

Onkologische Sicherheit der hydrostatischen Hochdruckbehandlung zur Devitalisierung von
Tumorzellen

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Impact of high hydrostatic pressure on the cytokine profile and head and neck cancer cell behavior: implications for oncological safety

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Introduction: The devitalization of tissue using high hydrostatic pressure (HHP) is an advanced method for processing tumor-infiltrated cartilage to treat tissue defects. This approach preserves the structural and biomechanical properties of the graft while effectively eliminating tumor cells. However, HHP induces the release of cytokines, which may influence the behavior of residual tumor cells in the surrounding tissue. This study characterizes cytokine profiles of HHP-treated head and neck squamous cell carcinoma (HNSCC) cell lines and evaluated its biological effects on intact tumor cells to further assess the oncological safety of the method.

Methods and results: HHP- treatment resulted in a dose-dependent release of pro-inflammatory cytokines, primarily IL-1 α and IL-1 β , in all investigated cell lines, while IL-6 and IL-8 concentrations were higher in untreated samples. Functional assays demonstrated that supernatants from HHP-treated HNSCC cells significantly enhanced proliferation, migration, and invasion of HNSCC cells relative to control conditions, with these effects being most pronounced at 200 MPa, a pressure associated with incomplete tumor cell devitalization. At 300 MPa, HHP achieved complete devitalization, correlating with intensified necrotic processes and increased intracellular cytokine release.

Discussion: Our findings indicate that while HHP significantly influences the cytokine profile and tumor cell behavior, pressures of ≥ 300 MPa ensure complete tumor cell devitalization, supporting its oncological safety for clinical applications. Further *in vivo* studies are needed to validate these observations and confirm the clinical safety of HHP-treated materials.

KEYWORDS

high hydrostatic pressure (HHP), cytokine release, head and neck cancer, whole cell vaccination, immune modulation, squamous cell carcinoma

1 Introduction

High Hydrostatic Pressure (HHP) is an emerging technique in tissue engineering and regenerative medicine, offering a cost-effective approach for tissue devitalization while preserving structural integrity and biomechanical properties (1–6). By applying pressures up to 600 MPa, HHP disrupts cellular membranes, leading to cell devitalization without compromising the extracellular matrix, a critical factor for the success of grafts and implants in reconstructive surgery (1, 7). Due to its uniform pressure distribution across complex tissues according to Pascal's law, HHP ensures standardized treatment, making it especially valuable for the preparation of autologous, tumor-infiltrated tissues such as cartilage for reimplantation (1, 3, 8).

While HHP is extensively used in food safety application for bacterial inactivation, its potential in regenerative medicine and oncology remains an active area of research (1, 9–12). Initial approaches from our working group have explored the use of advanced tissue grafts in head and neck surgery, where reconstruction is performed utilizing HHP-treatment (13). In head and neck surgery, especially for squamous cell carcinomas (SCC), functional and aesthetic preservation is a crucial aspect (14–18). SCCs in the head and neck region often cause significant tissue damage, have high recurrence rates, and often respond inadequately to secondary treatments such as immunotherapy (19–24). This challenge is particularly evident in cases of laryngeal infiltration, where partial laryngeal resection may be necessary for organ preservation. However, effective reconstruction is often limited by the loss of structural support within the laryngeal skeleton. Autologous reconstruction of tumor-surrounding or even partially affected cartilage might provide a tissue-preserving option that addresses both functional and aesthetic concerns (25–27).

Beyond tissue devitalization, HHP-treatment of HNSCC cells has demonstrated its capacity to induce immunogenic cell death, marked by calreticulin translocation and elevated ATP release at higher pressures, which can stimulate an antigen-specific immune response (28). This positions HHP as a valuable tool in developing whole-cell cancer vaccines. HHP provides an efficient and non-toxic method for devitalizing tumor cells, serving as an alternative to conventional methods such as chemical treatment, irradiation, or freeze-thaw cycles (29, 30). Importantly, HHP-treatment preserves the antigenicity of tumor cells, as well as the primary and secondary structures of proteins (28, 30, 31). Studies have shown that dendritic cell-based vaccines, pulsed with HHP-inactivated tumor cells, hold promise as an immunotherapy approach for solid tumors (30, 32–35). Additionally, it was observed that HHP-treated melanoma cells, in combination with irradiation, could significantly suppress tumor growth in mice (8, 36–38).

A previous work has shown that HHP-treatment at 315 MPa for 10 min effectively devitalizes head and neck SCC (HNSCC) cells while preserving the integrity of the cartilage matrix (28). While this safe devitalization of tumor cells is promising, ensuring the oncological safety of autografts for reimplantation remains crucial. In particular, residual cytokines and tumor antigens

within the treated cartilage could influence surrounding cells, including any remaining tumor cells, stem cells, and fibroblasts. Cytokines play a central role as mediators of the immune response and may be critical in the context of HHP-treatment. Pro-inflammatory cytokines such as Interleukin (IL)-1, IL-6, and IL-8 are of particular interest, as they can influence the tumor microenvironment and potentially stimulate immune responses (39–41). However, they may also contribute to chronic inflammation, a factor associated with tumor progression in SCCs (39). Conversely, certain cytokines can act as adjuvants in tumor vaccination by enhancing antigen presentation, promoting T-cell responses, or modulating the immune environment in ways that support anti-tumor immunity (42–45).

A detailed analysis of the cytokines released during HHP-treatment is crucial to evaluate how these factors impact the tumor microenvironment and oncological safety. In this study, relevant cytokines were identified through comprehensive screening, and their release during HHP-treatment was analyzed. Experiments were conducted to assess the effects of these cytokines on the proliferation, migration, and invasion of HNSCC cell lines. These investigations are essential to fully understand the potential of HHP-treatment for autologous reconstruction and the development of immunotherapies.

2 Materials and methods

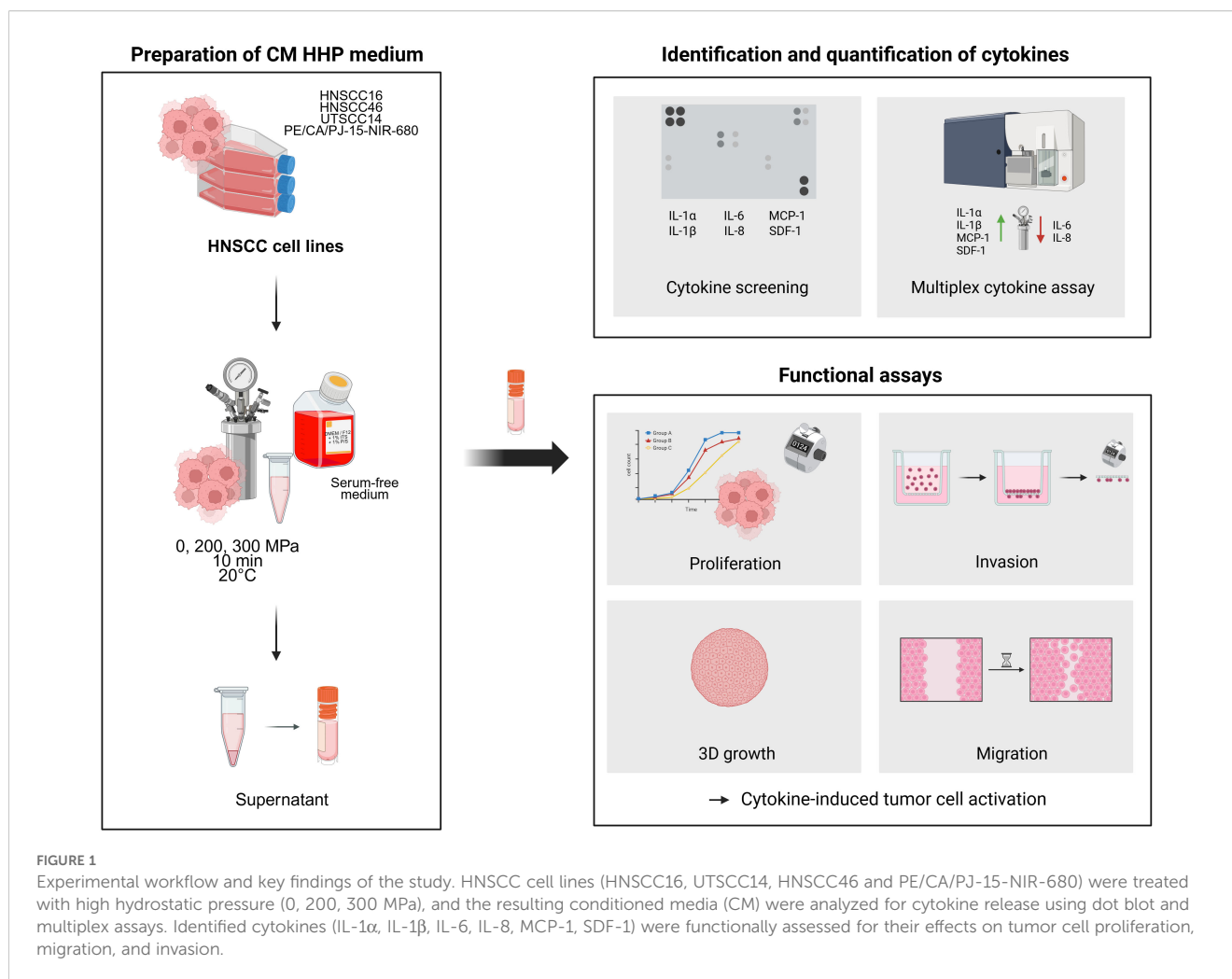
The experimental procedures used to investigate cytokine release following HHP treatment of HNSCC cells and the subsequent effects of conditioned media (CM) on tumor cell behavior are summarized in the graphical abstract shown in Figure 1.

