How protons pave the way to aggressive cancers

Pawel SWIETACH[1†], Ebbe BOEDTKJER[2†], Stine Falsig PEDERSEN[3†]

1. *Department of Physiology, Anatomy and Genetics, University of Oxford, Parks Road, Oxford, OX1 3PT, England*
2. *Department of Biomedicine, Aarhus University, Hoegh-Guldbergs Gade 10, DK-8000 Aarhus C, Denmark*
3. *Department of Biology, University of Copenhagen, University of Copenhagen, Faculty of Science, Universitetsparken 13, København Ø, Denmark*

[†]email: pawel.swietach@dpag.ox.ac.uk, eb@biomed.au.dk, sfpedersen@bio.ku.dk

Cancers undergo sequential changes to proton (H^+) concentration and sensing that are consequences of the disease and facilitate its further progression. The impact of protonation state on protein activity can arise from alterations to amino acids or their titration. Indeed, many cancer-initiating mutations influence pH balance, regulation, or sensing in a manner that enables growth and invasion outside normal constraints as part of oncogenic transformation. These cancer-supporting effects become particularly eminent when tumours develop an acidic microenvironment due to metabolic reprogramming and disordered perfusion. The ensuing intra- and extracellular pH disturbances impact multiple aspects of tumour biology, ranging from proliferation to immune surveillance, and can even facilitate further mutagenesis. As a selection pressure, extracellular acidosis accelerates disease progression by favouring acid-resistant cancer cells that are typically associated with aggressive phenotypes. Although acid-base disturbances in tumours often occur alongside hypoxia and lactate accumulation, there is now ample evidence for a distinct role of H^+-operated responses in key events underpinning cancer. The breadth of these actions presents therapeutic opportunities to swerve the trajectory of disease.

[H1] INTRODUCTION

Research over the past decade has strengthened the case that pH **[G]**, its regulation, and sensing mechanisms participate in carcinogenesis and disease progression. An implicit link between acidity and cancer was first made in the 1920s by Otto Warburg, who postulated that a shift from respiration to lactic acid fermentation allowed cancer cells to overcome constraints on proliferation normally set by oxygen availability.[1,2] At that time, the oxygen partial pressure **[G]** (pO_2) was considered the most critical limitation on growth, but a pro-oncogenic effect of lactic acidosis could not be excluded. After tumour acidosis was confirmed using pH-sensitive electrodes,[3,4] it received considerable attention in the context of resistance to weakly basic therapeutics.[5,6] However, with the discovery of oncogenic mutations, acidosis became relegated to an epiphenomenon rather than an active player in oncogenesis.[4] By the 1950s, mathematical models predicted that multiple oncogenic events are necessary to account for the incidence of human cancers.[7] These predictions highlighted a role for the tumour microenvironment in determining the trajectory of disease progression because the functional outcomes of individual genetic changes will depend on both intra- and extracellular context. The case for considering the microenvironment was further strengthened by models of somatic evolution that featured metabolites as selection pressures.[8] The discovery of hypoxia-inducible factors (HIFs)[9] in the 1990s put pO_2 at the forefront of efforts to explain how microenvironmental conditions affect genes, and *vice versa*. The role of dysregulated pH in cancer remained less studied, although cellular mechanisms that underpin acid production and pH homeostasis in transformed cells, and the effects of the ensuing microenvironmental acidosis on processes relevant to cancer, were recognized[10] . The role of pH, its regulators and sensors in oncogenic transformation, acid-adaptation of cells, and acid-driven selection of cancer phenotypes is increasingly being explored and recent progress has also disentangled effects of pH from concurrent factors, including hypoxia.

This Review first provides an overview of how pH influences protein functions and presents a framework for understanding the principles that determine the spatio-temporal dynamics of intracellular pH (pHi) and extracellular pH (pHe). We next describe the typical changes in pHi and pHe during cancer development and explain how disturbances to pH regulation or shifts in pH sensitivity can alter biological functions to make early oncogenic changes more likely. We then discuss how the later stages of cancer progression, including invasion and metastasis, are intertwined with acidosis. We end by highlighting the vulnerabilities arising from pH-related processes and discuss how this offers therapeutic opportunities, some of which are already in clinical trials.

[H1] CAUSES OF DYSREGULATED pH IN CANCERS

[H2] pH is a broad-spectrum modifier of protein function

Biological functions emerge from the ensemble activity of protein networks. Enzyme-assisted post-translational modifications, such as phosphorylation[11] or acylation,[12,13] regulate proteins by influencing charge distribution.[14,15] The charge of proteins can also be altered without enzymes, such as by the coordination of metal ions, including Ca^{2+} ref[16] or by the reversible H^+ binding, referred to as protonation **[G]**[17], to amino acid side-chains, carboxy- or amino termini. The degree of protonation depends on the concentration of H^+ (that is pH) and the H^+ affinity of titratable sites (that is the pK_a **[G]**). Strikingly, protein moieties of functional significance, such as enzyme active sites or receptor binding sites, tend to be enriched in histidine (His), an amino acid characterised by a pK_a near the physiological pH range.[18] This provides a powerful means of controlling protein function by pH changes and is commonly exploited in biological systems. For example, proteins belonging to a particular pathway may share a pH optimum so that overall activity can be orchestrated by compartmentalisation inside organelles maintained at a distinct pH, such as acidic lysosomes. Alternatively, cells can dynamically offset pH in order to activate biological events, such as proliferation, which features pH-dependent cell cycle transitions,[19] or cell migration and invasion that depend on changes in pHi and pHe.[20-22] One challenge in establishing these pH-sensitivities is that (de)protonation reactions do not require enzymes, and therefore routine experimental approaches, such as inhibitors or genetic ablation, for untangling causality are not available.

In addition to the reversible and immediate effects of protein protonation, more sustained consequences are possible if these trigger a cascade. In addition to the *bona fide* H^+ receptors (**Box 1**), H^+-sensitive signalling proteins include receptors such as the insulin receptor-related receptor (INSRR), which can be activated in the absence of ligand by elevated pHe, that induces conformational changes, and engage insulin receptor substrate (IRS) and AKT signalling.[23,24] Several protein kinases, including the AMPK–mTORC2 axis[25] and focal adhesion kinase (FAK)[26], become activated at high pHi. An interesting example is the tyrosine phosphatase suppressor of T-cell receptor signaling-1 (STS-1) which has a histidine-based active site that renders it hyperactive at low pHi and results in the inactivation of T-cell receptor (TCR) signalling[27]. Further, (de)protonation events can drive changes in gene expression when the target is a transcriptional regulator or epigenetic modifier. For example, protonation of the His residue His196 in oestrogen receptor α, which is conserved in ~50 members of the nuclear steroid receptor family, stabilizes binding to DNA.[28,29] Similarly, protonated His554 is critical for DNA-binding of the transcription factor forkhead box P2 (FOXP2).[30] In contrast, early growth response (EGR)- and Krüppel-like factor (KLF)-family transcription factors bind more strongly to DNA under alkaline conditions due to His residues in key functional sites.[31] Another interesting example is that of the death-inducer obliterator protein (DIDO1), which shows enhanced interaction with tri-methylated histone H3 (H3K4me3) at raised pH due to a regulatory His in the binding site.[32] Long-term epigenetic consequences of

reduced pHe, that presumably occur via changes in pHi have been attributed to altered histone deacetylase and DNA methylase activity.[33-35] Other examples of more sustained effects involve the recently demonstrated impact of pH on mRNA processing[33] and ribosomal gene splicing.[36] For example, exposure to low pH was associated with transcriptome rewiring involving altered RNA splicing and increased expression of targets of RNA binding proteins with specificity for AU-rich motifs such as the ELAV-like protein (also known as HuR) family of RNA binding proteins[33]. However, the most persistent changes to protein protonation arise from genetic alterations that shift the pK_a of functionally important motifs in the protein-product. This process occurs naturally as part of evolution; for example, a change from Ala to His in troponin-I allows skeletal muscle to be more resistant to lactic acidosis, compared to cardiac muscle.[37] Due to the inherent genetic instability of cancer cells, similar evolutionary processes are expected to occur in tumours on a much-accelerated time-scale.

