INTRODUCTION

The carcinogenic effect of bile reflux in hypopharyngeal mucosa has been recently documented by our established in vitro and in vivo models. We have shown that acidic bile (BA) can cause a progressive mutagenic effect characterized by a dramatic activation of a characteristic molecular phenotype, assigned as ‘BA-induced mRNA oncogenic phenotype’, including central molecules in head and neck squamous cell carcinoma (HNSCC), such as nuclear factor kappa B (NF-κB) and signal transducer and activator of transcription 3 (STAT3). Exploration of the BA effect using pharmacologic and dietary inhibitors of NF-κB revealed the key role of NF-κB in this process by activating early neoplastic molecular events. However, the role of STAT3 in bile reflux-related hypopharyngeal carcinogenesis remains unclear. We hypothesized that STAT3 contributes substantially to the BA-related oncogenic effect, by inducing transcriptional activation of inflammatory and cancer-related events, which can be dramatically prevented by STAT3 silencing. STA-21, SI3-201 or Nifuroxazide effectively inhibited STAT3 and cancer-related inflammatory phenotype, encouraging their single or combined application in preventive or therapeutic strategies of bile reflux-related hypopharyngeal carcinogenesis.

KEYWORDS
bile reflux, gastro-oesophageal reflux, head and neck cancer, hypopharyngeal cancer, laryngopharyngeal reflux, Nifuroxazide, SI3-201, STA-21, STAT3, STAT3 inhibition
genes, according to previously established BA-induced ‘mRNA onco-
genetic phenotype’,¹⁻⁸,¹²⁻¹⁸ in exposed human hypopharyngeal primary
cells (HCs) and preserving cell survival.

The STAT3 is considered an oncogene⁶,¹⁹ and its upregulation
has been associated with development and progression of head
and neck cancer,⁹,²₀,²¹ and especially of HPV-negative HNSCC.¹⁰
The mechanism by which STAT3 is activated has been described
by others.⁸,²²,²³ STAT3 proteins, as transducers of cytoplasmic
signals from extracellular stimuli, including cancer-related cyto-
kines and growth factors, can be activated through Janus-kinase
family members (JAK).²⁴,²⁵ JAKs after their dimerization can
phosphorylate a tyrosine residue (Tyr705) of STAT3 within its
Src homology 2 (SH2) domains. Then, STAT3 can homodimerize
or heterodimerize with other STAT proteins to translocate to
the nucleus and activate the transcription of inflammatory and
cancer-related genes.²⁶,²⁷ JAK/STAT3 activation plays a crucial
role in inflammatory-related carcinogenesis.⁸,²⁵,²⁶ EGFR can also
activate STAT3 by its intrinsic tyrosine kinase activity.⁹,²⁷ IL-6/STAT3
signalling¹¹ has also been previously suggested to play a
role in bile reflux-related oncogenesis, through NF-κB activated
pathway.¹²⁻¹⁸

To investigate whether STAT3 actually contributes to early on-
cogenic molecular events, previously linked to bile-induced hypo-
pharyngeal carcinogenesis,²⁴ we targeted the STAT3 pathway by
knocking down STAT3 gene expression, as well as by inhibiting its
activation using specific STAT3 pharmacologic inhibitors. STAT3
pharmacologic inhibition consisted of blocking (i) the upstream
extracellular receptor JAK2, which affects STAT3 phosphorylation;
or (ii) the SH2 domain of phosphorylated STAT3, which blocks its
dimerization and DNA binding, and therefore preventing the sub-
sequent target-gene transcription.²²,²⁵,²⁸,²⁹ Identification of the
mechanism by which STAT3 affects acidic bile-induced oncogenic
molecular events may not only contribute to the characterization
of its role in hypopharyngeal carcinogenesis but may also be more im-
portant to the development of new effective targeted agents in the
prevention and therapy of this process.

