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Original Articles

Examining the connectivity between different cellular processes in the Barrett tissue microenvironment

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ABSTRACT

In Barrett associated tumorigenesis, oxidative phosphorylation and glycolysis are reprogrammed early in the disease sequence and act mutually to promote disease progression. However, the link between energy metabolism and its connection with other central cellular processes within the Barrett microenvironment is unknown. The aim of this study was to examine the relationship between metabolism (ATP5B/GAPDH), hypoxia (HIF1α), inflammation (IL1β/SERPINA3), p53 and obesity status using *in-vivo* and *ex-vivo* models of Barrett oesophagus. At the protein level, ATP5B ($r = 0.71$, $P < 0.0001$) and p53 ($r = 0.455$, $P = 0.015$) were found to be strongly associated with hypoxia. In addition, levels of ATP5B ($r = 0.53$, $P = 0.0031$) and GAPDH ($r = -0.39$, $P = 0.0357$) were positively associated with p53 expression. Moreover, we demonstrate that ATP5B ($r = 0.8$, $P < 0.0001$) and GAPDH ($r = 0.43$, $P = 0.022$) were positively associated with IL1β expression. Interestingly, obesity was negatively associated with oxidative phosphorylation ($r = -0.6016$, $P = 0.0177$) but positively associated with glycolysis ($r = 0.743$, $P = 0.0015$). Comparable correlations were exhibited in the *ex-vivo* explant tissue between metabolism, *p53*, hypoxia, inflammation and angiogenesis ($P < 0.05$). We have shown that metabolism is closely linked with many cellular processes in the Barrett tissue microenvironment.

Introduction

Barrett oesophagus is an inflammatory preneoplastic lesion defined as a condition in which the normal stratified squamous epithelium of the oesophagus is replaced by metaplastic columnar epithelium, specifically specialised intestinal metaplasia (SIM) [1]. Barrett oesophagus arises from chronic gastroesophageal reflux disease (GORD) of acid and bile, and can progress to oesophageal adenocarcinoma (OAC) through low-grade dysplasia (LGD) and high-grade dysplasia (HGD) [2,3]. The annual risk of OAC in individuals with Barrett oesophagus is approximately 0.12% [2]. At this time, no established biomarker has been uncovered to establish risk and patients undergo regular surveillance endoscopy with multiple biopsies to assess LGD or HGD. The medical management is predominantly acid suppression therapy with proton pump inhibitors, and no therapies that target the immune or inflammatory environment have been developed [4,5].

It is established that key cellular processes, with distinctive functional roles, are involved in promoting disease progression in Barrett oesophagus. Some of these processes include inflammation, hypoxia and angiogenesis [6–12]. Hypoxia, mediated through hypoxia-inducible factor-1 alpha (HIF1α), is associated with the inflammatory reaction in Barrett oesophagus, however, little is known on how it links with other cellular mediators [7]. Increased HIF1α and HIF2α expression has been demonstrated across the metaplastic-dysplastic-OAC sequence [6]. Barrett-associated inflammation, through canonical pro-inflammatory mediators such as interleukin-6 (IL6), IL1β and signal transducer and activator of transcription 3 (STAT3), has additionally been shown to exacerbate inflammation and promote OAC [9,11]. A recent study showed that neoplastic and non-neoplastic Barrett cells expressing vascular endothelial growth factor (VEGF) and VEGFR2 mRNA and protein, promoted cell proliferation through a phospholipase C gamma1-protein kinase C-ERK pathway subsequent to VEGF-VEGFR2 activation [12]. Moreover, sunitinib-induced VEGF inhibition reduced the weight and volume in mouse xenografts tumours from transformed Barrett cells [12].

Obesity has further been implicated in disease progression in Barrett oesophagus [13,14]. Individuals with central or visceral

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obesity, measured by waist circumferences, are at an increased risk of developing Barrett oesophagus [13]. Furthermore, individuals with a greater propensity to obesity have higher risks of intestinal metaplasia, thereby showing that obesity is independently associated with Barrett oesophagus and its subsequent progression to OAC [14]. Epidemiological studies have also shown a link between leptin and reduced adiponectin, both characteristic of obesity and Barrett oesophagus [15–18]. However, how obesity and associated changes in adipokines, cytokines, insulin and IGF1-axis, immune mechanisms and their downstream signalling pathways influences Barrett oesophagus and Barrett tumorigenesis at the cellular level is currently unknown.

Alterations in energy metabolism is one of the new emerging hallmarks of cancer and disease progression [19,20]. Previous research from our group has demonstrated that both oxidative phosphorylation and glycolysis are reprogrammed early in the inflamed Barrett disease sequence and may act mutually to promote disease progression in Barrett oesophagus [21]. In addition, levels of ATP5B, a marker of oxidative phosphorylation, in first time surveillance biopsy material, segregated Barrett patients who progressed to HGD/OAC from non-progressors, highlighting the possible prognostic advantage of assessing metabolic profiles in these preneoplastic patients [21]. Moreover, Barrett cells were shown to favour the more detrimental oxidative phenotype that may be selected for during the early stages prior to disease progression [21]. Various studies linking energy metabolism with inflammation, hypoxia and angiogenesis highlight how reciprocal processes act jointly to significantly alter the local microenvironment and attenuate disease progression [22–25]. However, despite recent insight into energy metabolism profiles in Barrett oesophagus, less is known about how energy metabolism and other key cellular processes cooperate in the Barrett microenvironment. Interestingly, p53 overexpression is associated with an increased risk of neoplastic progression in patients with Barrett oesophagus, however, the risk is greater with loss of p53 expression [26]. Moreover, in inflamed Barrett oesophagus patients with progressive disease, oxidative-induced damage results in telomere shortening and mutations in the p53 gene abrogate p53's role as the checkpoint of proliferation and apoptosis [27]. In addition to obesity and p53 status, the length of the Barrett segment has been shown to be a risk factor in the development of Barrett associated OAC [28–30].

The aim of this study was to examine the link between energy metabolism, hypoxia, inflammation, p53 and obesity in the Barrett tissue microenvironment *in-vivo* and *ex-vivo*. We report herein that oxidative phosphorylation and p53 status positively correlated with hypoxia. Moreover, levels of oxidative phosphorylation are positively linked to p53 expression whereas levels of glycolysis are negatively associated with p53 expression. In addition, oxidative phosphorylation and glycolysis are positively associated with inflammation. Interestingly, we demonstrated that obesity was negatively associated with oxidative phosphorylation but positively associated with glycolysis. Analogous correlations were exhibited in *ex-vivo* explant tissue between metabolism, p53, hypoxia, inflammation and angiogenesis.

Materials and methods

ATP5B, GAPDH, p53, IL1β, SERPINA3 and HIF1α immunohistochemical analysis using tissue microarrays

Ethical approval to conduct all aspects of this work was granted by the Adelaide and Meath Hospital (AMNCH), Tallaght, Dublin (REC 200110405). All cases were prospectively recruited at our national referral centre for upper GI malignancy and written informed consent was obtained in accordance with local institutional ethical guidelines. All patients attending with histologically confirmed Barrett oesophagus were considered for inclusion. Patients with a prior history of dysplasia or carcinoma, other malignancy of any type, or ablative therapy (radiofrequency ablation, argon plasma coagulation, cryoablation) were excluded. Endoscopic examination consisting of white light and chromoendoscopy (FICE (fujinon) or NBI (olympus)) was performed in all cases (DOT and FMC). The Barrett segment was assessed and measured as per the Prague classification system and biopsied using large capacity forceps.

Patients with intestinal metaplasia were identified from our Barrett tissue database and immunohistochemistry was performed. The median age of patients ($n = 29$) with intestinal metaplasia was 65 years, there was a 2.22-fold male predominance and all patients were followed for a median of 7.5 years. The areas of interest on the diagnostic biopsy blocks were marked by a pathologist. 0.6 mm cores were taken from the blocks and tissue microarrays (TMAs) were constructed and pathology of the tissue re-evaluated prior to antibody staining. Immunohistochemistry was performed utilising the Vectastain Kit (Elite) as per manufacturer's instructions. All tissue microarray sections were processed and stained on the same day at room temperature. Endogenous peroxidases were quenched in 3% hydrogen peroxide (in methanol) for 30 mins and slides blocked with serum for 30 mins. Primary antibodies used were a mouse anti-ATP5B IgG (SantaCruz Biotechnology) (1:1000), a rabbit anti-GAPDH IgG (AbDserotec Division of MorphoSys) (1:300), a rabbit anti-p53 IgG (Abcam) (1:150), a rabbit anti-IL1β IgG (Abcam) (1:200), a rabbit anti-SERPINA3 IgG (Abcam) (1:200) and a rabbit anti-HIF1α IgG (Abcam) (1:200) diluted in PBS. Staining was undertaken with adequate negative controls (PBS only without primary antibody). Slides were incubated with primary antibody for 1 hour, then biotinylated antibody for 30 minutes, avidin–biotin complex for 30 minutes and DAB substrate for 2–15 minutes. DAB was rinsed off slides upon colour development and haematoxylin added for 30 seconds. Lastly, slides were dehydrated and mounted using DPX. Slides were scanned (Philips Digital Pathology Solutions) and immunoreactivity was assessed digitally under 40× magnification in a semi-quantitative manner for each protein by observers who were blinded to the pathology of all patients in the study. For each protein, both epithelial and stromal cells were evaluated for both percentage positivity and intensity of cytoplasmic staining. A third score, designated I × P, was obtained by multiplying intensity by positivity. Intensity was graded as 0 (negative), 1 (weak), 2 (moderate) and 3 (strong) and positivity was evaluated as 0%, 10%, 25%, 50%, 75%, 90% or 100%. The scoring was undertaken by consensus evaluation (JP and JOS) and the average value was calculated.

