated cells stimulated increased cell viability and proliferation in WHCO6, but increased cell death in MCF-7. This study highlights the importance of acidic tumour microenvironment in controlling apoptosis, cell proliferation, and immune evasion which may be different at different anatomical sites. Immunomodulatory molecules and growth factors provide therapeutic targets to improve the prognosis of individuals with cancer.

Abbreviations: 5-PL, 5 prime logistic; 7-AAD, 7-Amino Actinomycin D; ANOVA, analysis of variance; APC, allophycocyanin; BrdU, Bromodeoxyuridine; CM, conditioned media; CO_2, carbon dioxide; DMEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethyl sulfoxide; ECM, extracellular matrix; EDTA, ethylenediaminetetra-acetic acid; EGF, epidermal growth factor; EMT, epithelial-mesenchymal transition; EV, extracellular vesicle; FAK, focal adhesion kinase; FCS, foetal calf serum; FGF, fibroblast growth factor; FITC, fluorescein isothiocyanate; G-CSF, granulocyte colony-stimulating factor; GDF-15, growth/differentiation factor 15; GM-CSF, granulocyte-macrophage colony-stimulating factor; GRO-α, growth-regulated oncogene-alpha; HCl, hydrochloric acid; hr, hour; HREC, Human Research Ethics Committee; IFN-γ, interferon-gamma; IL-10, interleukin-10; IL-1β, interleukin-1 beta; IL-2, interleukin-2; IL-4, interleukin-4; IL-5, interleukin-5; IL-6, interleukin-6; IL-8, interleukin-8; IP-10, IFN-γ inducible protein-10; LFA-1, lymphocyte function-associated antigen-1; MCP-1, monocyte chemoattractant protein-1; MCP-2, monocyte chemoattractant protein-2; MCP-3, monocyte chemoattractant protein-3; MDSC, monocyte derived suppressor cell; MIG, monokine induced by gamma interferon; min, minute; MIP-1α, macrophage inflammatory protein-1 alpha; MIP-1β, macrophage inflammatory protein-1 beta; ml, millilitre; MPO, myeloperoxidase; NK, natural killer; NKG2D, natural killer group 2D; NO, nitric oxide; OSCC, oesophageal squamous cell carcinoma; PBS, phosphate buffered saline; PCD, programmed cell death; pg, picogram; pHe, extracellular pH; PI, propidium iodide; PI3K, phosphatidylinositol 3-kinase; PS, phosphatidylserine; rpm, revolutions per minute; ROS, reactive oxygen species; SA-PE, streptavidin-phycoerythrin; SDF-1α, stromal-derived factor 1 alpha; sICAM-1, soluble intracellular cell adhesion molecule-1; STS, staurosporine; sVCAM-1, soluble vascular cell adhesion molecule-1; TAM, tumour-associated macrophage; TE, trypsin EDTA; TME, tumour microenvironment; TNBC, triple-negative breast cancer; TNF-α, tumour necrosis factor alpha; Treg, regulatory T cell; μl, microliter; VEGF, vascular endothelial growth factor.

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1. Introduction

Cancer is a leading cause of death globally especially in individuals under 70 years of age [1]. Breast cancer is the most commonly diagnosed cancer with the highest mortality rate in women, while oesophageal carcinoma has the 6th highest mortality rate globally [1]. In South Africa, breast cancer is most common [1], while oesophageal squamous cell carcinoma (OSCC) is the most common cancer in men, and second most common cancer in woman in certain regions [2]. Tumours are typically detected at advanced stages and patients have a poor 5-year survival rate [3].

Darwinian evolution drives somatic evolution of cell populations with uncontrolled replication, and the evolution of genes that influence cancer risk [4,5]. The failure of multicellular systems to suppress somatic evolution, causes the breakdown of cooperative behaviours and regulatory networks in cancer cells [6]. Tumours are dynamic ecosystems in which cell clones face selective pressures and competition and must develop strategies to enhance fitness [7]. As the tumour microenvironment (TME) is resource-limited, cancer cells face trade-offs among competing energy demands and life-history traits such as development, survival, proliferation, dispersal, and cell plasticity [8].

Understanding tumour cell behaviour is key to developing effective treatment. Tumours modulate cytokine, chemokine, and growth factor secretion in response to environmental stimuli such as hypoxia, acidosis, chemotherapeutic drugs, and ionizing radiation [9–12]. These soluble factors also affect other non-tumour cells within the TME, and may determine survival or apoptosis in these bystanders [9]. They may also drive evasion of immune destruction by targeting both innate and adaptive immune cells. Tumourigenic cytokines inhibit cytotoxic T cell and natural killer (NK) cell activity, recruit immunosuppressive cells like regulatory T cells (Tregs), and promote the development of tumour associated macrophages (TAMs), neutrophils, and myeloid derived suppressor cells (MDSCs) [13].

Studies conducted in unicellular organisms may further aid in understanding tumour behaviour. Durand *et al.* showed that when apoptosis or programmed cell death (PCD) was induced in the unicellular chlorophyte *Chlamydomonas reinhardtii,* cellular components were released by apoptotic cells that promoted the growth of cells of the same species, while inhibiting the growth of competing species [14]. Additionally, cellular components released by cells undergoing necrosis or non-PCD forms of death were harmful [15]. Tumour cells display unicellular-like characteristics, and this model may be extrapolated to tumour cell behaviour. Within tumours, apoptosis may promote proliferation in neighbouring cells to compensate for cell loss induced by stress or treatment, allowing for expansion of more resistant clones [16–19], while necrosis may promote death in neighbouring cells through the bystander effect [20,21]. In addition, how tumour cells die, and the specific type of cell death they undergo has different immunomodulatory effects that may be therapeutically important [22].

A key feature of the tumour microenvironment is that it is acidic. It ranges from pH 6.5 and 7.0, but pH can drop even lower in regions with high glycolytic activity, low oxygen levels, and poor removal of waste and H^+ ions [23]. Lactic acid and low extracellular pH (pHe) in the TME block effector immune cell functions while promoting tolerogenic phenotypes, contributing to immune escape and cancer progression [13]. Constant exposure to acidic pH could result in the selection of more aggressive tumour phenotypes [24]. Both breast cancers and OSCC are characterized by high levels of genetic instability [25,26], and it is likely that the acidic TME impacts on this to drive tumour progression in a Darwinian model.

Extracellular acidity may impact on survival of cancer cells, selecting for clones adapted to extreme environments. To investigate the effects of environmental acidity on tumour cells, OSCC and breast carcinoma cell lines were exposed to acidic pH, and the effects of acute acid exposure on PCD, proliferation, and secretion of cytokines, chemokines, and growth factors were determined. The effects of conditioned media on PCD, proliferation, and survival of related cancer cells was also measured. The results of this study highlight the acidic tumour microenvironment as well as immunomodulatory factors detected as potential therapeutic targets.

2. Materials and methods

2.1. Cell culture

This study was approved by Human Research Ethics Committee (HREC) of the University of the Witwatersrand (approval M120205). The human OSCC cell line WHCO6, and the breast carcinoma MCF-7 cell lines were obtained from the Cell Biology Laboratory in the School of Molecular Cell Biology at the University of the Witwatersrand. Both cell lines were free of *Mycoplasma spp*. Cells were maintained in a solution of Dulbecco's Modified Eagle's Medium (DMEM)/Hams F12 (Sigma Aldrich, USA) (3:1) supplemented with 10% foetal calf serum (FCS) (Sigma Aldrich, USA) with 2% penicillin/streptomycin (Sigma Aldrich, USA), at 37 °C with humidity and 5% CO_2 in air. Cells were grown in a monolayer to 80% confluency, before being harvested using trypsin and ethylenediaminetetra-acetic acid (EDTA) (TE) (Merck Millipore, USA). Cells were counted and seeded into culture dishes and were incubated in DMEM/Hams F12 with 10% FCS for 24 h as described above before acid or chemical treatment.

2.2. Cell treatment with acidic and conditioned media

Culture media was removed after 24 h, and cells were washed with phosphate buffered saline (PBS) (Merck Millipore, USA). Cells were treated with culture media adjusted to moderate acidity pH 6.5 (representing a typical pH for solid tumours) and severe acidity pH 3.2 (mimicking the effects of bile and gastric acids within the oesophagus) as described in previous work [27]. Hydrochloric acid (HCl) (Sigma-Aldrich, USA) (1 N) was used to adjust the pH of the culture media, and the pH was measured using the PH50 Benchtop pH meter (Lasec, South Africa). Cells were incubated in acid-adjusted media for 15 min and 1 h. Cells were washed with PBS and reconditioned with serum-free DMEM/Hams F12 medium of pH 7.4 at 37 °C for 24 h. An untreated control culture incubated in serum-free DMEM/Hams F12 medium at pH 7.4 was also included. Conditioned media (CM) was removed and stored for further work below. Dimethyl sulfoxide (DMSO) (Sigma Aldrich, USA) and Staurosporine (STS) (Immunochemistry Technologies, USA) were included as positive controls as these induce apoptosis [28,29]. Additional cultures of WHCO6 and MCF-7 cells were incubated in CM from acid-exposed cells for 24 h.

2.3. Flow cytometric analysis of apoptosis

After treatment with acidic media, DMSO, and STS, or CM, cells were harvested and stained with the fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection Kit II (BD Pharmingen, BD Biosciences, USA) [27]. Conditioned media (CM) was removed and stored at −20 °C for further analysis. Adherent cells were harvested using TE mixture. Cells that were no longer adherent and were floating in the medium were combined with the adherent cell/TE mixture and the combination was centrifuged at 1 000 rpm for 5 min to pellet floating cells. Cells were washed and resuspended in PBS, then centrifuged. The cell pellet was resuspended in Annexin V Binding buffer, and cells were stained with Annexin V FITC antibody and propidium iodide (PI) for 15 mins. Binding buffer was added, and cells were acquired on the BD LSR II flow cytometer (BD Biosciences, USA).

2.4. Cell cycle analysis by bromodeoxyuridine incorporation

Cell cycle analysis was determined by bromodeoxyuridine (BrdU) incorporation into nuclei during the S phase of the cell cycle using the

allophycocyanin (APC) BrdU Flow Kit (BD Pharmingen, BD Biosciences, USA). BrdU was added to overnight cultures (1 μl BrdU per 1 ml culture media). Floating cells were collected, and adherent cells were harvested. Supernatants were removed, and the cells were washed in PBS and centrifuged (Allegra X-22, Beckman Coulter) to form a cell pellet which was then incubated in cytofix/perm buffer at 4 °C for 30 min, 10 min, 10 min and 5 min following washing in PBS. Following the final incubation, the pellet was resuspended in DNAse, incubated for 1 hr at 37 °C and resuspended50μl perm/wash buffer. The cells were incubated with anti-BrdU APC antibody for 20 min in the dark at room temperature. Cells were washed in perm/wash buffer at 300g for 5 min. The pellet was resuspended in 7-Amino Actinomycin D (7-AAD) viability dye and PBS and acquired on the BD LSR II flow cytometer (BD Biosciences, USA).

2.5. Gating strategy for data analysis

Gating was applied to flow cytometry dot plots as described previously [27]. Debris were excluded based on light scatter properties in the untreated control tube, while the remaining cells were used for further analysis of apoptosis. In the Annexin V FITC/PI dot plots, spider gates divided the discrete cell populations into 4 quadrants, namely live (Annexin V-, PI-), early apoptosis (Annexin V+, PI-) late apoptosis (Annexin V+, PI+), and dead (Annexin V-, PI+). The same gates were applied to all subsequent samples for each cell line.

For cell cycle analysis, the following discrete populations were gated in the untreated control, according to the kit manufacturer's recommendations: G_0/G_1 (BrdU-, 7-AAD-), S phase (BrdU+, 7-AAD+/-), and G_2/M phase (BrdU-, 7-AAD+). The same gates were applied to all subsequent samples. All data were analyzed using FlowJo™ v10.8 software (BD Life Sciences).

2.6. Protein expression by xMAP Luminex assays

Protein concentrations in CM from untreated and acid exposed cells were measured using the assays listed in Table 1. All assays were supplied by Invitrogen (USA) unless otherwise stated, and manufacturer's instructions were followed. CM was thawed on ice, assay standards and samples were prepared and tested in duplicate and were incubated with micro beads coated in the antibodies to the analytes of interest. A fluorescent conjugate of streptavidin–phycoerythrin (SA-PE) was added to bind to antibody-analyte complexes. A fluorescent readout was obtained by the Luminex Bio-Plex 200 analyzer (Bio-Rad, USA) using Bio-Plex Manager v 5.0 software (Bio-Rad, USA). Analyte concentrations of samples were determined from the 5-prime logistic (5-PL) standard curve and the means of the replicates were used for analysis.

Table 1
Luminex assays and analytes tested.

Assay	Analytes
Human Cytokine 10-Plex Panel (Cat # LHC001)	IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, GM-CSF, IFN-γ, TNF-α
Human Chemokine 10-Plex Panel (Cat # LHC6001)	IP-10, Eotaxin, Rantes, MCP-1, MCP-2, MCP-3, GRO-α, MIG, MIP-1α, MIP-1β
Human Growth Factor 4-Plex Panel (Cat # LHC0004)	VEGF, G-CSF, EGF, FGF
Stromal-derived factor 1 alpha (SDF-1α) Human ProcartaPlex™ Simplex Kit (Cat # EPX01A-12138–901)	SDF-1α
Human Cardiovascular Disease (CVD) Magnetic Bead Panel (Cat # HCVD2MAG-67 K, Merck Millipore, USA)	ADAMTS13, GDF-15, Lipocalin-2, MPO, myoglobin, sICAM-1, sVCAM-1, P-selectin

Abbreviations used in Table 1: EGF – epidermal growth factor; FGF – fibroblast growth factor; G-CSF – granulocyte colony-stimulating factor; GDF-15 – growth/differentiation factor 15; GM-CSF – granulocyte–macrophage colony-stimulating factor; GRO-α, growth-regulated oncogene-alpha; IFN-γ – interferon-gamma; IL-10, interleukin-10; IL-1β – interleukin-1 beta; IL-2, interleukin-2; IL-4 – interleukin, 4; IL-5, interleukin-5; IL-6, interleukin-6; IL-8, interleukin-8; IP-10 – IFN-γ inducible protein-10; MCP-1 – monocyte chemoattractant protein-1; MCP-2 – monocyte chemoattractant protein-2; MCP-3 – monocyte chemoattractant protein-3; MIG – monokine induced by gamma interferon; MIP-1α – macrophage inflammatory protein-1 alpha; MIP-1β – macrophage inflammatory protein-1 beta; MPO – myeloperoxidase; SDF-1α – stromal-derived factor 1 alpha; sICAM-1 – soluble intracellular cell adhesion molecule-1; sVCAM-1 – soluble vascular cell adhesion molecule-1; TNF-α – tumour necrosis factor alpha; VEGF – vascular endothelial growth factor.

2.7. Statistical analysis

Treatments with acidic medium were repeated at least 3 times for each cell line. Cell cycle analysis on WHCO6 was performed 4 times, while treatment with conditioned medium (for both apoptosis and cell cycle analysis) was performed 3 times to confirm findings. Descriptive statistics including mean concentrations and standard deviations were determined for all samples where relevant, and one- and two-way analysis of variance (ANOVAs) followed by post-hoc Dunnett's tests were used for multiple comparisons. All statistical analyses were performed using GraphPad Prism for Windows version 9.4 (California, USA).

3. Results

3.1. WHCO6 and MCF-7 cell lines secrete immunomodulatory proteins and growth factors at baseline

The WHCO6 and MCF-7 cell line were maintained in culture for a week to assess protein secretion. This was the maximum amount of time that these cell lines could be maintained in the same culture medium without mass cell death. Culture medium was not replaced during this time as we wanted to detect as many secreted factors as possible, especially those expressed at very low concentrations. Of the 33 analytes assayed, 11 proteins were detected in WHCO6 and 10 in MCF-7. Concentrations of the analytes varied, with interleukin-8 (IL-8) being the most highly expressed in WHCO6 (mean 31 058 pg/ml) (Fig. 1). Interferon-γ inducible protein-10 (IP-10), growth/differentiation protein-15 (GDF-15), Lipocalin-2, granulocyte colony-stimulating factor (G-CSF), vascular endothelial growth factor (VEGF), soluble intercellular cell adhesion molecule-1 (sICAM-1), and fibroblast growth factor (FGF) were also highly expressed, while interleukin-6 (IL-6), epidermal growth factor (EGF), and myoglobin were expressed at lower levels by WHCO6 (Fig. 1). In the MCF-7 cell line, GDF-15 had the highest expression level followed by myoglobin (Fig. 1). The growth factors VEGF, EGF, FGF, and G-CSF were expressed at moderate concentrations, while concentrations of IP-10, Lipocalin-2, sICAM-1, and IL-6 were all lower (Fig. 1).

3.2. Moderate and severe extracellular acidity induces different degrees of PCD in tumour cell populations

Both acute and chronic acid exposure may cause apoptosis in oesophageal epithelial cells [30,31]. To mimic the effects of acute acid exposure *in vivo*, the WHCO6 and MCF-7 cell lines were cultured in moderate (pH 6.5) and severe (pH 3.2) acidosis and apoptosis was measured. These exposure intervals were selected based on previous work where morphological changes such as cell rounding were observed at 1 h, and cell detachment was observed at 2.5 h [27]. As phosphatidylserine (PS) exposure on the cell surface is an early marker of apoptosis and occurs before morphological changes become evident [32], timepoints of no morphological changes (15 mins) and observed morphological changes (1 hr) were selected for comparison. When combined with PI staining, early and late apoptotic populations can be distinguished from one another [27,32]. These short exposure intervals also ensured that the pH of the medium was not affected by cancer cell metabolism.