2 | MATERIALS AND METHODS

2.1 | Cell culture and treatment conditions

Normal human hypopharyngeal cells (HCs; Celprogen Inc.) were
cultured, in Human Hypopharyngeal Normal Cell Culture Media
(Celprogen Inc.), at 37°C in humidified air and 5% CO₂, as previously
described.⁷,¹³,¹⁶ An intermittent exposure of HCs was performed, to
experimental and control media for 7 min, twice per day, for 4–5 days,
as previously described.⁹ Experimental media included (i) ‘BA’, acidic
bile at pH 4.0, (ii) ‘Nil’, BA plus 10 μM Nifuroxazide (CAS 965-52-6;
Santa Cruz Biotechnology Inc.), (iii) ‘SI3-201’, BA plus 50 μM STAT3
Inhibitor VI, S3I-201 (CAS 19983-44-9; Santa Cruz Biotechnology
Inc.) and (iv) ‘STA-21’, BA plus 20 μM STA-21 (CAS 28882-53-3;
Santa Cruz Biotechnology Inc.; Supplementary Material; Table S1).

All experimental groups were repetitively exposed to 400 μM of a
mixture of conjugated primary bile acids (Sigma Aldrich) as previ-
ously described,¹,¹²,¹³,¹⁶ in serum-free medium (Dulbecco modified
Eagle’s medium/F12, 1% pen/strep, Gibco®). pH at 4.0 was adjusted
by 1 M HCl (using a pH meter). ‘BA’ or acidic bile at pH 4.0, the cut-
off of reflux disease⁷⁰ was used according to our prior findings.¹,¹²,¹⁷
Control media included serum-free medium, as used in experimental
groups, at either pH 4.0 (acid control) or neutral pH 7.0 (control in-
cluding vehicle) (Supplementary material; Table S1). After each treat-
ment, experimental or control media were replaced by serum-free
media until the next exposure cycle.

Experimental and control groups were cultured in parallel. All the
experiments were independently repeated three times. Cells were
harvested immediately after the last treatment by 0.05% trypsin-
EDTA (Gibco®).

2.2 | STAT3 knockdown

To knockdown STAT3, STAT3 siRNA (STAT3 siRNA(h); sc-29493,
Santa Cruz Biotechnology Inc.) and Control siRNA, as a negative
control (Control siRNA-A; sc-37007, Santa Cruz Biotechnology Inc.)
were used. STAT3 and Control siRNAs were diluted to a final concen-
tration of 5 nM in serum-free culture medium (Opti-MEM® serum-
reduced growth medium, Gibco™ by Thermo Fisher Scientific),
including HiPerFect® Transfection Reagent (3 μl/well; Qiagen), ac-
cording to the manufacturers’ instructions, and incubated for 10 min
at room temperature. Cells were mixed with transfected complexes,
seeded at 1.5 × 10⁵ cells/well of six-well plates and incubated under
normal growth conditions (at 37°C and 5% CO₂) (Supplementary
Material; Table S2). Sixteen hours after transfection, media were changed with
complete growth medium and 4 h later were treated with ‘BA’
for 7 min. The media were removed and replaced with serum-
free medium until the next day, we repeated the 7-min treatment
two times with an interval time 6 h. After 12 h, cells were treated
for 7-min and immediately after this last treatment media were
removed, the cells were washed once with PBS and harvested
either (i) for total protein isolation, using M-PER reagent (mamma-
lian protein extraction reagent; Thermo Scientific), or (iii) for total
RNA isolation using RNA mini kit (Qiagen). Assays were carried
out according to the manufacturer’s instructions and performed
in triplicate. All experiments were independently repeated two
times.

2.3 | Luciferase assay

Luciferase assay was performed to measure the transcriptional activ-
ity of STAT3 dimers (homodimers or heterodimers) in HCs exposed
to (a) BA with knockdown of STAT3 or (b) BA with pharmacologic
inhibitors of STAT3, Nifuroxazide, S3I-201 or STA-21, compared
to BA alone and controls, as described in Supplementary Material
(Tables S1 and S2). STAT3 dual-luciferase reporter assay was used (Cignal reporter assay by Qiagen), including (i) a firefly luciferase reporter for STAT3 and a constitutively expressing Renilla luciferase construct (Creport-STAT3), and (ii) a control negative control with a non-inducible reporter construct and a constitutively expressing Renilla luciferase construct (Creport-NC). A reverse transfection was performed, using Lipofectamine® 2000 (Invitrogen™), according to manufacturer’s procedure.