Expression of all protein markers was subsequently correlated to waist circumference and to the length of the Barrett segment. Waist circumference and Barrett segment length demographics were available on 15 and 22 patients respectively from the 29 patients who participated in the study. 15 patients were used to assess the link between ATP5B, GAPDH and waist circumference; the median age of patients with intestinal metaplasia was 65 years, there was a 2.75-fold male predominance and patients had a median waist circumference of 104 cm. 22 patients were used to assess the link between ATP5B, GAPDH and the length of the Barrett segment; the median age of patients ($n = 22$) with intestinal metaplasia was 61.5 years, there was a 2.14-fold male predominance and patients had a median Barrett segment length of 5 cm.

Assessing the effect of hypoxia in an in-vitro *model of Barrett oesophagus*

Cell culture

The QH (Barrett metaplasia) cell line was sourced from American Type Culture Collection (ATCC) (LGC Standards, Middlesex, UK). QH cells were grown to 70% confluency in BEBM medium supplemented with BEBM SingleQuots (2 mL BPE, 0.5 mL insulin, 0.5 mL HC, 0.5 mL GA-1000, 0.5 mL retinoic acid, 0.5 mL transferring, 0.5 mL triiodothyronine, 0.5 mL adrenaline and 0.5 mL hEGF per 500 ml media). QH cells were seeded 50,000 per well in 12 well plates and subsequently cultured under normoxia (21% oxygen) and hypoxia (0.5%) conditions for 24 hours. RNA extraction was subsequently performed using RNeasy Mini Kit (Qiagen) following manufacturer's instructions. RNA content and quality was quantified and assayed respectively (Nanodrop®, 8-Sample Spectrophometer, ND-800) and RNA reverse transcribed using Bioscript enzyme (Bioline).

Gene expression analysis

Gene primer probes for *VEGFA*, *ATP5B*, *p53*, *PKM2*, *IL1β*, *AMPK*, *GAPDH*, *HSP60*, *mTOR* and *18S* (Applied Biosystems) were purchased and realtime PCR was performed using Taqman mastermix following manufacturer's instructions on a 7900HT Fast Realtime PCR Light-Cycler System (Applied Biosystems). PCR data was analysed utilising the $2^{-\Delta\Delta Ct}$ method [21]. First, threshold cycle (Ct) values were converted to 2^{-Ct} in order to be proportional to the amount of transcripts in cell line samples. Next, $2^{-\Delta Ct}$ values were calculated by normalising the data to a housekeeping gene, 18S, as follows; $2^{-\Delta Ct} = 2^{-Ct}$ (QH 21%)/2^{-Ct} (18S). In order to compare relative gene expression between cells exposed to normoxia and hypoxia, $2^{-\Delta\Delta Ct}$ values were calculated by normalising the experimental data by reference data. For example, data from one sample was normalised to another sample as follows: $2^{-\Delta\Delta Ct} = 2^{-\Delta Ct}$ (QH 21%)/$2^{-\Delta Ct}$ (QH 0.5%).

Assessment of secreted inflammatory and angiogenic markers

QH cell supernatant, previously exposed to normoxia and hypoxia conditions, was screened for a panel of secreted inflammatory and angiogenic markers. The inflammatory markers assessed were interleukin-10 (IL10), IL12p70, IL13, IL2, IL6, IL4, IL8 and tumour necrosis factor alpha (TNFα). The angiogenic markers assessed were angiopoietin 2 (ANG2), basic fibroblast growth factor (bFGF), intracellular cell ad-

hesion molecule-1 (ICAM-1), plasminogen activator inhibitor-1 (PAI-1), vascular cell adhesion molecule-1 (VCAM-1) and VEGF (Meso Scale Discovery Multi-Array technology). Secreted lactate levels were additionally assessed (Sigma). All *in-vitro* data was normalised to cell number using the crystal violet assay.

Barrett ex-vivo *explant culture*

All fresh *ex-vivo* Barrett intestinal metaplasia tissues were prospectively recruited at our national referral centre for upper GI malignancy and obtained as above. The median age of patients ($n = 9$) with intestinal metaplasia was 62 years and there was an 8-fold male predominance. Barrett explant tissue was subsequently transferred into 1 mL M199 medium (10% FBS, 1% Pen/Strep, 1 mg/mL insulin) (Gibco) and cultured for 24 hours at 5% CO_2 at 37 °C. Following 24 hour incubation, the Barrett conditioned medium was collected and explant tissues snap frozen in liquid nitrogen. Both media and tissue were stored at –80 °C for future use.

Ex-vivo *qRT-PCR analysis*

Barrett explant tissue was homogenised using a Tissue-Lyser (Qiagen) for 2.5 min at a frequency of 25 pulses per second, RNA and protein extracted simultaneously (Qiagen), RNA quantified and RNA quality assessed (Nanodrop®, 8-Sample Spectrophometer, ND-800). RNA was reverse transcribed using Bioscript enzyme (Bioline). Gene primer probes for *ATP5B*, *HSP60*, *GAPDH*, *PKM2*, *p53*, *HIF1A* and *18S* (Applied Biosystems) were purchased and realtime PCR was performed using Taqman mastermix (Applied Biosystems). Data was analysed utilising the $2^{-\Delta\Delta Ct}$ method as above. All 9 Barrett explant tissue was characterised by assessing the expression of the columnar epithelium molecular marker villin using RT-PCR; all Barrett explant tissue was positive for the presence of villin and all matched adjacent normal squamous tissues were negative for villin.

Ex-vivo *MSD multiplex ELISA analysis*

Barrett conditioned medium was screened for the levels of the multiplex inflammatory and angiogenic proteins (Meso Scale Discovery Multi-Array technology). The inflammatory proteins assessed were GRO-alpha (GROα), IL1β, IL10, IL2, IL6, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein 3 alpha (MIP3α), matrix metallopeptidase 2 (MMP2), MMP9 and TNFα. The angiogenic proteins assessed were ANG1, bFGF, PAI-1, ICAM-1, VCAM-1 and VEGF. Secreted lactate levels were also assessed in the Barrett conditioned medium. Subsequent to screening for these secreted inflammatory and angiogenic proteins, *HIF1α* and *p53* expression levels were evaluated through RT-qPCR and all factors were subsequently correlated to metabolism (*ATP5B*, *HSP60*, *GAPDH* and *PKM2*) at the gene level. Total protein extracted above (Qiagen) was assayed utilising a bicinchoninic acid assay (Thermo Scientific) and secreted protein levels of these secreted factors in all samples were normalised to total protein.

Statistical analysis

Data was analysed using Graph Pad Prism software (Graph Pad Prism, San Diego, CA). Paired *t*-tests were utilised to assess if hypoxia had a metabolic or inflammatory effect using the *in-vitro* QH Barrett cell line at the gene and protein level. *In-vivo* immunohistochemical expression profiles of ATP5B and GAPDH were correlated to p53, IL1β, SERPINA3, HIF1α, waist circumference and to the length of the Barrett segment using Spearman ρ analyses. Multiple regression analyses were undertaken to investigate associations between two factors taking into account other factors *in-vivo* (R statistical software, package 'abn'). In addition, additive Bayesian networks were implemented to assess the dependencies between factors taking into account the combined effect of all factors *in-vivo* (R statistical software, package 'abn'). Majority consensus was constructed over 5000 iterations to select a best-fitting network representation of the relations between markers. Secreted protein levels and gene expression levels in *ex-vivo* explant tissue were correlated similarly (Spearman ρ correlation). Moreover, BMI was correlated to all *ex-vivo* factors (Spearman ρ correlation). Differences of $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***) were considered statistically significant.

Results

Linking energy metabolism, p53 and inflammation in the Barrett tissue microenvironment in-vivo

To assess oxidative phosphorylation and glycolysis at the protein level, levels of ATP5B and GAPDH respectively were assessed in *in-vivo* Barrett patient tissue. Fig. 1 shows immunohistochemical expression of these metabolic markers and investigates the link between energy metabolism and p53 in the Barrett tissue microenvironment. ATP5B intensity is positively correlated with p53 intensity in the epithelium (Fig. 1A) ($r = 0.53$, $P = 0.0031$). This association, illustrated by representative images, shows that tissue from Barrett patients exhibiting low p53 expression (Fig. 1C) showed low ATP5B intensity (Fig. 1E), however, low p53 expression was associated with high I × P GAPDH expression levels (Fig. 1G). Stromal GAPDH I × P expression significantly negatively correlated to p53 I × P expression (Fig. 1B) ($r = –0.39$, $P = 0.0357$). This association, illustrated by representative images, shows that Barrett patients exhibiting high p53 expression (Fig. 1D) displayed high ATP5B intensity (Fig. 1F), however, high p53 expression was associated with low I × P GAPDH expression levels (Fig. 1H).