Although moderate acidosis did not impact significantly on overall

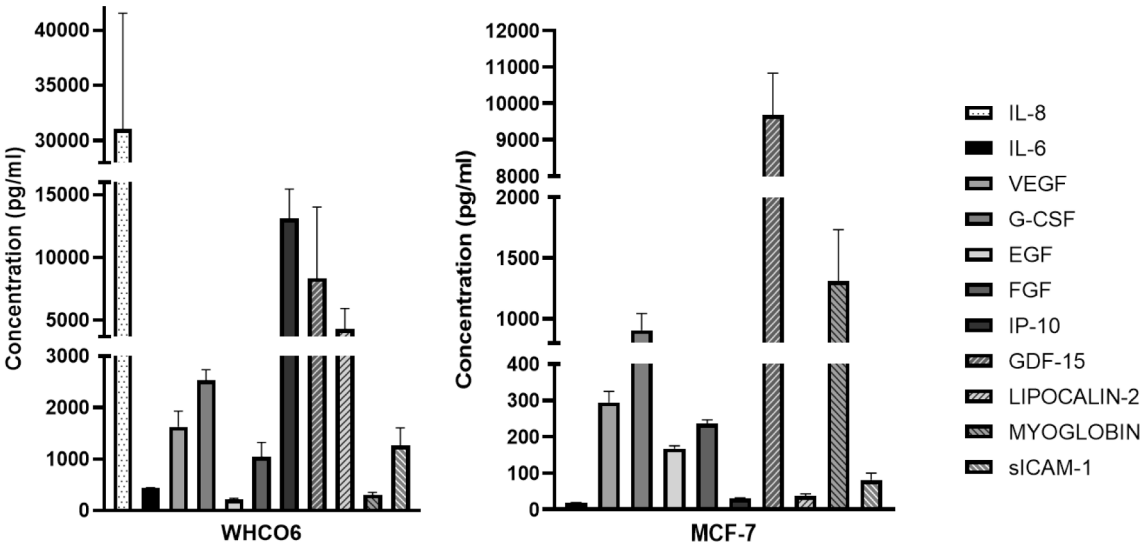

Fig. 1. Humoral factors secreted by tumour cell lines at baseline.

cell viability, both severe acidosis and incubation with DMSO and STS induced significant death compared to the untreated control ($p < 0.0001$) (Fig. 2). In both cell lines, the percentage of cell populations in early and late PCD were higher in cells treated with moderate acidosis for 15 min compared to both the untreated control, and to those treated for 1 h in both cell lines (Figs. 3 and 4).

Compared to the untreated control (A), DMSO (B) and STS (C) induced significant cell death. Early and late apoptosis was observed in cells incubated with pH 6.5 media for 15 min (D) and 1 hr (E), whereas most cells were in late PCD in cells treated with pH 3.2 media (F and G).

3.3. Effects of low pH on cell cycle progression

To measure the effects of acute moderate and severe acid treatment on the cell cycle of the WHCO6 cell line, BrdU incorporation was measured by flow cytometry. Fewer tumour cells treated with pH 6.5 were in S-phase and more cells were in the G_2/M fraction compared with the untreated control (Fig. 5). Cells treated with severe acidosis were predominantly non-viable and different cell phases could not be accurately differentiated. (Fig. 5).

3.4. Secretion of cytokines, chemokines, and growth factors is differentially affected by acid treatment

To investigate the effect of acidic conditioning on secretion by tumour cells, cytokines, chemokines, and growth factors were measured in cell supernatant. Both cell lines produced similar secretion patterns except for IL-6, which was not detected in MCF-7 (Fig. 6). Conditioning with acid did not promote secretion of any additional factors not detected at baseline. Following treatment with acidic medium for 15 min, there was a significant decrease in the production of most factors ($p < 0.001$) for both cell lines, while protein levels were not as significantly affected when WHCO6 cells were treated with pH 6.5 for 1 h (Fig. 6). Concentrations of GDF-15 and myoglobin were significantly increased at pH 6.5 (1 hr treatment) in the MCF-7 cell line ($p < 0.0001$) (Fig. 6B).

3.5. Conditioned media from acid-treated cells differentially affects cell survival

The effects of CM from WHCO6 and MCF-7 cells treated with acidic

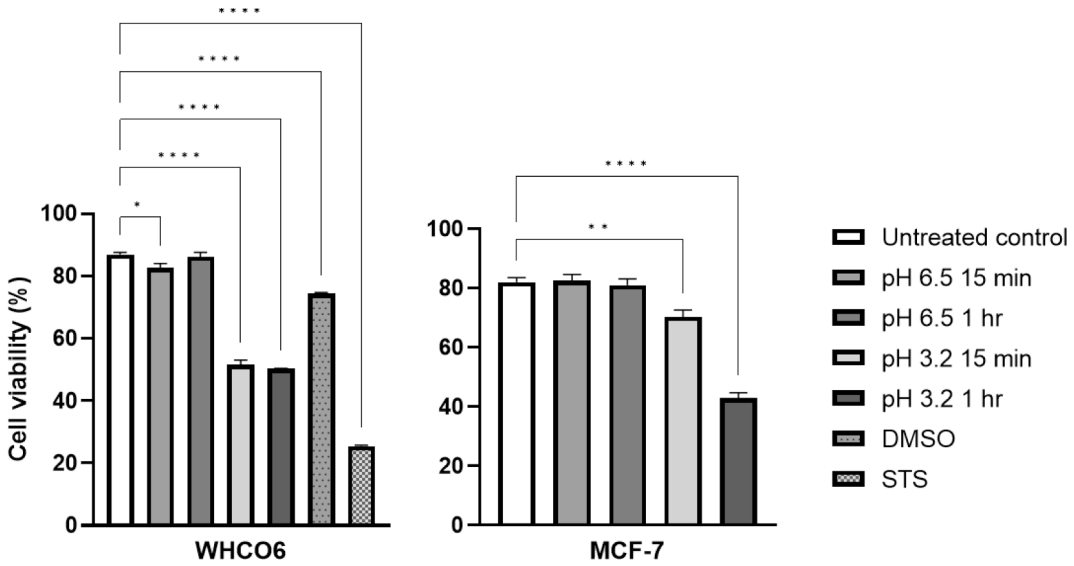

Fig. 2. Cell viability after chemical and low pH treatment. Cells treated with moderately acidic medium pH 6.5 had similar viability levels to the untreated control, whereas cells treated with pH 3.2, DMSO, and STS had far lower viability levels (ANOVA, $p < 0.0001$).

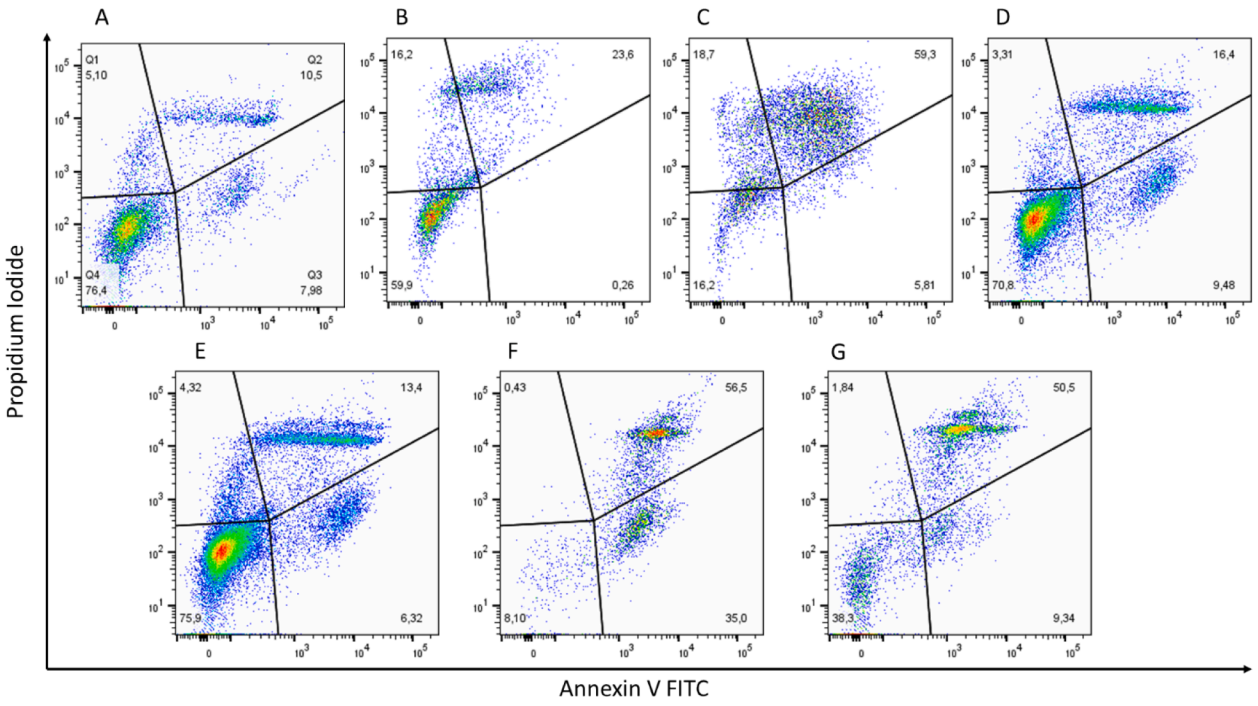

Fig. 3. Programmed cell death is induced by DMSO, STS, and low pH in the WHCO6 cell line.

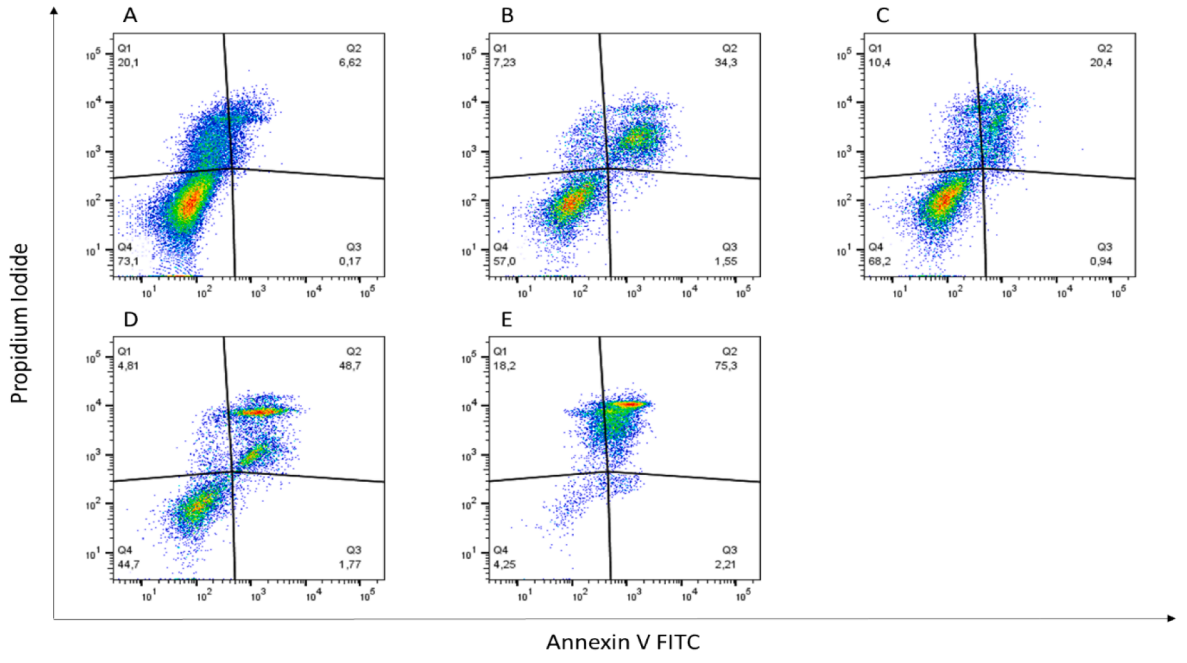

Fig. 4. PCD induced by acidic treatment in the MCF-7 cell line. (A) Untreated control (B) pH 6.5 (15 min) (C) pH 6.5 (1hr) (D) pH 3.2 (15 min) (E) pH 3.2 (1hr).

media on related cell cultures was assessed after 24 h. CM from all treatments resulted in a higher percentage of viable cells compared to the untreated control in WHCO6 (Fig. 7A&C). In contrast, treatment with CM caused an increase in apoptosis for all treatments in MCF-7 (Fig. 7B&C).

CM from cells that had undergone acute acid exposure, promoted a decrease in cells in G_0/G_1 phase, with an increase in cells in G_2/M phase. S phase fractions remained similar between samples tested (Fig. 8). In cultures treated with CM from moderate acidosis, an increase in cells in S phase was observed with increased incubation time (Fig. 9).

4. Discussion

Under normal conditions, cells inhabit a highly regulated environment in which homeostatic processes control cell proliferation, differentiation, and cell death [33]. This regulation is mediated by communication with other cells, interaction with extracellular matrix (ECM) components, activation of DNA repair mechanisms and cell cycle checkpoints, and by signalling molecules including growth factors, cytokines, and chemokines. Dysregulation enables unchecked cellular proliferation, progressing towards a malignant phenotype. The inherent

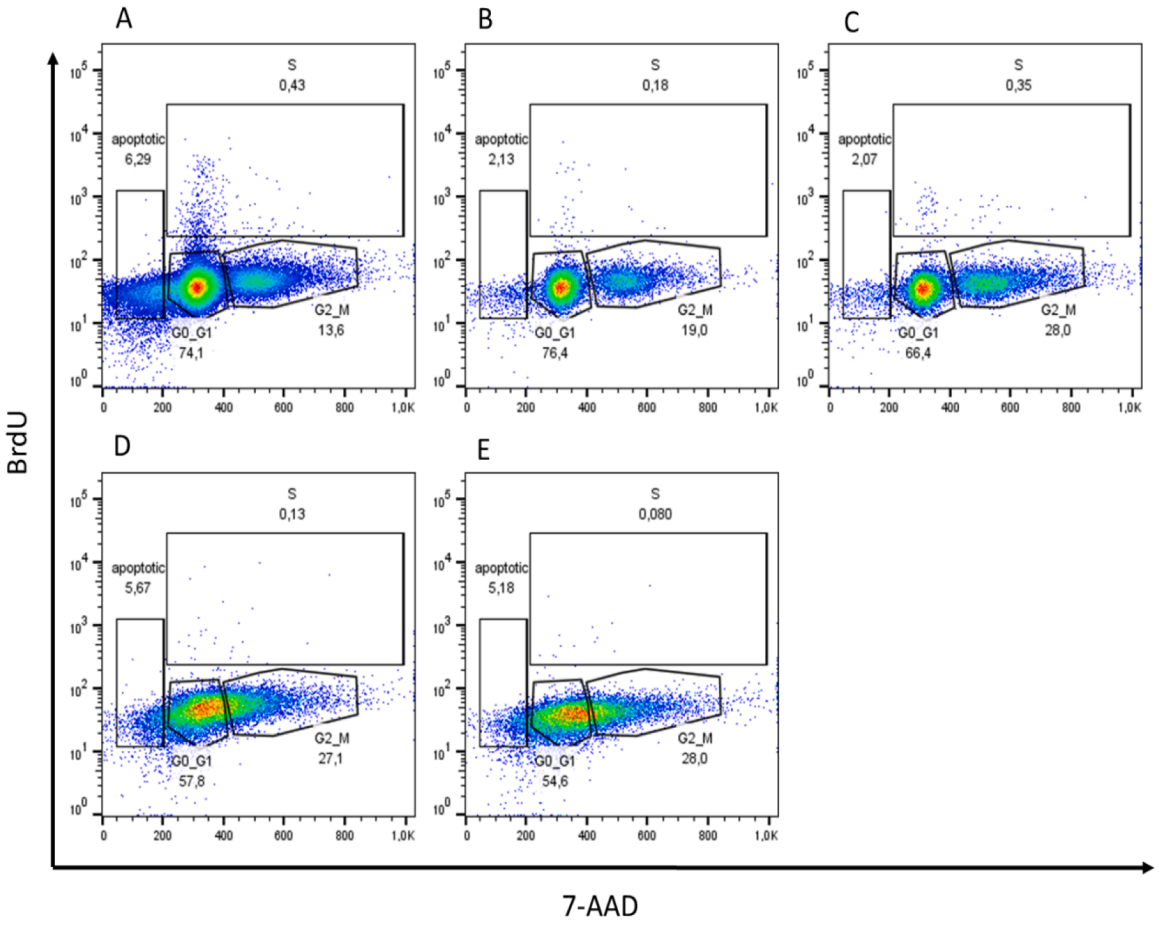

Fig. 5. Cell cycle assessment in WHCO6 cells treated with low pH. Compared to the untreated control (A), fewer cells were in S phase, while more were in G2/M phase after treatment with moderate acid treatment (B and C), while severe acid treatment disrupts the cells, preventing cell cycle progression (D and E).

genetic instability of tumour cells, coupled with microenvironmental pressure, can drive tumour progression [33]. Key determinants of tumour cell interaction and survival within the tumour microenvironment include a relatively acidic environment and the programmed cell death of individual tumour cells. Both factors impact on immunomodulation through the secretion of pro-tumourigenic factors.

The WHCO6 OSCC and MCF-7 breast carcinoma cell lines were selected to determine the effect of the acidic microenvironment on tumour cells from different origins. Genetic instability is prominent in OSCC cell lines including WHCO6 [25,34], and is coupled with the expression of key oncogenes and loss of tumour suppressor genes [25]. MCF-7 is a commonly studied breast cancer cell line that expresses both oestrogen and progesterone receptors, is only weakly metastatic under normal cell culture conditions, and is highly aneuploid, with elevated levels of genetic instability [26]. The inherent genetic instability in these cell lines may increase phenotypic plasticity and enhance the ability of these cells to adapt to environmental stressors such as changes in pH.