2.4 | Immunofluorescence cell staining

Immunofluorescence (IF) assay was performed for p-STAT3 (Tyr705) and p-NF-κB (p65 S536), as previously described7,10 and in Supplementary Material. Briefly, HCs were grown on multiwell chamber slides (Lab-Tek®; Thermo Fisher Scientific) and treated with BA, with or without pharmacologic inhibitors of STAT3, Nifuroxazide, chamber slides (Lab-Tek®; Thermo Fisher Scientific) and treated with BA, with or without pharmacologic inhibitors of STAT3, Nifuroxazide, Si3-201 or STA-21, acid at pH 4.0 alone. Primary antibodies were used, including anti-p-STAT3 (Tyr705) (rabbit mAb, D3A7 XP®, Cell Signaling Technology, Inc.) or anti-phospho-NF-κB (rabbit polyclonal anti-phospho-p65 Ser536, AbD Serotec, BIO-RAD). Secondary antibodies were also used, including anti-rabbit or anti-mouse DyLight®488 (green; Vector Labs). Prolong Gold Mountant with DAPI; Life Technologies, Thermo Scientific) was also used for nuclear staining (blue colour).

Zeiss Confocal microscope and imaging software by Zen were used to examine stained slides and captured images, respectively (Zen imaging software, Carl Zeiss, microscopy GmbH).5,15 Expression levels of p-STAT3 and p-NF-κB were assessed by fluorescence intensity (mean ± SD bin count) from at least two independent images (>10 cells; Zen imaging software).

2.5 | Protein expression analysis

Western blot analysis and a direct enzyme-linked immunosorbent assay (ELISA) were performed, as previously described7,12,16,31 and in Supplementary Material, to monitor the successful knockdown of STAT3 expression, and to determine the effect of STAT3 knockdown or its pharmacologic inhibition on BA-induced STAT3, NF-κB and Bcl-2 protein levels. We used primary antibodies for p-STAT3 (Tyr705) (clone B-7), STAT3 (clone F-2), p-NF-κB (p65 Antibody 27. Ser 536), bcl2 (Clone N-19), Histone 1 (AE-4) and β-actin (C4) (Santa Cruz Biotechnology Inc.). Protein levels obtained by Western blot analysis were quantified by the Gel imaging system (Bio-Rad) in each nuclear or cytoplasmic cellular compartment (Image Lab 5.2 analysis software, Bio-Rad). Protein levels obtained by ELISA were quantified by Gen5™ software reading the absorbance values using a microplate reader (Sunergy1, BIOTEK; Gen5™ software; BioTek Instruments Inc.). Assays were carried out according to the manufacturer’s instructions and performed in triplicates and repeated two times, independently.

2.6 | Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (qPCR) analysis (Bio-Rad real-time thermal cycler CFX96™; Bio-Rad) was performed, as previously described7,12,16 and in Supplementary Material, to evaluate the effect of STAT3 knockdown or pharmacologic inhibition on transcriptional levels of EGFR, TNF-α, IL6, STAT3, RELA(p65), REL, BCL2 and WNT5A, previously associated with HNSCC7,13 and in particular with bile carcinogenesis2-5 and HSCC.41 Specific primers were used for target genes and reference housekeeping gene, human glyceraldehyde 3-phosphate dehydrogenase (hGAPDH) (QuantiTect Primers Assays; Qiagen; Supplementary Materials; Table S3) and data were analysed by CFX96™ software.7,13,16 Relative mRNA expression levels were estimated for each target gene compared to the reference control gene (ΔΔCt).

2.7 | Cell viability assay

Cell Titer-Glo® Luminescent Cell Viability Assay (Promega Corp.) was used as described in Supplementary Material. All values were normalized to mean value of untreated controls. All experimental groups and controls were performed in triplicates.

2.8 | Statistical analysis

GraphPad Prism 7 software and multiple t test analysis (GraphPad Prism 7 software; t test; multiple comparisons by Holm-Sidak; GraphPad Prism 7.0, GraphPad Software Inc.) were used to demonstrate the differential expression (p-values < 0.05) for each analysed gene, protein expression and cell viability between different experimental and control groups.