Fig. 2 investigates the relationship between p53 and inflammation in the Barrett tissue microenvironment. IL1β I × P expression significantly positively correlated to levels of p53 I × P expression in the epithelium (Fig. 2A) ($r = 0.43$, $P = 0.02$). This association, illustrated by representative images, shows that Barrett patients exhibiting low epithelial p53 expression (Fig. 2C) demonstrated low I × P IL1β expression (Fig. 2E) and low SERPINA3 positivity levels (Fig. 2G). SERPINA3 positivity significantly positively correlated to p53 positivity in the epithelium (Fig. 2B) ($r = 0.536$, $P = 0.0033$). This association, illustrated by representative images, shows that Barrett patients exhibiting high epithelial p53 expression (Fig. 2D) displayed high I × P IL1β expression (Fig. 2F) and high SERPINA3 (Fig. 2H) positivity levels.

Next, the association between energy metabolism and inflammation in the Barrett tissue microenvironment was investigated. Fig. 3 investigates the association between energy metabolism and inflammation in the Barrett tissue microenvironment. Epithelial ATP5B positivity significantly positively correlated to epithelial IL1β positivity (Fig. 3A) ($r = 0.8$, $P < 0.0001$). Furthermore, epithelial GAPDH positivity significantly positively correlated to epithelial IL1β positivity (Fig. 3B) ($r = 0.43$, $P = 0.022$). In addition, GAPDH expression did not significantly correlate to levels of SERPINA3 in the epithelium, however, ATP5B I × P expression did significantly correlate to I × P expression levels of SERPINA3 in the epithelium ($r = 0.66$, $P = 0.0001$) (data not shown).

Linking hypoxia to energy metabolism, inflammation and p53 in Barrett oesophagus in-vitro *and* in-vivo

Firstly, to investigate if hypoxia is potentially associated with mediating energy metabolism and inflammation, QH cells, an *in-vitro* model of an intestinal metaplasia culture, were exposed to normoxic (21% oxygen) and hypoxic (0.5% hypoxia) conditions. Levels of genomic *ATP5B* (Fig. 4A), *GAPDH* (Fig. 4B), *PKM2* (Fig. 4C), *VEGFA* (Fig. 4D) and *IL1β* (Fig. 4E) were significantly differentially expressed in Barrett QH cells under normoxic and hypoxic conditions. In addition, secreted levels of lactate (Fig. 4F), VEGF (Fig. 4G), IL-8 (Fig. 4H) and PAI-1 (Fig. 4I) were differentially altered in those Barrett cells exposed to normoxia (21% oxygen) and hypoxia (0.5% hypoxia). Hypoxia was shown to significantly affect cell viability (see Supplementary Fig. S1). As we found differential hypoxia-induced alterations in various metabolic and inflammatory profiles *in-vitro*, we next investigated the affect of hypoxia *in-vivo*.

To assess if the relationship between hypoxia and these cellular processes can be recapitulated *in-vivo*, protein levels of HIF1α were assessed. Fig. 5 investigates the effect of hypoxia on energy metabolism, inflammation and p53 in the Barrett tissue microenvironment. Focusing on the exact same area within the Barrett tissue, Barrett patients with low I × P HIF1α expression (Fig. 5A) exhibited low I × P expression levels of ATP5B (Fig. 5C). Similarly, patients with high HIF1α I × P expression (Fig. 5B) exhibited high I × P expression levels of ATP5B (Fig. 5D). HIF1α I × P expression significantly positively correlated to ATP5B I × P expression in the epithelium (Fig. 5E) ($r = 0.71$, $P < 0.0001$). However, HIF1α I × P expression ($r = 0.06$, $P = 0.7$) did not significantly correlate to levels of GAPDH I × P expression in the epithelium as shown in Fig. 5F. HIF1α I × P expression significantly

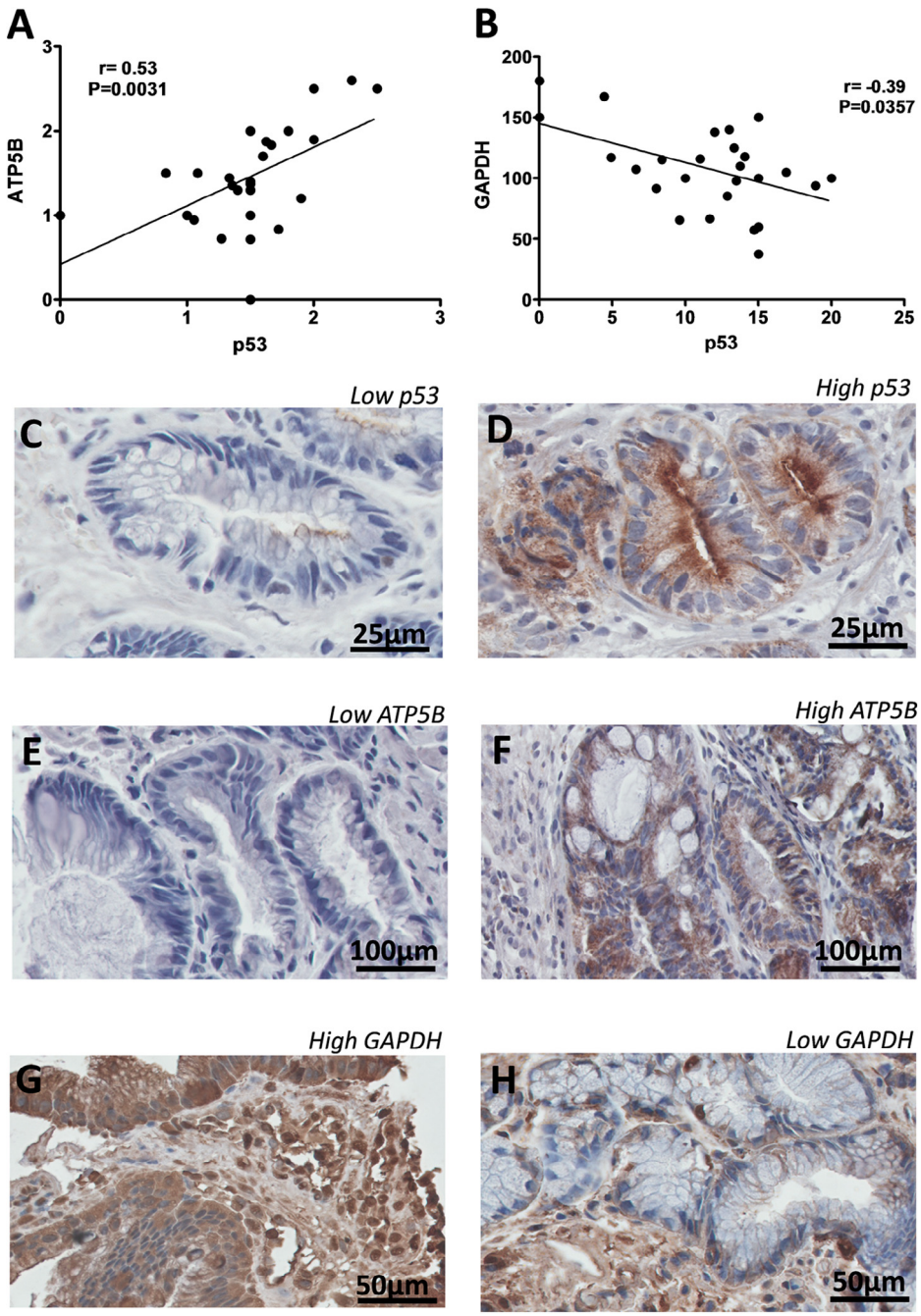

Fig. 1. The link between energy metabolism and p53 in Barrett oesophagus *in-vivo*. (A) Epithelial ATP5B intensity significantly positively correlated to epithelial p53 intensity (r = 0.53, *P* = 0.0031). (B) GAPDH I × P expression significantly negatively correlated to levels of p53 I × P expression in the stroma (r = –0.39, *P* = 0.0357). (C) Barrett patients displaying low levels of p53 expressed low intensity levels of ATP5B (E) and high I × P expression levels of GAPDH (G) respectively. (D) Barrett patients displaying high levels of p53 expressed high intensity levels of ATP5B (F) and low I × P expression levels of GAPDH (H) respectively. r denotes Spearman ρ correlation (I × P = intensity by positivity).

positively correlated to levels of SERPINA3 I × P expression in the epithelium (Fig. 5G) (r = 0.522, *P* = 0.0044). Epithelial HIF1α I × P expression significantly positively correlated to levels of epithelial p53 I × P expression (Fig. 5H) (r = 0.455, *P* = 0.015).

Linking energy metabolism to obesity and to the length of the Barrett segment in the Barrett tissue microenvironment in-vivo

Fig. 6 investigates the link between energy metabolism, obesity and the length of the Barrett segment in the Barrett tissue microenvironment. Stromal ATP5B I × P expression significantly negatively correlated to waist circumference (Fig. 6A) (r = –0.6016, *P* = 0.0177). Epithelial GAPDH intensity significantly positively correlated to waist circumference (Fig. 6B) (r = 0.743, *P* = 0.0015).