Both tumour cell lines secreted immunomodulatory substances at baseline including the cytokines GDF-15, IL-6 and G-CSF, growth factors VEGF, EGF, and FGF, the soluble endothelial marker sICAM-1, as well as Lipocalin-2 and myoglobin. Both IL-6 and GDF-15 inhibit natural killer (NK) cell activity [35]. IL-6, working in concert with IL-8, reduces natural killer group 2D (NKG2D) expression [36], although the function of the low concentration IL-6 produced by the MCF-7 cell line, which does not typically secrete IL-6 [37], is unclear. IL-6 and GDF-15 also modulate dendritic cell activation and IL-6, through the production of reactive oxygen species (ROS), has been linked to the differentiation of MDSCs [35,36]. GDF-15 contributes to resistance to targeted therapy in breast cancer by activating the phosphatidylinositol 3-kinase (PI3K) pathway [38].

G-CSF is secreted in triple-negative breast cancer (TNBC) [39] and the MCF-7 cell line [40], and enhances epithelial-mesenchymal transition (EMT), migration, and invasion [35]. In OSCC tumours, G-CSF production is uncommon but where documented, it causes increased neutrophil production with peripheral leucocytosis [41,42], metastasis to distant organs [42], and aggressive tumour growth [41]. Myoglobin is co-expressed with the oestrogen receptor and is regulated by oestrogen signaling in some breast cancers [43], though it is expressed also by the oestrogen-negative MCF-7 cell line [44]. Myoglobin acts by scavenging ROS and nitric oxide (NO), protecting cancer cells against damage [45]. The significance of sICAM-1 presence is unclear, though it competitively binds to lymphocyte function-associated antigen-1 (LFA-1) present on T cells, NK cells, monocytes, and neutrophils and may play an immunosuppressive role [46,47].

Both cancer cell lines produced growth factors which support iron access to tumour cells (lipocalin-2) [48,49], and angiogenesis (VEGF and FGF) [26,50,51]. EGF is associated with proliferation and invasion in OSCC [52–54], while both EGF and FGF are associated with drug resistance in breast cancer by stimulating both the PI3K and focal adhesion kinase (FAK) pathways and modulating anti-VEGF resistance respectively [38,55]. IL-8 was secreted by the WHCO6 cell line only. It promotes angiogenesis, tumour development, and cell migration and metastasis [36], as well as immunomodulation linked to increased presence of TAMs, lymph node metastasis, and poor patient prognosis in OSCC [56]. IP-10 has pleiotropic roles in cancer development [57] but may facilitate recruitment of Tregs to the TME [58].

Cells were treated with moderate (pH 6.5) and severely (pH 3.2) acidic medium for 15 min and 1 h. These periods were selected to ensure

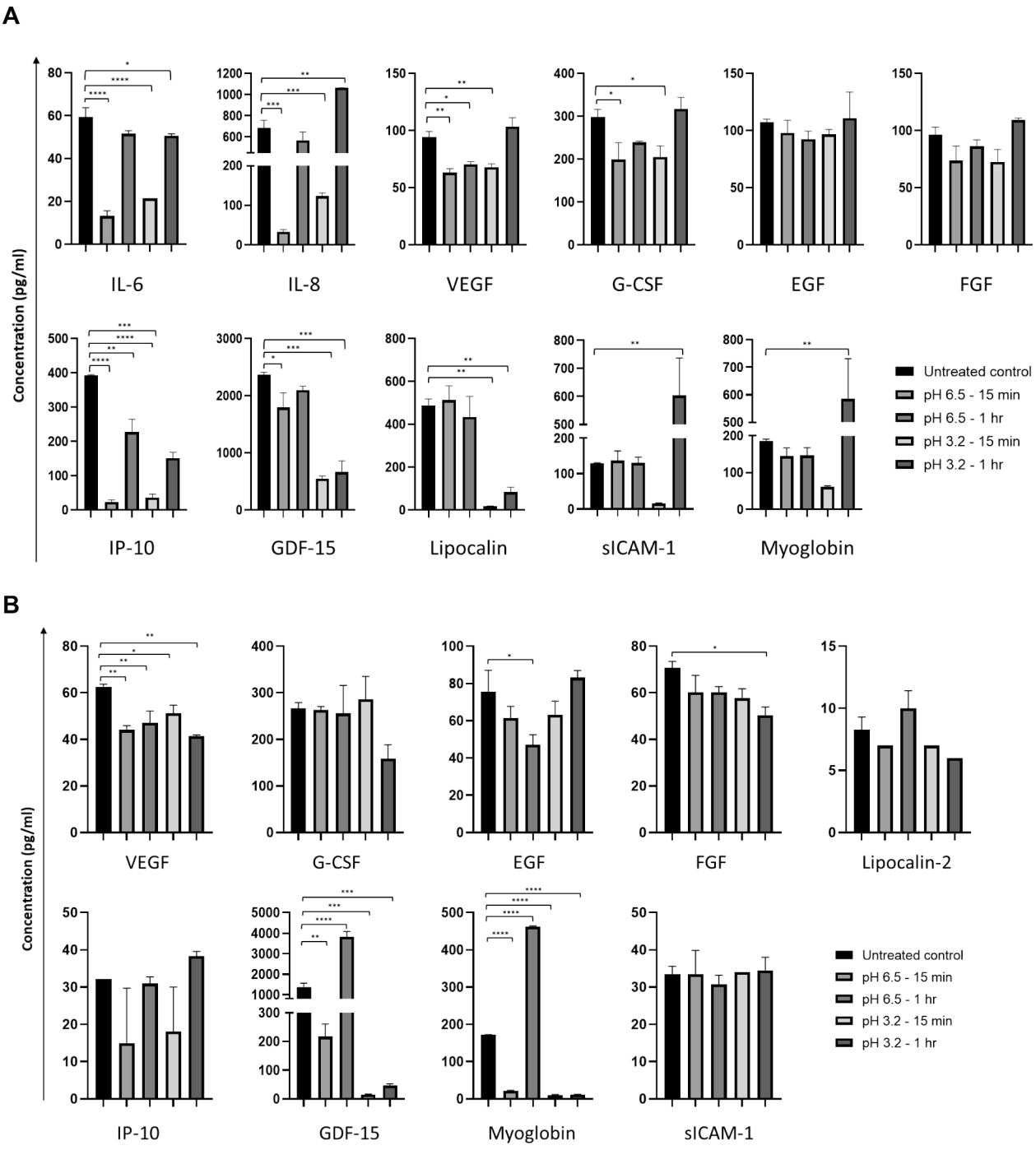

Fig. 6. Acidic treatment affects the secretion of humoral factors by the WHCO6 and MCF-7 cell lines. Protein secretion levels of (A) WHCO6 (B) MCF-7.

that the pH level was maintained, and to avoid pH changes induced by tumour cell metabolism. Shi *et al.* (2017) reported that in human pulmonary epithelial cells exposed to various pH's the pH of the medium returned to physiological pH after 48 h in most instances [59]. There is lack of consensus in the literature when classifying acidity with some studies classifying pH 5.5 as weakly acidic [60,61], while others classify this pH as moderate [62,63] or severely acidic [64]. These differences are likely owing to differences in tumour cell types and anatomical locations. The oesophagus in particular is continuously exposed to very low pH < 2 [65,66] and by comparison, pH 6.5 would not be considered as severe at this anatomical site. Similarly, pH 6.5 has been classified as moderately acidic in breast cancer [63]. This manuscript has therefore followed Davern *et al.* (2022) and Yang *et al.* (2013) by calling pH 6.5 moderate, and Sasaki *et al.* (2021) calling ~ pH 3.0/3.2 strongly or severely acidic.

Treatment with acidic pH impacted on cell viability and secretion of proteins. Moderate acidity resulted in a temporary reduction in secretion of factors by the WHCO6 cell line when treated for 15 min, although many analytes recovered almost to the level of the untreated control when treated with pH 6.5 for 1 h. This suggests rapid tumour cell adaption to a moderately low pH. Although the concentrations of G-CSF, VEGF, GDF-15, IP-10, sICAM-1, and Lipocalin-2 did not fully recover, moderate acidosis did not significantly impact the ability of the tumour cells to produce pro-tumorigenic compounds. Exposure to severe acidosis significantly reduced protein secretion in WHCO6, except for IL-8, sICAM-1 EGF and FGF, and myoglobin which suggests some

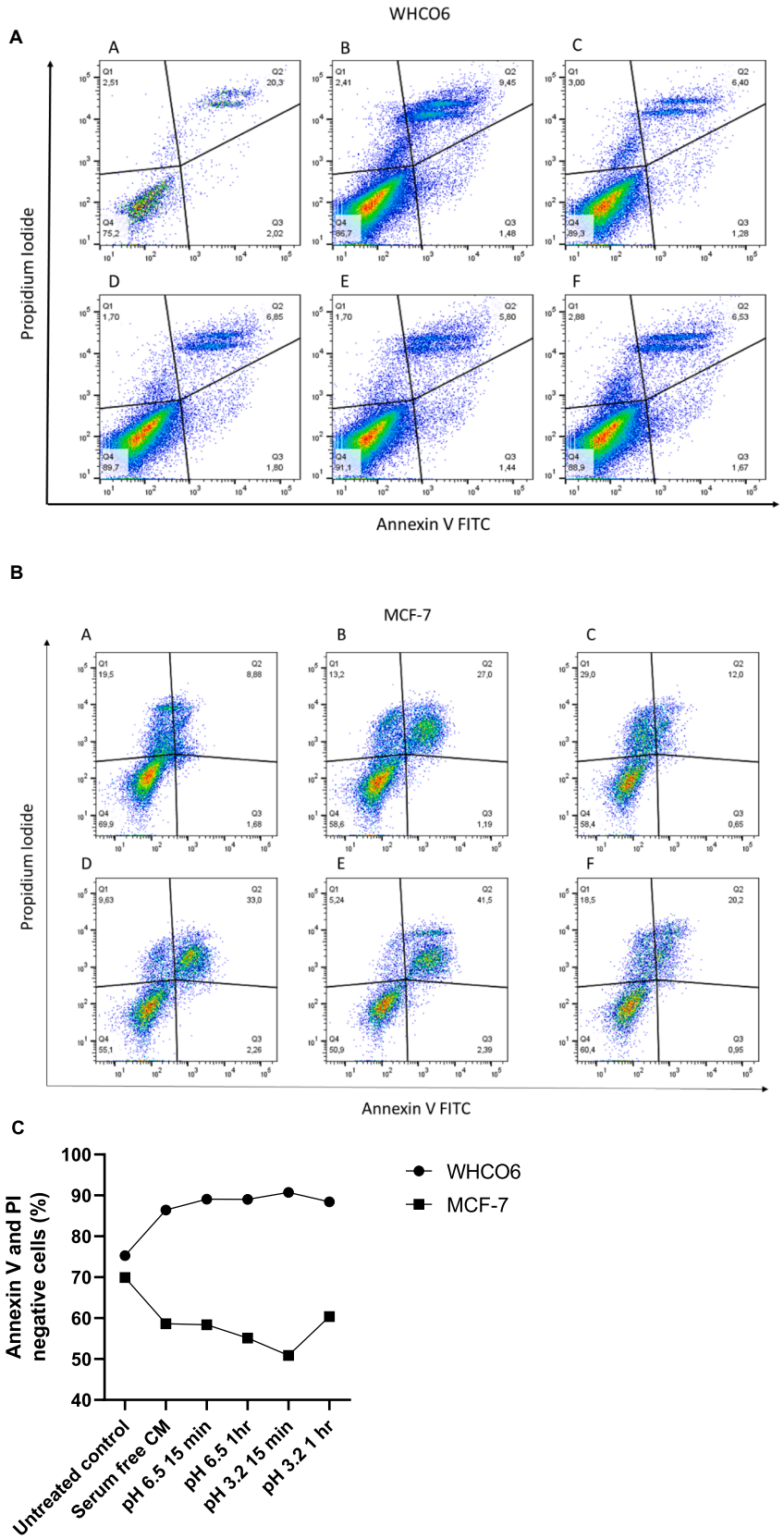

Fig. 7. Treatment with conditioned media confers a survival advantage in WHCO6 but not in MCF-7. (A) WHCO6 (B) MCF-7 (C) Treatment with CM from acid-exposed cells resulted in a higher percentage of viable cells in WHCO6, but a reduced percentage of viable cells in MCF-7 when compared with the untreated control.

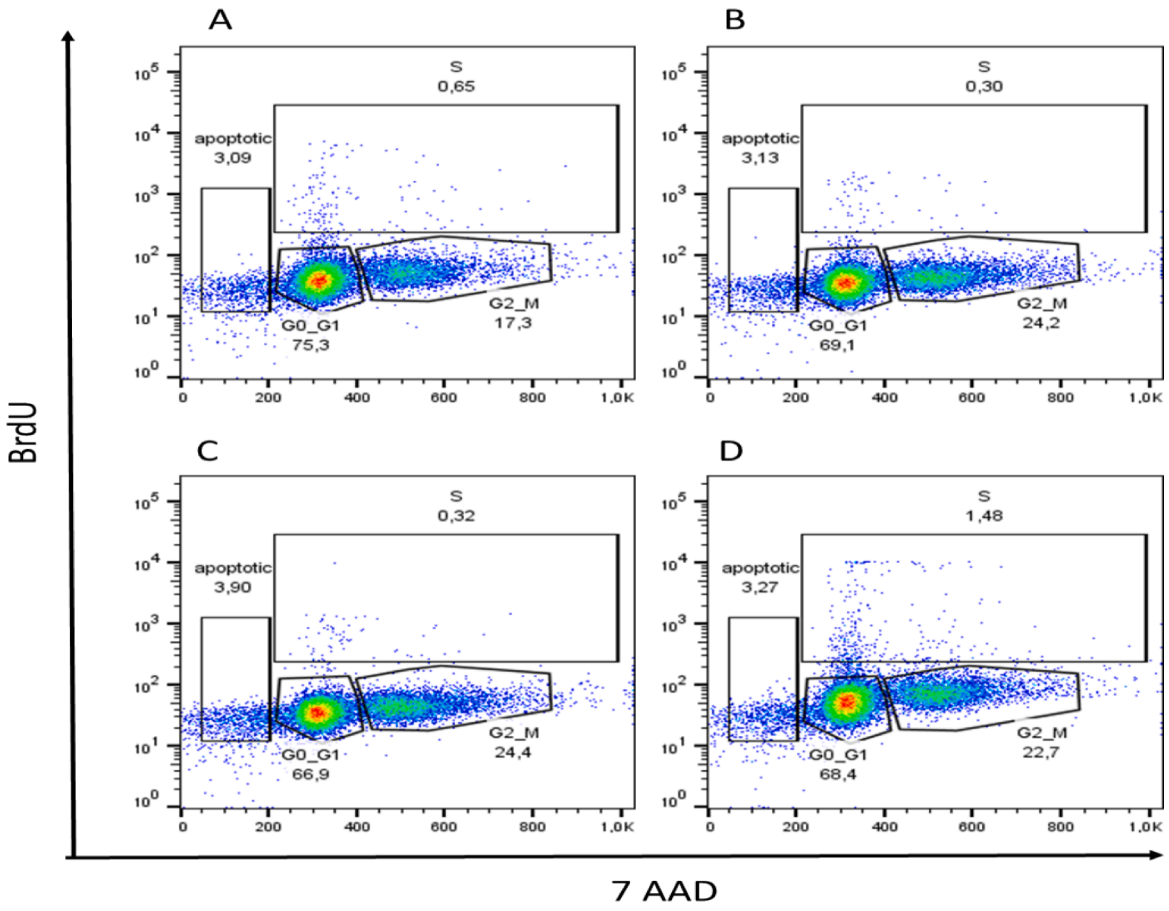

Fig. 8. Effect of conditioned media on cell cycle progression in WHCO6 (A) pH 6.5 15 min (B) pH 6.5 1 hr (C) pH 3.2 15 min (D) pH 3.2 1 hr.

tumourigenic processes including angiogenesis, invasion and migration may be preserved even under these conditions. Secretion of GDF-15 and myoglobin dropped significantly in MCF-7 cell line, when exposed to pH 6.5 medium for 15 min, but then increased when exposed to the same pH 6.5 for a longer period. Levels of other analytes did not show significant changes with acid exposure. These cytokines may provide a selective advantage to MCF-7 cells exposed to moderate acidity for prolonged periods, by removing damage-inducing free radicals and promoting an immunosuppressive microenvironment. Severe acid exposure, by contrast, reduced expression of several factors including VEGF, suggesting that this cell line adapts less easily to severely acidic pH conditions. VEGF is pro-angiogenic and its loss may impact MCF-7 tumour growth.

A key hypothesis of this study was that programmed cell death of tumour cells may modify the TME to impact on the survival of other cells. Apoptotic cells release signals that affect their local environment, including factors that promote proliferation and growth, or those that that trigger additional apoptosis [67]. Cellular viability following exposure to low pH was investigated in both cell lines. Exposure to moderate acidity (pH 6.5) did not significantly alter viability when compared with the untreated control, with some cells entering PCD in WHCO6, whereas more cells were driven into late PCD in MCF-7 at this pH. Severe acidity (pH 3.2) pushed a high percentage of cells into late PCD or death in both cell lines. More cells entered the G_2/M phase in the WHCO6 cell line than the untreated control when exposed to moderate acidosis suggesting continued cell cycle progression. This suggests that moderate acidosis within the TME does not affect tumour viability in this cell line, giving tumour cells an adaptive advantage, but adaptation at very low pH is poorer. CM from acid treated cells, in contrast, improved viability of WHCO6 cells when compared with the untreated control. Although it could not be shown that the cellular origin of the cytokines was from cells undergoing PCD instead of the remaining viable cells, since there was between 24 and 92% apoptosis in the acid-treated cells, it is possible that these were responsible for some secretion of factors into the medium. This suggests that growth-promoting signals are released by tumour cells undergoing PCD which confer a survival advantage on other WHCO6 tumour cells. This is in keeping with observation in other studies where low pH induced tumour cells to release extracellular vesicles (EVs) that contained lipids, nucleic acids, and proteins that can be selectively taken up other stromal or tumour cells [68]. In cells treated with CM, while cells in S phase were similar to the untreated control after 24 h, increases in the S phase fraction were noted over time over longer incubation periods.