3 | RESULTS

3.1 | Knockdown of STAT3 reduces BA-induced total STAT3, p-STAT3 and Bcl-2 protein levels

Immunofluorescence (IF) assay revealed that STAT3 knockdown prevented the BA-induced activation of STAT3, as indicated by weak nuclear staining of p-STAT3 (Tyr705) in treated HCs compared to BA alone (Figure 1A-a). IF also revealed that STAT3 knockdown had a minimal effect on BA-induced activation of NF-κB, as shown by a less intense nuclear staining of p-NF-κB (p65 S536) relative to BA alone (Figure 1A-b). Western blot analysis confirmed that silencing of STAT3 effectively suppressed STAT3 protein levels produced by BA exposure in HCs (Figure 1B). Quantification by ELISA also showed that STAT3 knockdown significantly reduced the BA-induced total p-STAT3 (Tyr705; Figure 1C-a) and Bcl-2 protein levels (Figure 1C-b). STAT3 knockdown also induced a decrease, although not statistically
Figure 1  Silencing of STAT3 suppressed total STAT3, p-STAT3 (Tyr705) and bcl2 protein levels, with a minimal suppressive effect on p-NF-κB (p65 S536), in BA-treated HCs. (A) (a) Immunofluorescence staining for p-STAT3 (Tyr705) (green: p-STAT3; blue: nuclear DNA staining with DAPI; scale bar 20 μm; Zen imaging software). (b) Immunofluorescence staining for p-NF-κB (p65 S536) (green: p-NF-κB (p65 S536); blue: nuclear DNA staining with DAPI; scale bar 20 μm; Zen imaging software). (B) Western blot analysis for total STAT3 in BA-treated HCs and controls (Control: media at pH 7.0, including vehicle; Acid: media at pH 4.0) with knockdown of STAT3 gene. Graph depicts the total STAT3 protein levels, in BA and control-treated HCs after STAT3 knockdown. (C) Graphs depict the total protein levels, by ELISA, of p-STAT3 (Tyr705), Bcl-2 and p-NF-κB (p65 S536) in BA-treated HCs after STAT3 knockdown. (from left to right) si-C: media at pH 7.0 plus Control siRNA; si-STAT3: media at pH 7.0 plus STAT3 siRNA; BA+si-C: Bile at pH 4.0 plus Control siRNA; BA+si-STAT3: Bile at pH 4.0 plus STAT3 siRNA; Acid+si-C: media at pH 4.0 plus Control siRNA; Acid+si-STAT3: media at pH 4.0 plus STAT3 siRNA (β-actin was used to normalize total protein extracts; t test; multiple comparisons by Holm-Sidak, **p < 0.005; ***p < 0.0005; GraphPad Prism 7.0; means ± SD of three independent experiment)
significant, in total p-NF-κB levels, compared to BA alone (Figure 1C-c). IF, Western blot and ELISA analyses showed that controls produced low levels of STAT3 and its activated form (p-STAT3), and so silencing of STAT3 induced slight changes (Figure 1A-a,B,C-a). Similarly, controls presented low p-NF-κB (p65 S536) levels, mostly located in the cytoplasm (Figure 1A), accompanied by low Bcl-2 levels, while silencing of STAT3 had a minimal effect on their expression (Figure 1B,C-b,c).

**3.2 | Pharmacologic inhibition of STAT3 prevents BA-induced STAT3 activation and Bcl-2 overexpression**

Immunofluorescence (IF) assay presented that BA induced an intense nuclear staining of p-STAT3 (Tyr705), as it was expected.7,14 However, application of Nifuroxazide, which blocks the upstream extracellular receptor JAK2, affecting STAT3 phosphorylation,28 or application of SI3-201 or STA-21 that block the SH2 domain of phosphorylated STAT3, inhibiting dimerization and DNA binding,22,29 successfully suppressed BA-induced activation of STAT3, as shown by a less intense nuclear staining of p-STAT3 (Tyr705) in treated HCs, compared to BA alone (Figure 2A-a). Scoring of IF staining revealed significantly higher nuclear levels of p-STAT3 (Tyr705) in BA-treated HCs, compared to controls (Figure 2A-b). The application of Nifuroxazide, SI3-201 or STA-21 resulted in significantly lower nuclear levels of p-STAT3, compared to BA. In particular, Nifuroxazide was found to induce the lowest p-STAT3 nuclear levels among SI3-201 and STA-21 (Figure 2A-b).