ATP5B and GAPDH were also correlated to the length of the Barrett segment. Stromal GAPDH intensity significantly positively correlated to the length of the Barrett segment (Fig. 6C) (r = 0.5192, *P* = 0.0133), however, ATP5B did not. Note that no additional statistically significant associations were found *in-vivo* between all protein markers within the stromal compartment (data not shown).

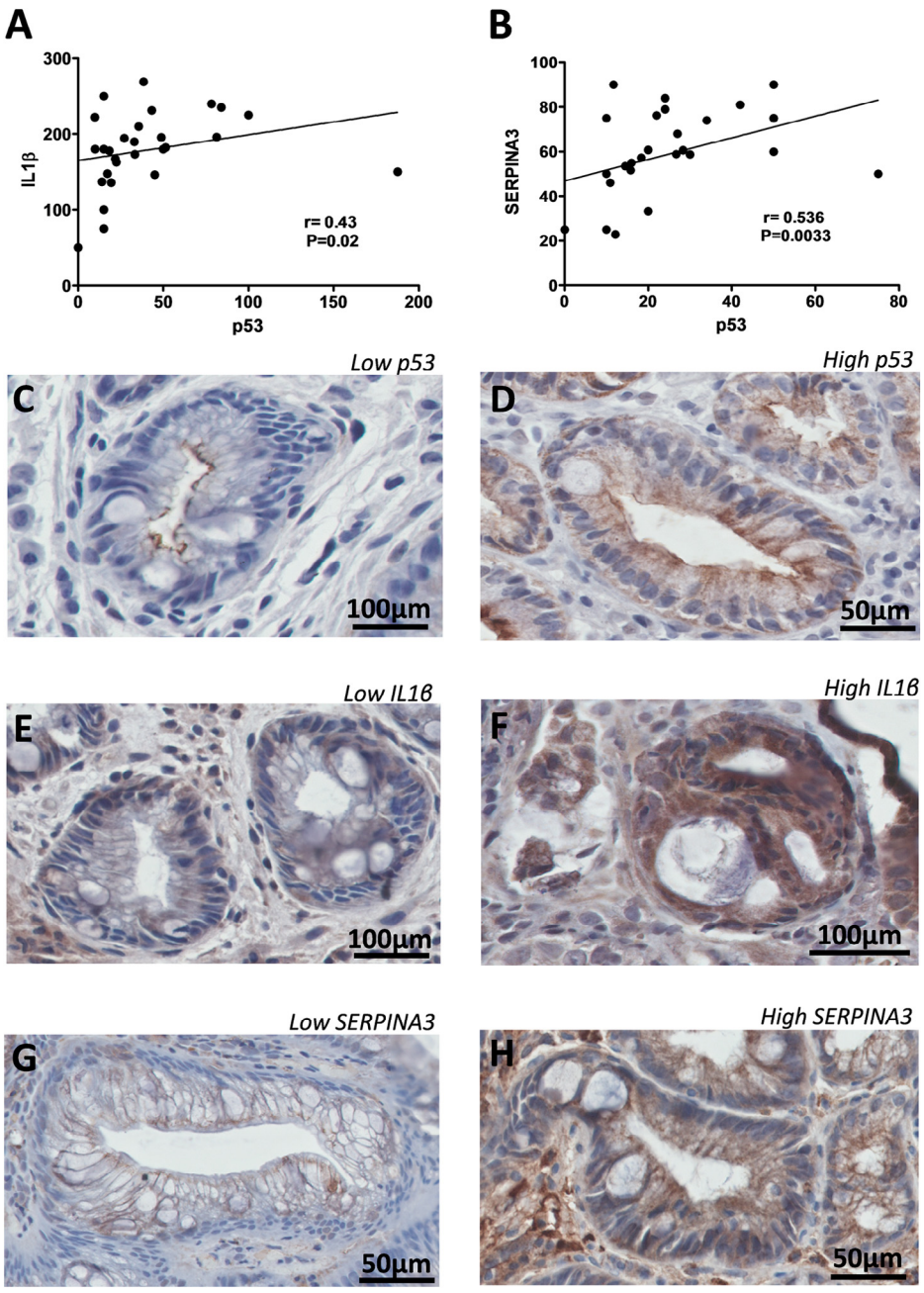

Fig. 2. Linking p53 and inflammation in Barrett oesophagus *in-vivo*. (A) Epithelial IL1β I × P expression significantly positively correlated to levels of epithelial p53 I × P expression (r = 0.43, *P* = 0.02). (B) SERPINA3 positivity significantly positively correlated to p53 positivity in the epithelium (r = 0.536, *P* = 0.0033). (C) Barrett patients displaying low epithelial levels of p53 expressed low epithelial I × P expression levels of IL1β (E) and low epithelial positivity levels of SERPINA3 (G). (D) Barrett patients displaying high epithelial levels of p53 expressed high epithelial I × P expression levels of IL1β (F) and high epithelial positivity levels of SERPINA3 (H). r denotes Spearman ρ correlation (I × P = intensity by positivity).

Importantly, to ascertain a more comprehensive understanding between factors without the influence of other endogenous factors *in-vivo*, multiple regression analyses were undertaken. Table 1 compares the associations between metabolism, inflammation, hypoxia, p53 obesity and the length of the Barrett segment *in-vivo* using both Spearman ρ and multiple regression analyses. ATP5B was significantly associated with p53 (*P* = 0.03), IL1β (*P* = 0.019) and HIF1α (*P* = 0.002). GAPDH was significantly linked with p53 (*P* = 0.007) and waist circumference (*P* = 0.003). All other pairings did not reach statistical significance. In addition, construction of Additive Bayesian Networks from multiple regressions showed some evidence of interdependencies between markers (data not shown).

Linking energy metabolism to p53, inflammation, hypoxia and angiogenesis in the Barrett tissue microenvironment ex-vivo

To elucidate the relationship between energy metabolism and these processes, we used Barrett *ex-vivo* explant tissue in an effort to utilise a model that mimicked the Barrett tissue microenvironment linking gene expression profiles and protein secretion profiles associated with the above cellular processes. Table 2 summarises the association between metabolism and inflammation, hypoxia, p53 and angiogenesis in Barrett *ex-vivo* explant tissue. *ATP5B* expression significantly positively correlated to TNFα (r = 0.79, *P* = 0.027), IL6 (r = 0.93, *P* = 0.002), *HIF1α* (r = 0.88, *P* = 0.003) and *p53* (r = 0.72,

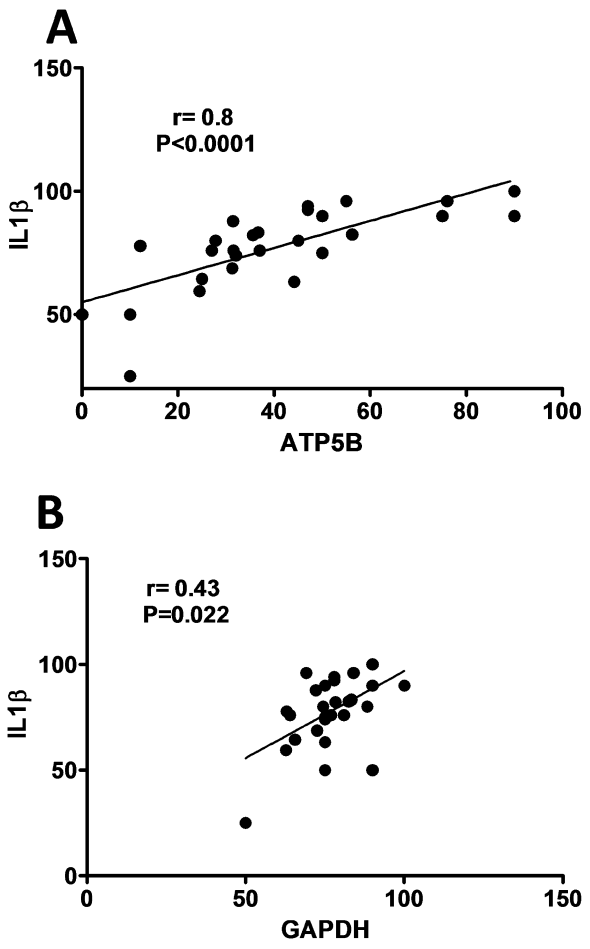

Fig. 3. The association between energy metabolism and inflammation in Barrett oesophagus *in-vivo*. (A) Epithelial ATP5B positivity significantly positively correlated to epithelial IL1β positivity (r = 0.8, $P < 0.0001$). (B) Epithelial GAPDH positivity significantly positively correlated to epithelial IL1β positivity (r = 0.43, $P = 0.022$).