2.1 HNSCC cell line cultivation

The head and neck squamous carcinoma cell lines HNSCC16 (larynx P1 M1), UTSCC14 (tongue, RRID: CVCL_7810), HNSCC46 (hypopharynx P0 M2) and PE/CA/PJ-15 (tongue, RRID: CVCL_2678) were utilized in this study.

PE/CA/PJ-15, originally obtained from the German collection of cell cultures (DSMZ; Braunschweig, Germany) was transduced to stably express the fluorescent near-infrared protein iRFP680 (NIR). The primary tumor cells HNSCC16 and HNSCC46 were established by the research group (Schoenwalder et al. (46)) and authenticated by STR profiling (against the respective patient tumor and patient-derived xenograft (PDX)). All cell lines were Human papillomavirus (HPV) negative, and all experiments were performed with mycoplasma-free cells.

Cells were grown in Dulbecco's modified Eagle medium (DMEM)/Ham's F12 (1:1) (Pan Biotech Germany) supplemented with 10% fetal calf serum (FCS, Pan Biotech, Germany) and 1% Penicillin/Streptomycin (P/S, Sigma-Aldrich, US) and cultured in 75 cm² culture flasks at 37°C with 5% CO₂ in a humidified incubator. The medium was replaced every 2–3 days. Upon reaching 80–90%



confluence, cells were washed with Dulbecco's Phosphate Buffered Saline (DPBS, Gibco, US), detached with 0.25% Trypsin-ethylenediaminetetraacetic acid (EDTA, Pan Biotech, Germany), and seeded in new flasks or treatment wells.

For the experiments DMEM/Ham's F12 (1:1) supplemented with 1% Insulin-Transferrin-Selenium (ITS, Gibco, US) and 1% P/S was used to eliminate interfering effects of FCS and is labelled as serum-free medium or control (CTRL) medium in this study.

2.2 High hydrostatic pressure treatment and preparation of conditioned medium

HHP-treatment was performed in cryotubes (1.8 mL Nunc, Thermo Fisher Scientific, US). 1×10^6 HNSCC cells (HNSCC16, UTSCC14, HNSCC46, PE/CA/PJ-15) were suspended in serum-free medium (DMEM/F12 + 1% ITS + 1% P/S) and added to the cryotube. The cryotubes were centrifuged at $120 \times g$ for 8 min, filled with serum-free medium, and closed without air bubbles. The cryotubes were sealed with Parafilm M Laboratory Film (Pechiney Plastic Packaging Inc., US) and placed into water-filled, air-bubble-free centrifuge tubes. The sealed centrifuge tubes containing the

samples were then placed in the glycol-filled pressure chamber of the high hydrostatic pressure device (Dustec Hochdrucktechnik GmbH, Germany). Cells were treated for 10 min at 0, 200 or 300 MPa respectively, with the temperature maintained at 20°C. The pressurization phase consisted of an initial pressure increase at +25 bar/s, followed by a slower ramp at +10 bar/s until the target pressure was reached. The target pressure was maintained for 10 min, after which decompression occurred at -25 bar/s. After treatment, the cryotubes were centrifuged again, incubated for 1 hour (h) at 37°C, and then the CM was transferred to a new cryotube. The CM was frozen at -80°C until further use in experiments.

CM of the different cell lines is labelled $CM_{HNSCC16}$, $CM_{UTSCC14}$, $CM_{HNSCC46}$ and $CM_{PE/CA/PJ-15}$ in this study.

2.3 Cytokine screening using the dot blot assay

The Human Cytokine Array C3 (RayBiotech Inc., USA) dot blot assay was used as a qualitative method to analyze the secretion of 42 cytokines, including key pro-inflammatory cytokines, chemokines,

and growth factors. The assay was performed on thawed CM collected from pressure treated HNSCC16 (200 MPa and 300 MPa) and untreated control cells (0 MPa). The assay was conducted following the manufacturer's protocol. Labeled proteins were visualized via enhanced chemiluminescence, using detection buffer and X-ray film exposure. Cytokines appeared as dots of varying intensity and size. Dot blot assay was performed once for qualitative cytokine screening to identify relevant targets for subsequent quantitative analysis.

2.4 Cytokine quantification in conditioned medium

Cytokine levels (IL-1 α , IL-1 β , Monocyte Chemoattractant Protein-1 (MCP-1), Stromal cell-derived factor 1 (SDF-1), IL-6, IL-8) of CM of treated cells (200 and 300 MPa) and control cells (0 MPa) were determined using the LEGENDplex Human Custom Panel (BioLegend, US) according to the manufacturer's instructions. Briefly, CM were incubated with capture beads over night at 4°C on an orbital shaker. Next, detection antibody was added, and beads were incubated for 1 h at room temperature. After washing the beads, samples were measured using a BD Canto flow cytometer (BD Biosciences, US). Cytokine concentration was calculated of triplicates based on a standard curve using the BioLegend LEGENDplex data analysis software (version 2023-02-15, BioLegend, US). Quantification of cytokines was repeated at least four times per sample in technical triplicates.

2.5 Quantification of cellular proliferation

Cell counts over time were analyzed as a measure of cell proliferation using CASY Cell Counter (OMNI Life Science GmbH & Co. KG, Germany). HNSCC16 or UTSCC14 cells respectively were seeded in duplicates in a 24-well cell culture plate in serum-free medium. Medium was changed after 5 h and CM or control medium respectively were applied. Cells were incubated for 96 h and counted every 24 h. Each experiment was repeated at least four times in technical duplicates.

2.6 Analysis of cell migration potential

Tumor cell migration of HNSCC16 and UTSCC14 cells was evaluated by the scratch assay using Ibidi culture-insert 2 well system (Ibidi, Germany). Inserts were placed in a 24-well plate and 4×10^4 HNSCC16 or 5×10^4 UTSCC14 cells respectively were seeded in 100 μ L serum-free medium in each insert chamber. After allowing the cells to attach overnight, the culture insert was removed to create a cell-free gap, and the cells were covered with CM or control medium respectively. Migrating cells were documented immediately after creation of the gap (0 h) at 10x magnification via live-cell-imaging (Carl Zeiss AG, Germany)

under stable pressure of 5% CO₂ in air at 37°C. The cell migration was recorded at the whole area of the gap at 2 h time intervals for 16 h. Evaluation was performed by measuring the cell-free area with ImageJ software (version 1.54 f, National Institutes of Health, US). Data was collected from at least three independent experiments each performed with four to six technical replicates.