IF assay also revealed that application of Nifuroxazide, SI3-201 or STA-21, diminished the BA-induced activation of NF-κB, as shown by a less intense nuclear staining of p-NF-κB (p65 S536) relative to BA alone (Figure 2B-a). However, scoring of IF staining did not reveal a significant difference between p-NF-κB (p65 S536) nuclear levels measured in HCs exposed to BA plus STAT3 pharmacologic inhibitors, compared to BA alone (Figure 2B-b).

In order to confirm the above IF data, and further determine if targeting JAK/STAT3 phosphorylation can affect the acidic bile-induced STAT3 activation, Bcl-2 overexpression and NF-κB activation, we performed Western blot analysis. Our analysis revealed that Nifuroxazide inhibited the activation of STAT3 and Bcl-2 overexpression, caused by acidic bile. This was shown by significantly reduced nuclear levels of p-STAT3(Tyr705) and cytoplasmic levels of Bcl-2 in HCs exposed to BA plus Nifuroxazide compared to BA alone (Figure 2C-a,b). However, our analysis showed that Nifuroxazide did not reduce BA-induced nuclear levels of p-NF-κB (Figure 2C-c).

**3.3 | STAT3 knockdown or pharmacologic inhibition of STAT3 reduces BA-induced STAT3 transcriptional activity**

Our analysis revealed a significant reduction of STAT3-reporter luciferase activity in BA-treated HCs with STAT3 knockdown, compared to BA alone (BA plus STAT3 siRNA vs. BA plus Control siRNA; Figure 3A). Acid and neutral control-treated groups appeared with low levels of STAT3 luciferase activity; however, silencing of STAT3 gene also suppressed its levels (STAT3 siRNA vs. Control siRNA).

Our analysis, using STAT3-reporter luciferase assay and Nifuroxazide, SI3-201 or STA-21, also revealed that pharmacologic inhibition of STAT3 affected the BA-induced STAT3 transcriptional activity in HCs, as similarly shown by knocking down of STAT3. Specifically, our analysis showed that BA-treated HCs presented significantly higher STAT3-transcriptional activity, compared to controls (Figure 3B). However, application of all three pharmacologic inhibitors significantly reduced BA-induced STAT3-transcriptional activity. This was indicated by significantly lower levels of luciferase activity for STAT3-reporter, in HCs exposed to BA plus STAT3 pharmacologic inhibitors, compared to BA alone (Figure 3B).

**3.4 | STAT3 knockdown suppresses transcriptional changes caused by acidic bile**

qPCR analyses showed that knockdown of STAT3 reversed the BA-induced transcriptional changes of genes, previously assigned as BA-induced ‘mRNA oncogenic phenotype’ and linked to malignant transformation of murine hypopharyngeal mucosa caused by BA and HNSCC2,4,7,9,11,26,27,32-41 (Figure 4). As shown in Figure 4 and Table 1, BA-treated HCs with STAT3 knockdown produced significantly lower mRNA levels of IL6, TNF-α, BCL2, REL(Ap65), STAT3, REL, WNT5A and EGFR, compared to BA alone (Supplementary Material; Table S4). Acid and neutral control groups presented with low mRNA levels of the analysed genes and STAT3 knockdown had a minimal effect on them, except STAT3 and IL6 in acid control group (Supplementary Material; Table S4).

Overall, STAT3 knockdown induced a pronounced suppression of BA-induced transcriptional activation of all the analysed genes (Table 1).

**3.5 | Pharmacologic inhibition of STAT3 inhibits BA-induced transcriptional activation of antiapoptotic and cancer-related inflammatory genes**

qPCR analyses showed that application of Nifuroxazide prevented the BA-induced mRNA profile of antiapoptotic gene BCL2,32,33 cancer and inflammatory-related genes, IL6, TNF-α, REL(Ap65), oncogenic STAT3 and EGFR, as well as of cell proliferation or tumour-promoting factor WNT5A,8,11,19,26,27,33-40 as shown in Figure 5 and Table 1 (Supplementary Material; Table S5). Specifically, targeting JAK2/STAT3 phosphorylation, by Nifuroxazide, induced significantly lower mRNA levels of these genes, compared to BA alone.