$P = 0.037$). *HSP60* expression was significantly positively associated with *HIF1α* (r = 0.73, $P = 0.031$). *GAPDH* expression significantly positively correlated to TNFα (r = 0.81, $P = 0.021$), IL6 (r = 0.9, $P = 0.004$) and *HIF1α* (r = 0.87, $P = 0.002$). *PKM2* expression significantly positively correlated to IL6 (r = 0.73, $P = 0.025$), *HIF1α* (r = 0.92, $P = 0.001$) and *p53* (r = 0.9, $P = 0.002$). Moreover, *HSP60* (r = –0.7, $P = 0.036$) and *PKM2* (r = –0.68, $P = 0.042$) were significantly negatively associated with angiogenesis (ANG-1). No significant correlations were found with secreted lactate levels ($P > 0.05$).

Linking inflammation, hypoxia, p53, BMI and angiogenesis in the Barrett tissue microenvironment ex-vivo

Table 3 summarises the link between inflammation, hypoxia, p53, BMI and angiogenesis in the Barrett *ex-vivo* explant tissue. Angiogenesis (ICAM-1) significantly positively correlated to MCP-1 (r = 0.76, $P = 0.036$), MMP9 (r = 0.72, $P = 0.03$), IL2 (r = 0.78, $P = 0.013$) and TNFα (r = 0.79, $P = 0.028$). In addition, secreted levels of bFGF significantly positively correlated to IL1β (r = 0.76, $P = 0.036$) and IL10 (r = 0.71, $P = 0.047$). Moreover, BMI significantly positively correlated to secreted levels of VCAM-1 (r = 0.8, $P = 0.01$), ICAM-1 (r = 0.73, $P = 0.025$) and ANG1 (r = 0.72, $P = 0.03$). *HIF1α* significantly positively correlated to expression levels of *p53* (r = 0.88, $P = 0.003$), GROα (r = 0.75, $P = 0.025$), IL6 (r = 0.88, $P = 0.003$), MIP3α (r = 0.75, $P = 0.025$), MMP2 (r = 0.81, $P = 0.022$), TNFα (r = 0.72, $P = 0.037$) and IL1β (r = 0.67, $P = 0.049$). Furthermore, *p53* status significantly positively correlated with IL6 (r = 0.75, $P = 0.025$) and MMP2 (r = 0.76, $P = 0.037$).

Discussion

An understanding of the complex processes in the inflammation to tumour model of Barrett oesophagus and OAC may provide some insight into both biomarkers of risk progression, and the potential for the development of novel targeted therapies. We have shown for the first time that mitochondrial energy metabolism is strongly associated with inflammation and hypoxia in the Barrett oesophagus tissue microenvironment. The study also highlights that mitochondrial energy metabolism is aligned with p53 status, obesity status and to the length of the Barrett segment, further demonstrating that various cellular mechanisms may operate interdependently fuelling progression within the Barrett microenvironment to HGD and OAC.

To assess oxidative phosphorylation and glycolysis respectively, levels of the protein markers ATP5B and GAPDH were assessed as increased ATP5B and GAPDH is known to be associated with neoplastic progression across the Barrett disease sequence [21]. Levels of ATP5B and GAPDH are commonly used as surrogate markers of both oxidative phosphorylation and glycolysis [21,31–35]. The aberrant expression of the tumour suppressor protein, p53, commonly associated with aneuploidy is associated with an increased risk of neoplastic progression in Barrett oesophagus [26]. In this study, we have shown *in-vivo* and *ex-vivo* that ATP5B, a marker of oxidative phosphorylation, was significantly positively correlated to p53 status in Barrett oesophagus. We also demonstrate *in-vivo* that GAPDH, a marker of glycolysis, was significantly negatively associated with p53. At the gene level *ex-vivo*, *p53* was shown to be positively associated with another marker of glycolysis, *PKM2*. p53 has an important role in cellular metabolism, lowering glycolysis, an anti-Warburg affect, and promoting oxidative phosphorylation [9,36–38]. p53 promotes oxidative phosphorylation through the p53-inducible protein Mieap and also through the master transcription regulator nuclear factor kappa B (NFκB)-mediated upregulation of mitochondrial synthesis of cytochrome c oxidase 2 (SCO2) [39,40]. p53 negatively regulates glycolysis by downregulating key components of glycolysis such as glucose transporter 1 (GLUT1), GLUT3, phosphofructose kinase 1 and PKM2 [9,41–43]. It may be plausible that the increased oxidative microenvironment that p53 promotes in Barrett oesophagus supports metaplastic progression as one study has found that oxidative-induced damage in Barrett patients results in telomere shortening and mutations in the p53 gene itself [27]. Therefore, with an oxidative-induced abrogation of p53 function, glycolytic levels are no longer negatively regulated and this may explain why levels of glycolysis may increase early in the progression of OAC [21]. Alternatively, p53 may simultaneously promote oxidative phosphorylation, producing large amounts of ATP and shunt high energy glycolytic intermediates into the pentose phosphate pathway [44]. The NFκB family member RelA is transported into mitochondria and recruited to the mitochondrial genome where it can repress mitochondrial gene expression, oxygen consumption and cellular ATP levels, thereby contributing to the switch to glycolysis. Conversely, upon low cellular ATP levels, NFκB can upregulate mitochondrial respiration through SCO2, a key component of complex IV of the electron transport chain [40]. Despite some evidence in glioma cells suggesting that p53 localisation is associated with p53 mutational status, whether mutant p53 localises to the cytoplasm and mediates cellular function and metabolism warrants future investigation in the setting of Barrett oesophagus and in other preneoplastic and neoplastic tissues [45].

Few studies have investigated the direct association between oxidative phosphorylation and inflammation. NFκB is a key inflammatory mediator of both oxidative phosphorylation and gly-

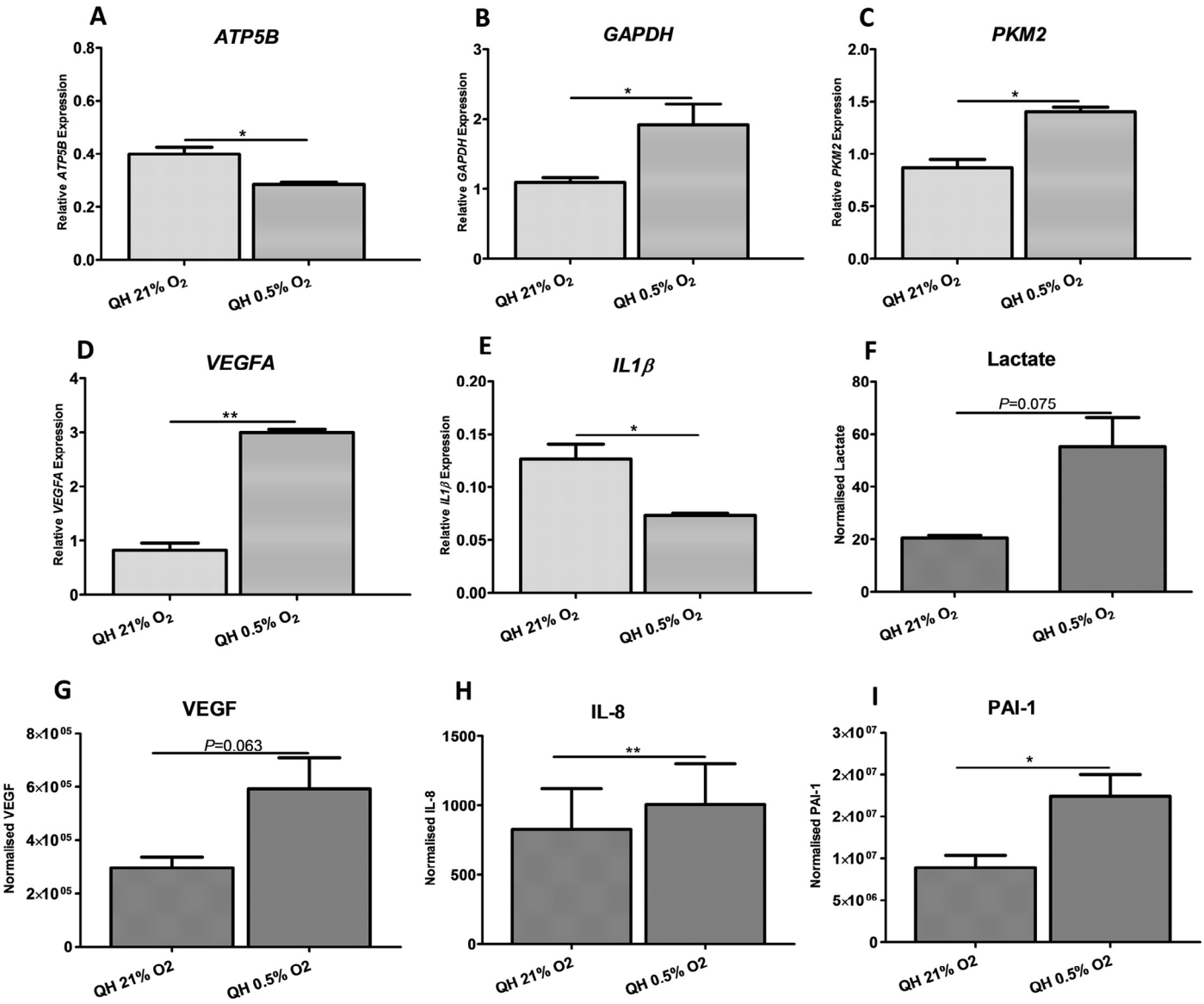

Fig. 4. Investigating the effect of hypoxia on metabolic, inflammatory and angiogenic profiles in an *in-vitro* model of Barrett oesophagus. Levels of genomic *ATP5B* (A), *GAPDH* (B), *PKM2* (C), *VEGFA* (D) and *IL1β* (E) were significantly differentially expressed in Barrett cells exposed to normoxia (21% oxygen) and hypoxia (0.5% hypoxia). Secreted levels of lactate (F), VEGF (G), IL-8 (H) and PAI-1 (I) were found to be differentially altered in Barrett cells exposed to normoxia (21% oxygen) and hypoxia (0.5% hypoxia). ($^{*}P < 0.05$, $^{**}P < 0.01$; paired *t*-test; $n = 3–7$).