[H2] Acid-base fluxes set the level of pH in cells and tissues

To understand the role of pH in cancer, it is first necessary to describe the processes that determine pHi and pHe in normal tissues. Whilst some biochemical reactions generate chemical bases, the metabolism of most cells is a net source of acid. In particular, cellular pH homeostasis is continuously challenged by lactic acid production by fermentation and CO_2 production by mitochondrial respiration (**Fig 1a**). Lactic acid dissociation and CO_2 hydration release H^+, thereby reducing pHi to a lower steady-state level. To offset this metabolic acid production, a matching flux of acid must be removed from the cell (**Fig 1a**). Membranes are typically highly permeable to CO_2, but the export of lactic acid as H^+ and lactate requires facilitation by monocarboxylate transporters (MCTs), notably MCT1 (also known as SLC16A1) and MCT4 (also known as SLC16A3).[38] These isoforms differ in their substrate affinity; for example, the lower lactate affinity of MCT4 favours lactate efflux whereas MCT1, with its higher lactate affinity, is often considered an influx pathway.[39] However, the net direction of transport via MCTs depends on the sum of the concentration gradients for lactate and H^+, and in many glycolytic cancer cells, this is outward-directed unless extracellular lactic acidosis is extreme[40,41]. When the outward transmembrane gradients and permeability are large enough, efflux of CO_2 and lactic acid can match their production and pHi will be steady. An intracellular excess of H^+ may also be re-distributed into organelles, such as lysosomes, but this only removes acidity from the cell if the organellar contents are ultimately exocytosed. For the same reason, pH buffering **[G]**, the reversible binding of H^+ to buffers, does not remove acidity from the cell but, instead, stores the excess acidity in a saturable chemical reservoir that will eventually need emptying. The most intuitive way of removing H^+ from a cell is by direct H^+ transport across the plasma membrane (**Fig 1a**). An alternative strategy exploits the chemical equilibrium between H^+ and its buffers, which allows the selective transport of the acidic or basic form of a buffer to change

pHi. The body's most abundant buffer is CO_2/HCO_3^-, facilitated by carbonic anhydrases **[G]** (CAs), and selective transport of HCO_3^- (or possibly CO_3^{2-})[42] is a widespread mechanism of regulating pHi. In this instance, HCO_3^- is an H^+ equivalent **[G]**. Coupling the transport of H^+ or H^+-equivalents to a source of energy, such as ATP hydrolysis or a flux of another ion, can energise uphill net acid transport and regulate pHi to a desirable set-point. Notable examples of such transporters are Na^+/H^+ exchangers (NHEs), Na^+/HCO_3^- cotransporters (NBCs), and H^+-ATPases.

Following transport across the plasma membrane,[43] acid must be transmitted across the aqueous extracellular compartment (**Fig 1b**). This diffusive process is passive but often facilitated by mobile buffers. Since the majority of H^+ is buffered, the apparent diffusivity of acid relates to the properties of buffers, namely their mobility and (de)protonation kinetics. This is problematic for the most abundant buffer, CO_2/HCO_3^-, because of its inherently slow spontaneous reaction kinetics.[44] To overcome this kinetic disadvantage, most cells express CAs[45]. Eventually, the excess acidity is carried away with the capillary blood flow, which in most tissues are close to metabolically active cells in order to limit the diffusion path length. With good capillary perfusion, short interstitial diffusion distances, and feedback-operated membrane transport, most cells and tissues experience only small fluctuations of pHe or pHi. Typically, pHe is between 7.3 and 7.4 and thus effectively clamped to plasma pH, and pHi ranges from 7.0 to 7.3.[46,47] . However, functionally relevant dynamics in pHi take place during normal development[21,48]. Moreover, pHe fluctuations occur physiologically, for example, in skeletal muscle during exercise[49] and in epithelia responsible for high capacity acid-base transport, such as the kidney, pancreas, and stomach.[50] Low pHe has also been reported in the T zones of lymph nodes[51] and in inflammatory states, such as chronic pancreatitis or cystic fibrosis.[50]

[H2] Regulation of pHi and pHe in tumours

Disruption at any point in the acid-handling cascade – from metabolism, membrane transport, diffusion, to perfusion – can result in deviations of pHi and pHe from their physiological levels. Tumours, compared with most normal tissues, have dramatically altered metabolism, accelerated biomass growth, and aberrant perfusion. These modifications invariably alter the balance of acid-fluxes, and hence pH (**Fig 1c**).[52,53] Additionally, normal homeostatic mechanisms for acid transport across membranes or acid sensing can undergo changes as a direct or indirect consequence of mutations.[54] For instance, in leukaemia, several oncogenic mutations confer epigenetic upregulation of MCT4 through change in histone acetylation[54]. Furthermore, it is well established that activating KRAS mutations upregulate glucose uptake and promote glycolytic activity.[55,56] The range of pHi and pHe within tumours will depend on cancer type and stage of disease progression. For example, the overall metabolic rate, the relative dependency on oxidative phosphorylation **[G]** (OXPHOS) and glycolysis, the cellular capacity for net acid extrusion **[G]**, and the distance to the nearest perfused

blood vessels vary between tumours and influence the tendency for metabolites to accumulate in the interstitial space. Relatively little is known about pHe in early hyperplastic stages, such as ductal carcinoma in situ (DCIS) or pancreatic intraepithelial neoplasia (PanIN), ostensibly because of their small size. A recent study of pre-malignant lesions of the breast described higher net acid extrusion capacity, and hence elevated pHi.[57] Disturbances of pH homeostasis are better characterised in cancers at the stage of attaining a solid tumour mass, with low pHe emerging as a common feature.[4,58] Nonetheless, *in vivo* studies of tumour pH must be interpreted with care, because different methods report either pHe, pHi, or a combination thereof. Certain magnetic resonance-based methods have been designed to explicitly probe pHi[3,59,60] or pHe[61] whereas others, such as positron emission tomography (PET) with carbon-11-labeled dimethyloxazolidinedinone (^{11}C-DMO), report a weighted average of pHi and pHe.[62] Measurements using pH-sensitive electrodes typically access extracellular spaces (although some cellular damage can be inflicted) and were the first to indicate that the tumour microenvironment is acidic.[3,4] However, variation in tumour pHe measurements is substantial, with values reported in the range 5.6 to 7.4 in squamous cell carcinomas, 6.5 to 7.2 in gliomas, and 6.5 to 7.5 in mammary cancers.[3,4] These ranges may reflect variation between tumours of the same cancer type or differences arising from disease subtypes (e.g., highly and less glycolytic subtypes of mammary cancer)[63]. Alternatively, at least some of this variation may be explained by intra-tumoural spatial heterogeneity, as revealed subsequently by imaging methods.[33,64] The range of pHe during invasive stages likely depends on the pattern of dissemination, for example local spread *versus* circulating cancer cells. During intravasation, spread and extravasation, pHe will, at least initially, resemble that of normal interstitium, which is more alkaline than in the primary tumour. However, as the secondary tumour grows in mass, pHe is expected to fall for the same reasons as in the primary lesion.