2.7 Characterization of the tumor cell invasion potential

Tumor cell invasion potential of HNSCC16 and UTSCC14 cells was evaluated using the Boyden chamber assay. For this purpose, 5×10^4 cells were seeded in triplicates in 800 μ L serum-free medium on top of gelatine-coated filter (pore size 8 μ m; it4ip S.A., Belgium). The bottom chamber was filled with 290 μ L of CM or control medium respectively. The cells were incubated for 4 or 24 h in the chambers at 37°C and 5% CO₂. After incubation time the filters were removed, and non-migrated cells were wiped away. Filters were then placed upside down on a glass slide and cells were fixed with 4% paraformaldehyde (Otto Fischar GmbH & Co. KG, Germany) for 10 min. Afterwards, filters were washed in DPBS and stained with Hoechst reagent (0.5 μ g/mL; Sigma-Aldrich, US) for 10 min and placed on glass slides. The invaded cells were counted using a fluorescence microscope (Leica Microsystems GmbH, Germany) at 10x magnification, evaluating ten fields of view per filter. Each experiment was repeated at least four times in technical triplicates.

2.8 Spheroid growth assay

Three-dimensional (3D) spheroid formation was assessed using HNSCC16 cells. Cells were seeded into 12-well plates and treated with CM_{HNSCC16} (0 MPa and 300 MPa) or control medium respectively for priming the cells for 48 h. Subsequently, cells were detached and 1×10^5 were seeded in a custom-fabricated polydimethylsiloxane (PDMS) μ -well insert made from 1600 kPa-stiff silicone. For this, Sylgard[®] 184 (PDMS) (VWR, USA) was used and mixed in various base (A) to crosslinker (B) ratios (R) (R; A to B, (w/w)). Mixing ratios were calculated from existing calibrations and substrates were prepared as described previously (47). Cross-linking occurred at 50°C overnight. After curing, respective elasticities were measured with nanoindentation. Effective Young's modulus (E_{eff}) of 1600 kPa (R; 10 to 1 – cartilage) were validated and used up to a deviation of 10%. Sterilization of elastomer substrates was performed by washing once with 1 mL of 98% isopropanol (IPA) and 1 mL of 1x DPBS. Afterwards, substrates were dried completely under sterile conditions for at least 1 h before use. The insert provides 64 uniform microcavities, each 500 μ m in diameter, allowing for reproducible spheroid formation and quantitative analysis of 3D cell growth. Either CM or control medium was added to each chamber. Spheroid growth was monitored over a period of five days. Images were acquired at 24

h, 48 h, and 120 h using a fluorescence microscope (Carl Zeiss AG, Germany) at 10x magnification. Spheroid area and diameter were quantified using ImageJ software (version 1.54f, National Institutes of Health, USA). A total number of 62 spheroids per condition was analyzed.

2.9 Statistical analysis

Statistical analysis was performed using GraphPad PRISM software, version 8.0.1 (GraphPad Software, US). A p-value <0.05 was considered statistically significant. After proving the assumption of normality (Shapiro-Wilk test), one-way ANOVA with Tukey's multiple comparison *post hoc* test was performed. If the normality test failed, the Kruskal-Wallis test with Dunn's multiple comparison test was performed.

3 Results

This study identified and quantified cytokines associated with HHP-treatment of tumor cells. Additionally, the cytokine effects on

the biological behavior of HNSCC tumor cells were analyzed, focusing on cell proliferation, migration, and invasion.

3.1 HHP-induced cytokine secretion

After treatment with HHP (0, 200, and 300 MPa), the CM from the tumor cells was analyzed for 42 cytokines using an antibody membrane assay, with a focus on pro-inflammatory cytokines (Supplementary Figure 1). All groups showed signals for IL-6, IL-8, MCP-1, and SDF-1. Additionally, the samples treated with 200 and 300 MPa showed signals for IL-1 α and IL-1 β .

3.2 Quantification of secreted cytokines

For detailed quantification of the identified cytokines, concentrations of IL-1 α , IL-1 β , MCP-1, SDF-1, IL-6, and IL-8 were determined using multiplex analysis.

IL-1 α (Figure 2A) levels increased significantly after HHP-treatment, particularly at 300 MPa, across all analyzed cell lines. Compared to the untreated control, IL-1 α concentrations were

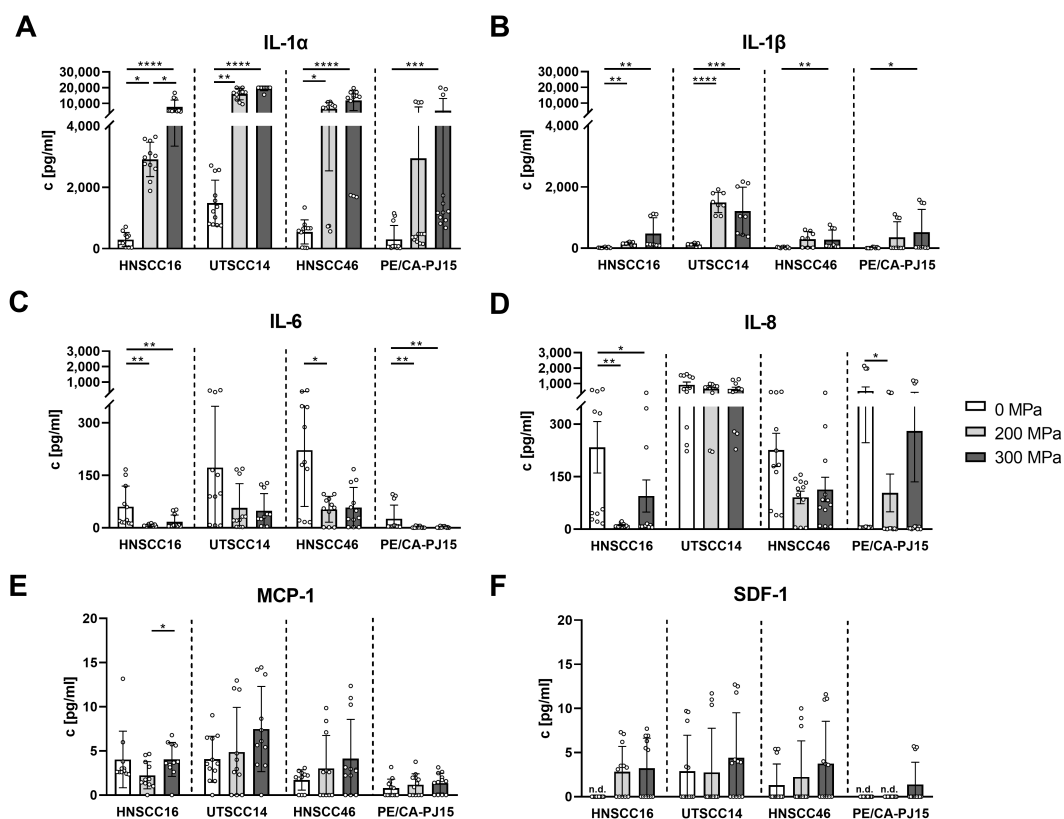


FIGURE 2

Cytokine quantification in conditioned medium (CM) of high hydrostatic pressure-treated tumor cells using multiplex analysis. HNSCC cells HNSCC16, UTSCC14, HNSCC46 and PE/CA/PJ15 were treated with 0, 200, 300 MPa. CM was analyzed in a multiplex assay for IL-1 α (A), IL-1 β (B), IL-6 (C), IL-8 (D), MCP-1 (E), and SDF-1 (F). Results are shown as means and standard deviations; n=8-12 (from four independent experiments). Statistical analysis was performed via one-way ANOVA and Tukey's multiple comparison *post hoc* test; *p<0.05; **p<0.01, ***p<0.001; ****p<0.0001. n.d., not detected.

elevated by 26-fold in CM_{HNSCC16} ($p<0.0001$), 13-fold in CM_{UTSCC14} ($p<0.0001$), 22-fold in CM_{HNSCC46} ($p<0.0001$), and 18-fold in CM_{PE/CA/PJ-15} ($p=0.001$). Treatment at 200 MPa also led to significant IL-1 α increases in CM_{HNSCC16} (10-fold, $p=0.0229$), CM_{UTSCC14} (11-fold, $p=0.0019$), and CM_{HNSCC46} (12-fold, $p=0.0254$) compared to untreated controls. The concentration in the 300 MPa treated samples was higher than in the 200 MPa treated samples, with significant differences observed in CM_{HNSCC16} (2.7-fold increase, $p=0.0229$).