colysis, and promotes IL1β and TNFα production [40,42,46]. To assess inflammation, levels of the inflammatory markers IL1β and SERPINA3 were assessed. IL1β and SERPINA3 have previously been utilised as markers of inflammation and have previously been identified as playing an important role in neoplastic progression [9,47–52]. In this study, ATP5B was significantly positively correlated to levels of IL1β and SERPINA3 *in-vivo*, and with TNFα and IL6 in Barrett *ex-vivo* explant tissue. Furthermore, GAPDH was significantly positively correlated to levels of IL1β, TNFα and IL6 in *in-vivo* and *ex-vivo* Barrett tissue. IL1β, IL6 and TNFα may have key roles in the development and subsequent progression of OAC, consistent with a murine model where overexpression of IL1β induced intestinal metaplasia and OAC, an effect abrogated by IL6 deficiency [9]. In this context, the effector functions of IL1β-dependent T_H17 cells have been shown to be related to their glycolytic capacity [53]. TNFα has also been shown to stimulate glycolysis in colorectal cancer cells [24]. Moreover, increased expression of the key glycolytic enzymes, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 and hexokinase 2, have been shown to be orchestrated by the IL6-STAT3 pathway further providing a mechanism for inflammation-associated oncogenesis in Barrett oesophagus [54]. Inflammation is also known to mediate metabolism through NFκB in a p53-dependent manner [40,46].

Hypoxia and related genes, and their interface with inflammation and metabolism, are also relevant to this model. Subsequent to demonstrating differential hypoxia-induced effects on inflammation, angiogenesis and metabolism *in-vitro*, we found that both oxidative phosphorylation (*ATP5B* and *HSP60*) and glycolysis (*GAPDH* and *PKM2*) significantly positively correlated to HIF1α at the gene level *ex-vivo* and *in-vivo* ATP5B protein levels significantly positively correlated with HIF1α protein expression; however, HIF1α showed no correlation with GAPDH. Interestingly, various studies in the cancer setting have implicated glycolysis as one of the main distinctive factors in the adaptation to hypoxic stress, as hypoxia mediates metabolism by inducing the expression of various glycolytic components [55]. No studies to our knowledge have showed such a strong positive association between hypoxia and oxidative

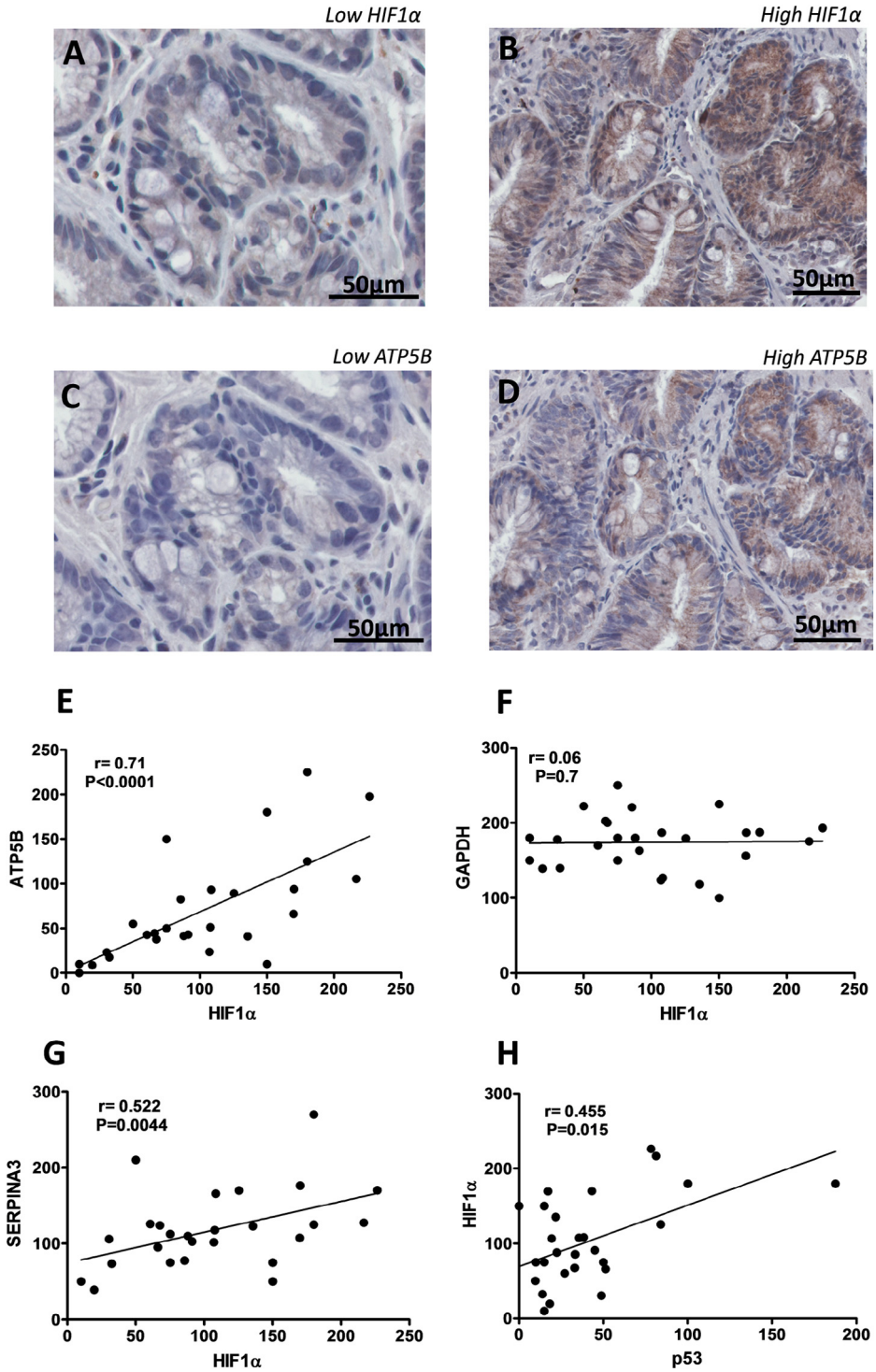

Fig. 5. Linking hypoxia to energy metabolism, inflammation and p53 in Barrett oesophagus *in-vivo*. (A) A Barrett patient, displaying low I × P expression levels of HIF1α also expressed low epithelial I × P expression levels of ATP5B (C) within the same tissue area of the Barrett tissue microenvironment. (B) A Barrett patient, displaying high epithelial I × P expression levels of HIF1α also expressed high epithelial I × P expression levels of ATP5B (D) within the same tissue area of the Barrett tissue microenvironment. (E) Epithelial ATP5B I × P expression significantly positively correlated to levels of epithelial HIF1α I × P expression (r = 0.71, $P < 0.0001$). (F) HIF1α I × P expression did not correlate to levels of GAPDH I × P expression in the epithelium (r = 0.06, $P = 0.7$). (G) HIF1α I × P expression significantly positively correlated to levels of SERPINA3 I × P expression in the epithelium (r = 0.522, $P = 0.0044$). (H) p53 I × P expression significantly positively correlated to levels of HIF1α I × P expression in the epithelium (r = 0.455, $P = 0.015$). r denotes Spearman ρ correlation (I × P = intensity by positivity).

phosphorylation in the preneoplastic or neoplastic setting. Even though this adaptation to oxidative phosphorylation results in the production of large amounts of ATP produced per glucose molecule compared to glycolysis, it is plausible, however, that this positive relationship reflects more of an indirect relationship between hypoxia and other complex constitutive processes in the Barrett microenvironment, such an inflammation and p53 status. Therefore, we investigated if there was an association between hypoxia, inflammation and p53 status in Barrett tissue microenvironment. We found that hypoxia significantly positively correlated to levels of p53 *in-*