The pHe of solid tumours is reduced because a large flux of acid is released from metabolically active cells into an extracellular space that has poor diffusive coupling with aberrant capillary perfusion[4,53,65]. Nonetheless, certain adaptive mechanisms in cancer cells address these bottlenecks. For example, CA isoforms with extracellular-facing catalytic sites, such as CA9 and CA12,[66-70] have an important role in facilitating acid diffusion across the extracellular compartment in tumours (**Fig 1**). Moreover, their abundance is increased under hypoxia, that is in regions of poor diffusive coupling, to match availability of enzyme activity with the need to facilitate diffusion. CA9 and CA12 activity has sometimes been proposed as the cause of extracellular acidosis, but this is potentially misleading because the net direction of catalysis depends on the concentrations of H^+, CO_2 and HCO_3^-.[53,70,71] Thus, CA activity will reduce pHe[71] if the net reaction is in the direction of CO_2 hydration, which is likely in respiring regions of tumours. The converse will be true in extracellular spaces into which HCO_3^- or H^+ are secreted; here, CA activity will raise pHe.[70] Irrespective of the net direction of catalysis and pHe change, extracellular-facing CA isoforms facilitate CO_2 or H^+ diffusion over long distances. Some experimental findings have been interpreted in terms of a role of CAs in

supporting H^+ or HCO_3^- transport through a so-called metabolon **[G]** involving direct physical interaction between the transporter and the CA molecule.[72,73] However, calculations and subsequent studies have questioned the physiological relevance of such physical interactions.[44,74] A second adaptive response to curtail the fall of pHe in solid tumours is to stimulate the expansion of a well perfused capillary network.[53] To that end, many cancers secrete pro-angiogenic factors such as vascular endothelial growth factor (VEGF).[75] Finally, cells along the path from the acid-producing cancer cells to capillaries can minimize pHe drops, for instance, by lactate/H^+ uptake and subsequent lactate oxidation[76]. This is possible in tumours with large radial pO_2 gradients because there will be regions with sufficient access to oxygen for oxidative metabolism to proceed.[77]

The aforementioned adaptations will facilitate acid venting in tumours, without impacting transport across the cancer cell membrane. However, various feedbacks are activated at low pHe to influence cell metabolism and membrane transport. For example, the build-up of lactate and H^+ extracellularly will hinder MCT-facilitated lactate/H^+ efflux from cells, which in turn can reduce pHi and inhibit fermentative metabolism **[G]**. This feedback reduces lactic acid fermentation to protect the extracellular and intracellular fluids from deleterious acidification, at the expense of a lower fermentative rate[78]. Acid-base transporters also receive pH feedback from either side of the plasma membrane to fine-tune their activity. This feedback arises from thermodynamic constraints on the transport cycle and allosteric pH-sensing domains. Whereas a fall in pHi activates acid-extruders, for example the Na^+/H^+ exchanger NHE1 (also known as SLC9A1) and inhibits acid-loaders such as the Cl^-/HCO_3^- anion exchange protein 2 (AE2, also known as SLC4A2), a fall in pHe generally has the opposite effect.[79,80] The latter influence is important because it causes pHi to be responsive to changes in pHe. The strength of this coupling can be determined by plotting the pHe-pHi relationship **[G]** and calculating its slope (**Fig 2a-c**). A study using a panel of 66 colorectal cancer cells reported a range of slopes between 0.18 and 0.50,[46] but slopes as high as 0.8 have been described in other systems.[80,81] Whilst pHi responds to pHe changes in the short term, the pHi-regulatory apparatus can adapt to chronically low pHe and return pHi to its physiological level. In colorectal cancer and pancreatic ductal adenocarcinoma cells, for example, exposure to low pHe for several days reduces the abundance of the acid-loader AE2 at the cell surface,[46] raising pHi and flattening the apparent pHe-pHi relationship (**Fig 2a-c**).

In contrast to the consensus that pHe of solid tumours is remarkably low, available studies have indicated that the bulk of the tumour intracellular compartment is near the physiological pHi range.[59,60] This is somewhat counter-intuitive, because low pHe is also expected to reduce pHi, but the explanation lies in considering changes to the pHe-pHi relationship; for example, a higher capacity to extrude acid from cancer cells will raise pHi. Many studies investigating cancer cell pHi under notionally physiological extracellular conditions (pHe 7.4 with 5% CO_2 and 22 mM HCO_3^-) have reported pHi levels that overshoot the physiological range. This observation is likely to be a

consequence of upregulated net acid extrusion capacity, which becomes even more activated when pHe is raised as part of the experimental manoeuvre.[81]Reports of elevated pHi in cancer cells at the physiological pHe of ~7.4 have often been extrapolated to claim that the pHi of cancers *in situ* is also above physiological pHi.[82] However, this fails to consider that cancer cells in a solid tumour are typically bathed in an acidic milieu, which hinders net acid extrusion and reduces pHi *per se*, possibly back to the physiological range.

It is plausible that sensing mechanisms (**BOX 1**) in cancer cells of solid tumours fine-tune pHi regulation to generate the most favourable steady-state pHi. One example is that the loss of the HCO_3^- sensor receptor protein tyrosine phosphatase γ (RPTPγ) in breast epithelium raises expression levels of the net acid extruder NBCn1 (also known as SLC4A7). [57] This then elevates pHi during early transformation to support proliferation.[57] Considering the heterogeneity and dynamics of the chemical environment of tumours, the balance of acid-base fluxes will vary greatly between tumour regions. Consequently, a tumour is likely to harbour cancer cells with either reduced or raised pHi in different regions at any one point in time.[81,83]

[H1] ACIDOSIS MODULATES ONCOGENIC TRANSFORMATION

[H2] Mutagenic effects of acidic conditions

During oncogenic transformation, cells accumulate activating mutations in oncogenes and inactivating mutations in tumour suppressor genes. These genetic changes confer a malignant phenotype characterised by sustained proliferation, evasion of growth suppressors, resistance to cell death, and replicative immortality.[84] Under *in vitro* conditions, exposing cells to profoundly acidic conditions can cause the structure of DNA to become unstable by promoting double-stranded DNA breaks and inhibiting DNA damage repair.[85-88] This clastogenic effect **[G]** is unlikely to be a significant player in cancer initiation because most tissues do not experience the magnitude of extracellular acidosis necessary for such DNA changes. Nonetheless, mutagenic effects of low pHe may contribute to the cancer-promoting influences of inflammatory conditions, such as pancreatitis and inflammatory bowel disease.[50,89]

[H2] Mutations that stimulate net acid-extrusion from cells

Whereas mutagenic effects of acidosis are unlikely to underpin early oncogenic transformation, genetic changes that affect pHi homeostasis can realistically influence cell behaviours at this point and possibly facilitate carcinogenesis (**Fig 2d**). Early in the disease process, when cells undergo transformation in a milieu close to physiological pHe, overexpression or activation of net acid extruders at the plasma membrane can raise pHi[57] and evoke a myriad of downstream changes.

Strikingly, many oncogenes increase the expression and/or activity of net acid-extruding proteins, notably of NHE1 by oncogenic Ras[90] and papillomavirus E7 oncogene,[91] and of NBCn1 by oncogenic ERBB2 (also known as HER2).[92,93] There are multiple examples of how pHi accelerates carcinogenesis.[57,83,94-96] Specifically, human and mouse data indicate that susceptibility to breast cancer is increased by genetic variants that raise the expression or activity of net acid extruders, for example NBCn1, directly[63,96,97] or through relevant sensors, such as RPTPγ[57] (**BOX 1**). The upregulation of net acid extrusion may be necessary to offset a concurrent increase in metabolic rate also conferred by oncogenes; for example, ERBB2 activity stimulates both glycolysis and NBCn1 overexpression.[92] In these scenarios, there may be little or no overall effect on pHi, but increased net acid extrusion should be viewed as permissive for the higher metabolic rate.[19,94]

[H2] Mutations that alter the pH-sensitivity of pro-oncogenic processes

For transformation to proceed, mutations must produce a meaningful pro-survival benefit that is selected positively by somatic evolution. A distinct means of changing protein activity is to re-tune its pH-sensitivity through amino acid substitutions, insertions or deletions that affect the pK_a of relevant motifs (**Fig 2e**).[98] Such modifications may cause oncogene-coded proteins to become abnormally active, or tumour-suppressors to become inactive, even at constant pHi. It is therefore notable that substitutions of arginine (Arg; pK_a ~12) to His (pK_a ~6.1, but often nearer to ~7.0 in the folded protein) occur at a frequency of 6%, which is markedly higher than other amino acid substitutions, in cancer-driving genes such as epidermal growth factor receptor (*EGFR)* and *TP53.*[98-100] The Arg776His mutation in the gene-product of the oncogenic *EGFR* accentuates pH-sensitivity and stimulates downstream ERK signalling, proliferation, and adhesion-independent colony formation at higher pH values, whereas the Arg273His mutation in the gene-product of the tumour suppressor *TP53* reduces its transcriptional activity at higher pH values, in turn reducing its anti-tumorigenic effect.[99] Synergy between mutations affecting pHi-sensitivity and those disrupting pHi homeostasis would be a powerful means of facilitating positive selection. Whilst the over-representation of Arg-to-His and His-to-Arg somatic mutations in human cancers is striking and argues for a role of pH-sensing in cancer initiation, only a handful of these mutations have been studied in terms of the pH-sensitivity of protein function. Considering that somatic evolution selects by phenotype rather than genotype *per se*, such investigations are urgently needed, and their outcomes may influence our appreciation of the role of pH in early oncogenic transformation.