PCD was induced in the breast carcinoma cell line exposed to low pH as in other studies where low pH induced apoptosis in MCF-7 and other luminal cell lines, although TNBC and basal-like breast cell lines were less affected [69]. Low pH alone decreases glycolysis in MCF-7 while lactic acidosis promotes it [69]. Acidosis-induced ROS production may also contribute to cell death in MCF-7 [70]. CM treatment of MCF-7 cells induced higher levels of PCD compared to the control possibly because of reduced growth factor production. This highlights the importance of the anatomical location of a tumour and suggests that tumours from distinct locations may adapt to pH changes differently. Understanding the metabolic reprogramming induced by low pH in different tumours may prove valuable for future research.

This study has limitations. Mimicking exact conditions in cell culture is difficult, and it is unclear if the findings can be extrapolated to tumour behaviour *in situ*. Cells were exposed to abrupt but acute pH changes to control experimental conditions, while acidity in the TME may rather increase gradually as a result of cancer cell metabolism. It would also be

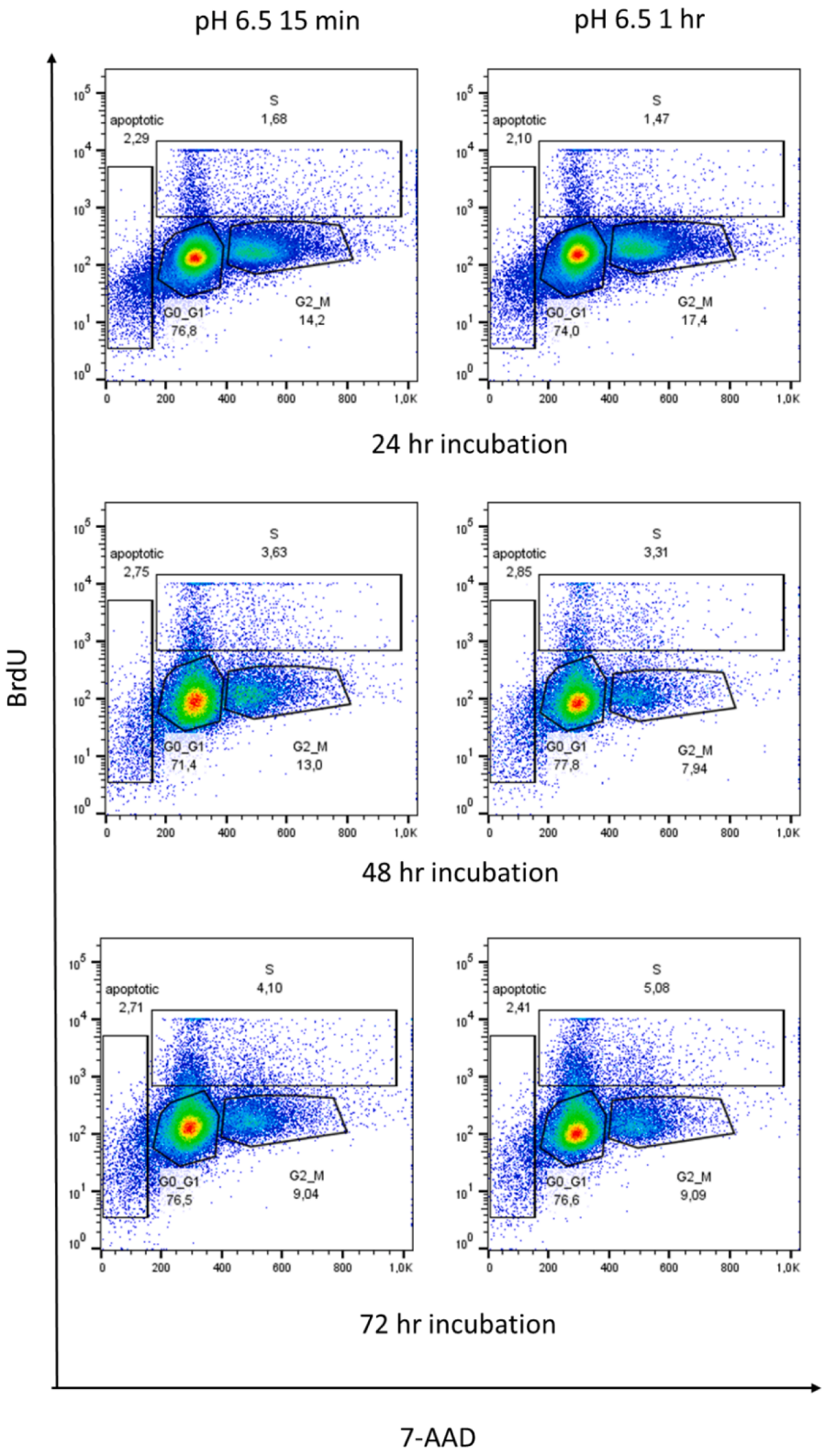

Fig. 9. Cell cycle analysis of WHCO6 cells incubated with conditioned media over 24, 48, and 72 h. Treatment with CM from cells treated with moderate acidosis promotes an increase in cells in S phase over time.

of interest to measure protein expression and changes in cell viability at different time points in future experiments. In addition, determining which factors are specifically released by cells undergoing PCD compared to the remaining viable cells would help in understanding the possible effects released factors on neighbouring or related tumour cells. Improved models such as developing 3-dimensional cancer spheroids may aid in better understanding the effects of low pH on cancer cells [71,72]. These, and cell lines adapted to low pH may be used to screen for cytotoxic drug compounds and tumour-targeted antibodies that can function at low pH [73,74]. Future studies looking at the influence on

immune cell trafficking and activation would also provide insight into the immunomodulatory effects induced by low pH.

In conclusion, this study compared the effects of low pH on different cell lines. Treatment with moderately acidic medium caused an acute decrease in secretion of factors by both cell lines, while many analytes recovered when exposed for a longer time. These factors may promote tumour cell survival at this pH. Severe acid exposure, by contrast, reduced expression of most factors. Acidosis had divergent effects on apoptosis in these cell lines with WHCO6 being better suited to survival at moderate acidity, and treatment with CM promoting survival in WHCO6, but not MCF-7. This suggests that the effects of PCD on tumour cells may be both site and tumour dependent. Further investigation into the effects of low pH on different tumour types may provide valuable insight that can be used to inform drug trials as well as therapeutic manipulation of the tumour microenvironment.

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CRediT authorship contribution statement

Catherine M. Worsley: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Validation, Writing – original draft, Writing – review & editing. **Rob B. Veale:** Conceptualization, Resources, Supervision, Writing – review & editing. **Elizabeth S. Mayne:** Conceptualization, Funding acquisition, Methodology, Resources, Supervision, Writing – review & editing.

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.

Data availability

Data will be made available on request.

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Supplementary data

The aim of these experiments was to determine whether treatment with conditioned media (CM) obtained from acid-treated apoptotic cell cultures affected protein secretion. Cells were incubated in CM for 24 hours and the effects on cell viability and cell cycle progression were documented. Medium was removed, and Luminex assays were performed as described [94]. Protein expression and mean overall concentration was compared before and after treatment with CM (see Appendix G for raw data and statistical analyses).

The protein concentrations in CM and post conditioned medium (PCM) were measured using the Luminex assays listed in Table 1 in Worsley *et al.* 2023 [94]. Both assay standards and specimens were prepared and tested in duplicate. A fluorescent readout was compared to the 5-PL standard curve to determine the concentrations of each analyte in each specimen tested. Mean concentrations of the replicates for each analyte were determined, and concentrations in CM and PCM were compared using 2-way ANOVA tests followed by post-hoc Dunnett's tests for multiple comparisons. Significance was reported ($p<0.05$). All statistical analyses were performed using GraphPad Prism for Windows version 9.4 or 9.5 (California, USA).

All analytes that were present in CM were detected in the PCM in both cell lines (Fig. S1). G-CSF was significantly increased in WHCO6, while other growth factors including VEGF, epidermal growth factor (EGF), and fibroblast growth factor (FGF) increased to a lesser extent. Together these factors may contribute to promoting angiogenesis (VEGF), as well as to the increased viability and proliferation observed in this cell line (FGF, EGF, G-CSF) [94-98]. While decreases in interleukin-8 (IL-8), interferon-γ inducible protein-10 (IP-10), GDF-15, and soluble intracellular cell adhesion molecule-1 (sICAM-1) may promote destruction by cytotoxic immune cells, elevated G-CSF levels may mitigate this by promoting neutrophil and MDSC recruitment and activation, with the resultant ROS production causing T cell and NK cell inhibition [99-103].

The factors detected in the PCM from the MCF-7 cell line correlate with the decrease in cell viability [94]. The secretion of growth factors remained consistently low with levels similar to those in the CM, suggesting limited uptake by cells (Fig. S1). Myoglobin and GDF-15 secretion increased significantly (Fig. S1). Upregulated myoglobin expression correlates with apoptotic markers in several cancers, and it is thought to promote apoptosis by increasing ROS release [104], while GDF-15 expression is reported to increase in response to stress, inflammation, and tissue injury [105].

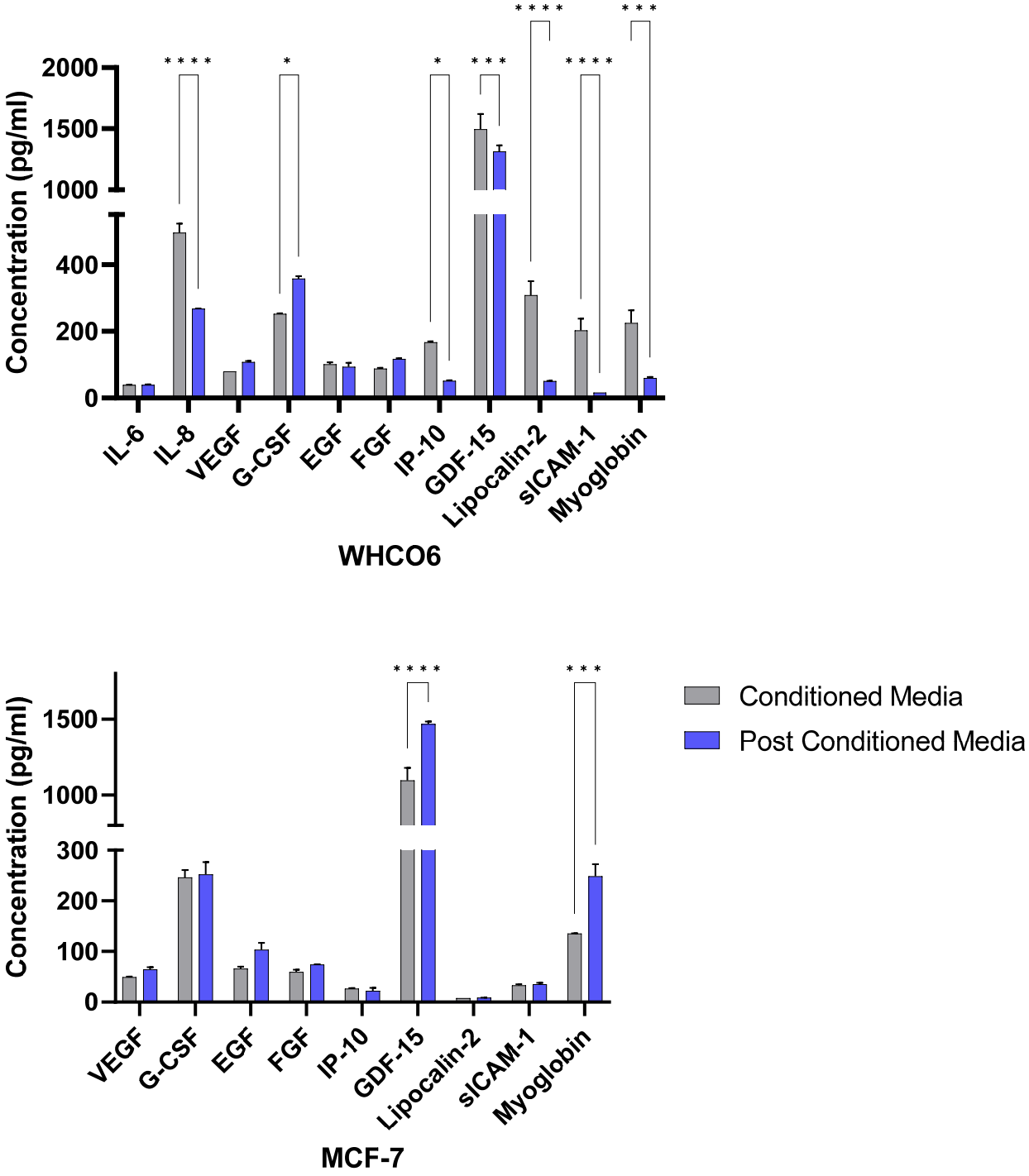

Figure S1. Comparison of secreted factors in WHCO6 and MCF-7 cell lines pre- and post-treatment with conditioned media.

(**** p< 0.0001, *** p< 0.001, ** p < 0.01, * p < 0.05).

Taken together, these data support the published findings where treatment with CM from apoptotic cells improved cell viability and proliferation with the stimulation of supportive growth factors in WHCO6, but caused apoptosis in MCF-7 with a significant increase in myoglobin and GDF-15 which may be linked to cell damage. This supports the hypothesis that the WHCO6 will prioritise cell viability at low pH.

Chapter 6: Discussion and Conclusion

The stability of multicellular organisms depends on cooperation and conflict resolution between several mechanisms that police normal cellular functions and suppress somatic mutations. Within a particular ecological setting, mutations and genetic instability provide subpopulations of cells with a fitness advantage over normal somatic cells resulting in cancer [14]. Cancer is a leading cause of death globally [1], and occurs when several cooperative multicellular systems fail including those that control cell adhesion, cell-cell communication, proliferation, apoptosis, and immune activation [14]. This leads to increased fitness at the single cell level at a cost to the multicellular organism [14]. As many cancers are genetically unstable, there in an increased likelihood of acquiring mutations that provide opportunities for adaptation that drive tumorigenesis [28].

Solid tumours are comprised of heterogeneous cell populations with variable phenotypes and levels of fitness [106]. Uncontrolled proliferation (a breakdown in cooperation) and dysregulated apoptosis (a breakdown in self-policing) cause tumours to grow, placing limitations on available nutrients and space which cell clones then compete for in a selective Darwinian process [10]. Glycolytic metabolism and hypoxia create acidic areas within the tumour microenvironment which inhibit immune cells, promote immune editing, and affect tumour cell proliferation, apoptosis, and intracellular signalling [50, 107]. Acidity, hypoxia, ROS, and nutrient deprivation in the TME drive genomic instability and select for aggressive clones that cause metastatic disease [107]. Either tumour expansion or anti-cancer treatment can create complex environmental conditions that drive the selection of cancer clones with enhanced fitness or resistance. Ultimately, this can lead to tumour cell migration and the establishment of new outgrowths at distant sites [41].

Life history theory provides a foundation for understanding how variability in the TME drives trade-offs between tumour cell survival, proliferation, migration, and immune evasion. As resources are finite, not all fitness components can be maximized simultaneously meaning that increased investment in one component leads to a

reciprocal decrease in investment in another [108]. Investment in tumour cell survival and growth at a cost of decreased proliferation is an example of a fitness trade-off in a tumour with a slow life history, whereas a tumour with a fast life history may be characterized by rapid cell turnover, but with cells that are less resistant to treatment [8, 24]. Multiple tasks can also trade-off simultaneously including cell proliferation, growth, energy production, invasion, and immune interactions [37]. Within heterogeneous tumours, cells in different subsections may be exposed to different environmental challenges which drive life history decisions. Cancer cells with the most successful adaptive strategies will likely be selected and maintained, allowing them to pass these advantages onto their progeny [33, 108].

This study aimed to investigate how life history trade-offs impact on tumour cell survival using apoptosis in an acidic environment as a model. A key hypothesis was that the acidic TME influences cell dynamics to promote tumour growth and survival. To achieve this aim, a comprehensive literature review was undertaken, and a protocol was validated to optimize exposure of cancer cells to low extracellular pH and to study apoptosis in breast and oesophageal squamous cell carcinoma cell lines. The effect of acidic pH on the trade-offs including cell survival, proliferation, and immune evasion was investigated.

6.1 Cancer cell line selection

This study was conducted using well established breast carcinoma (MCF-7) and oesophageal squamous cell carcinoma (OSCC) (SNO and WHCO6) cell lines. These were selected based on the high incidence rates of these cancers globally, and in South Africa more specifically. The tumours they are derived from are found in different anatomical locations, and coupled with their different life history strategies, they reflect a continuum of cancer development. Additionally, these cell lines have elevated levels of genetic instability and phenotypic plasticity which make them key candidates to explore possible adaptations to low extracellular pH.