Application of SI3-201 similarly prevented the BA-induced mRNA profile of TNF-α, IL6, BCL2, REL(Ap65), STAT3, EGFR and WNT5A, as well as of cell proliferation or tumour-promoting factors, REL,38 as shown in Figure 5 and Table 1 (Supplementary Material; Table S5). Specifically, targeting STAT3 dimerization, by
FIGURE 2. Pharmacologic inhibition of STAT3 prevents BA-induced nuclear localization of p-STAT3 and Bcl-2 overexpression, with a minimal effect on p-NF-κB activation, in treated HCs. (A) Immunofluorescence staining for p-STAT3 (Tyr705) (green: p-STAT3; blue: nuclear DNA staining with DAPI; scale bar 20 μm; Zen imaging software). (B) Immunofluorescence staining for p-NF-κB (p65 S536) (green: p-NF-κB (p65 S536); blue: nuclear DNA staining with DAPI; scale bar 20 μm; Zen imaging software). (C) Nuclear and cytoplasmic protein levels of p-STAT3 (Tyr705), Bcl-2 and p-NF-κB (p65 S536) in BA-treated HCs, with or without pharmacologic inhibition of STAT3, by Western blot analysis. Acid media at pH 4.0; Nif: acidic bile plus Nifuroxazide; S3I-201: BA plus STAT3 inhibitor VI (S3I-201); STA-21: BA plus STA-21. (Histone 1 and β-actin were used to normalize nuclear and cytoplasmic protein extracts, respectively; by Image Lab 5.2 analysis software, Bio-Rad; t-test; multiple comparisons by Holm-Sidak, *p < 0.05; **p < 0.005; ***p < 0.0005; GraphPad Prism 7.0; means ± SD of three independent experiments.)
SI3-201, induced significantly lower mRNA levels of these genes, compared to BA.

Finally, as presented in Figure 5 and Table 1, STA-21 similarly to other two inhibitors prevented the BA- induced transcriptional changes of IL6, TNF-α, BCL2, RELA(p65), STAT3 and EGFR (Supplementary Material; Table S5). Targeting STAT3 dimerization and its DNA binding, by STA-21, induced a pronounced reduction of transcriptional levels of IL6, RELA(p65), STAT3 and EGFR, compared to BA.

3.6 | Silencing or pharmacologic inhibition of STAT3 affects cell viability of BA-treated HCs

We performed a cell viability assay to explore the effect of STAT3 knockdown or its pharmacologic inhibition on BA-induced cell survival rates. Our analysis revealed that silencing STAT3 (siRNA STAT3; Figure 6A) or blocking STAT3 dimerization and DNA binding, by SI3-201 or STA-21, or targeting STAT3 phosphorylation, by Nifuroxazide (Figure 6B) significantly reduced the BA-induced survival rates of treated HCs (p < 0.05; by t test; multiple comparisons by Holm-Sidak). Blocking STAT3 by STA-21 presented similar to STAT3 knockdown changes in cell survival of BA-treated HCs (Figure 6).

4 | DISCUSSION

Chronic exposure to BA has recently been shown to cause malignant transformation of hypopharyngeal epithelial cells which preceded by significant transcriptional activation of cancer and inflammatory-related genes, previously assigned as ‘BA-induced mRNA oncogenic phenotype’, including oncogenic STAT3.2-5 Interestingly, the oncogenic molecular phenotype induced by BA in vitro and in vivo was similarly identified in clinical specimens from bile-related HSCC, demonstrating an aberrant overexpression of STAT3 compared to controls.39 The role of STAT3 as a crucial transcription factor in HNSCC has been discussed extensively by others.9,10,20-22,27 Although other STAT factors, such as STAT1 and STAT5, have also been associated with cancers of the upper aerodigestive tract,42,43 we focused our current exploration on STAT3, based on our previous findings.2-4,7,12,14-18 Investigating whether STAT3 contributes substantially to BA-induced molecular oncogenic profile may elucidate a better understanding to the mechanism of bile reflux-related hypopharyngeal carcinogenesis and demonstrate useful key molecules for its early detection or targeted treatment. Also, using pharmacologic inhibitors that can block different steps of STAT3 activation process may provide us with insightful information about the upstream signalling of its activation under the BA
Our novel findings conclude that silencing of STAT3 expression had a dramatic effect on BA-induced oncogenic molecular phenotype. Specifically, STAT3 knockdown induced a strong suppression of total p-STAT3 and Bcl-2 protein levels, and significantly reduced STAT3 transcriptional activity and the transcriptional levels of anti-apoptotic BCL2,32,33 cancer and inflammatory-related...
Upstream signal, but also to alternative signalling, such as EGFR. BA, which does not seem to be exclusively depended on JAK/STAT3 may support a constitutive activation of STAT3 in HCs, caused by reduction of BA-related mRNA phenotype and cell survival. This view finds strong support the hypothesis that STAT3 plays an important role in bile reflux-related oncogenic molecular events in HCs.