[H1] CANCER PROGRESSION IN AN ACIDIC ENVIRONMENT

[H2] Towards a microenvironment that produces acid-selection

Once transformed, cancer cells become proliferative and establish the primary tumour mass. When the tumours are very small, net acid venting is still within the physiological limits of diffusive coupling, and it is unlikely that pHe falls substantially. However, with further growth and as perfusion becomes inadequate, tumours will attain a low pHe (**Fig 2d**).[101,102] This extracellular acidosis then acts as a selection pressure that favours the expansion of acid-resistant clones[101-103], which are capable of surviving in the harsh, acidic microenvironment, while non-resistant clones succumb. Furthermore, genetic instability in cancer cells could be exacerbated by profound acidosis, due to its mutagenic effect, thereby establishing a vicious cycle that accelerates the emergence of more aggressive phenotypes and disease progression.[85-88] Darwinian dynamics widen the phenotypic gap between transformed and normal cells, allowing the former to dominate. If acid-stress increases gradually, cells may have time to adapt through an iterative process and acquire heritable advantages that enable survival at low pHe. In contrast, although signalling and posttranslational mechanisms, for example through acid-sensing receptors (see below and **Fig. 3**), can elicit adaptive responses to acidic stress, a rapidly imposed acid-stress is more likely to eliminate large swathes of cells, making way for a minority of fitter clones that had acquired an acid-resistant phenotype *a priori* by means of random mutagenesis. These acid-resistant cells will, as a consequence of their increased net acid extrusion capacity, attain a higher pHi when invading non-tumour tissue of physiological pHe. This elevation of pHi will increase their capacity for proliferation, translation, and motility, and limit their vulnerability to apoptotic cell death.[19,104]. A profoundly acidic pHe is not expected until the secondary tumours reach a certain size (**Fig 2d)** with implications for pH-directed cancer treatments, as described below.

[H2] Cancer cell-autonomous mechanisms of acid-driven oncogenic progression

A routinely used experimental approach for studying cancer progression is to mimic tumour microenvironment acid-stress *in vitro*.[105,106] Supporting that acidity drives phenotypic changes in cancer cells, month-long culture at low pHe (~6.5) produces transcriptional changes that resemble those distinguishing cancer from normal tissues.[107] Although the mechanisms that underpin acid-driven phenotypic changes are not well understood, three common traits stand out.

The first trait is that cancer cells cultured under acidic conditions undergo extensive metabolic reprogramming, such as a partial reversal of the carcinogenesis-stimulated fermentative phenotype,[78] increased consumption of nutrients other than glucose (such as fatty acids and glutamine), lipid droplet accumulation, increased β-oxidation, and greater reliance on OXPHOS (**Fig 3**).[105,106,108-112] In addition to the pH-sensitivity of glycolytic enzymes (**Fig 2e**),[113] the decrease in fermentative glycolysis at low pHe has been suggested to involve the upregulation of the glycolysis suppressors, thioredoxin-interacting protein (TXNIP) and arrestin domain-containing protein 4 (ARRDC4), driven by the transcription factor MondoA (also known as MLXIP) downstream from

acid-sensing mechanisms.[114] A recent study points to the involvement of the H^+ sensor GPR68 (also known as OGR1, **BOX 1**) in lipid droplet accumulation in cancer cells at low pHe.[106] Acid-resistant cancer cells have increased reliance on peroxisome proliferator-activated receptor α (PPARα), which is activated by fatty acids and drives upregulation of mitochondrial and peroxisomal fatty acid β-oxidation and also upregulates TXNIP.[112] The mechanism of increased cellular fatty acid entry at low pHe is unclear; a role for CD36-facilitated transport is suggested,[108] but the effect could simply be driven by fatty acid protonation to a more membrane-permeable form.[115] An acidic environment can also impact cancer cell metabolism via mTOR inhibition at low pHi.[116] Low pHi leads to lysosome translocation toward the plasma membrane and away from the perinuclear mTOR activator, the small GTPase RHEB, thereby preventing mTOR activation (**Fig. 3**).[116] The resulting inhibition of translation blunts circadian rhythms, a notable example of a process that was initially thought to be hypoxia-driven, but subsequently confirmed to be regulated by acidosis.[116] The translocation of lysosomes towards the cell periphery is, indeed, a trait of aggressive cancer cells that is triggered by acidosis (**Fig. 3**).[117] To explain the aforementioned metabolic changes, it is important to consider the sources of ATP and their yield. Whilst 'bulk' ATP production is believed to be lower in solid tumours than in the corresponding normal tissue,[118] measurements of steady-state intracellular ATP concentration *in vitro* suggest raised levels, particularly in chemo-resistant cancer cells.[119] This conundrum can be explained by a reduction in ATP demand, which would imply that the hyper-proliferative and de-differentiated phenotype of cancer cells diverts energetic resources and building blocks to cell division and growth, at the expense of tissue-specific functions inherited from parental cells.[118] The carcinogenesis-associated *relative* shift from OXPHOS to fermentation, first described by Warburg, reduces ATP production but raises its H^+ yield in solid tumours. This shift is important for generating the acid-stress that then contributes to somatic evolution.[78,109,111,112] However, cells cannot perpetually increase their energetic reliance on fermentation because the ensuing acidosis will, eventually, inhibit glycolytic enzymes (**Fig 2e**). Alongside competition for a finite supply of glucose, this acid-evoked inhibition of glycolysis forces cancer cells to resort to alternative nutrients, notably lipids and glutamine that require the tricarboxylic acid (TCA) cycle and OXPHOS for processing.[105,109,111,112] Conveniently, respiration is pH-insensitive and persists even at low pHe[110,120] as an essential means of generating the majority of ATP in cancer cells.[78,121] The capacity for lipid metabolism will, however, depend on oxygen availability[122] and under restricted oxygenation, the lipids required for growing biomass will be obtained by uptake to circumvent nicotinamide adenine dinucleotide (NAD^+) insufficiency.[123]

The second common trait relates to amplified net acid-extrusion arising from the upregulation of acid-extruders, including NHE1 and NBCn1,[57,83] and induction of MCT4 (**Fig 3**).[124-127] Enhanced net acid extrusion promotes cell proliferation, particularly in tumour regions of high glycolytic activity and acid loading.[83,96]These adaptations can help maintain pHi homeostasis under acidic conditions, but will overshoot the physiological pHi range upon exposure to higher pHe, such

as metastatic cells or invasion into new niches (**Fig. 2d**).[81,83,112] Interestingly, acid-adapted cancer cells acquire greater invasiveness and a growth advantage over normal cells when entering an environment of physiological pHe (~7.4).[128] This reflects that the acid-induced aggressive phenotype is at least partially a product of selection and can be further amplified by the raised pHi observed upon exposure to physiological pHe[128,129].

The third trait relates to increased invasiveness and metastatic potential (**Fig 3**).[108,128,130,131] The underlying mechanisms include: acidosis-induced changes to the tumour matrisome **[G]** and capacity for motility, such as epithelial-to-mesenchymal transition (EMT) driven by transforming growth factor-β (TGFβ)[108] or Ca^{2+}–RhoA[132] signalling, the impact of low pHe on interactions between cells and their matrix,[22,133] and CA9 overexpression, which promotes EMT and metastasis.[134] Pericellular zones of localised acidity are likely to favour extracellular matrix degradation because matrix metalloproteinases (MMPs) show an acidic optimum and elevated expression in acidic tumour regions.[33,135] It is also interesting to note that elevated pHi or disrupted acid-base sensing correlates with more aggressive histopathologies;[57] for example, malignant breast tumours have increased pHi compared to lower grade tumours.[63]