IL-1 β (Figure 2B) followed a similar pattern, with significant increases at 300 MPa across all cell lines. Compared to controls, IL-1 β concentrations increased by 24-fold in CM_{HNSCC16} ($p=0.0021$), 10-fold in CM_{UTSCC14} ($p=0.0006$), 11-fold in CM_{HNSCC46} ($p=0.0042$), and 34-fold in CM_{PE/CA/PJ-15} ($p=0.0247$). At 200 MPa, IL-1 β levels were significantly elevated in CM_{HNSCC16} (7.5-fold, $p=0.0021$) and CM_{UTSCC14} (13-fold, $p<0.0001$) compared to untreated samples. Although IL-1 β levels were higher at 300 MPa than 200 MPa in all cell lines, the differences were not statistically significant.

In contrast, IL-6 (Figure 2C) concentrations were lower in HHP-treated samples compared to controls across all cell lines. At 200 MPa, IL-6 levels decreased by 8-fold in CM_{HNSCC16} ($p=0.0015$), 4-fold in CM_{HNSCC46} ($p=0.0211$), and 14-fold in CM_{PE/CA/PJ-15} ($p=0.0064$). At 300 MPa, significant reductions were observed in CM_{HNSCC16} (3.6-fold, $p=0.0042$) and CM_{PE/CA/PJ-15} (14-fold, $p=0.0100$). No significant differences were detected between 200 MPa and 300 MPa treatments.

A similar trend was observed for IL-8 (Figure 2D), with significant decreases at 200 MPa in CM_{HNSCC16} (20-fold, $p=0.0024$) and CM_{PE/CA/PJ-15} (1.8-fold, $p=0.0377$) compared to controls. At 300 MPa, IL-8 levels were significantly lower than in untreated controls in CM_{HNSCC16} (2.5-fold reduction, $p=0.0101$).

MCP-1 (Figure 2E) showed a dose-dependent increase in CM_{UTSCC14}, CM_{HNSCC46}, and CM_{PE/CA/PJ-15}. However, statistical significance was observed only between 200 MPa and 300 MPa in CM_{HNSCC16} (1.8-fold increase, $p=0.0458$).

SDF-1 (Figure 2F) levels remained low across all samples, showing a non-significant trend towards increased release with higher pressure in all cell lines.

3.3 Proliferation analysis of HNSCC cells after treatment with conditioned medium

The effect of cytokines released by HHP-treated HNSCC tumor cells on the proliferation of HNSCC16 and UTSCC14 cells was analyzed by cell counting.

HNSCC16 cell proliferation (Figure 3A) increased when cultured with CM_{HNSCC16}, compared to the medium control. Cells cultured with 200 MPa-CM exhibited the highest proliferation rates, with significant increases of 1.54-fold after 48 h ($p=0.0002$) and 1.87-fold after 72 h ($p<0.0001$) compared to the medium control. Similarly, cells cultured with 300 MPa-CM showed significantly higher proliferation, with a 1.35-fold increase at 48 h ($p=0.0368$) and a 1.50-fold increase at 72 h ($p=0.0261$) relative to the medium control. 0 MPa-CM_{HNSCC16} treated cells also promoted cell growth, with significant proliferation increases of 1.43-fold at 48 h ($p=0.0032$), 1.54-fold at 72 h ($p=0.0071$), and 1.54-fold at 96 h ($p=0.0320$) compared to the control.

In contrast, no significant differences in the growth behavior of UTSCC14 cells were observed after culturing with their own CM (Figure 3B).

To evaluate the relevance of cytokine effects under more physiologically representative conditions, a 3D spheroid growth assay was performed using HNSCC16 cells (Supplementary Figure 2). Cells were preconditioned with 0 MPa-CM, 300 MPa-CM, or control medium for 48 h prior to seeding in custom-fabricated μ -well inserts. During five days of observation, spheroid diameter and area were monitored. While spheroid growth was slightly reduced in the 300 MPa-CM group compared to control and 0 MPa-CM, the overall differences were moderate, and no proliferative enhancement was observed, in contrast to the 2D proliferation data.

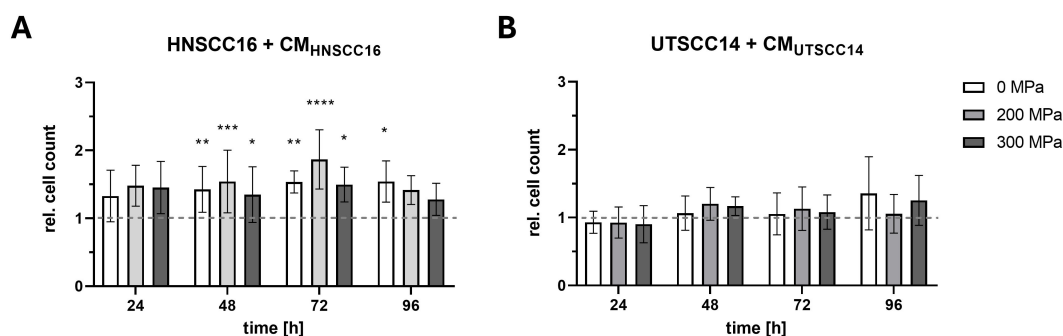


FIGURE 3

Proliferation analysis of HNSCC cells exposed to conditioned medium (CM). HNSCC16 (A) and UTSCC14 (B) cells were cultured for 96 h with CM (0, 200, and 300 MPa). Serum-free culture medium served as reference and was set to 1 and all experimental conditions were normalized to this control. Data are presented as means and standard deviations; $n=5-8$ (from four independent experiments). Statistical analysis: one-way ANOVA, Tukey's multiple comparison *post hoc* test; * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

3.4 Migration analysis of HNSCC cells after treatment with conditioned medium

The effect of cytokines following HHP-treatment on HNSCC cell migration was evaluated using a scratch assay.

The migration speed of HNSCC16 cells (Figure 4A) was highest when cultured with the 200 MPa-CM_{HNSCC16}. After 16 h, the gap was nearly closed (97.08% ± 5.33%). The same was observed with the 0 MPa-CM (98.46% ± 2.56%). In contrast, the control and the 300 MPa-CM still showed an open gap after 16 h (85.45% ± 11.97% for CTRL; 82.52% ± 17.70% for 300 MPa) (Figure 4B). Significant differences between the 200 and 300 MPa conditions were observed after 8 h (57.71 ± 20.55% vs. 35.88 ± 9.95%; $p=0.0119$), 10 h (79.99 ± 18.08% vs. 48.79 ± 13.93%; $p=0.0019$), 12 h (89.42 ± 13.84% vs. 63.28 ± 15.64%; $p=0.0094$) and 14 h (94.70 ± 9.22% vs. 74.48 ± 18.69%; $p=0.0047$). After 14 h HNSCC16 cells treated with 200 MPa-CM showed significantly increased migration activity compared to cells treated with 0 MPa-CM (94.70 ± 9.22% vs. 79.64 ± 14.00%; $p=0.0252$).

Migration analysis of UTSCC14 cells (Figure 4C) following exposure to CM revealed no substantial differences between the various HHP-treatments. However, significant differences were observed between 0 MPa and 200 MPa treatments (9.75 ± 9.65% vs. 0.30 ± 5.26%; $p=0.0277$) within 2 h of migration analysis (Figure 4D). Between 8 and 16 h of incubation with CM, cells incubated with 0 MPa-CM exhibited reduced migratory activity compared to other conditions, although these differences were not statistically significant.

3.5 Invasion analysis of HNSCC cells after treatment with conditioned medium

The influence of cytokines in the CM after HHP-treatment on the invasion potential of HNSCC tumor cell lines was investigated using a Boyden chamber assay.