Breast cancer is the most common cancer both worldwide and within South Africa [1], and is a heterogeneous disease with several distinct sub-types. Sub-classification is based on the expression of human epidermal growth factor receptor 2 (HER2), oestrogen receptor (ER), and progesterone receptor (PR), as well as presence or absence of BReast CAncer gene (*BRCA*) mutations for molecular sub-typing [109]. The four categories are recognized as luminal A (ER+, PR+, HER2-), luminal B (ER+, PR+/-, HER2+), HER2-positive (ER+/-, PR+/-, HER2+), and triple-negative breast cancer (TNBC) where none of the hormone receptors are expressed [110]. ER and PR expression are important diagnostic and prognostic markers, with ER-positive breast cancers accounting for approximately 70% of invasive breast carcinomas, with over half of those cases co-expressing PR [110]. Up to 25% of breast cancers express HER2, which is expressed early in the carcinogenic process [110]. TNBC is highly aggressive with the worst prognosis and there are fewer available treatment options compared to hormone-positive breast cancers [111].

The MCF-7 cell line used in this study was derived from epithelial cells present in a pleural effusion taken from a 69-year old female Caucasian patient with metastatic breast carcinoma [112]. The pleural effusion also contained a few mature erythrocytes, granulocytes, and lymphocytes [112]. MCF-7 cells have features of differentiated mammary epithelium, including the expression of E-cadherin, β-catenin, and cytokeratin 18 (CK18) [113]. This cell line is highly aneuploid with heightened genetic instability [113]. MCF-7 belongs to the luminal A sub-type, and is both ER and PR positive [113]. Luminal A breast cancers are typically low grade and slow growing, but respond well to anti-hormonal therapy [110].

Oesophageal cancer is the 8th most common cancer globally, and is highly aggressive and metastatic with a poor patient survival [6, 114]. The two most common histological subtypes, adenocarcinoma (AC) and squamous cell carcinoma (SCC), have different aetiologies and are predominant in different geographical locations [1]. AC is prevalent in high-income countries and risk factors for its development include excess body weight, gastrointestinal reflux disease, and Barrett's oesophagus, whereas OSCC occurs predominantly in parts of Asia and sub-Saharan Africa and is linked to poor

nutrition, alcohol consumption, smoking, HPV infection, and chronic inflammation [1, 115-117]. OSCC is the most common cancer in South African men [93], with high mortality rates globally largely attributed to late detection [1, 115]. High levels of genetic instability are influenced by these risk factors which together promote uncontrolled tumour growth and progression and have adverse effects on treatment response in this cancer type [115-117].

The SNO and WHCO6 cell lines, which are well- and moderately-differentiated, respectively, were derived from male African patients with locally invasive OSCC [97, 118]. High levels of genetic instability have been reported with upregulated oncogene and loss of tumour suppressor gene expression [115, 119]. The genetic instability in these breast and OSCC cell lines may promote phenotypic plasticity, making them key candidates to explore life history strategies and possible adaptations to low extracellular pH. Importantly, both tumours represent unique ecological niches where selective pressures are differentially applied.

6.2 Optimization of exposure to low extracellular pH

The TME is acidic owing to poor vascular access and high rates of glycolytic metabolism by tumour cells [51]. While the pH of TME is generally reported to be around pH 6.5 – 7.0 [51], compartments of varying acidity are observed throughout the tumour mass [57], especially in necrosing solid tumours. In addition, sites like the oesophagus are continuously exposed to bile acids, gastric fluids (pH < 2), and even food and drink of low pH [120, 121]. This chronic acidosis then drives an inflammatory phenotype that contributes to the activation of oncogenes, redox pressure, release of exosomes, immune cell inhibition, and other tumorigenic processes [61]. While some cells within the tumour may die from acid exposure, others evolve with traits that result in highly aggressive tumours that are resistant to treatment [44, 46, 56].

There is little consensus in the literature on the pH to use or the length of exposure to acidic pH, with pH levels (between pH 4.5 – 6.8) and time of exposure varying greatly

(between 30 minutes and 3 months) with contrasting results [107, 122-126]. For instance, breast cancer cell lines exposed to pH 6.5 for 48 hours had reduced viability and inversely correlated with the malignancy of the cell line [126], whereas treatment with pH 6.7 for 3 months caused cells to become mesenchymal in appearance, with increased proliferation and migration [107]. In contrast, oesophageal cell lines treated with pH 4.5 for 1 week were resistant to apoptosis [122]. Melanoma cell lines exposed to pH 6.7 for 24 hours showed increased exosome secretion which stimulated pH-naïve cells to become invasive and migratory [124], while exposure over 3 months caused cells to adapt to this pH and express stem-related markers [123].

A method was validated as a component to this project to determine the effects of low pH on apoptosis. SNO cells were treated with media of decreasing pH (ranging between pH 7.5 and 3.2). Morphological changes such as cell rounding and membrane blebbing were seen at 1 hour incubation and cell detachment at 2.5 hours after exposure to low pH, with increasing levels of apoptosis as pH decreased [73]. As morphological changes are only noticeable after intracellular changes occur, confirmation of the mechanisms of cell death were explored.

There is little agreement in the literature when classifying acidity with different studies classifying pH 5.5 variably as weakly [58, 127], moderately [128, 129], or severely acidic [130]. These discrepancies reflect differences in tumour cell types and anatomical locations as well as heterogeneity within the tumours themselves [94]. The oesophagus in particular is continuously exposed to very low pH [120, 121], so a pH of 6.5 was considered moderate for this tumour and a pH of 3.2 was classified as severely acidic [128]. For comparison purposes, the MCF-7 cell line was exposed to similar pH levels in this study. The WHCO6 and MCF-7 cell lines were exposed to pH 6.5 and 3.2 for short intervals of 15 minutes and 1 hour [94]. Short intervals of exposure of 15 minutes and 1 hour were selected to observe any cellular changes of early apoptosis induction, and to ensure cancer cell metabolism had a limited impact on the pH of the medium. Continuous exposure for longer time periods may yield different results.

6.3 Detection of apoptosis

Cell death is a key feature of cell physiology and can occur in either a well-controlled sequence or in response to substantial cell injury. Apoptosis occurs when survival signals are removed, or in response to DNA damage, and is a genetically organized process in which cellular components are packaged into membrane-bound apoptotic bodies, or into vesicles that can be taken up by other cells [71]. In contrast, extensive cell damage results in necrosis with cell swelling, membrane rupture, mitochondrial dysfunction, and the release of inflammatory molecules into the local environment [71].

Apoptosis is detected by observing morphological changes, DNA fragmentation, caspase activation, and mitochondrial activity changes [73]. In this study, a flow cytometric assay using Annexin V and propidium iodide (PI) to assess cell viability and distinguish between early and late apoptosis was optimized using untreated cells (negative control), and staurosporine (STS) and dimethyl sulfoxide (DMSO) (positive controls) [73, 94]. Viable cells did not bind Annexin V or PI, while those in early apoptosis with PS redistribution from the cytoplasmic to the exterior membrane surface bound Annexin V. PI was excluded by the intact nuclear membrane. As apoptosis progressed, PS was completely exposed on the plasma membrane and PI bound to nuclear DNA [73, 94]. Cell lines were then treated with low pH medium, and the effects on cell viability and apoptosis were measured.

6.4 Apoptosis in the acidic TME influences life history trade-offs

Aberrations in apoptosis have been widely reported in malignancies with pleiotropic effects. Apoptosis may retard cancer growth and progression, and cells often block apoptosis by inhibiting cell cycle checkpoints, as well as by promoting immune evasion and inducing lymphocyte apoptosis [131]. Individual cancer cell apoptosis can, however, also promote proliferation of neighbouring tumour cells and tumour progression [85]. This highlights the importance of apoptosis in the curation of both the tumour and the surrounding TME. As all mammalian cells can sense and react to apoptosis in

neighbouring cells [89], a better understanding of the signalling mechanisms involved in this process could help predict cellular responses and tumour progression.

As an ecological niche, the tumour and the TME are impacted by variable selective pressures which can drive or retard overall tumour survival and progression. Apoptosis is a factor which may influence both the humoral and cellular composition of the TME.

Both the MCF-7 and WHCO6 cell lines secrete a number of signaling molecules that can mediate communication with other tumour or stromal cells [94]. These factors play important roles in driving life history strategies in these cell lines as they are involved in trade-offs that may reflect many of the hallmarks of cancer including sustained proliferation (due to both proliferative signals and lack of response to growth suppressors), replicative immortality (including de-differentiation and EMT), invasion and migration, resisting cell death (by promoting cell maintenance and growth), reprogramming transport systems (angiogenesis), and evading immune-mediated destruction (Table 6.1). They are also influenced by genetic instability and environmental stimuli such as acidity or inflammation [14]. Also, each factor may not act alone but rather cooperates with others to promote specific effects.

At baseline, where there is little environmental pressure, the MCF-7 cell line secretes factors involved in promoting cell growth and maintenance, immune evasion, and EMT, while proliferation may be a lower priority (Table 6.1). This is suggestive of a tumour with a slow life history, which supports the evidence of this cell line being non-aggressive and only weakly metastatic [113]. In contrast, the WHCO6 cell line collectively secretes molecules that support key aspects of all four trade-offs and may contain a mixed population with cells across the slow-fast life history continuum. This suggests that some cells within this population may prioritize cell maintenance, growth, or proliferation, while others are more capable of recruiting suppressive cells like MDSCs and TAMs and of suppressing cytotoxic cells. These cell types may cooperate with one another to promote development of the oesophageal tumour.

Table 6.1. Factors secreted by the WHCO6 and MCF-7 cell lines.

Roles in trade-offs	Factors responsible	References
Immune evasion		
Suppression of immune cell activation (e.g., NK cells, T cells, monocytes)	GDF-15, interleukin-6 (IL-6), IL-8, sICAM-1	[101, 103, 132, 133]
Downregulation of dendritic cell activation, migration, antigen presentation, and T cell activation	GDF-15, IL-6	[101, 134]
Drives MDSC differentiation	IL-6	[101, 132]
Polarization of M2 macrophages	IL-6, Lipocalin-2	[50, 135]
TAM recruitment	IL-8, EGF	[134, 136]
Neutrophil production	G-CSF, IL-8	[88, 98, 137]
Treg recruitment	IP-10	[138]
Cell maintenance and/or growth		
Stimulate ROS production	IL-6	[101, 132]
Angiogenesis	IL-6, IL-8, FGF, VEGF	[95, 96, 113, 132, 139]
Tumour growth	EGF, FGF, G-CSF, IL-6, Lipocalin-2	[98, 139, 140]
Preventing cell damage by scavenging ROS and nitric oxide	Myoglobin	[141]
Proliferation		
Increased cell proliferation	EGF, GDF-15, IL-6, IP-10	[97, 139, 142-145]
Invasion and metastasis		
Migration and metastasis	EGF, GDF-15, G-CSF, IL-6, IL-8, IP-10	[97, 101, 132, 137, 139, 142-145]
EMT	GDF-15, G-CSF, VEGF	[101, 146, 147]

A number of other analytes were tested for but were not detected in either the WHCO6 or MCF-7 cell lines despite numerous reports of their involvement in many cancer types including those of the breast and oesophagus (see Table 1 in [94]). While the reasons for this are unclear, it may be that these factors are not selected for because the functions they perform are redundant, are better supported by the factors that are secreted, or because they do not support the life history strategy of the tumour at that

timepoint under those conditions. Evidence suggests that different cell lines from the same cancer type can exhibit dissimilar cytokine expression patterns despite being grown under the same conditions [148]. This impacts on the evaluation of drug candidates which may produce contrasting results in different cell lines.

Exposure to acidic pH affected each cell line differently. In severely acidic conditions, however, most cells died in both cell lines, indicating that there are constraints to adaptation to extremely low pH, although as expected, the oesophageal cell line adapted better than the breast carcinoma cell line [94]. Exposure to moderate acidity for 15 minutes induced apoptosis in more cells compared to the untreated control and to those treated for 1 hour in both cell lines. In the case of WHCO6, with moderate acidity the cell viability was slightly lower than the untreated control, with a reduced S-phase fraction but increased G_2/M fraction [94]. This supports reports of other cancer cell lines that adapt to moderate acidity [122, 149].

Moderate pH did not significantly affect the secretion of most proteins in the WHCO6 cell line, except for VEGF, G-CSF, and IP-10 which were reduced [94]. While it was unclear whether the dying or viable cells were responsible for the secreted analytes, it is likely that these factors support cell survival, growth, and some mechanisms of immune escape at the expense of accelerated angiogenesis, proliferation, and metastasis. This again reflects the life history of this cell line as well as its origin in that it was derived from a tumour that was locally invasive and not highly metastatic [97]. Taken together, these data suggest that while some cells undergo apoptosis, most WHCO6 cells were able to adapt to survive at pH 6.5. This may indicate adoption of a slower life history strategy under these conditions. With cell recovery over time, these surviving cells may adopt fast life history strategies again when conditions change.

In the MCF-7 cell line, moderate acidity induced apoptosis in more cells [94]. This supports findings of some studies [126], while others report resistance to apoptosis at pH 6.7 [150], and partial resistance to low doses of cytotoxic drugs paclitaxel and doxorubicin at pH 6.6 [151]. These differences may reflect the duration of acid exposure

which differed between studies. With moderate acidity, MCF-7 secreted significantly less VEGF, FGF, and IP-10, while GDF-15 and myoglobin were increased [94]. This suggests that under these acidic conditions, angiogenesis, growth, and proliferation are constrained as efforts are put into protecting remaining cells from damage and immune destruction. These cells may recover and survive with time, potentially adopting a slow life history. These data support the findings of other studies where initial acid exposure caused extensive apoptosis, but where extended periods of acid exposure caused cells to adapt and to protect their cells membranes from acid hydrolysis, and to acquire mesenchymal-like phenotypes, stem cell markers, enhanced migration capacity, and increased chemo- and radio-resistance [62, 125].

6.5 Apoptosis impacts on other cell populations

Numerous reports describe the beneficial effects of apoptosis in preventing and treating cancer. Work in unicellular organisms has however shown that apoptosis benefits related cell populations [72, 78]. To test this hypothesis in cancer, cell lines were treated with conditioned medium from cells treated with low pH and different responses were induced in the two cell lines. Direct treatment with acidic pH resulted in 15 – 20 % (at pH 6.5) and ~ 50 % (pH 3.2) of WHCO6 cells undergoing cell death. Utilising this medium from these treated cells on additional cultures showed paradoxical enhancement of viability as well as secretion of factors that could stimulate proliferation.

A comparison between tumorigenic and anti-tumorigenic factors produced suggested that growth factors were prioritised under these conditions rather than immunomodulation. Growth factors that enhance cell growth, survival, angiogenesis, and proliferation were increased (VEGF, G-CSF, FGF) after conditioned medium treatment, while other factors involved in protection from the immune system (IL-8 and IP-10) were reduced (Chapter 5). This suggests that a trade-off between immune modulation and proliferation was induced under these conditions. As G-CSF is elevated however, the recruitment and activation of myeloid cells such as neutrophils and MDSCs, followed by increased ROS production and subsequent T cell and NK

cell suppression may still protect WHCO6 cells from immune-mediated destruction [99]. GDF-15 and EGF levels were not significantly affected however, indicating that these cells may still be resistant to treatment (Chapter 5). In contrast, treatment with conditioned medium had a detrimental effect on MCF-7 cells which were induced to undergo apoptosis [94]. The increases in GDF-15 and myoglobin released may be due to cell damage or loss during in this process (Chapter 5).

The data presented here suggest that apoptosis is responsible for various effects that may be tumour-promoting rather than suppressive in different tumour types. Apoptosis-induced proliferation (AiP), also known as compensatory proliferation, has been reported in developmental and wound healing processes of different organisms. For example, cell death in a restricted compartment of the *Drosophila* wing triggered enhanced proliferation in cells in a neighbouring compartment, and apoptosis induced by ionizing radiation in mice caused compensatory proliferation and the restoration of tissue function [152]. In mammals, AiP is well described in epithelial cells as it is critical for wound repair and tissue regeneration as it enables tissues to eliminate damaged or proinflammatory cells and replace them with healthy progeny of neighbouring cells [87, 152].

Apoptosis promoted increased cell viability and proliferation in other tumour cells in the WHCO6 cell line [94]. This has also been seen where radiotherapy induced death in some tumour cells but caused overall regrowth of the tumour driven by caspase-3 activation and production of PGE2 [153]. Caspase-dependent AiP in cancer also involves c-jun N-terminal kinase (JNK) activation, ROS production, and the recruitment of macrophages [87, 154].

Caspase-dependent AiP can also extend to other cell types in the TME. While cytotoxic radiotherapy targeted at tumour cells can cause AiP-mediated tumour repopulation, it can also trigger the release of factors that act on endothelial cells [87]. Both MCF-7 and WHCO6 cell lines secreted increased levels of VEGF after being exposed to conditioned medium from apoptotic cells, which may lead to angiogenesis

(Chapter 5). Studies show that apoptotic cells drive angiogenesis either through direct signalling with vascular progenitor cells, or indirectly through macrophage recruitment and activation [155, 156]. This possibly creates a dynamic feedback loop that supports tumour growth: tumour cell apoptosis promotes angiogenesis which in turn increases blood supply to the tumour. Increased nutrient availability drives tumour cell proliferation, which then causes increased acidity and hypoxia, which drives tumour cell apoptosis [74]. The extent to which apoptosis-induced-angiogenesis may impact on tumour development in different cancer types requires further investigation.