[H2] Cancer–stromal cell interactions impacted by extracellular acidity

Non-transformed cells of the tumour stroma are expected to be less resilient to acidosis compared to neighbouring cancer cells. Moreover, stromal cells can become co-opted by cancer cells to support tumour growth through mechanisms enhanced under acidosis (**Fig 4**).[136,137] Multiple studies have shown that tumours evade immune surveillance at low pHe, in synergy with lactate, itself a powerful immunomodulator.[136,137] Low pHe causes $CD8^+$ T-cells to enter an anergic state,[138,139] upregulates immune checkpoint proteins on T-cells,[140] switches macrophages to an anti-inflammatory tumour-associated phenotype,[141,142] and inhibits the activity of natural killer (NK) cells (**Fig. 4**).[139] In mammary cancer cells, upregulation of the immune checkpoint protein programmed cell death ligand-1 (PD-L1) has been implicated in acidosis-evoked immune evasion.[143] In T-cells at low pHe, enhanced engagement of the V-domain immunoglobulin suppressor of T-cell activation (VISTA) may also contribute to immune suppression.[144] In line with these findings, neutralizing tumour acidity through $NaHCO_3$ ingestion has been shown to potentiate anti-cancer immunotherapies.[145] However, a more recent study described how prolonged extracellular acidosis restricts one-carbon metabolism in CD8+ T-cells, thus preserving epigenetic T-cell stemness by reducing methionine levels and histone methylation.[146] This mechanism was found to limit T-cell exhaustion, such that acid-expanded lymphocytes had enhanced anti-tumour activity. A possible explanation for the apparent contradiction with the above-mentioned acid-induced $CD8^+$ T-cell anergy[138,139] may relate to the extent and duration of acidosis. While short-term extracellular acidosis reduces $CD8^+$ cell effector cytokine production without affecting transcription factor 1

(TCF1) expression or stemness, prolonged acidosis promotes TCF1 expression and alters T-cell metabolism, favouring a stem-like state.[146] Consistent with the role of net acid-extruders in acidifying the tumour microenvironment, interfering with the activity of NBCe1 (also known as SLC4A4) in pancreatic cancer cells restored T-cell function and curtailed macrophage-mediated immunosuppression.[127] Acidosis could also play a role in determining the fate of recruited monocytes by skewing their differentiation to dendritic cells. This effect involves mTORC1 inhibition at low pHi[147] and is consistent with other reports suggesting that acidosis stimulates the maturation and function of dendritic cells.[148,149]

In addition to the impact of pHe on immune surveillance, survival of cancer cells in the acidic tumour microenvironment can be supported by non-immune stromal cells (**Fig 4**). For instance, cancer-associated fibroblasts (CAFs) stimulated by TGFβ have greater capacity to absorb cancer cell-derived acid through upregulated anion exchanger AE2.[150] This effect could be particularly beneficial in dampening a rapid decrease in pHe. CAFs also upregulate CA9, which was proposed to increase the activity of MMP-2 and -9 released from CAFs, which in turn supports EMT in cancer cells.[151] The endothelium is another stromal component essential for tumour growth. Most prior studies, as well as the use of angiogenesis inhibitors in clinical cancer treatment, have focused on the newly-forming tumour vasculature.[152] However, interventions that influence the contractile activity of mature blood vessels and hence tumour perfusion more acutely provide an interesting alternative approach with potential to modify local pH, pO_2, and drug delivery and hence responses to therapy .[153] Similar to most normal blood vessels, acidosis causes vasorelaxation of the arteries that supply solid tumours.[154] However, these tumour feed arteries, compared to normal arteries, show dramatically different vasomotor responses, for example to α_1-adrenoceptor agonists[154] that can be exploited to maximize tumour perfusion *in vivo*.[70] In normal tissues, even modest acidosis can inhibit endothelial cell proliferation, migration, and differentiation.[155,156] The H^+ sensor GPR4 (**BOX 1**) is highly expressed in the endothelium and was recently reported to drive a stress response in the endoplasmic reticulum of normal endothelial cells, presumably eliciting a downstream inflammatory response.[157] However, tumour and normal endothelial cells differ in several ways; for instance, tumour endothelial cells are hyperglycolytic[158] and, unlike their normal counterparts, can proliferate under lactic acidosis in a manner dependent on VEGF-induced CA2 upregulation (**Fig. 4**).[159] Accordingly, acidosis stimulates secretion of VEGF and basic fibroblast growth factor (bFGF) in brain tumours, as well as from normal and immortalized endothelial cells.[52,155,160,161] Moreover, VEGF and nitric oxide (NO) potentiate each other's signalling cascades.[162] Whereas the overall influence of low pHe on NO release varies between vascular beds,[163,164] acidification of endothelial cells invariably inhibits NO synthesis.[165,166] The release of pro-angiogenic factors into the acidic tumour microenvironment may compensate the growth-restricting actions of acidosis that dominate most vasculatures.[156] Specifically, the inhibitory effect of tumour acidity on NO-mediated vasodilation and angiogenesis[167] is counteracted by upregulation of endothelial NO

synthase in human colon cancer feed-arteries.[168] In summary, the evidence, albeit not mechanistic, indicates that tumour endothelial cells acquire a degree of acidosis-resistance in order to maintain tumour perfusion.

[H1] THE RELATIONSHIP BETWEEN ACIDOSIS AND HYPOXIA

The relationship between tumour acidosis and hypoxia has been debated since the first measurements of pO_2[169,170] and pH[65,103,171-174] in human and experimental cancers. A noteworthy difference between O_2 and H^+ is membrane permeability, which is substantially lower for the latter. Consequently, it is possible for pHi to be different from pHe, whereas pO_2 is likely to equalise across membranes. Tissue hypoxia, at normal arterial oxygenation, reflects the imbalance between cellular respiration (the major consumer of O_2) and capillary perfusion. High respiratory rates require an adequate flow of O_2 down a diffusion gradient between blood and respiring cells. When capillary perfusion is compromised, the pO_2 gradient becomes steeper, resulting in hypoxia at the tissue core.[169] Since respiration also produces CO_2, a gas that yields H^+ upon hydration, an increase in respiratory rate or decrease in perfusion are expected to reduce pO_2 and pH in tandem.[66,175] The stoichiometry of O_2 consumption over CO_2 production is substrate-dependent and equal to the respiratory quotient (RQ). This mechanistic coupling implies a *causal* relationship between hypoxia and acidosis. However, a parallel source of acidity in tumours is lactic acid fermentation, which does not consume O_2 and therefore uncouples pO_2 from pH. Whilst there have been debates about the lactate-to-H^+ stoichiometry,[176] a simple consideration of charge balance indicates that glucose must produce two H^+-equivalents if ATP hydrolysis is in balance with ATP synthesis.[177] Acidosis is therefore a hallmark of all under-perfused and metabolically active tumours, whereas hypoxia is expected in tumours with high respiratory rates but restricted O_2 supply.

Most human cancers rely on a combination of respiration and fermentation, subject to local microenvironmental context and intracellular regulators.[95] For example, the fermentative rate is limited by acid-inhibition of glycolysis[78] (**Fig 2e**) . As a result, fermentative metabolism is unlikely to support cells adequately in acidic niches, unless these are exceptionally effective in maintaining a constant pHi. According to a recent CRISPR–Cas9 screen, survival of colorectal cancer cells under acidic conditions *in vitro* is contingent on adequate respiration.[110] Conversely, cells relying on respiration are limited by adequate oxygenation. In the fluctuating chemistry of the tumour microenvironment, selection pressures are likely to retain both fermentative and respiratory phenotypes.[95] This pragmatic survival strategy also makes it harder for single-target therapies to kill cancer cells.[102] Indeed, acid-selection was shown to drive cancer cells to a more respiratory metabolic phenotype, however, the cells retained a robust glycolytic capacity and switched to glycolysis when respiration was prevented[112]. Our understanding of cancer metabolism has

therefore shifted from Warburg's dogma of a predominantly fermentative phenotype, towards the notion of a co-habitation of respiring and fermentative cells. Whilst it is tempting to link pHe and pO_2 using simple metrics, their relationship is complex and requires a spatially resolved appreciation of metabolism, buffering, diffusion, and geometry. Not surprisingly, measurements at fine resolution have described the relationship between pHe and pO_2 as neither stoichiometric nor overlapping.[33]