HNSCC16 cells showed increased invasion when exposed to the CM compared to the medium control (Figures 5A, C). After stimulation with 300 MPa-CM_{UTSCC14}, HNSCC16 cells

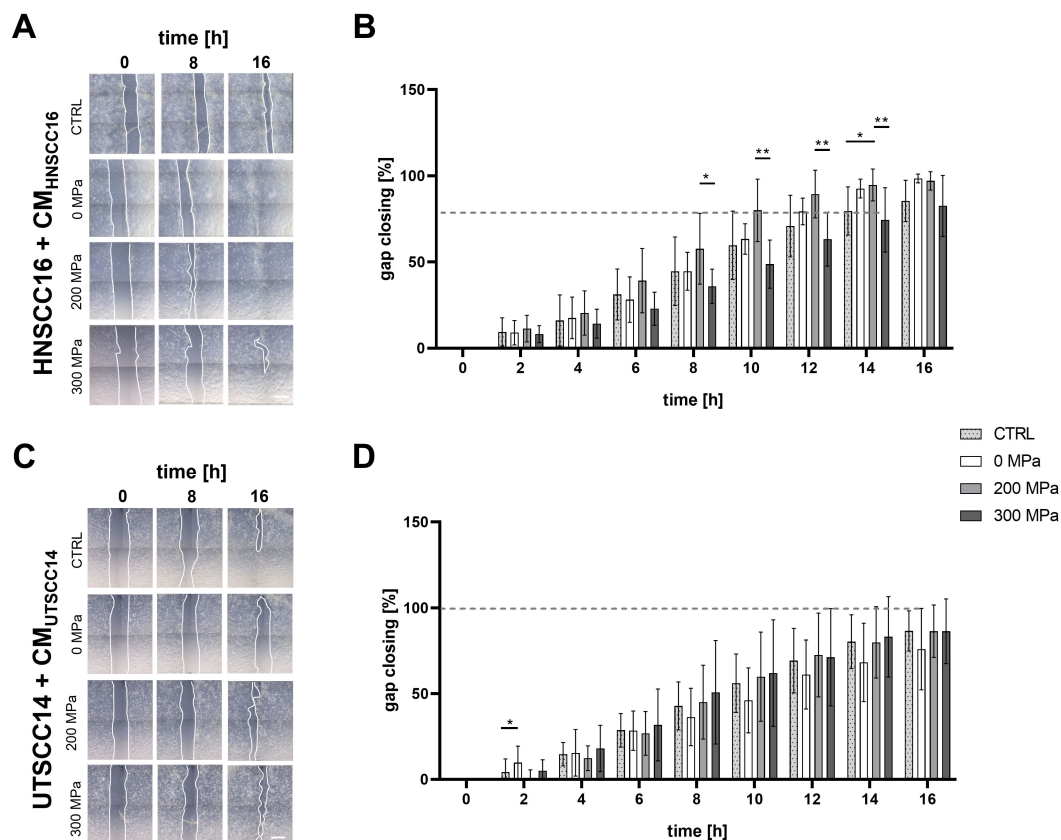


FIGURE 4

Migration analysis of HNSCC cells exposed to conditioned medium (CM). HNSCC16 and UTSCC14 cells were cultured for 48 h with their CM (0, 200, and 300 MPa). The conditioned cells were seeded into Ibidi wound healing inlays and allowed to adhere overnight. After inlays removal, the cells were cultured with the corresponding medium in an incubation chamber. Cell migration of HNSCC16 (A) and UTSCC14 (C) cells was documented via live-cell imaging. Migration speed was determined by measuring the area of the remaining gap, which was normalized to the area at time 0 h (B, D). Serum-free culture medium served as reference (CTRL). Results are shown as means and standard deviations; $n=10-11$ (from three to four independent experiments). Statistical analysis: one-way ANOVA, Tukey's multiple comparison *post hoc* test; * $p<0.05$; ** $p<0.01$; scale bar: 500 μm .

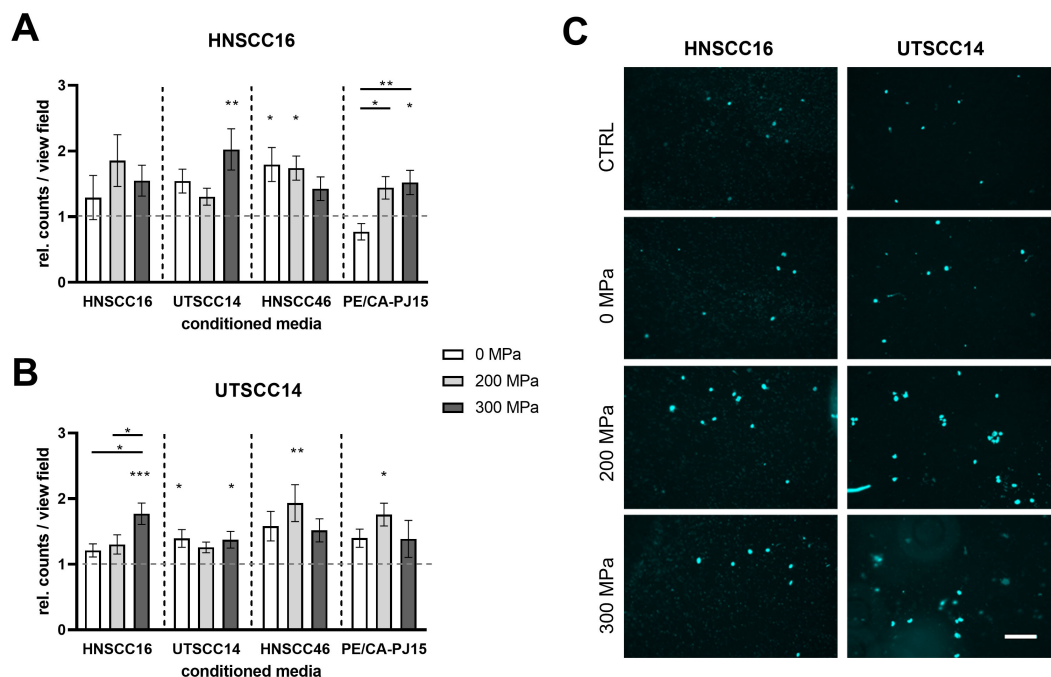


FIGURE 5

Invasion analysis of HNSCC cells exposed to conditioned medium (CM). HNSCC cell lines HNSCC16 and UTSCC14 were examined using a Boyden chamber with a pore size of 8 μ m. The lower compartment contains the CM from their own or another HNSCC cell line after high hydrostatic pressure-treatment (0, 200 and 300 MPa). Serum-free culture medium served as reference and was set to 1. The number of HNSCC16 (A) and UTSCC14 (B) cells that migrated through the membrane was determined and normalized to the control. Representative pictures of migrated HNSCC16 and UTSCC14 cells through the membrane are shown (C). Data are presented as means and standard deviations; $n=8-12$ (from four independent experiments). Statistical analysis: one-way ANOVA, Tukey's multiple comparison *post hoc* test; * $p<0.05$; ** $p<0.01$; *** $p<0.001$; scale bar: 100 μ m.

demonstrated a 2.02-fold increase in invasion compared to the control ($p=0.0036$). Additionally, HNSCC16 cells exhibited significant increases in invasion with 0 MPa-CM_{HNSCC46} and 200 MPa-CM_{HNSCC46}, showing 1.79-fold ($p=0.0236$) and 1.74-fold increases ($p=0.0337$), respectively. In the presence of CM_{PE/CA/PJ-15}, HNSCC16 cells showed 1.52-fold increased invasion activity with 300 MPa compared to the control ($p=0.0494$), as well as 1.87-fold increases between 0 MPa and 200 MPa and 0 MPa and 300 MPa ($p=0.0132$ in both cases). Boyden chamber assay with a prolonged incubation time of 24 h also revealed a trend toward increased invasion in response to HHP-conditioned medium across all cell lines (Supplementary Figure 3) with a statistically significant increase observed for HNSCC16 cells treated with 0 MPa-CM_{HNSCC46} compared to the control (1.59-fold increase; $p=0.0386$).