In the MCF-7 cell line, acid-induced apoptosis seemed to promote further apoptosis [94]. This event is also seen in other organisms which may provide valuable insight to understanding this phenomenon. While AiP has been described in *Drosophila*, apoptosis-induced apoptosis (AiA) also occurs where apoptosis in one wing compartment can stimulate apoptosis in other compartments through the release of signalling molecules by the dying cells [157]. Both AiA and AiP have been reported in vascular smooth muscle cells (VSMCs), though it was unclear how each process was selected for or regulated [158]. A possible mechanism is that AiA may increase the number of apoptotic cells, thereby amplifying the mitogenic stimuli needed to promote proliferation and vessel growth.

Apoptosis-induced apoptosis may also play a role in the bystander effect reported in cancer therapy, in which non-irradiated or treated cells die just as the targeted tumour cells do [159]. Many forms of cancer treatment rely on this bystander effect to cause mass cell death in tumours. This effect has also been reported in untreated tumours, where apoptotic cells can trigger DNA damage and inflammation in surrounding cells [160]. Other studies have reported that radiation-induced apoptosis caused apoptosis in bystander cells in one tumour type and proliferation in another [161]. The microenvironment in which these irradiated cells occur likely has an important role to play in driving different outcomes. Other reports showed that while extracellular vesicles (EVs) released by apoptotic cells that died from heat shock caused stress in neighbouring cells, these cells were more likely to survive subsequent heat shock stress as an adaptive response [162].

6.6 Different life history strategies for different tumours

In this study, the MCF-7 and WHCO6 cell lines responded differently to acidic stress and apoptosis. This reflects their different anatomical locations and environmental niches as well as life history strategies and trade-off decisions which may affect outcomes in patients with similar tumours. While they are both epithelial cell lines, they were derived from different anatomical locations in different patients and may have distinct capacities to adapt to low pH. Their ability or lack of ability to adapt to acidic stress impacts on their life history strategies which may affect treatment outcomes in patients with similar tumours.

At baseline, where there is no selective stress, the WHCO6 culture is likely composed of heterogeneous cell populations with various fitness levels along the slow-fast life history continuum, with some prioritizing proliferation, others promoting growth and cell maintenance, while others are involved in immune modulation (Fig 6.1). When exposed to low pH, the composition and life history strategy of this population may shift. Susceptible cells undergo apoptosis as their fitness is compromised, while other cells with greater fitness adapt to survive and adopt slow life history strategies. The WHCO6 cell line is better at adapting to low pH, probably because the oesophagus is continuously exposed to high levels of acidity compared to breast tissue. Slowly proliferating cells are often found in acidic areas of solid tumours and may be responsible for disease relapse owing to their resistance to apoptosis and therapy [163]. The factors released by either the apoptotic or surviving cells stimulate growth and proliferation in neighbouring cells, which adopt fast life history strategies and may become the dominant successful cell population (Fig. 6.1). Cancer cells that adapt to and become more resistant to acidity over time are driven to remodel the extracellular matrix and invade surrounding tissue [107, 164].

The MCF-7 cell line has a different life history strategy. At baseline, like WHCO6, the culture is likely comprised of different cell populations with variable fitness. As the pH

decreases however, many more sensitive cells undergo apoptosis in the MCF-7 cell line (Fig. 6.1). Fewer cells have the capacity to adapt to this pH, and those that do develop a slow life history strategy focussed on cell preservation and prevention of cell damage. Signals released by these cells are not supportive, and in fact promote death in most other MCF-7 cells (Fig. 6.1). Whether the remaining cells enter a state of dormancy and later recover to become active again requires further investigation.

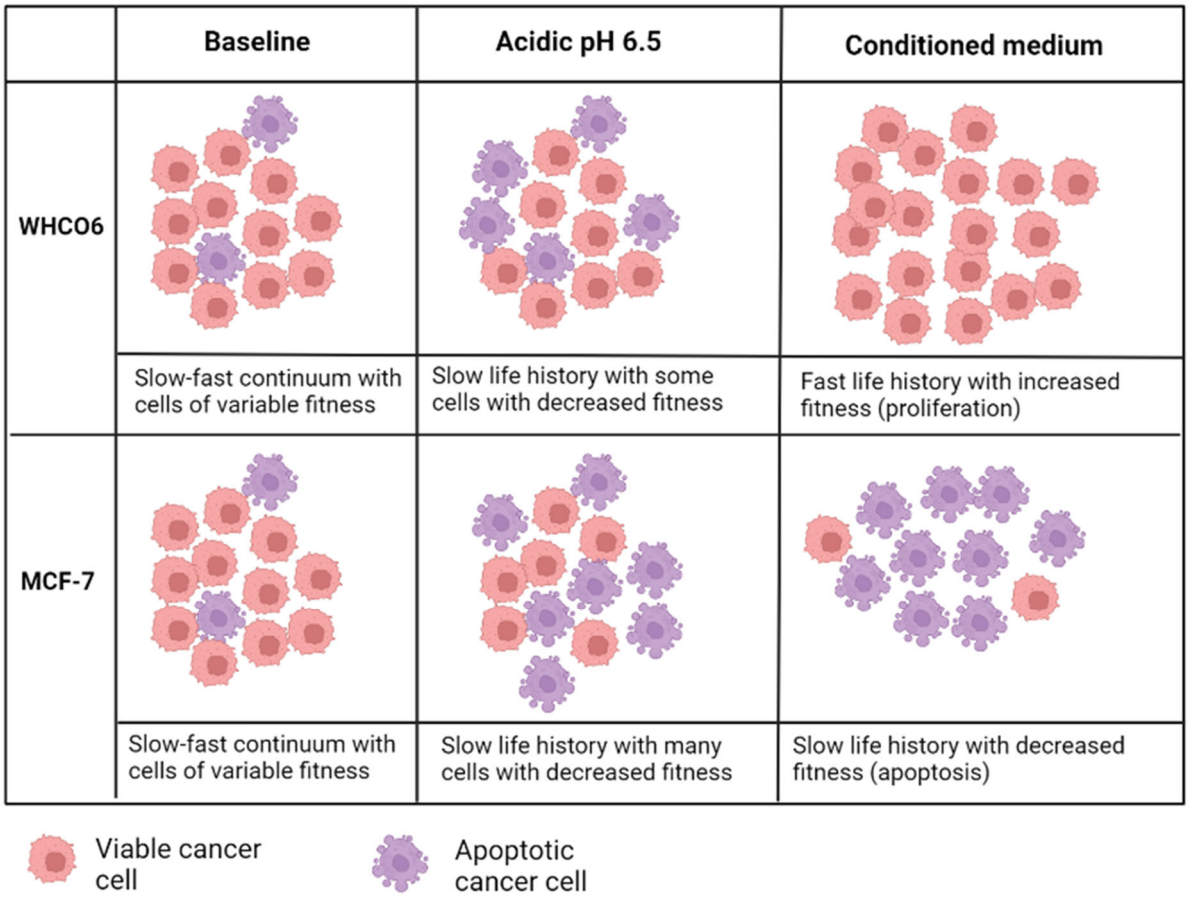

Figure 6.1. Graphical summary of life history strategies of WHCO6 and MCF-7 cell lines. (Figure created in BioRender).

These results demonstrate vastly different life history strategies employed by two different cancer types in response to low pH and apoptosis. This makes it difficult to extrapolate this broadly to other cancer types, requiring further research to understand these factors fully. Cancer progression may not only be regulated by the balance

between proliferation and apoptosis, but also by the balance between the pro- and anti-tumorigenic functions of apoptosis [85]. These apoptotic effects may be tumour-type or tumour-stage specific and are likely impacted on by the TME. These effects may also be different in tumours with either slow or fast life history strategies, which should be considered when treating these tumours. A focus on the selective pressures that drive life history strategies in tumours should allow for the development of new and improved therapeutic strategies that promote cancer control.

6.7 Darwinian solutions for cancer treatment and control

Cancer treatments can modify the life history characteristics of tumours through selection [24]. Current therapeutic approaches target tumour cells themselves either by directly removing them through surgery, or by killing them with chemotherapy and/or radiation. While these therapies have had some success, advanced and metastatic disease remains difficult to control or eradicate. Cytotoxic drugs kill off sensitive cells, leaving resistant cell populations behind [33]. This may initially select for slow life history characteristics that promote survival including quiescence, increased drug metabolism, efflux pumps, and DNA repair [24]. As the sensitive cells have been removed and there is no longer competition for space and resources, resistant clones use this advantage to adopt a fast life history strategy and start to proliferate profusely [24, 33]. Genetic variation and cell plasticity provide opportunities for cells to escape death, resulting in resistant populations that can proliferate and cause disease relapse [33]. Extensive efforts are placed on identifying dominant mutations for drug targets, and while some success has been seen with these in cancers such as chronic myeloid leukaemia (CML), in other cancers the benefits are temporarily and cancer clones with resistant phenotypes are selected for [11]. These clones may have additional mutations that allow them to bypass the signalling pathways of the original drug target [11].

Different approaches are needed to successfully treat cancer and prevent the development of resistance. As evolutionary dynamics determine the development and proliferation of resistant cells, evolutionary strategies should be used in conjunction with conventional treatment to target cancer.

One strategy would be to use an ecological approach to target the microenvironment, as in the context of Darwinian evolution, an efficient way to control cancer may be to destroy its niche by altering its environment [165]. This could include targeting hypoxia, angiogenic factors, nutrients, and other growth factors available to the tumour, and environmental pH. The data presented in this study highlights the role of the acidic microenvironment in driving tumorigenic processes and suggests it as a target to be exploited therapeutically. The acidity of the microenvironment reduces drug efficacy. The use of proton pump-inhibitors (PPIs) and proton extruder systems are being explored to increase extracellular pH. Approaches that exploit rather than treat acidic pH are also promising with the development of pH-sensitive drug delivery systems to ensure appropriate drug release at tumour sites [50].

Other elements of the TME provide promising therapeutic targets including immune cells, stromal cells, and signalling molecules that support tumour growth and survival. This study has identified several secreted factors that could be targeted therapeutically. For instance, targeting factors that promote proliferation and invasion could promote a slow rather than a fast life history in these tumours. Additionally, as some of these molecules promote immune evasion, inhibiting them could block the recruitment of suppressive immune cells while simultaneously promoting the activity of cytotoxic responders. Other studies have investigated blocking angiogenesis to restrict tumour growth by using the antibody bevacizumab to inhibit VEGF receptors [165].

Adaptive therapy is gaining traction as an alternative, where the goal is to control rather than eradicate cancer, and to avoid selecting for resistance cells [166, 167]. The strategy employed includes using short but repeated sessions of drug treatment that are interrupted by periods with no or minimal treatment. In this way, any resistant clones that develop compete with non-resistant cells (or cells with lower resistance) which usually have higher fitness in the absence of drugs [168]. This capitalizes on the trade-offs that develop when the mutations that confer drug resistance also compromise other biological functions (i.e., decrease fitness) of the cell [168]. Another option under

investigation is the use of cytostatic rather than cytotoxic drugs that could delay cancer progression. Sensitive competitor cells would not be killed, allowing them to compete for space and resources with resistant cells. This would not only limit the expansion potential of these clones, but would reduce the opportunities for acquiring new mutations [11]. Tumours with slow life histories are possibly more stable and grow more slowly and have a lower probability of causing patient death [24]. The danger however is that while cytostatic drugs may select for more dormant cancer cells with slow life strategies, these cells could switch to fast life history traits if environmental conditions change (for instance, if treatment is interrupted). This minimal residual disease could cause disease relapse years after treatment. Dosing and treatment regimens would have to be optimized to slow tumour progression and prevent the development of resistance or disease relapse [24]. Given the toxicity of drugs aimed at complete cancer destruction, however, less intensive strategies may be of benefit especially in patients with reduced tolerance for therapy.

6.8 Study limitations

This study successfully investigated how life history trade-offs impacted on tumour cell survival in different cancer cell lines. While apoptosis in an acidic microenvironment was used as a model, other factors in the TME including hypoxia and nutrient deprivation also affect cell adaptation and survival and should be considered in future studies. The acidic TME was shown to have a variable effect on the life history strategies of these breast and oesophageal SCC cell lines, and the mechanisms involved are being investigated further. The impact of acute acid exposure on life history strategies may be different under chronic acid exposure and should be explored. The impact of acidosis and apoptosis should also be investigated in other cell lines from other cancers and in 3-dimensional (3-D) models that better replicate tumour composition and dynamics as observed *in vivo*. For instance, in a 3-D model, MCF-7 cells that adapted to acidity reportedly moved to the outside of the sphere protecting cells on the interior [107], while in our data MCF-7 cells treated with low pH underwent apoptosis. Three-dimensional models such as tumour spheroids allow for gradients in pH as well as oxygen and nutrient concentrations so that responses

across different niches on the tumour can be investigated. This may improve our understanding of the trade-offs implicated in tumour dynamics as well as the life history strategies employed by different tumours under variable conditions.

Other cell death pathways were not investigated in this study that may be important in regulating tumour growth. Recent literature suggests that the induction of pyroptosis, ferroptosis, and necroptosis in combination with immune checkpoint inhibitors enhances anti-tumour activity in animal and cell culture models [68]. It is suggested that necroptosis may increase immunogenicity by releasing endogenous antigens not usually accessible to the adaptive immune system and may stimulate tumour clearance [169]. There is some evidence suggesting that crosstalk between these pathways is vital for controlling tumour growth and preventing progression [68], and this should be investigated further. There is, however, little information on the role that low pH plays in these cell death pathways in models, or whether this has relevance *in vivo*. In addition, distinguishing between these different mechanisms of cell death in the laboratory is currently challenging owing to similar morphological and cell signalling features [73].

Whether the findings obtained from *in vitro* studies bear relevance *in vivo* needs to be verified. By using new molecular technologies such as high-resolution sequencing and single cell technologies, gene and protein expression profiles and the activity of signalling networks can be compared. Computational modelling using information obtained from both *in vitro* and *in vivo* studies can be used to predict tumour evolution as well as response to treatment.

6.9 Conclusion

Understanding cancer evolution in its ecological context is important for predicting progression, metastasis, and treatment resistance, and in developing appropriate treatment. Life history theory provides a framework for understanding how the ecological niche drives trade-offs that affect cancer initiation, progression, and escape

from therapy. This study showed that the acidic TME influences life history trade-offs that affect adaptation in different tumour types. In response to environmental stimuli such as low pH, tumour cells either adopt slow or fast life history strategies that affect both short- and long-term survival, and impact on the development of persistent cell clones. Through metabolic reprogramming and glycolysis, tumour cells themselves drive extracellular acidity. This study shows that low pH impacts on several hallmarks of cancer including apoptosis, proliferation, evasion of growth suppression, and immune avoidance, and possibly replicative immortality. This highlights the central role that acidity plays as a driver of tumour plasticity. This has major implications for successful treatment of cancer and patient survival.

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Appendices

Appendix A – Ethics Approval

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG
Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Miss Catherine M Worsely

CLEARANCE CERTIFICATE	**M120205**
PROJECT	Life History Trade-Off Genes Associated with Evolution of Multicellularity and Cancer
INVESTIGATORS	Miss Catherine M Worsely.
DEPARTMENT	Dept of Molecular Medicine & Haematology
DATE CONSIDERED	24/02/2012
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 24/02/2012 **CHAIRPERSON** .....
(Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable
cc: Supervisor : Dr Pierre Durrand

DECLARATION OF INVESTIGATOR(S)
To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

Appendix B – Approval of Title Amendment

UNIVERSITY OF THE
WITWATERSRAND,
JOHANNESBURG

Private Bag 3 Wits, 2050
Fax: 027117172119
Tel: 02711 7172076

Reference: Mrs Sandra Benn
E-mail: sandra.benn@wits.ac.za

20 May 2022
Person No: 0104116J
TAA

Miss CM Worsley
89 Royal Oak Drive
Irene Farm Villages
Irene Farm Village
0133
South Africa

Dear Miss Catherine Worsley

Doctor of Philosophy: Change of title of research

I am pleased to inform you that the following change in the title of your Thesis for the degree of **Doctor of Philosophy** has been approved:

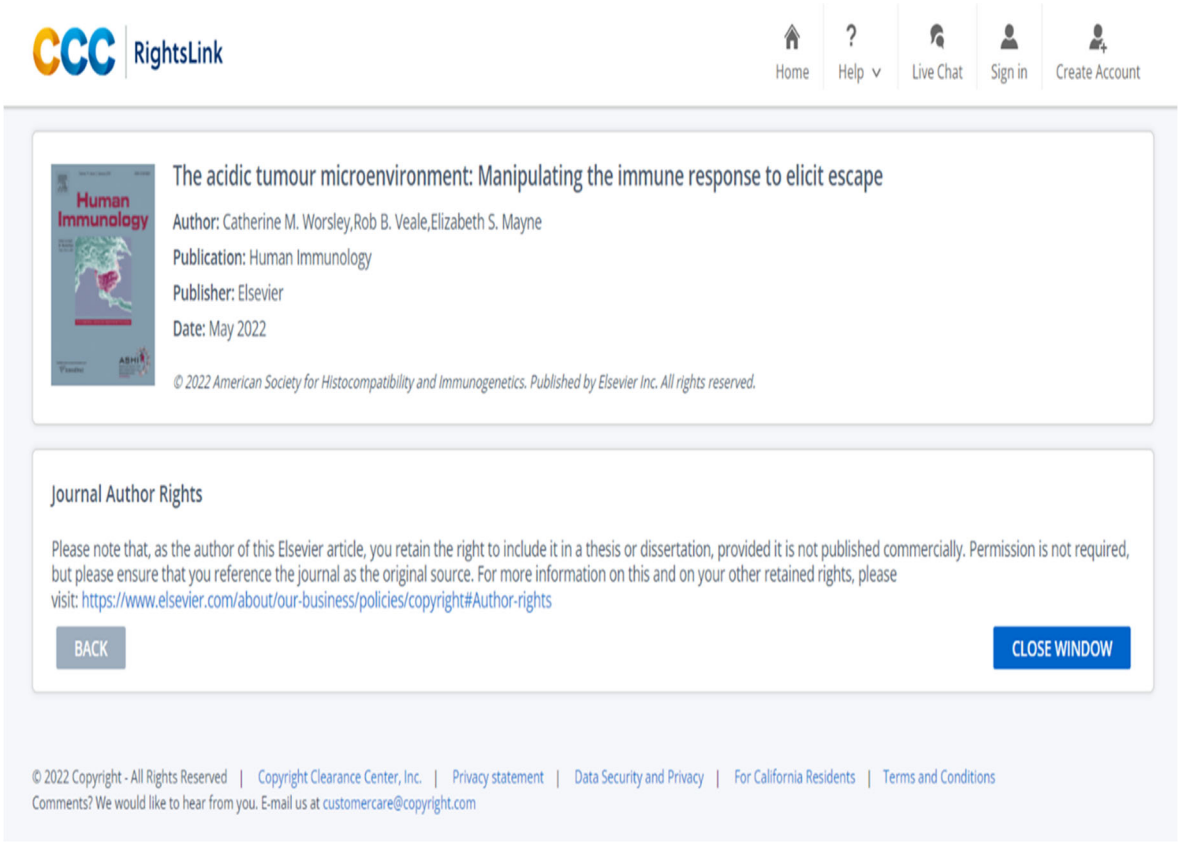
From:	**Life history trade-off genes associated with the evolution of multicellularity and cancer**
To:	**Life-history tradeoffs associated with evolution of cancer**