The association between hypoxia and acidosis in tumours has made it challenging to distinguish the contributions from pO_2 and pH to cancer biology. Hypoxia has historically been championed as the major microenvironmental factor shaping cancer progression, a bias strengthened by the discovery of HIF,[178] which provided a tractable pathway for experimental interrogation. In contrast, effects of acidosis are not transduced by a single pathway but are, instead, the ensemble of a myriad of pH-sensitivities that are less amenable to experimental studies. Without appropriate control measures, it is difficult to unambiguously attribute biological responses to pO_2 or pH when both vary concurrently. Furthermore, the signalling interplay between hypoxia and acidosis is dynamic. Imposing hypoxia onto cells stabilises hypoxic signalling, which then causes lactic acidosis. Conversely, there have been reports that low pH increases HIF signalling, via nucleolar sequestration of the von Hippel-Lindau (VHL) protein[179] that normally drives HIF degradation, or via a heat shock protein 90 (HSP90)-dependent pathway[180]. One way to differentiate these mechanisms is to manipulate pHe and/or pHi independently of other factors and then compare the outcomes to paired experiments where pH is allowed to change alongside other influences. Recent studies have taken this approach to identify pH-sensing pathways, ranging from surface-expressed receptors to nuclear transcription factors.[112,114] Inconsistent or inadequate reporting standards often prevent a retrospective review of the literature to distinguish pH effects from other actions. However, given the intensity of acid-production and omnipresence of pH-sensitivity, it is highly probable that pH has been a major contributor in multiple studies of signalling in cancer (**BOX 2**).

[H1] EXPLOITING TUMOUR pH FOR DIAGNOSIS AND TREATMENT

In terms of therapy, the acidic tumour microenvironment is recognised for its effect on reducing the efficacy of weakly basic chemotherapeutic drugs, such as doxorubicin due to reduced cellular uptake or increased compartmentalization[6,112,181]. However, the acidic tumour microenvironment also reveals new therapeutic opportunities. Druggable vulnerabilities include metabolic or ion transport processes that underpin acid-base disturbances, the mechanisms that endow cancer cells with acid-resistance, or the signalling pathways that become altered because of changes to pH or its sensing. Other interventions that affect metabolism (for example metformin)[182] or perfusion (such as anti-VEGF therapies)[183] inevitably affect pHi and/or pHe, but are outside the scope of this review.

Buffering of tumour extracellular acidosis has been explored as a means of limiting acid-driven growth and metastasis. Oral administration of sodium bicarbonate or the H^+ buffer Tris raised tumour pHe in mouse models of breast, pancreatic, and prostate cancer, which inhibited metastasis,[184,185] and enhanced the efficacy of immunotherapy.[145] Other studies have not confirmed these beneficial influences, or even described pro-malignant responses in primary tumours.[186] Buffer therapy **[G]** was initially trialled for cancer-related pain[187188] and in combination with gemcitabine in non-resectable pancreatic cancer[189]. However, those trials were discontinued due to poor patient compliance arising from the unpleasant side effects of sodium bicarbonate ingestion.[190] An alternative approach to raising tumour pHe proposes targeted delivery of an antibody-conjugated urease, which generates NH_4^+ and HCO_3^- locally in tumours, thus raising buffering capacity. This treatment appears to be well tolerated and show therapeutic promise in patients with non-small cell lung cancer.[191]

Cancer cells residing in acidic tumour microenvironments often rely on activated net acid-extrusion for survival. Interventions that block these adaptations and reduce pHi are therefore a therapeutic opportunity. Indeed, *in vivo* studies using a variety of mammary cancer models have demonstrate reduced tumour growth after genetic ablation of acid-extruding transporters, such as NBCn1 or NHE1, or of CA9-facilitated H^+ diffusion .[69,83,125,192] Several ongoing trials assess the potential of a chimeric IgG1 monoclonal antibody targeting CA9 (Girentuximab) mainly for diagnostic tumour imaging.[193-196] Because of redundancy in mechanisms of pH regulation, a promising therapeutic strategy is the simultaneous targeting of multiple acid-base regulatory proteins as well as the molecules that link tumour acidosis with increased cancer aggressiveness. The latter includes pathways that have altered pH-sensitivity as a result of mutations. The effect of targeting net acid extrusion is particularly promising in combination with immunotherapies, as illustrated recently by the successful relief of acid-induced immunotherapy resistance achieved by targeting of NBCe1.[127]

Since the 1980s, extracellular acidosis has been associated with chemotherapy resistance.[6] This effect is partly due to protonation-dependent extracellular trapping of weakly basic drugs, including doxorubicin, etoposide, mitoxantrone, and 5-fluorouracil. This ion trapping **[G]** also causes drugs to accumulate in acidic organelles, especially lysosomes, which limits their bioavailability at the intended targets.[181,197] Ion trapping can, however, also strengthen therapeutic actions; for instance, 5-(N-ethyl-N-isopropyl) amiloride (EIPA) and other pyrazinoylguanidine compounds that inhibit NHEs, accumulate in acidic compartments and induce paraptosis-like cell death **[G]** in addition to their inhibitory effect on NHE1.[198] Acidosis may also contribute to chemotherapy resistance by maintaining cancer cell stemness, a state associated with drug insensitivity.[180,199]

Extracellular acidosis also provides an avenue for targeting drugs selectively to tumours. This includes acid-triggered drug release[200], and the increased affinity of an antibody developed

against the immunosuppressive CD47 molecule at acidic pH[201], and the generation of mutated cytokines such as interleukin-2 with altered pH sensitivity that enables $CD8^+$ T-cells to function more effectively in the low pHe tumour environment.[202] Such approaches could partially restore anti-cancer immune function in solid tumours,[127,138] whilst protecting normal tissues that are not acidic. Critically, however, strategies that exploit extracellular acidity may not show satisfactory efficacy in metastases, where pHe, at least initially, may not be as low as in primary tumours (**Fig 2d**).

Finally, monitoring acidosis at tumour margins has been useful for guiding surgery, as demonstrated in mouse tumour models using pH-responsive probes[203,204], and in human cancers using pH-sensitive nanoparticles.[205] Tumour acidosis, measured non-invasively, can be exploited to monitor responses during radio-chemotherapy in human gliomas, for example by CEST-MRI.[206]

[H1] CONCLUSIONS AND PERSPECTIVES

The past two decades have delivered remarkable progress in our ability to measure tumour extracellular acidosis, to study its consequences on cancer and stromal cells, and to design therapies that exploit vulnerabilities associated with dysregulated pH. These breakthroughs have brought long-overdue recognition to pH as a factor in cancer initiation and progression. However, many aspects relating to pH in cancer remain to be clarified. For example, a priority is to map the exact timeline of pHe and pHi changes, starting from the earliest events in oncogenic transformation. These chemical changes cannot be inferred from studying solid tumour masses or already transformed cells, but will likely require high-resolution *in vivo* monitoring. Another matter that warrants further investigation is the designation of disturbed pH regulation as either cause or consequence of cancer. Thirdly, more effort is needed to describe the functional consequences of mutations that impact the pH sensitivity of proteins, specifically those involving histidine residues. It may turn out that acid-base disturbances are more important than previously anticipated because of the breadth of post-translational actions stemming from protonation. To fully appreciate the scope of actions, it is necessary to look beyond rapid-onset responses and consider the longer-term consequences of pH, such as those involving epigenetic changes or DNA-transcription factor interactions. Finally, there is growing awareness of the importance of untangling the effects of pH from concurrent chemical changes, such as hypoxia and conjugate anions. With better understanding of the scope and limitations of pH-related mechanisms in cancer, opportunities to exploit vulnerabilities generated by acid-base disturbances in tumours will become more realistic.

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HIGHLIGHTED REFERENCES

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The authors adapt cancer cell lines of various origins to acidosis and find that lipid β-oxidation and PPARα activity are raised in acid-resistant cells, thus identifying PPARα to convey resistance to low pHe.

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The authors demonstrate that loss of the HCO_3^--sensing protein RPTPγ facilitates pre-malignant changes in breast tissue by elevating pHi, which is attributed to an activation of net acid extrusion, principally through NBCn1.

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This study shows that tumour acidosis mediates melanoma immune evasion by inducing the transcriptional repressor ICER in macrophages, which converts these toward a non-inflammatory phenotype.

13. Cheng, H. *et al.* Extracellular acidosis restricts one-carbon metabolism and preserves T cell stemness. *Nat Metab* 5, 314-330 (2023).