Our findings from the pharmacologic inhibition of STAT3, using Nifuroxazide, S3I-201 or STA-21 demonstrated a very similar effect to its gene silencing, supporting their effective application on reducing the BA-induced oncogenic profile. Using three different inhibitors that each one can block a different step of STAT3 upstream signalling, we gained information that improve our knowledge regarding the mechanism of bile reflux-related carcinogenesis in hypopharynx. In theory, Nifuroxazide inhibits the JAK/STAT3 phosphorylation, while S3I-201 and STA-21 inhibit STAT3 dimerization and its subsequent translocation to the nucleus. As we know, STA-21 can block STAT3 dimerization, DNA binding and STAT3 dependent transcription, when STAT3 is constitutively active. Herein, we show that all three inhibitors were similarly effective to suppress STAT3 activation, with STA-21 presenting the most pronounced reduction of BA-related mRNA phenotype and cell survival. This view may support a constitutive activation of STAT3 in HCs, caused by BA, which does not seem to be exclusively depended on JAK/STAT3 upstream signal, but also to alternative signalling, such as EGFR. EGFR cross-talk with STAT3 has previously been identified to affect activation of STAT3 via tyrosine kinase, while Bhat et al., recent findings supported EGFR and STAT3 interactions in oesophageal precanerous and cancerous cells under the acidic bile effect. Further investigation is required to elucidate possible similar EGFR-STAT3 interactions in acidic bile-related hypopharyngeal squamous cell cancer.

Notably, both S3I-201 and STA-21 appeared to produce a relatively more pronounce suppressive effect on BA-induced mRNA phenotype than Nifuroxazide (Table 1). A possible explanation to this view could be that BA-induced constitutive activation of STAT3 through JAK/STAT3 pathway may lead to interactions of STAT3 with other STAT factors, like STAT1, contributing to this process. Future exploration of STAT1 in combination with STAT3 inhibitors may clarify the role of STAT in BA-induced oncogenic effect in hypopharynx.

Besides the above variations, our data demonstrated that similarly to STAT3 silencing, all three pharmacologic drugs suppressed the transcriptional activation of IL6, TNF, STAT3, BCL2, RELAp(65) and EGFR (Table 1). This observation strongly supports that the constitutive activation of STAT3 can maintain the continuous production of inflammatory and cancer-related molecules that are central to the carcinogenic process. Therefore, we confirm that BA-induced molecular oncogenic events can be effectively prevented by the application of either Nifuroxazide, S3I-201 or STA-21.

IL6 plays a central role in inflammatory and cancer promotion of HNSCC and is considered as a promising therapeutic target. Our current findings showed that BA effect can induce an abundant overexpression of IL6 in exposed HCs, as previously shown in premalignant or malignant murine hypopharyngeal mucosa and in bile-related HSCC. IL6 overexpression can promote a persistent activation of STAT3, by the IL6/STAT3 pathway. Our data show that silencing or pharmacologic inhibition of STAT3 can effectively reduce IL6 transcriptional levels and thus limit potential positive feedback.

Although we show that targeting STAT3, either by its knockdown or its pharmacologic inhibition, had a minimal effect on total p-NF-κB (p65 SS36) levels, our findings strongly support a role of STAT3 in promoting the transcriptional activation of NF-κB. NF-κB inhibition, using BAY 11-7082, had previously supported the role of NF-κB in BA-induced activation of STAT3, among other factors. In view of all the above, we propose a possible molecular cross-talk between the NF-κB and STAT3 transcription factors signatures in bile reflux-related inflammation and tumorigenesis in hypopharynx, as has been proposed by others in HNSCC that requires further exploration.