Yours sincerely

Mrs Sandra Benn
Faculty Registrar
Faculty of Health Sciences

Appendix C - Permission to use articles in this thesis

Co-authored articles



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The acidic tumour microenvironment: Manipulating the immune response to elicit escape

Author: Catherine M. Worsley,Rob B. Veale,Elizabeth S. Mayne

Publication: Human Immunology

Publisher: Elsevier

Date: May 2022

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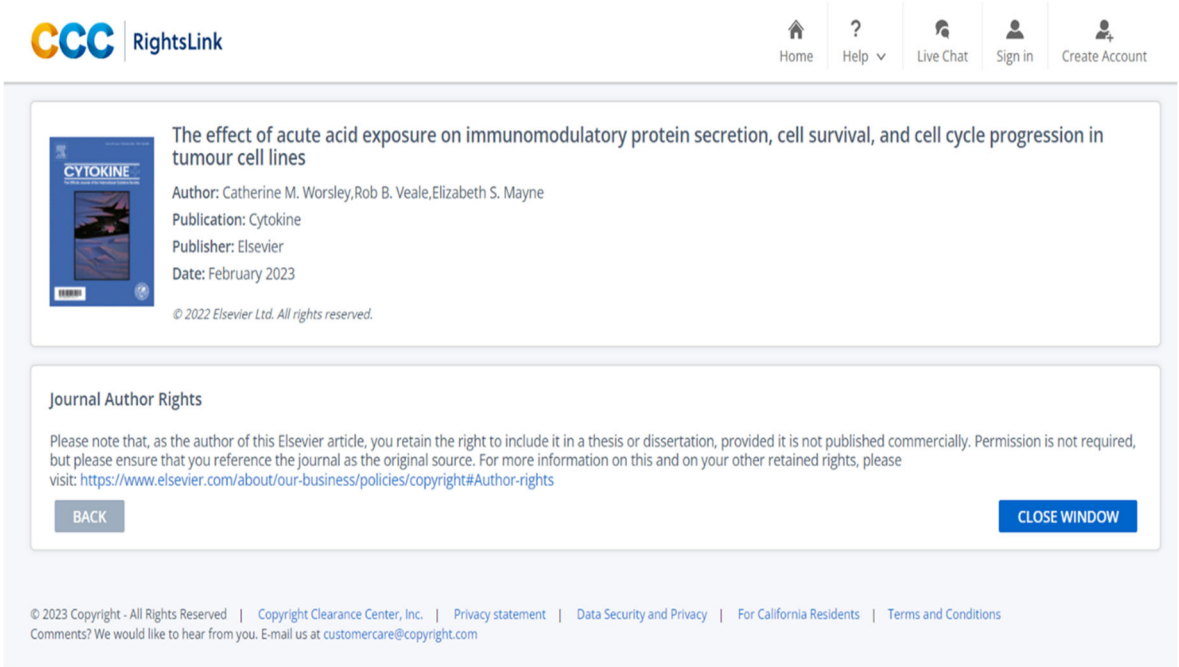

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The effect of acute acid exposure on immunomodulatory protein secretion, cell survival, and cell cycle progression in tumour cell lines

Author: Catherine M. Worsley,Rob B. Veale,Elizabeth S. Mayne

Publication: Cytokine

Publisher: Elsevier

Date: February 2023

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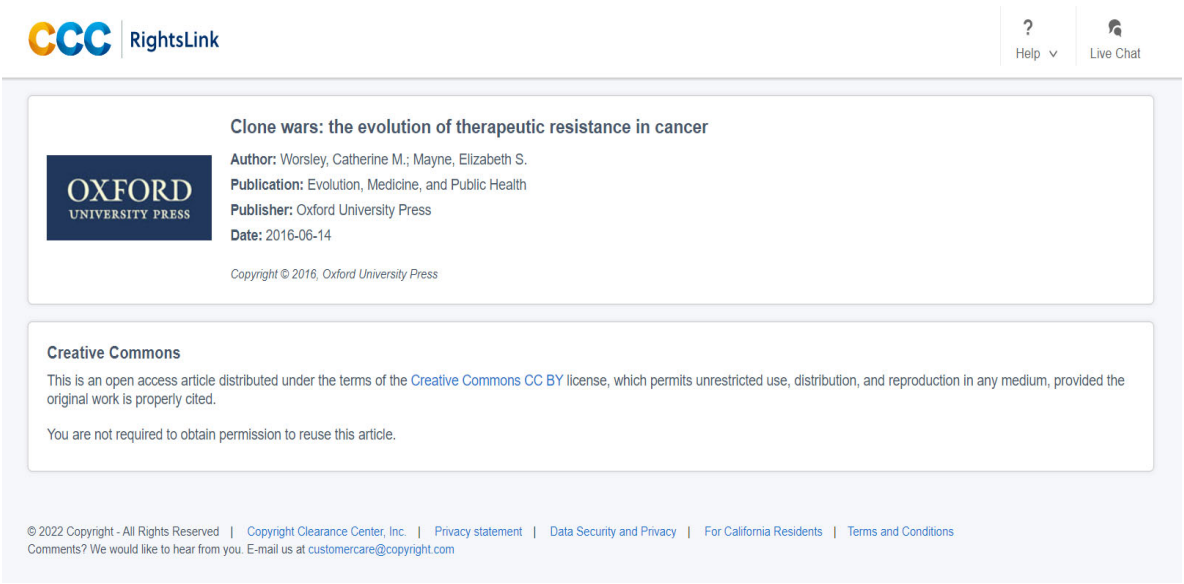

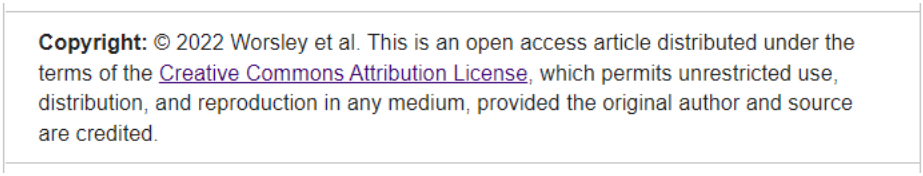

Other articles

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Appendix D – Declaration of Student's Contribution to Articles and Co-author Agreements

Declaration: Student's contribution to article(s) and agreement of co-author(s)

I, Catherine Worsley, student number 0104116J, declare that this Thesis is my own work and that I contributed adequately towards research findings published in the article(s) stated below which are included in my Thesis.

Signature of Student ..... **Date** 10/01/2023

Name of Primary Supervisor Elizabeth Mayne

Signature of Primary Supervisor ..... **Date** 10/01/2023

Agreement by co-authors: By signing this declaration, the co-authors listed below agree to the use of the article(s) by the student as part of his/her Thesis/Dissertation/Research Report. In cases where the student is not the 1st author of a published article, the primary supervisor must explain (under comments) why the student is entitled to use the paper for his/her degree purposes.

Article 1: Title: The acidic tumour microenvironment: manipulating the immune response to elicit escape.

Journal name, year, volume and page numbers: Human Immunology, 2022, 83: 399-408

Authors	Name	Signature	Date
1st author	Catherine M. Worsley		10/01/2023
2nd author	Rob B. Veale		11/01/2023
3rd author	Elizabeth S. Mayne		10/01/2023

Comments by primary supervisor: Supported

Article 2: Title: Inducing apoptosis using chemical treatment and acidic pH, and detecting it using the Annexin V flow cytometric assay.

Journal name, year, volume and page numbers: PLoS ONE, 2022, 17(6): e0270599

Authors	Name	Signature	Date
1st author	Catherine M. Worsley		10/01/2023
2nd author	Rob B. Veale		11/01/2023
3rd author	Elizabeth S. Mayne		10/01/2023

Comments by primary supervisor: Supported

Article 3: Title: The effect of acute acid exposure on immunomodulatory protein secretion, cell survival, and cell cycle progression in tumour cell lines.

Journal name, year, volume and page numbers: Cytokine, 2023, 162: 156118

Authors	Name	Signature	Date
1st author	Catherine M. Worsley		10/01/2023
2nd author	Rob B. Veale		11/01/2023
3rd author	Elizabeth S. Mayne		10/01/2023

Comments by primary supervisor: Supported

Article 4: Title: Clone wars: the evolution of therapeutic resistance in cancer.

Journal name, year, volume and page numbers: Evolution, Medicine, and Public Health, 2016, pp 180-181

Authors	Name	Signature	Date
1st author	Catherine M. Worsley		10/01/2023
2nd author	Elizabeth S. Mayne		10/01/2023
3rd author	Rob B. Veale		11/01/2023

Comments by primary supervisor: Supported

Appendix E – Plagiarism Declaration

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

FACULTY OF HEALTH SCIENCES

PLAGIARISM DECLARATION TO BE SIGNED BY ALL HIGHER DEGREE STUDENTS

SENATE PLAGIARISM POLICY: APPENDIX ONE

I CATHERINE WORSLEY (Student number: 0104116J) am a student registered for the degree of PHD in the academic year 2023.

I hereby declare the following:

- I am aware that plagiarism (the use of someone else's work without their permission and/or without acknowledging the original source) is wrong.
- I confirm that the work submitted for assessment for the above degree is my own unaided work except where I have explicitly indicated otherwise.
- I have followed the required conventions in referencing the thoughts and ideas of others.
- I understand that the University of the Witwatersrand may take disciplinary action against me if there is a belief that this is not my own unaided work or that I have failed to acknowledge the source of the ideas or words in my writing.
- I have included as an appendix a report from "Turnitin" (or other approved plagiarism detection) software indicating the level of plagiarism in my research document.

Signature: C Worsley Date: 11/01/2023

Appendix F – Supplementary Data for Chapter 4

In support of open science and data availability, this section contains the protocol published as supplementary data to the article in Chapter 4 which can also be accessed at protocols.io (dx: doi.org/10/17504/protocols.io.b2dfqa3n).

b2p9qdr6

Inducing apoptosis using chemical treatment and acidic pH, and detecting it using the Annexin V flow cytometric assay V.(b2p9qdr6)

Catherine M. Worsley[1,2,3], Rob B. Veale[4], Elizabeth S. Mayne[5,3]

[1]Department of Molecular Medicine and Haematology, Faculty of Health Sciences, University of the Witwatersrand;

[2]Department of Immunology, Faculty of Health Science, University of Pretoria;

[3]National Health Laboratory Service;

[4]School of Molecular and Cell Biology, Faculty of Science, University of the Witwatersrand;

[5]Department of Immunology, Faculty of Health Sciences, University of the Witwatersrand

1

worsleycm

Cell death is important in physiology, and can happen as a result of structural damage, or as a sequence of programmed cellular processes known as apoptosis. Pathogenic alterations in apoptosis occur in a number of diseases, including cancer, viral infections, autoimmune diseases, immunodeficiencies, and degenerative conditions. Developing accurate and reproducible laboratory methods for inducing and detecting apoptosis is vital for research into these conditions. A number of methods are employed to detect cell death, including DNA fragmentation, the TUNEL assay, and electron microscopy although each has its limitations. Flow cytometry allows for the distinction between live, early apoptotic, late apoptotic and necrotic cells. In this protocol we successfully induce apoptosis using chemical treatment and treatment with low pH in solid tumour cell lines, and have optimized detection using the Annexin V/PI apoptosis assay.

Catherine M. Worsley, Rob B. Veale, Elizabeth S. Mayne . Inducing apoptosis using chemical treatment and acidic pH, and detecting it using the Annexin V flow cytometric assay. **protocols.io** https://protocols.io/view/inducing-apoptosis-using-chemical-treatment-and-ac-b2p9qdr6

National Research Foundation
Grant ID: TTK20110727000022309

Dec 08, 2021

Dec 08, 2021

55777

Annexin V FITC Apoptosis Detection kit **BD Biosciences Catalog #BD/556570**

Cytometer Setup and Tracking beads **BD Biosciences Catalog #BD/641319**

Dimethyl sulfoxide (DMSO) **Sigma – Aldrich Catalog #D8418**

Dulbeccos Modified Eagles Medium (DMEM) **Sigma Aldrich Catalog #D5030**

FACSFlow Sheath fluid **BD Biosciences Catalog #BD/342003**

Fetal Bovine Serum **Sigma – Aldrich Catalog #F2442**

Hams F12 **Sigma – Aldrich Catalog #N6658**

Hydrochloric acid **Sigma – Aldrich Catalog #H1758**

Phosphate Buffered Saline (PBS) **Merck Millipore Catalog #524650**

Penicillin-Streptomycin **Sigma – Aldrich Catalog #P4333**

Staurosporine (STS) **Immunochemistry Technologies Catalog #6212**

Trypan blue **Sigma – Aldrich Catalog #T8154**

Trypsin EDTA **Merck Millipore Catalog #SM-2004-C**

LSR II
Flow Cytometer
BD **0000**

Cell culture of mammalian solid tumour cells lines

Bring all reagents to 37°C in a waterbath.

1 Note: ensure that all surfaces and equipment are cleaned with 70% ethanol. Perform all work in a laminar airflow cabinet.

2 In a 10cm culture dish, add 10ml of Dulbecco's Modified Eagle's Medium (DMEM)/Hams F12 (3:1) supplemented with 10% fetal calf serum (FCS) and 2% penicillin/streptomycin.

3 Add the cell suspension to the dish, then incubate at 37°C with humidity, and 5%C CO_2 in air to mimic *in vivo* conditions.

Cell detachment

4 Bring all reagents to 37°C in a waterbath.

5 Observe cell growth daily using an inverted light microscope

6 When cells reach 80% confluency, remove the culture media and wash the cells with 2ml phosphate buffered saline (PBS). Discard the PBS.

7 Add 2ml of trypsin/EDTA (TE) solution and incubate at 37°C for 5-10 mins or until cells start to detach from the culture dish. Note: do not leave cells in TE for too long as it can cause damage to the cells. To speed up detachment, lightly move the dish so that the TE covers the whole area.

Cell counting using Trypan Blue

8 Once cells are detached, add 2ml of the cell-TE mixture to 8ml of DMEM/HAMS F12 medium.

9 On a piece of parafilm, pipette 20µl of 0.4% trypan blue.

10 Add 20µl of the cell mixture to the trypan blue on the parafilm, and gently mix together by slowly pipetting up and down

11 Add 20µl of the cell/trypan blue mixture to the chamber of the haemocytometer, and cover with a coverslip. This will ensure that the liquid moves onto the counting chambers by capillary action. Ensure that there are no bubbles under the coverslip.