This study shows how low pHe impairs methionine metabolism, which alters histone methylation and preserves a stem-like memory state of T cells, thus revealing a mechanism how tumour acidosis suppresses T cell effector functions.

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Author contributions

The authors contributed equally to all aspects of the article.

Competing interests

E.B. is inventor on a patent addressing NBCn1 inhibition in cancer (EP-3271402). S.F.P. is co-founder of SOLID Therapeutics. The other author declares no competing interests.

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FIGURE 1: **Disturbances to the acid-handing cascade in tumours.**

a. Most cells are net acid producers and regulate intracellular pH (pHi) by membrane transport of H^+ and their equivalents. Glycolytic cells produce lactate and H^+, which cross the plasma membrane mostly through monocarboxylate transporters, such as MCT1. Respiring cells produce CO_2 that escapes across the cell membrane but can also partially hydrate to H^+ and HCO_3^- intracellularly through carbonic anhydrases (CAs). A favourable resting pHi is attained by fine-tuning acid influx and efflux pathways. Acid extruders include Na^+/H^+ exchangers such as Na^+/H^+ exchanger 1 (NHE1, also known as SLC9A1) and Na^+/HCO_3^- co-transporters (NBCs), such as NBCn1 (also known as SLC4A7). Acid loaders (not shown) include Cl^-/HCO_3^- exchangers like AE2 (also known as SLC4A2). **b.** Acid traffic across the membrane takes the form of CO_2, or H^+ coupled to, for example, lactate. Ultimately, this excess of acid is washed away with capillary flow or, in some circumstances, metabolised by cells *en route*. These ´sinks´ for acid are coupled to the acid-producing cell by diffusion, often facilitated by the mobile buffer CO_2/HCO_3^-, and catalysed by extracellular-facing CAs, such as CA9 or CA12. Once in the blood stream, capillary blood flow removes excess acid by means of convective transport. Ultimately, the lung and kidney ensure that blood pH is regulated through excretion of CO_2 and H^+, respectively. **c.** In cancer cells, the acid-handling cascade becomes altered or dysregulated at various points. Cancer cell metabolism can change the flux of acid and its relative composition (CO_2 versus H^+ or lactate). Membrane transport can be affected by mutations, changes in gene expression, or regulatory influences. Growth of the tumour mass can lead to large diffusion distances that cause larger extracellular pH (pHe) gradients to form at steady state, despite efforts to facilitate diffusion, for example by hypoxia-induced CA9 or CA12. Another confounding factor can be aberrant blood perfusion, which causes extracellular acid retention because of impaired washout. If the build-up of acid is substantial, it can feedback on the cell, dually by affecting trans-membrane driving forces and triggering pHe-sensitive cascades.

FIGURE 2: **The relationship between extra- and intracellular pH can be dynamic in cancer.**

a. Due to relatively low membrane permeability to H^+, it is possible for pH to be different on either side of surface membrane. Intracellular pH (pHi) can be offset relative to extracellular pH (pHe) by transporters, including secondary active transport **[G]** of H^+ or their chemical equivalents, either in the direction of acid-extrusion or acid-loading. Additionally, some finite permeability to H^+ can be conferred by passive pathways such as H^+ channels. Inadvertently, a coupling arises between pHe and pHi because they both affect the transport cycle of secondary active transporters and driving force across channels. However, the sensitivity of pHi to changes in pHe is highly dependent on the type of transporters present, and can be quantified in terms of the slope of the steady-state pHe-pHi relationship. **b.** In cancer cells, pHi control can become dysregulated by a change in the balance between acid-extruders and acid-loaders; for example, upregulation of acid-extrusion will raise pHi (indicated by (1)) from baseline. **c.** In the acidic tumour microenvironment, low pHe draws pHi (1) to a lower steady-state (2), at least in the short-term. The magnitude of this pHi response depends on the pHe-pHi slope and attained pHe. Over time, some cancer cells can adapt to their new milieu; for example, they can raise pHi (3) by reducing the levels of acid loaders such as AE2 at the surface membrane. This adaptive response raises pHi, and may fully or partially compensate for the initial pHi drop. Thus, there can be short- and long-term responses to low pHe, and the final level of pHi attained in cancer cells is difficult to predict and must be measured, with appropriate consideration to pHe and duration of acid-exposure. **d.** Changes in pHe and pHi during tumour initiation and progression. Colour coding illustrates the level of pH, relative to the physiological range of the relevant compartment. From top left: A layer of epithelial cells, including cells undergoing transformation as a consequence of oncogenic mutations. Once cells hyper-proliferate, the tumour mass undergoes metabolic changes and becomes inadequately perfused. This generates tumour extracellular acidosis, which can also acidify the intracellular compartment of cells. Some cells will succumb to the low pHe, but others may upregulate net acid-extrusion mechanisms and emerge as acid-resistant cells, with an apparently physiological or even supra-physiological pHi. Transforming cells from premalignant lesions, characterised by enhanced net acid-extrusion,[57] can overshoot the physiological pHi range. This can also be the case when acid-resistant cells escape the primary tumour microenvironment, during transit in fluids of physiological pHe, such as blood or the interstitium of remote organs, as part of metastasis and invasion. Only when micro-metastases grow to secondary tumours of a certain size pHe will decrease again, but this will have limited impact on a priori acid-resistant cells. **e**. Some mutations may affect pHi regulation, causing cells to alkalinise, referred to as ´pHi offset´ and affecting protein activity. Datapoints are reproduced from[78]. Arrow indicates direction of pH offset that triggers a change in pathway activity (here, glycolytic enzymes) ; other mutations may shift the pHi sensitivity of key proteins, causing some processes to become

aberrantly activated or inhibited at normal pHi. Common mutations affecting pH-sensitivity involve Arg-to-His or His-to-Arg mutations.

FIGURE 3: **The acidic tumour microenvironment alters cancer cell phenotypes.**

a. Cancer cells can sense changes in extracellular pH (pHe) through membrane-localized sensors, including H^+ sensing G protein-coupled receptors (GPCRs), such as GPR68, GPR4, and GPR65, acid-sensing ion channels (ASICs), and HCO_3^- sensing receptors, such as receptor protein tyrosine phosphatase γ (RPTPγ); and indirectly through pHe-pHi coupling, which transfers the pHe change onto a pHi change, thereby accessing a myriad of intracellular H^+ and HCO_3^- sensors. Responses to pHe trigger intracellular signalling events, such as the activation of transcription factors on peroxisome proliferator-activated receptor α (PPARα) and MondoA (also known as MLXIP). **b**. Cancer cells in low pHe environments undergo extensive metabolic changes. The accelerated glycolysis and lactate production is slowed down because low pHi inhibits glycolytic enzymes and via signalling events including upregulation of thioredoxin-interacting protein (TXNIP). Fatty acid uptake, lipid droplet accumulation, peroxisome mass, and lipid β-oxidation are increased, accompanied by a relative increase in the oxidative phosphorylation (OXPHOS)-dependence of cell metabolism. **c**. Low pHe can also activate net acid extrusion through transcriptional upregulation and allosteric regulation of multiple transporters, including Na^+/H^+ exchangers (NHEs) and Na^+/HCO_3^- cotransporters (NBCs). Intra- and extracellular carbonic anhydrases (CAs) facilitate intra- and extracellular H^+ and CO_2 diffusion. **d**. Low pHe activates epithelial–to–mesenchymal transition (EMT) through processes activated by pH sensors and pH-sensitive transcription factors. Invadopodia express net acid extruders, such as NHE1, whose activity produces low pHe locally to facilitate matrix metalloproteinase (MMP) activation. Lysosomes translocate toward the cell periphery, increasing cathepsin release. Collectively, these events increase migration and invasiveness.