Similarly, UTSCC14 cells demonstrated increased invasion potential when exposed to the CM, with all CM conditions (0, 200, and 300 MPa) showing higher invasion rates than the medium control (Figures 5B, C). When exposed to 300 MPa-CM_{HNSCC16}, UTSCC14 cells showed a 1.77-fold increase in invasion activity compared to the control ($p=0.0002$). Significant differences were also observed between 0 MPa and 300 MPa (1.21-fold vs. 1.77-fold; $p=0.0110$) and 200 MPa and 300 MPa (1.30-fold vs. 1.77-fold; $p=0.0498$). Treatment with CM_{UTSCC14} resulted in a 1.39-fold increase for 0 MPa ($p=0.0332$) and 1.37-fold increase for 300 MPa ($p=0.0462$) compared to the control. Additionally, exposure

to CM_{HNSCC46} at 200 MPa resulted in a 1.93-fold increase compared to the control ($p=0.078$). CM_{PE/CA/PJ-15} (200 MPa) led to a 1.76-fold increase in invasion compared to the control ($p=0.0239$). No significant differences in invasion activity were found between the 200 MPa and 300 MPa treatments for CM_{PE/CA/PJ-15}.

4 Discussion

4.1 Oncological safety of HHP-treatment

Oncological safety is paramount in both autologous reconstruction of cartilage-infiltrating tumors and the development of whole-cell vaccines. This includes addressing the potential retention of cytokines and tumor antigens after HHP-treatment, which may influence surrounding cells, including residual tumor cells at the resection margin. In the context of whole-cell vaccines, certain cytokines may enhance immune responses, improving therapeutic outcomes. By identifying and characterizing cytokines and analyzing their effects on tumor cell proliferation, migration, and invasion, we aimed to evaluate the safety of HHP-treatment for clinical applications.

A critical factor in ensuring oncological safety is the choice of pressure levels used for devitalization. Previous findings demonstrated that 315 MPa is the threshold for effective

devitalization of HNSCC cells (28). In unpublished data, this threshold was refined to 300 MPa, the pressure used in this study. Notably, when a pressure of 200 MPa is used, complete devitalization of tumor cells is not achieved. In this study, 200 MPa was also investigated to compare its effects on cytokine release and the associated biological responses with those observed at 300 MPa.

4.2 Structural integrity and autologous reimplantation

Cartilage subjected to HHP retains its structural integrity and can be revitalized with stem cells, maintaining both biomechanical properties and immunological compatibility (6, 48). These properties form the basis for the safe use of HHP-treated cartilage in reconstructive procedures after tumor resection. The ability to preserve cartilage integrity is crucial for autologous reimplantation, ensuring that the tissue retains its original function without compromising mechanical stability.

Compared to other tissue regeneration methods, HHP offers the advantage of maintaining extracellular matrix structures without introducing foreign materials or relying on decellularization processes. Other approaches, such as scaffold-based cartilage regeneration or synthetic implants, often lack the biomechanical fidelity required for long-term integration. HHP-treated cartilage, in contrast, allows for seamless incorporation into the surgical site, supporting functional restoration while eliminating the risk of reintroducing viable tumor cells.

4.3 Cytokine profiles and impact on tumor cells

Screening for 42 cytokines was performed using a dot blot assay, focusing primarily on pro-inflammatory cytokines with potential immunomodulatory effects. The dot blot identified IL-1 α , IL-1 β , MCP-1, SDF-1, IL-6, and IL-8 as key cytokines released from HHP-treated HNSCC cells, primarily pro-inflammatory in nature. IL-1 α and IL-1 β were found to promote epithelial-to-mesenchymal transition (EMT), invasion, and an immunosuppressive microenvironment through recruitment of Myeloid-derived suppressor cells (MDSCs) and tumor associated macrophages (TAMs), while IL-6 and IL-8 were associated with chronic inflammation, cellular proliferation, and tumor progression (49–51). Both IL-1 and IL-6 play pivotal roles in creating a tumor-promoting microenvironment, with chronic inflammation fostering DNA damage, cellular proliferation, and immune suppression, all of which contribute to cancer progression (52–54). The multiplex analyses demonstrated a dose-dependent release of IL-1 α and IL-1 β with increasing pressure, likely due to intracellular storage and membrane disruption caused by HHP. It was shown that upon proapoptotic signals, IL-1 α migrates to the nucleus, binds chromatin, and remains non-inflammatory (55). In contrast,

necrotic signals cause IL-1 α to move to the cytosol, where its release acts as a damage-associated molecular pattern (DAMP), triggering inflammatory responses via IL-1 receptor type I (IL-1R1) (56). IL-6 and IL-8 concentrations were higher in untreated samples, likely due to continuous cytokine secretion by intact cells. HNSCC cells exhibit basal IL-6 expression driven by the constitutive activation of the NF- κ B and STAT3 signaling pathways, while basal IL-8 expression is regulated primarily through the NF- κ B pathway (57–59). Under stress conditions, such as inflammation or, in this study, hypoxia resulting from incubation in sealed cryo vials during and after HHP-treatment, as well as DNA damage induced by pressure treatment, a significant increase in IL-6 and IL-8 production was observed (60–62). SDF-1 and MCP-1, which are also associated with migration, invasion and tumor progression, showed comparatively very low concentrations in the multiplex analysis (63–65).

Functional assays revealed distinct biological effects of the cytokines released during HHP-treatment on other HNSCC cells. In scratch assays with HNSCC16 cells, CM from 300 MPa-treated cells slowed migration compared to 200 MPa-treated CM, consistent with the need for complete devitalization to minimize residual tumor vitality (28). In Boyden chamber assays, increased invasion potential was observed in both cell lines exposed to CM, regardless of the pressure applied. These observations were consistent after 4 h and 24 h incubation times in the Boyden chamber. However, no direct correlation between HHP amplitude and invasion rates could be established. HNSCC16 cells exposed to CM exhibited significantly increased proliferation, whereas UTSCC14 cells were not affected. This differential response likely reflects both the distinct biological origins and the genetic backgrounds of the two cell lines. UTSCC14, a long-established tongue carcinoma cell line, likely shows reduced microenvironmental sensitivity due to prolonged *in vitro* passaging (66). In contrast, PDX-derived cell lines, such as HNSCC16, preserve tumor heterogeneity and cytokine responsiveness (67, 68). Beyond origin, genetic differences further contribute to the observed functional divergence. While both cell lines harbor mutations in the *TP53* gene, UTSCC14 additionally carries a homozygous loss-of-function mutation in *CDKN2A*, whereas this gene is wild type in HNSCC16 (46, 69). The loss of *CDKN2A* disrupts the G1/S checkpoint, resulting in constitutive cell cycle progression and reduced sensitivity to external cues such as proinflammatory cytokines (70). Interestingly, while the proliferative effects of HHP-conditioned medium were clearly detectable in 2D monolayer cultures, these effects were not observed within the timeframe of our 3D spheroid assay. Spheroid growth of HNSCC16 cells pretreated with 300 MPa-CM remained comparable to the control group. This observation highlights the distinct characteristics and limitations of both systems. 2D cultures provide uniform exposure to soluble factors and allow sensitive detection of early, cytokine-driven effects on proliferation, making them particularly well suited for assessing short-term tumor cell activation and oncological safety. In contrast, 3D models more closely reflect *in vivo*-like tissue architecture, including diffusion barriers, gradients of oxygen and nutrients, and cell–matrix interactions, which may delay or attenuate

cytokine effects. It is therefore possible that longer observation periods or additional 3D assay formats may be required to fully capture functional changes in such settings. Nevertheless, the absence of proliferative stimulation in the 3D model, even under cytokine-rich conditions, further supports the interpretation that HHP treatment at 300 MPa does not promote tumor cell regrowth. Together, these complementary findings strengthen the conclusion that HHP-treated tissues meet key oncological safety criteria *in vitro*.