12 Focus the microscope on the gridlines using the 10x objective.

13 Count the lives cells (live cells exclude trypan blue) in one set of 16 squares.

14 Move the haemocytometer to count the cells in the next set of 16 squares. Do this 4 times.

15 1. To calculate the number of viable cells/ml:
a.Add the total number of cells together
b.Divide by the number of sets of squares counted to get the average number of cells.
c.Multiply by the dilution factor (2)
d.Multiply by 10 000 (10^4) (this is the volume of the squares)
e.Calculate the volume needed to get the required number of cells per dish (in this case 150 000)
Example:
Number of cells (84)
Number of block sets (4)= 21 (average number of cells per block)

21 x dilution factor (2) x 10 000 = 420 000 cells/ml

Number of cells wanted (150 000)
Cells/ml (420 000)= 0.357ml of cell suspension to each dish

16 Pipette the volume of cell suspension calculated above into the number of 6cm dishes required (7 dishes).

17 Add culture medium to the dish to make the total volume up to 1ml

18 Place dishes into the incubator and leave overnight at 37°C with humidity, with 5% CO_2 in air.

Cell death induction

19 Prepare the Staurosporine (STS) and pH-adjusted media.

20 STS preparation:
a.Dissolve STS powder in tissue culture grade DMSO to obtain a 1mM concentration

21 pH-adjusted media preparation:
Adjust medium pH to required pH using hydrochloric acid (HCl) and measure with a pH meter

22 Remove all the dishes from the incubator, and wash the cells with 2ml of PBS.

23 Treat the 7 dishes as follows:

Dishes	Dish 1 - untreated control	Dish 2 - DMSO	Dish 3 - STS+DMSO	Dish 4 - pH 6.5 15min	Dish 5 – pH 6.5 1hr	Dish 6 – pH 3.2 15min	Dish 7 – pH 3.2 1hr
Initial steps	Add 6ml of serum-free DMEM/Hams F12 medium	Add 10µl DMSO per ml of culture media (i.e. 60µl DMSO in 6ml culture media)	Add 10µl STS+DMSO per ml of culture media	Add 6ml of pH 6.5 media. Place back into the incubator for 15 minutes.	Add 6ml of pH 6.5 media. Place back into the incubator for 1 hour.	Add 6ml of pH 3.2 media. Place back into the incubator for 15 minutes.	Add 6ml of pH 3.2 media. Place back into the incubator for 1 hour.
Additional steps	None	None	None	Remove media after 15min and wash cells in 2ml PBS. Remove PBS and add 6ml serum-free media	Remove media after 1 hr and wash cells in 2ml PBS. Remove PBS and add 6ml serum-free media	Remove media after 15min and wash cells in 2ml PBS. Remove PBS and add 6ml serum-free media	Remove media after 1 hr and wash cells in 2ml PBS. Remove PBS and add 6ml serum-free media
Incubation	Incubate overnight at 37°C						

Annexin V FITC and Propidium Iodide staining

24 Remove medium (with floating cells) and place in a 15ml tube. Centrifuge at 1000rpm for 5 min.

25 Remove the conditioned media supernatant and store at -20°C.

26 Trypsinize adherent cells in 1 ml TE and add to a tube containing 4ml of DMEM/HAMS F12 culture media.

27 Centrifuge at 1000rpm for 5 min. Discard the supernatant.

28 Resuspend pellets of both tubes in PBS and combine into 1 tube.

29 Add 5ml of PBS. Centrifuge at 1000rpm for 5 min. Discard supernatant.

30 Resuspend cells in 1-2ml of 1x binding buffer

31 To 100µl of cell suspension, add 5µl of Annexin V FITC and 5 µl of propidium iodide (PI)

32 Incubate at room temperature in the dark for 15 min

33 Add 200 µl of binding buffer and acquire on the BD LSR II flow cytometer.

Flow Cytometer setup

34 Switch the BD LSR II flow cytometer on and open FACSDiva software. Allow the instrument to

warm up for at least 30 minutes. Ensure that maintenance has been performed and that the instrument is in good working order.

35 Verify instrument performance by running BD Cytometer Setup and Tracking (CST) beads

36 Create an experiment in FACSDiva software

37 To adjust photon multiplier tube (PMT) voltages for compensation, prepare unstained and single stained tubes as follows:
a.Tube 1 - 100µl of unstained cells
b.Tube 2 - 100µl of cells stained with 5µl Annexin V FITC
c.Tube 3 - 100µl of cells stained with 5µl PI

38 Acquire unstained cells. Adjust PMT voltages to ensure that cells are in the negative quadrant for all fluorophores
Note: this needs to be done as some cells autofluoresce which can interfere with the assay

39 Acquire tube 2. Adjust PMT voltages so that there is a well separated positive and negative population in the FITC channel. Adjust PMT voltages so that all other channels only have a negative population.

40 Acquire tube 3. Adjust PMT voltages so that there is a well separated positive and negative population in the PerCP-Cy5.5 channel. Adjust PMT voltages so that all other channels only have a negative population

41 Once compensation has been set up, acquire experimental tubes
Note: acquire and record as many cells as possible for analysis.

42 Perform maintenance and shutdown of the flow cytometer as per manufacturer's instructions

Analysis

43 Export the FCS data files in FSC 2.0 and 3.0 to a folder or external harddrive

44 Import FSC files into FlowJo software

45 Select the untreated control tube and double click. A forward scatter (FSC) vs. side scatter (SSC) plot should open

46 1. Select the cell population of interest and using the gating tool to draw a gate around the population. Name or label that population of cells.

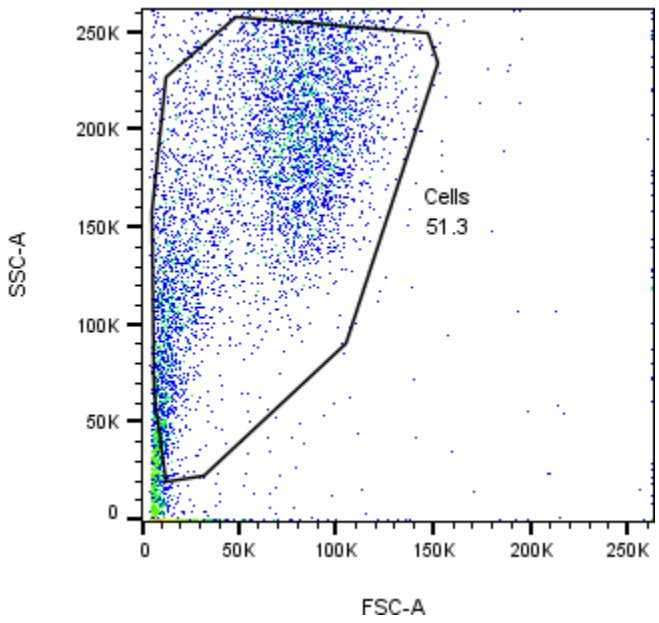

Figure 1. FSC vs. SSC plot

47 Double click on that gate and another FSC/SSC plot should appear.

48 Click on the X–axis and change it to Annexin V or the FITC channel

49 Click on the Y-axis and change it to the PI or PerCP-Cy5.5 channel

50 1. Insert a quadrant or spider quadrant gate as shown below in Figure 2. Gates should separate live cells (Q4: Annexin V-/PI-), early PCD (Q3: Annexin V+/PI-), late PCD (Q2: Annexin V+/PI+) and dead/necrotic (Q1: Annexin V-/PI+) from each other. The numbers in the quadrants indicate the percentage of cells in that quadrant

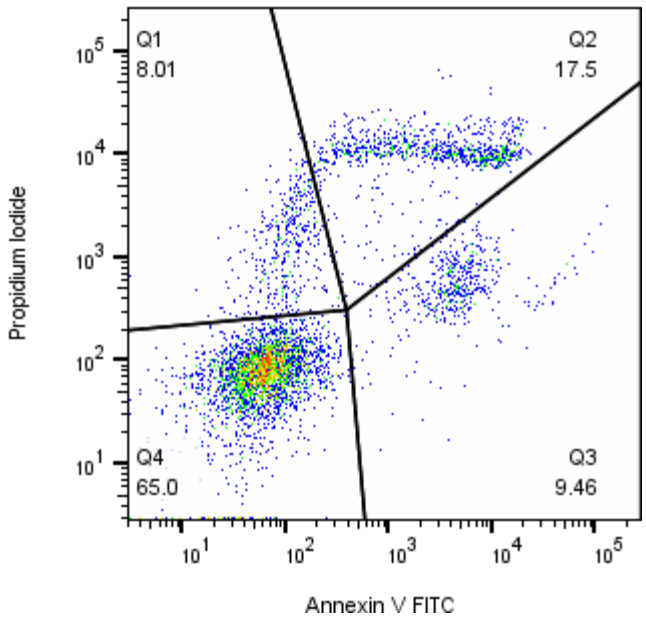

Figure 2. Annexin V FITC vs. Propidium Iodide showing cells undergoing PCD

Appendix G – Supplementary Data for Chapter 5

This section includes raw data obtained as well as statistical analyses performed in keeping with the principles of open access science. All statistical analyses were performed using GraphPad Prism for Windows versions 9.4 or 9.5 software (California, USA).

Table S1. Luminex determination of protein concentrations sampled in duplicate (pg/ml)

Sample ID	IL-8		IL-6		VEGF		G-CSF		EGF		FGF		IP-10		GDF-15		Lipocalin-2		sICAM-1		Myoglobin	
WHCO6																						
Baseline	38480	23635	447	438	1416	1847	2392	2677	237	204	844	1245	14768	11468	4360	12353	3169	5471	1020	1510	277	342
Serum free	638	737	62	56	98	91	311	285	109	105	91	101	392	394	2331	2400	466	510	130	127	182	189
pH 6.5 - 15 min	37	30	14	11	65	60	227	172	90	105	82	64	28	18	1612	1978	467	561	117	156	129	160
pH 6.5 – 1 hr	512	624	50	52	72	69	241	237	97	87	82	90	254	201	2058	2150	368	503	118	142	132	161
pH 3.2 – 15 min	129	117	21	21	66	70	187	223	94	99	64	80	30	43	517	585	20	17	17	15	63	59
pH 3.2 – 1 hr	1064	1065	50	51	98	109	298	336	95	127	108	110	139	164	530	803	69	100	509	698	483	688
Serum free PCM	125	148	17	22	92	91	258	268	70	65	98	102	52	52	1388	1712	74	71	29	26	52	57
pH 6.5 – 15 min PCM	149	145	20	19	117	89	345	317	104	85	124	105	49	57	1683	1627	54	45	17	14	61	64
pH 6.5 – 1 hr PCM	238	256	49	51	107	86	348	336	137	79	96	109	46	25	1616	1804	47	55	13	14	65	68
pH 3.2 - 15 min PCM	100	91	31	30	132	136	427	441	102	78	115	130	38	28	826	613	35	35	9	15	22	25
pH 3.2 – 1 hr PCM	721	700	74	79	100	126	385	453	92	116	140	142	78	86	885	991	47	46	14	12	90	95
MCF-7																						
Baseline			18	19	273	315	809	1002	162	173	243	227	30	31	8868	10495	32	41	1008	1609	69	95
Serum free					61	63	258	275	83	67	68	72	32	32	1252	1505	9	7	171	172	35	32
pH 6.5 – 15 min					42	45	268	258	65	57	54	65	4	25	248	186	7	7	22	20	38	29
pH 6.5 – 1 hr					50	43	298	213	50	43	58	61	29	32	3634	4013	9	11	464	461	29	32
pH 3.2 – 15 min					53	48	320	251	58	68	54	60	26	9	13	16	7	7	9	11	34	34
pH 3.2 – 1 hr					41	40	136	180	85	80	47	52	39	37	42	51	6	6	11	12	37	32
Serum free PCM					6	63	258	275	83	67	68	72	29	29	2010	2136	10	9	326	313	38	38
pH 6.5 – 15 min PCM					63	53	244	230	147	88	75	67	28	15	1206	1259	8	8	256	230	42	33
pH 6.5 – 1 hr PCM					67	63	261	220	118	86	77	81	29	17	912	937	9	7	179	151	41	32
pH 3.2 – 15 min PCM					69	66	281	244	71	81	72	78	35	19	977	917	7	9	219	146	29	33
pH 3.2 – 1 hr PCM					65	63	248	244	139	111	61	76	29	28	2183	2144	10	9	345	315	36	31

Table S2. Cell viability (1-way ANOVA and post hoc Dunnett's test comparisons of treatments compared to the serum free (untreated) control.

	Mean 1 (% of parent)	Mean 2 (% of parent)	Mean difference	95% confidence interval (CI) of difference	Adjusted p-value
WHCO6 (1-way ANOVA p < 0.0001**)**					
Serum free control vs. pH 6.5 15 min	86.90	82.60	4.300	1.035 to 7.565	0.0135*
Serum free control vs. pH 6.5 1 hr	86.90	86.20	0.7000	-2.565 to 3.965	0.9421
Serum free control vs. pH 3.2 15 min	86.90	51.60	35.30	32.03 to 38.57	<0.0001****
Serum free control vs. pH 3.2 1 hr	86.90	50.30	36.60	33.33 to 39.87	<0.0001****
Serum free control vs. DMSO	86.90	74.40	12.50	9.235 to 15.77	<0.0001****
Serum free control vs. STS	86.90	25.45	61.45	58.18 to 64.72	<0.0001***
MCF-7 (1-way ANOVA p<0.0001**)**					
Serum free control vs. pH 6.5 15 min	82.00	82.50	-0.5000	-6.533 to 5.533	0.9996
Serum free control vs. pH 6.5 1 hr	82.00	81.05	0.9500	-5.083 to 6.983	0.9847
Serum free control vs. pH 3.2 15 min	82.00	70.25	11.75	5.717 to 17.78	0.0015**
Serum free control vs. pH 3.2 1 hr	82.00	42.85	39.15	33.12 to 45.18	<0.0001****
Serum free control vs. DMSO	82.00	86.35	-4.350	-10.38 to 1.683	0.1711
Serum free control vs. STS	82.00	38.80	43.20	37.17 to 49.23	<0.0001****

Table S3. 1-way ANOVA and Dunnett's test per analyte after low pH treatment

Analyte	1-way ANOVA p-value	Dunnett's adjusted p-value			
		Untreated vs. pH 6.5 15 min	Untreated vs. pH 6.5 1hr	Untreated vs. pH 3.2 15 min	Untreated vs. pH 3.2 1hr
WHCO6					
IL-6	<0.0001****	<0.0001****	0.0588	<0.0001****	0.0379*
IL-8	<0.0001****	0.0001***	0.1433	0.0002***	0.0016**
VEGF	0,0012**	0.0036**	0.0115*	0.0076**	0.2840
G-CSF	0.0174*	0.0340*	0.1833	0.0427*	0.8598
EGF	0.5580	0.8454	0.5895	0.7966	0.9922
FGF	0.0319*	0.1265	0.6274	0.1073	0.4458
IP-10	<0,0001****	<0.0001****	0.0010**	<0.0001****	0.0002***
GDF-15	0.0002***	0.0363*	0.3488	0.0002***	0.0003***
Lipocalin-2	0.0006***	0.9644	0.7470	0.0011**	0.0021**
sICAM-1	0.0013**	0.9998	>0.9999	0.3138	0.0017**
Myoglobin	0.0030**	0.9215	0.9325	0.2996	0.0054**
MCF-7					
VEGF	0.0045**	0.0042**	0.0094**	0.0340*	0.0022**
G-CSF	0.0987	0.9999	0.9935	0.9553	0.0958
EGF	0.0260*	0.2839	0.0325*	0.3711	0.6997
FGF	0.0443*	0.1644	0.1671	0.0891	0.0163*
IP-10	0.1512	0.2519	0.9994	0.3805	0.8791
GDF-15	<0.0001****	0.0015**	<0.0001****	0.0007***	0.0008***
Lipocalin-2	0,0239*	0.4137	0.2022	0.4137	0.0985
sICAM-1	0,8448	>0.9999	0.8516	0.9997	0.9947
Myoglobin	<0.0001****	<0.0001****	<0.0001****	<0.0001****	<0.0001****

Table S4. Percentage of viable cells after treatment with PCM

PCM treatment	Viable cells (% of total cell population)	
	WHCO6	MCF-7
Untreated control	75.3	69.9
Serum free PCM	86.4	58.6
pH 6.5 - 15 min PCM	89.1	58.4
pH 6.5 – 1 hr PCM	89.0	55.1
pH 3.2 – 15 min PCM	90.7	50.9
pH 3.2 – 1 hr PCM	88.4	60.4

Table S5. Comparison of analyte concentrations in CM compared to PCM by 2-way ANOVA (p<0.0001) and post hoc Dunnett's test (MCF-7 cell line).

Analyte	Mean concentration in CM (pg/ml)	Mean concentration in PCM (pg/ml)	95% confidence interval (CI) of difference	Adjusted p-value
VEGF	49.25	64.74	-84.35 to 53.38	0.9976
G-CSF	246.1	252.3	-75.03 to 62.69	>0.9999
EGF	66.14	103.6	-106.3 to 31.40	0.6315
FGF	59.83	74.28	-83.31 to 54.42	0.9986
IP-10	26.93	22.64	-64.58 to 73.15	>0.9999
GDF-15	1096	1468	-441.0 to -303.3	<0.0001****
Lipocalin-2	7.650	8.650	-69.86 to 67.86	>0.9999
sICAM-1	33.25	35.35	-70.96 to 66.76	>0.9999
Myoglobin	135.6	248.3	-181.6 to -43.89	0.0006***

Table S6. Comparison of analyte concentrations in CM compared to PCM by 2-way ANOVA (p<0.0001) and post hoc Dunnett's test (WHCO6 cell line).

Analyte	Mean concentration in CM (pg/ml)	Mean concentration in PCM (pg/ml)	95% confidence interval (CI) of difference	Adjusted p-value
IL-6	39.34	39.57	-102.1 to 101.6	>0.9999
IL-8	495.8	267.8	126.2 to 329.9	<0.0001****
VEGF	80.07	108.1	-129.9 to 73.84	0.9961
G-CSF	252.1	358.3	-208.0 to -4.324	0.0367*
EGF	101.3	93.40	-93.99 to 109.7	>0.9999
FGF	87.69	116.6	-130.8 to 72.90	0.9948
IP-10	166.9	51.61	13.41 to 217.1	0.0189*
GDF-15	1497	1315	80.10 to 283.8	0.0001***
Lipocalin-2	308.3	51.05	155.4 to 359.1	<0.0001****
sICAM-1	203.1	16.30	84.90 to 288.6	<0.0001****
Myoglobin	225.0	60.25	62.90 to 266.6	0.0005***

* $p < 0.05$

** $p < 0.01$

*** $p < 0.001$

**** $p < 0.0001$

Appendix H - Corrigendum

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Corrigendum

Corrigendum to "The effect of acute acid exposure on immunomodulatory protein secretion, cell survival, and cell cycle progression in tumour cell lines" [Cytokine 162 (2023) 156118]

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The authors regret that part of the caption was left out of Figure 3. The figure caption should read: "**Figure 3.** Programmed cell death is induced by DMSO, STS, and low pH in the WHCO6 cell line. Compared to the untreated control (A), DMSO (B) and STS (C) induced significant cell death. Early and late apoptosis was observed in cells incubated with pH 6.5 media for 15 min (D) and 1 hr (E), whereas most cells were in late PCD in cells treated with pH 3.2 media (F and G).".

The authors would like to apologise for any inconvenience caused.

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