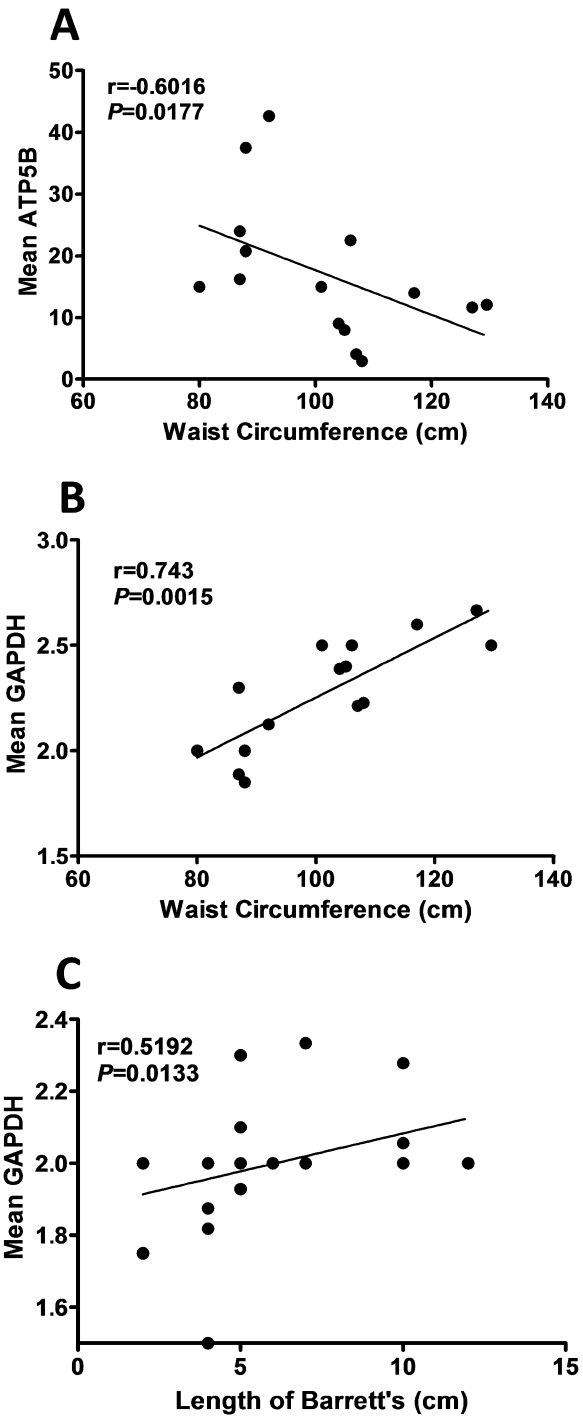

Fig. 6. Linking energy metabolism to obesity and to the length of the Barrett segment in Barrett oesophagus *in-vivo*. (A) Stromal ATP5B I × P expression significantly negatively correlated to waist circumference (r = –0.6016, *P* = 0.0177). (B) Epithelial GAPDH intensity significantly positively correlated to waist circumference (r = 0.743, *P* = 0.0015). (C) Stromal GAPDH intensity significantly positively correlated to the length of the Barrett segment (r = 0.5192, *P* = 0.0133). Waist circumference and Barrett segment length demographics were available on 15 and 22 patients respectively from the 29 patients who participated in the study. r denotes Spearman ρ correlation (I × P = intensity by positivity).

vivo and *ex-vivo*. No studies to our knowledge have linked hypoxia to p53 in the Barrett setting. Some studies, however, have shown p53 to encompass an important role in hypoxic microenvironments [56,57]. Under hypoxic conditions, the p53-inducible phosphatase, *TP53*-induced glycolysis and apoptosis regulator, forms

Table 1
Comparing the association between metabolism, inflammation, hypoxia, p53, obesity and the length of the Barrett segment *in-vivo* using Spearman ρ and multiple regression analyses.

	Spearman Correlation		Multiple Regression	
Correlation	r[†]	*P*	Estimate	*P*
Metabolism vs p53				
ATP5B vs p53[1A]	0.53	0.0031	0.3847	0.03
GAPDH vs p53[3B]	–0.39	0.0357	–3.78245	0.007
p53 vs Inflammation				
p53 vs IL1β[3A]	0.43	0.02	–0.1452	0.464
p53 vs SERPINA3[2A]	0.536	0.0033	0.1397	0.537
Metabolism vs Inflammation				
ATP5B vs IL1β[2A]	0.8	<0.0001	0.5118	0.019
GAPDH vs IL1β[2A]	0.43	0.022	0.155	0.378
Metabolism vs Hypoxia				
ATP5B vs HIF1α[3A]	0.71	<0.0001	0.4838	0.002
GAPDH vs HIF1α[3A]	0.06	0.7	–0.24441	0.112
Hypoxia vs Inflammation				
HIF1α vs SERPINA3[3A]	0.522	0.0044	0.08036	0.744
p53 vs Hypoxia				
p53 vs HIF1α[3A]	0.455	0.015	0.1915	0.253
Metabolism vs Waist Circumference				
ATP5B[3B] vs Waist Circumference	–0.6016	0.0177	–0.89	0.399
ATP5B[3A] vs Waist Circumference	–0.354	0.195	–2.19	0.059
GAPDH[1A] vs Waist Circumference	0.743	0.0015	49.06	0.003
Metabolism vs Length of Barrett's Segment				
GAPDH[1B] vs Barrett Segment	0.5192	0.0133	6.0504	0.181

[†] Correlation Coefficient (r).
[1] Intensity.
[2] Positivity.
[3] Intensity × Positivity.
[A] Epithelium.
[B] Stroma.

a complex with hexokinase 2 at the mitochondria [56]. This complex subsequently shunts glycolytic intermediates into the pentose phosphate pathway reducing glycolytic flux and generating NADPH, ribose-5-phosphate and erythrose-4-phosphate for the production of fatty acids, nucleotides, nucleic acids and amino acids [56]. In the neoplastic setting, suppression of HIF2α has been shown to restore p53 activity reversing chemoresistance in renal carcinoma cells [57]. Moreover, it is also noteworthy to mention that making direct comparisons between the *in-vitro* Barrett cell line and the *in-vivo* and *ex-vivo* tissue would be inaccurate as they are biologically differ-

Table 2
The association between metabolism and inflammation, hypoxia, p53 and angiogenesis in Barrett *ex-vivo* explant tissue.

Correlation	Correlation Coefficient, r	*P*
Metabolism vs Inflammation		
ATP5B vs TNFα*	0.79	0.027
ATP5B vs IL6*	0.93	0.002
GAPDH vs TNFα*	0.81	0.021
GAPDH vs IL6*	0.9	0.004
PKM2 vs IL6	0.73	0.025
Metabolism vs Hypoxia		
ATP5B vs *HIF1α*	0.88	0.003
HSP60 vs *HIF1α*	0.73	0.031
GAPDH vs *HIF1α*	0.87	0.002
PKM2 vs *HIF1α*	0.92	0.001
Metabolism vs p53		
ATP5B vs *p53*	0.72	0.037
PKM2 vs *p53*	0.9	0.002
Metabolism vs Angiogenesis		
HSP60 vs ANG1	–0.7	0.036
PKM2 vs ANG1	–0.68	0.042

Spearman ρ, $n = 9$ ($n = 8^*$).

Table 3
The association between inflammation, hypoxia, p53, BMI and angiogenesis in Barrett *ex-vivo* explant tissue.

Correlation	Correlation Coefficient, r	*P*
Inflammation vs Angiogenesis		
ICAM-1 vs MCP-1*	0.76	0.036
ICAM-1 vs MMP9	0.72	0.03
ICAM-1 vs IL2	0.78	0.013
ICAM-1 vs TNFα*	0.79	0.028
bFGF vs IL1β*	0.76	0.036
bFGF vs IL10*	0.71	0.047
Angiogenesis vs BMI		
VCAM-1 vs BMI	0.8	0.01
ICAM-1 vs BMI	0.73	0.025
ANG1 vs BMI	0.72	0.03
Hypoxia vs p53		
HIF1A vs *p53*	0.88	0.003
Hypoxia vs Inflammation		
HIF1A vs GROα	0.75	0.025
HIF1A vs IL6	0.88	0.003
HIF1A vs MIP3α	0.75	0.025
HIF1A vs MMP2*	0.81	0.022
HIF1A vs TNFα	0.72	0.037
HIF1A vs IL1β	0.67	0.049
p53 vs Inflammation		
p53 vs IL6	0.75	0.025
p53 vs MMP2*	0.76	0.037

Spearman ρ, $n = 9$ ($n = 8$*).

ent; *in-vivo* and *ex-vivo* tissue is complex primarily due to their rich cell diversity, however, the Barrett cell line are solely epithelial cells.

We have further demonstrated that hypoxia significantly positively correlated with inflammation in the Barrett setting *in-vivo*. *Ex-vivo*, we illustrate that *HIF1α* is positively associated with various secreted inflammatory proteins including GROα, IL6, MIP3α, MMP2, MMP9, TNFα and IL1β. HIF1α protein expression has previously been documented to be associated with inflammation in Barrett oesophagus, however, this study utilised the Sydney System to assess inflammation and not an inflammatory molecular marker analogous to this current study [7]. Another study found increased expression of the hypoxia-inducible proteins across the metaplasia-dysplasia-OAC sequence *in-vivo* [6]. Therefore, the greater degree of inflammation known to be associated with the latter stages of the Barrett sequence may in part be hypoxia-induced inflammation in origin [58,59]. Moreover, T-cells are known to play an important role in the initiation and subsequent progression of Barrett oesophagus [60]. Interestingly, hypoxia is known to stimulate increased levels of IL1β, IL10 and IL8 in human $CD4^+$ T cells, but the absence of glucose reduces secretions of these cytokines implying that $CD4^+$ T cells can be highly metabolically adaptable in highly fluctuating bioenergetic microenvironments [61]. Since both oxidative phosphorylation and glycolysis were found to be strongly associated with increased inflammation in the Barrett tissue microenvironment in this study, hypoxia-induced secretions of various pro-inflammatory proteins may in theory alter the metabolic profile in the Barrett microenvironment. In addition to its close link with hypoxia, we have shown *in-vivo* that p53 significantly positively correlated with both IL1β and SERPINA3 in Barrett oesophagus. *Ex-vivo*, we illustrate positive links between *p53*, IL6 and MMP2. Few studies have linked p53 to IL1β and SERPINA3 in the literature but p53's role in the regulation of the key central inflammatory and metabolic regulator, NFκB, has been documented [42,46]. Consequently, the intrinsic relationship between hypoxia, inflammation and p53 early in the Barrett microenvironment may be sufficient to mediate metabolism in support of metaplastic transformation.