4.4 Pro- and anti-tumorigenic effects

This study is the first to examine the cytokine profile in HHP-devitalized HNSCC. Our findings suggest that cytokines retained within devitalized tumor cells may interact with residual tumor cells at the resection margin. On the one hand, these cytokines could contribute to a pro-tumorigenic microenvironment by promoting tumor regrowth through mechanisms such as enhanced cell proliferation, angiogenesis, or EMT (71). On the other hand, it is conceivable that pro-inflammatory cytokines might attract immune effector cells, including cytotoxic T cells and natural killer (NK) cells, potentially supporting immune surveillance and aiding in the destruction of residual tumor cells (72, 73). However, this interpretation remains speculative in the absence of direct functional evidence. To clarify potential effects on immune cell recruitment, activation, or modulation, future investigations should incorporate functional assays, such as co-culture systems or *in vivo* models. The overall effect of the cytokines likely depends on multiple factors, including the specific cytokine composition, the viability of remaining tumor cells, and the immune status of the patient (74–76). Furthermore, the potential clinical impact of cytokines released from HHP-treated tumor cells must be considered in the context of their biological degradation kinetics and the procedural handling of devitalized tissue. Given the short half-life of cytokines (intracellular half-life for IL-1 α : 15 h; IL-1 β : 2.5 h (77), half-life in serum for IL-6: 15 h (78)), and additional washing steps during the preparation and implantation of devitalized grafts are likely to further reduce cytokine concentrations. Moreover, since the devitalized tissue is no longer metabolically active, no further cytokine production occurs post-implantation. Therefore, any paracrine influence of residual cytokines on surrounding viable tumor cells in the clinical setting is expected to be minimal and transient.

4.5 Clinical applications and perspectives

Standard treatments for squamous cell carcinoma include partial or complete organ removal, which significantly impacts function and aesthetics. When surgery is not feasible, radiotherapy is used, though it carries its own side effects. Combining surgery with radiotherapy is also an option. In the future, systemic therapies that target cancer cells throughout the body may be incorporated. Our approach to organ-preserving

surgery with autologous cartilage reuse aims to minimize functional and aesthetic limitations by using HHP-treated autologous cartilage for reconstruction. The use of HHP-treated cartilage eliminates the risk of reintroducing viable tumor cells while maintaining safety for the patient.

Another possible field of application for tumor cells devitalized by HHP is whole cell vaccination, as it has already been shown that devitalization by HHP triggers immunogenic cell death (8, 35). Tumor cells devitalized by HHP can be used to generate immunogenic vaccines that elicit anti-tumor responses while maintaining a structurally intact antigenic profile (28, 30). In this context, cytokines released from HHP-treated tumor cells may positively influence the immune response, serving as adjuvants to enhance vaccine efficacy (79, 80). Pro-inflammatory cytokines play a crucial role as adjuvants in tumor vaccination strategies, enhancing the immune response against cancer cells. These cytokines improve the anti-tumor immune response enhancing antigen presentation, stimulating T cell responses or modulating the tumor microenvironment. In mouse models of subcutaneous melanoma and Lewis lung carcinoma, an IL-1 β -based targeted cytokine was shown to safely enhance antitumor CD8 $^{+}$ T cell responses and synergize with other immunotherapies (81). Additionally, IL-1 α and IL-1 β have shown efficacy as mucosal adjuvants, promoting strong systemic and mucosal immune responses, which could be crucial for vaccines targeting pathogens like Human immunodeficiency virus (HIV) that infect mucosal surfaces (82). This approach aligns with current immunotherapeutic strategies, potentially complementing checkpoint inhibitors and adoptive T-cell therapies.

4.6 Limitations

This study investigated the oncological safety of HHP-treatment with regard to the autologous reimplantation of the treated tissue. Our findings offer a comprehensive overview of relevant, particularly pro-inflammatory, cytokines associated with HHP-treatment and their influence on other tumor cells. However, the potential influence of surrounding stem cells and fibroblasts that may transdifferentiate into cancer-associated fibroblasts (CAFs), supporting tumor progression, remains unclear and requires further exploration to fully confirm oncological safety (83, 84).

The use of CM following HHP-treatment allowed detailed cytokine profile analysis, identifying key factors involved in tumor progression. While our study provides a robust characterization using both commercially available and PDX-derived cell lines, these models may not fully recapitulate the tumor microenvironment or patient-specific heterogeneity. Further research involving primary tumor cells and proteome analysis could uncover additional mediators relevant to reimplantation and tumor vaccination. Expanding the cytokine screening approach could help identify previously unknown factors influencing clinical applications. Our findings are limited to *in vitro* models, which do not account for the complexity of the *in vivo* tumor microenvironment, immune interactions, and systemic factors. As an initial step toward more

physiologically relevant modeling, we included a 3D spheroid assay using HNSCC16 cells. Further refinement and expansion of 3D models, including invasion assays and tumor-stroma co-culture systems, will be essential to fully assess the translational relevance of our findings and to confirm the oncological safety of HHP-treated materials under tissue-like conditions. To complement these efforts, future studies will incorporate *in vivo* models, including xenograft HNSCC mouse models, to investigate the oncological safety and immunological consequences of pressure-treated tumor material in a systemic context. These experiments will be crucial to determine whether the observed cytokine alterations influence tumor progression, or immune cell recruitment, thereby providing critical validation for the clinical use of HHP-treated tissue in oncological reconstructive surgery.

The oncological safety of HHP-treatment was the primary focus in this study, but its immunogenicity, crucial for whole-cell vaccine development, requires further study. While HHP-induced immunogenic cell death in HNSCC cells is established, the role of cytokines in immune activation remains unclear, necessitating follow-up research.

5 Conclusion

In summary, cytokines present in the conditioned medium were found to enhance the proliferation, migration, and invasion of HNSCC cells. However, our findings demonstrate that cytokines released during the HHP-treatment process, under a pressure of 300 MPa, do not promote tumor regrowth, as evidenced by functional assays. This underscores the oncological safety of HHP-treatment *in vitro*. The rapid degradation of these cytokines, along with their likely removal from tissues during post-treatment washing steps, suggests a favorable safety profile for HHP-treated cartilage in autologous reimplantation. However, further investigations are needed to validate these findings in clinical settings. Interestingly, the cytokines released during HHP-treatment may hold potential as adjuvants in whole-cell vaccines, contributing to enhanced immune responses. To fully understand the clinical implications of these findings and to explore the therapeutic potential of HHP-treated materials in oncology, further *in vitro* and *in vivo* studies are essential.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The establishment of the PDX-derived cell lines HNSCC16 and HNSCC46 was conducted in accordance with the guidelines of the Declaration of Helsinki and was approved by the local Ethics Committee of the corresponding German authority (reference

number A2018-0003), as previously described by Schoenwaelder et al. (2022).

Author contributions

JB: Methodology, Writing – original draft, Conceptualization, Data curation, Investigation, Software, Visualization, Formal Analysis. VF: Methodology, Formal Analysis, Writing – review & editing. DS: Conceptualization, Formal Analysis, Funding acquisition, Writing – review & editing, Project administration. FK: Methodology, Writing – review & editing. AJ-H: Conceptualization, Visualization, Writing – review & editing, Formal Analysis, Supervision. RB: Funding acquisition, Resources, Writing – review & editing. MH: Formal Analysis, Writing – review & editing, Supervision. AS: Formal Analysis, Writing – review & editing, Conceptualization. MW: Resources, Writing – review & editing. SH: Conceptualization, Formal Analysis, Funding acquisition, Resources, Supervision, Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Generative AI statement

The author(s) declare that Generative AI was used in the creation of this manuscript. ChatGPT-4 (OpenAI, L.L.C., San Francisco, CA,