TAGs, triacylglycerols; TCA, tricarboxylic acid; sAC, soluble adenylate cyclase

FIGURE 4: **The acidic tumour microenvironment shapes the interactions between cancer and stromal cells.**

a. The myeloid and lymphoid branches of the immune system are both regulated by extracellular pH (pHe). In a low pHe environment, created for instance by the collective activity of carbonic anhydrase 9 (CA9) and Na^+/HCO_3^- cotransporters (NBCs), monocyte differentiation is skewed toward dendritic cells rather than the anti-tumorigenic M1-like macrophages; cancer cell immune escape is driven by upregulated PD-L1–PD1 interactions, which limit macrophage-induced cancer cell phagocytosis and tumour immunity[207] ; macrophage polarization to tumour-associated macrophages is favoured; and the activity of natural killer (NK) cells and anti-tumorigenic $CD8^+$ T cells is inhibited. **b.** In cancer-associated fibroblasts (CAFs), low pHe favours release of latent transforming growth factor-β (TGFβ) from the ECM[208]; TGFβ activates Cl^-/HCO_3^- exchange in CAFs to increase cellular acid uptake; CA9 is upregulated to favour local MMP activation when catalysis is in the direction of net CO_2 hydration, and cancer cell EMT, and facilitates H^+ and CO_2 diffusion across the extracellular compartment. **c.** Tumour endothelial cells are highly glycolytic, which contributes to local acidification. They may sense environmental acidosis through the H^+-sensing receptor GPR4. Their proliferation in this pHe environment is facilitated by vascular endothelial growth factor (VEGF)-induced upregulation of CA2. Release of VEGF and basic fibroblast growth factor (bFGF) from tumour endothelial cells is increased at low pHe, which partially offsets the growth-inhibiting effects of extracellular acidosis.

BOX 1: Cellular sensors of environmental pH

Changes in extracellular pH (pHe) can be sensed by two major classes of proteins at the plasma membrane: *bona fide* H^+-receptors and pH-sensitive ion channels. The best described H^+ receptors are the G protein-coupled receptors (GPCRs) GPR68 (also known as OGR1), GPR4, and GPR65 (also known as TDAG8).[209] These plasma membrane proteins act via multiple Gα subunits and, depending on the cell type and context, mediate downstream signalling via Ca^{2+}-, 3′,5′-cyclic AMP (cAMP)-, ERK- and other regulatory pathways.[209] More recently, receptor protein tyrosine phosphatase γ (RPTPγ) has been identified as a plasma membrane sensor of extracellular HCO_3^-,[210,211] the loss of which increases net acid extrusion from breast epithelial cells and favours breast cancer development, aggressiveness, and recurrence.[57] pH-sensitive ion channels include acid-sensing ion channels (ASICs),[212] and a variety of K^+ channels and transient receptor potential (TRP) channels.[213] Moreover, pHe signals can gain access to the internal milieu as a consequence of the coupling between pHe and intracellular pH (pHi). This way, extracellular acid-base disturbances can evoke cellular responses through proteins that are sensitive to pHi changes, including a myriad of intracellular sensors implicated in the control of protein synthesis, metabolism, cell cycle progression, and apoptosis.[17] Among the many intracellular targets, the FAK-related kinase PYK2 is considered a *bona fide* pHi-sensor,[214] whereas soluble adenylate cyclase (sAC) is reported to sense intracellular HCO_3^- ref[215] **(Fig. 3).**

BOX 2: Good practice in controlling and interpreting pH experiments. A particular difficulty in studying the effects of acidosis on biological processes concerns adequate control over pH under culture conditions and *in vivo*. pH is challenging to maintain because many of the acids produced metabolically are non-volatile and accumulate in culture media. Whilst incubators are designed to clamp CO_2 close to blood-borne levels, that is 5 kPa, technology is not widely available for offsetting the continuous production of non-volatile acids, notably lactic acid. Medium HCO_3^- titrates at least some non-volatile acids to CO_2, which can then escape to equilibrate with the incubator's atmosphere, but pH invariably decreases in the process because HCO_3^- becomes progressively depleted. We speculate that inadequate pH control has disguised *bona fide* pH responses in the noise of recordings in many prior studies. In an attempt to standardise procedures and encourage detailed reporting, guidelines for pH control have been formulated.[216] It is also important to disentangle the effects of H^+ from the conjugate anions that dissociate from acids. Recent studies have indicated important actions of the latter that may occur independently of pH; for example, through the epigenetic consequences of histone lactylation **[G]** or the role of lactate in regulating protein-zinc interactions.[217] Although acids release H^+ and their conjugate anions in a stoichiometric ratio, the former are highly buffered and their accumulation as free ions will inevitably be much smaller than the conjugate anion. The actions of H^+ can be identified by comparing biological responses to pH changes brought about using strong acids such as HCl versus weak acids such as lactic acid. The former strategy would identify *bona fide* pH signals, whereas the latter would resolve additional contributions from the conjugate anion.

GLOSSARY

pH: A dimensionless, logarithmic scale that describes the acidity/basicity of a solution, where 7.0 denotes a neutral solution, below 7 is an acidic solution, and above 7 is an alkaline solution.

Protonation: The reversible binding of H^+ to a chemical moiety, such as a carboxylate, amine or imidazole, that in the case of proteins can affect structure and function.

H^+-equivalent: A species that is in chemical equilibrium with H^+, such as CO_2, lactic acid, HCO_3^-, CO_3^{2-} and OH^-, the membrane transport of which produces a change in pH in the abutting aqueous compartments.

Net acid extrusion: A membrane transport process that results in an increase in intracellular pH, arising from the export of H^+ or import HCO_3^- or similar H^+-equivalents.

pK_a: the negative logarithm of the acid dissociation constant ($K_a=[A^-]\times[H^+]/[HA]$) which is used to categorise substances as weakly acidic ($3<pK_a<7$), strongly acidic ($pK_a<3$), weakly basic ($7<pK_a<11$) or strongly basic ($pK_a>11$).

pH buffering: A chemical reaction involving a weak acid and its conjugate base (in equilibrium with H^+) which reduces – but does not eliminate – a pH change in response to the addition of acids or bases, for example from a metabolic or membrane transport source.

Buffer therapy: An intervention aimed at raising the extracellular buffering capacity of tumours, achieved by delivering an exogenous buffer (e.g., Tris) or raising the level of a physiological buffer (notably CO_2/HCO_3^-).

Fermentative metabolism: The glycolytic processing of carbohydrates that ends in the production and excretion of lactate and H^+, yielding two molecules of ATP per glucose.

Oxidative phosphorylation: (OXPHOS) The mitochondrial process through which oxidation of substrates produces CO_2 and an H^+ gradient across the inner mitochondrial membrane that drives ATP production, yielding up to 38 molecules of ATP per glucose.

Ion trapping: A phenomenon in which weakly-basic drugs become protonated in acidic compartments and less permeable across membranes, resulting in the accumulation of the drug.

Oxygen partial pressure: (pO_2) A measure of the concentration of oxygen dissolved in a solution, related to the pressure that oxygen gas exerts.

Carbonic anhydrases. A widely expressed family of enzymes that catalyse CO_2 hydration and its reverse reaction, with catalytic sites that can be intracellular or extracellular.

Clastogenic effect. A mutagenic action that causes structural changes in DNA, resulting in the deletion, insertion or rearrangement of entire segments of a chromosome.

pHe-pHi relationship. A graphical representation of the effect that changes in pHe have on steady-state pHi, from which sensitivity can be inferred by measuring the slope.

Matrisome: The complete set of extracellular matrix proteins that in mammals comprises about 1000 proteins, and is usually divided into the core matrisome, including collagens, glycoproteins and proteoglycans, and the matrisome-associated proteins, such as regulators and secreted factors.

Metabolon: A spatial arrangement of enzymes and/or transporters representing sequential steps in a more complex biological pathway, which in the case of pHi regulation has been suggested to involve carbonic anhydrases and H^+/H^+-equivalent transporters.

Paraptosis-like cell death: A type of programmed cell death distinct from apoptosis and necrosis, characterized by vacuolation and damage to mitochondria and endoplasmic reticulum.

Secondary active transport: A form of membrane transport that leads to uphill movement of a solute and is energized by a coupled downhill movement of another solute, typically sodium ions.

Lactylation: A post-translational modification in which lactate undergoes a condensation reaction with an amino acid residue such as lysine on proteins e.g. histones.

TABLE OF CONTENTS SUMMARY:

In this Review, Swietach and colleagues discuss how the pH balance is deregulated in tumours and how alterations in intracellular and extracellular pH impact tumour biology to accelerate disease progression, providing a rationale for therapeutic targeting of acid-base disturbances in cancer.