Obesity is strongly associated with the increase in incidence of Barrett oesophagus and OAC [13,14]. Detailed studies of the Barrett and tumour microenvironment in this model are, however, scarce; therefore, we assessed if there was an association between obesity and both oxidative phosphorylation and glycolysis in our Barrett cohort. Interestingly, we found a significant negative association between waist circumference and ATP5B expression but a significant positive association between waist circumference and GAPDH expression. No studies have shown such a reciprocal association between obesity and metabolism in preneoplastic or cancer tissue. It is possible, however, that such obesity-induced metabolic alterations are the functional result of various adipokines known to be secreted by adipose tissue [62]. The fatty acid, palmitic acid, has been shown to increase concentrations of fructose 2,6-biphosphate, a key glycolytic intermediate [63]. Obesity has also been shown to promote aerobic glycolysis in prostate cancer cells [64]. Conversely, a high fat diet has been shown to downregulate genes required for oxidative phosphorylation [65]. Moreover, after a 6-month exercise programme, extensive alterations in the transcriptome profile of healthy human adipose tissue has shown increases in a variety of genes associated with oxidative phosphorylation [66]. Adiponectin is also known to promote mitochondrial biogenesis [67]. Serum adiponectin levels are known to decline with obesity in Barrett oesophagus which may decrease mitochondrial biogenesis in the process [62]. In addition, it has also been shown that a palmitic acid-induced decrease in adiponectin is responsible for mitochondrial dysfunction in adipocytes [68]. The degree of glycolysis may also depend on the oxidative capacity of the tissue, thus, increases in glycolysis may be a compensatory mechanism as oxidative metabolism is inhibited. To support this, when we inhibit oxidative phosphorylation in *in-vitro* Barrett cells utilising Seahorse technology, we see a significant compensatory increase in glycolytic levels (see Supplementary Fig. S2). We also found a significant positive correlation between GAPDH and the length of the Barrett segment, previously linked to metaplastic progression in Barrett oesophagus [28–30].

Angiogenesis, a key hallmark of cancer, is linked to hypoxia and glycolysis and therefore is key to endothelial cell function [22]. We found that both *HSP60* and *PKM2* were significantly negatively associated with the angiogenesis marker, ANG1, in Barrett *ex-vivo* explant tissue. Although angiogenesis is positively associated with neoplastic progression in some disease entities, these findings may have some significant physiological implications; that is, high energy levels are associated with low angiogenic potential and low energy levels are associated with high angiogenic potential. In addition, we found *ex-vivo* that angiogenesis (ICAM-1 and bFGF) significantly positively correlated with levels of inflammation (MCP1, MMP9, IL2, TNFα, IL1β and IL10). Moreover, angiogenesis (ICAM-1, VCAM-1 and ANG1) significantly positively correlated with BMI status *ex-vivo*.

Overall, the *in-vivo* and *ex-vivo* findings in this study, as depicted in Fig. 7, effectively demonstrate the complexity of the cellular processes of the Barrett tissue microenvironment and give an insight into how these processes could potentially support metaplastic progression through the alteration of cellular energy metabolism. Despite showing links between specific markers in this study, these links represent the associations between the processes themselves and not the markers; therefore, future studies need to address the molecular mediators linking these processes. However, it is plausible that markers do regulate other markers in this study as evidenced by previous studies [69–72]. Identifying and exploring the underlying molecular mechanisms that link metabolism to these key cellular processes, and to each other, would significantly aid in understanding how these processes interact and may provide some insight into the development of targeted therapies influencing these processes.

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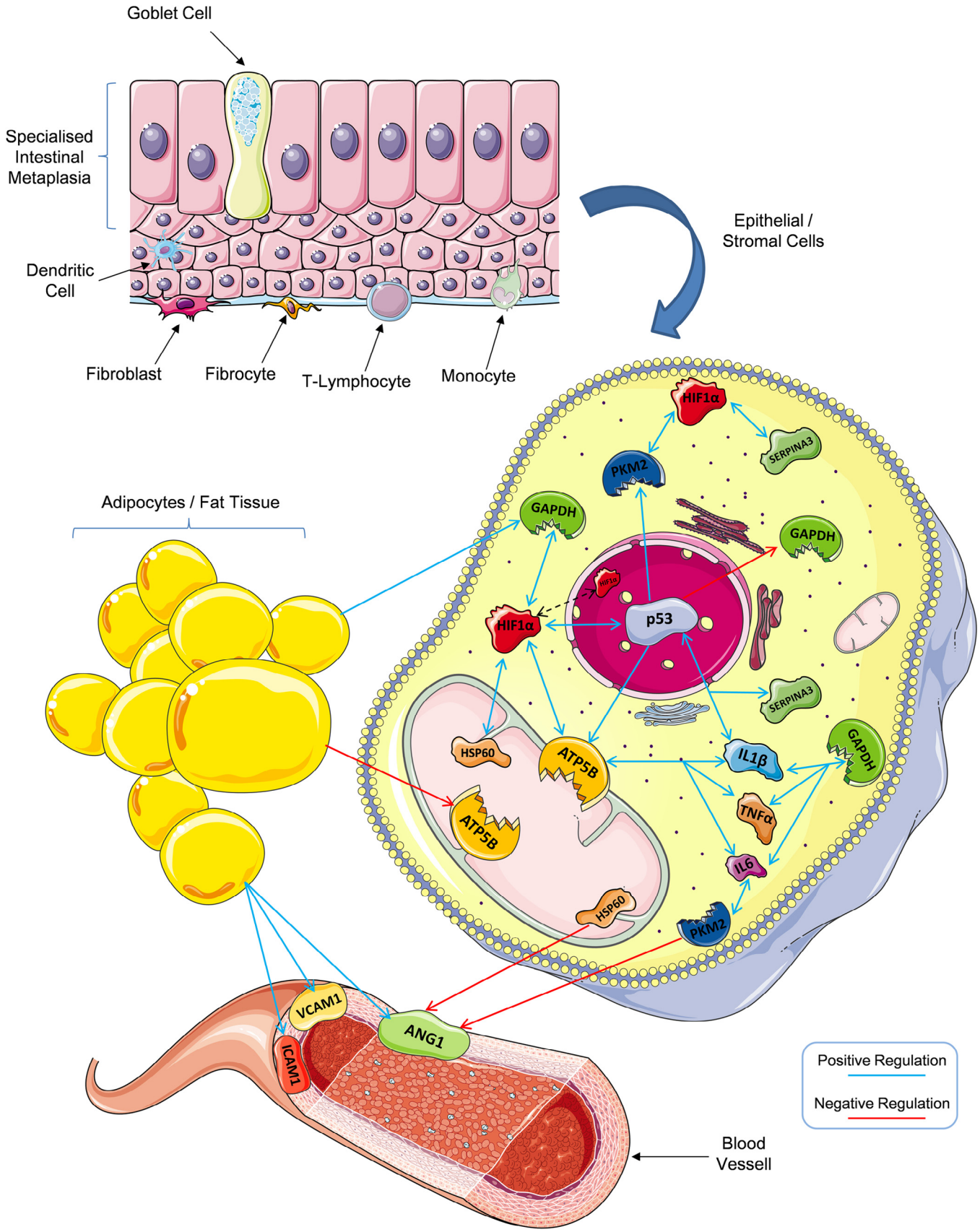

Fig. 7. The Barrett oesophagus microenvironment. The inflamed specialised intestinal metaplasia of Barrett oesophagus demonstrates many complex interdependencies. Within epithelial and stromal cells, p53 positively associates with ATP5B but negatively with GAPDH. p53 positively correlates with IL1β and SERPINA3. Both ATP5B and GAPDH are positively linked with IL1β, TNFα and IL6. p53 expression positively correlates with HIF1α expression. HIF1α expression positively correlates with ATP5B, PKM2 and SERPINA3. HSP60 and PKM2 negatively associate with ANG1, a marker of angiogenesis. Obesity status positively links with GAPDH but negatively with ATP5B. Obesity positively correlates with angiogenesis. Inflammation and angiogenesis are additionally interdependent.
Image produced with the aid of Servier Medical Art software.

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Conflict of interest

The authors declare no conflicts of interest.

Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.canlet.2015.11.041.

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