Given the central role of STAT3 pathway in HNSCC, despite its complexity, our data provide supportive evidence of STAT3 inhibition as a preventive and therapeutic approach in hypopharyngeal cancer. STAT3 is indeed a demanding target not only because of the complexity of its activating signal but also because of its resistance to standard treatment in HNSCC. However, recent preclinical studies have shown the antiproliferative effect of STAT3 inhibition, and the latest clinical trials have presented very promising anti-oncogenic phenotype under STAT3-target therapy. S3I-201 has been extensively tested as an antitumour target in HNSCC, since it is promising to abrogate resistance to anti-EGFR therapies. Nifuroxazide has also been proved to be a safe drug with antitumour and anti-inflammatory effects. Finally, STA-21 has been clinically tested and found to improve chronic inflammatory disorders. Our data from the use of the three pharmacological inhibitors of STAT3 strongly support their use as promising drugs for the prevention or treatment of inflammatory or neoplastic diseases associated with laryngopharyngeal reflux. It was very recently presented an association between bile acid-receptors, such as nuclear farnesoid X receptors (FXRs), and

| Table 1: The effect of STAT3-inhibition on oncogenic mRNA phenotype caused by acidic bile in HCs |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | Nifuroxazide    | S3I-201         | STA-21          | siSTAT3         |
| IL6            | -102            | -600            | -1554           | -313            |
| TNF- α         | -5.1            | -3.4            | -4              | -234            |
| BCL2           | -9              | -3.5            | -2.8            | -285            |
| STAT3          | -7.0            | -5.0            | -95.0           | -163            |
| RELAp(65)      | -8.0            | -26             | -96             | -130            |
| EGFR           | -13             | -38             | -40.5           | -76             |
| WNT5A          | -1.8            | -4.0            | 1               | -94             |
| REL            | -1.1            | -1.8            | -1.1            | -80             |

mRNA ratios BA + STAT3-inh versus BA.
JAK/STAT3 in colon,\textsuperscript{56} which inspires a further exploration of FXRs possible expression in the hypopharynx and their potential interaction with JAK/STAT3 or other oncogenic pathways in bile reflux-related HSCC. Other STAT factors, such as STAT1 and STAT5, have also been associated with epithelial cancers and their interaction with STAT3.\textsuperscript{42,43} Future experiments may clarify the role of STAT1 and STAT5 in acidic bile-induced hypopharyngeal carcinogenesis and how STAT3 silencing, or inhibition may affect their expression.
CONCLUSION

Our novel data strongly support that STAT3 contributes substantially to bile reflux-related molecular oncogenic events in HCs. This view was documented by silencing STAT3 gene which induced a strong suppression of total p-STAT3 and Bcl-2 protein levels, and a dramatic reduction of transcriptional levels of cancer-related cytokines, genes and cell survival. Using three inhibitors, each one targeting a different step of STAT3 upstream signalling, such as Nifuroxazide, S3I-201 or STA-21, we extracted novel findings supporting the constitutive activation of STAT3 under the acidic bile effect. This constitutive activation does not appear to be exclusively dependent on JAK/STAT3. We also show that all three STAT3 inhibitors, with STA-21 having the most profound effect, can effectively suppress the continuous production of cancer-related molecules, IL6, TNF, RELAp65), EGFR, BCL2 and STAT3, previously associated with hypopharyngeal cancer, caused by acidic bile. These findings document the important role of STAT3 in hypopharyngeal carcinogenesis associated with bile reflux, and also encourage the single or combined application of Nifuroxazide, S3I-201 or STA-21, in clinical studies for preventive or therapeutic approaches.

CONFLICT OF INTEREST

The authors whose names are listed in this article certify that they have no affiliations with or involvement in any organization or entity with any financial or non-financial interest in the subject matter or materials discussed in this manuscript.

AUTHOR CONTRIBUTIONS

Dimitra P Vageli: Conceptualization (lead); Data curation (lead); Formal analysis (lead); Investigation (lead); Methodology (equal); Project administration (equal); Resources (equal); Software (lead); Supervision (lead); Validation (equal); Visualization (lead); Writing-original draft (lead); Writing-review & editing (equal).

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DATA AVAILABILITY STATEMENT

Data are contained within the article or Supplementary Material.

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