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Supplementary material

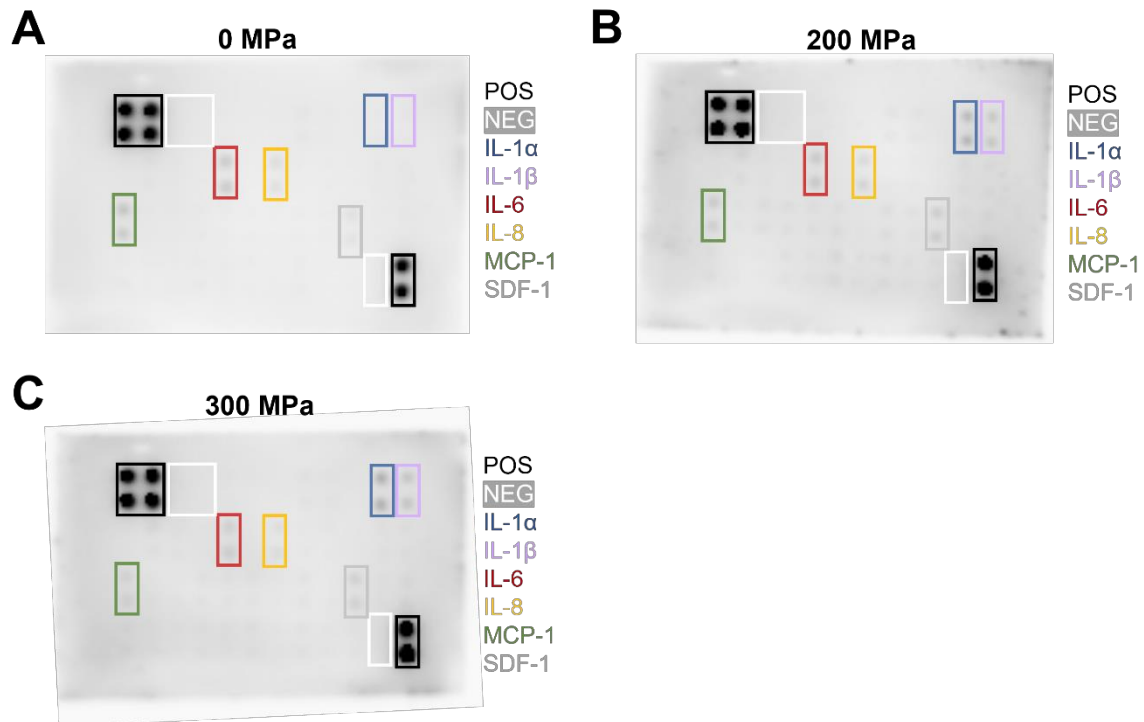
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References

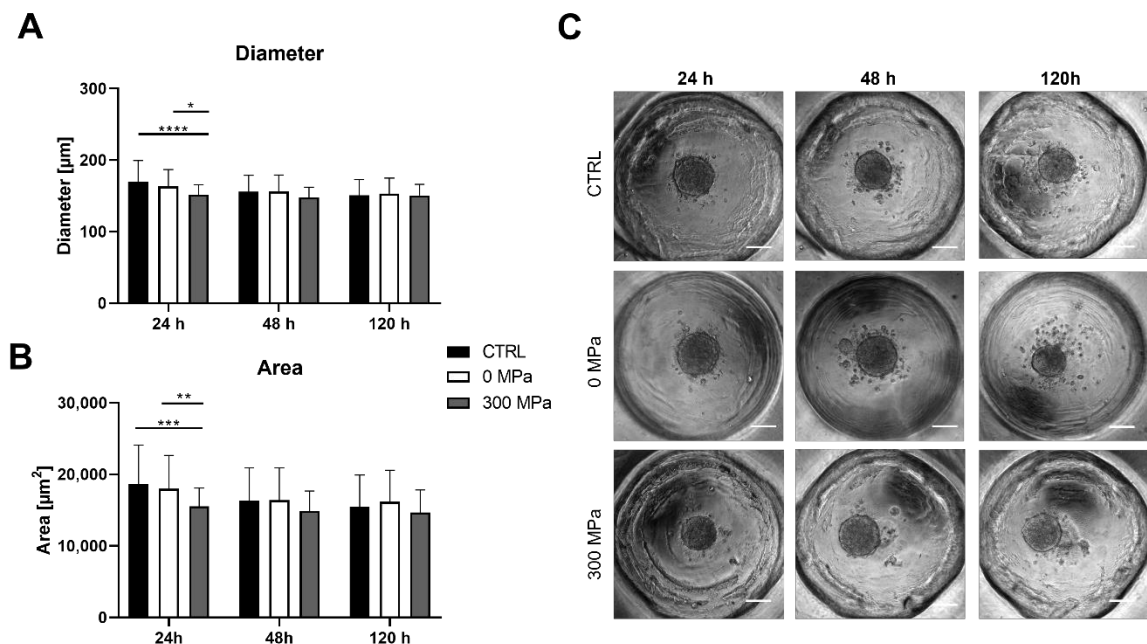
- Rivalain N, Roquain J, Demazeau G. Development of high hydrostatic pressure in biosciences: pressure effect on biological structures and potential applications in biotechnologies. *Biotechnol Adv.* (2010) 28:659–72. doi: 10.1016/j.biotechadv.2010.04.001
- Waletzko-Hellwig J, Pohl C, Riese J, Schlosser M, Dau M, Engel N, et al. Effect of high hydrostatic pressure on human trabecular bone regarding cell death and matrix integrity. *Front Bioeng Biotechnol.* (2021) 9:730266. doi: 10.3389/fbioe.2021.730266
- Weiss EM, Frey B, Rödel F, Herrmann M, Schlücker E, Voll RE, et al. Ex vivo- and in vivo-induced dead tumor cells as modulators of antitumor responses. *Ann N Y Acad Sci.* (2010) 1209:109–17. doi: 10.1111/j.1749-6632.2010.05743.x
- Frey B, Janko C, Ebel N, Meister S, Schlücker E, Meyer-Pittroff R, et al. Cells under pressure - treatment of eukaryotic cells with high hydrostatic pressure, from physiologic aspects to pressure induced cell death. *Curr Med Chem.* (2008) 15:2329–36. doi: 10.2174/092986708785909166
- van de Sande MAJ, Bovée JVMG, van Domselaar M, van Wijk MJ, Sanders I, Kuijper E. Successful disinfection of femoral head bone graft using high hydrostatic pressure. *Cell Tissue Bank.* (2018) 19:333–40. doi: 10.1007/s10561-017-9678-6
- Waletzko J, Dau M, Seyfarth A, Springer A, Frank M, Bader R, et al. Devitalizing effect of high hydrostatic pressure on human cells—Influence on cell death in osteoblasts and chondrocytes. *Int J Mol Sci.* (2020) 21:3836. doi: 10.3390/ijms21113836
- Diehl P, Schmitt M, Schauwecker J, Eichelberg K, Gollwitzer H, Grading R, et al. Effect of high hydrostatic pressure on biological properties of extracellular bone matrix proteins. *Int J Mol Med.* (2005) 16:285–9. doi: 10.3892/ijmm.16.2.285
- Seitz C, Rückert M, Deloch L, Weiss EM, Utz S, Izydor M, et al. Tumor cell-based vaccine generated with high hydrostatic pressure synergizes with radiotherapy by generating a favorable anti-tumor immune microenvironment. *Front Oncol.* (2019) 9:805. doi: 10.3389/fonc.2019.00805
- Rendueles E, Omer MK, Alyseike O, Alonso-Calleja C, Capita R, Prieto M. Microbiological food safety assessment of high hydrostatic pressure processing: A review. *LWT - Food Sci Technol.* (2011) 44:1251–60. doi: 10.1016/j.lwt.2010.11.001
- San Martín MF, Barbosa-Cánovas GV, Swanson BG. Food processing by high hydrostatic pressure. *Crit Rev Food Sci Nutr.* (2002) 42:627–45. doi: 10.1080/20024091054274
- Yamamoto K. Food processing by high hydrostatic pressure. *Biosci Biotechnol Biochem.* (2017) 81:672–9. doi: 10.1080/09168451.2017.1281723
- Diehl P, Schauwecker J, Mittelmeier W, Schmitt M. High hydrostatic pressure, a novel approach in orthopedic surgical oncology to disinfect bone, tendons and cartilage. *Anticancer Res.* (2008) 28:3877–83.
- Kalle F, Stadler VP, Brach JK, Grote VF, Pohl C, Schulz K, et al. High hydrostatic pressure treatment for advanced tissue grafts in reconstructive head and neck surgery. *J BioMed Mater Res A.* (2024) 18(1):e37791. doi: 10.1002/jbm.a.37791
- Firat C, Gurlek A, Aydin NE. Viability of cartilage grafts in various forms. *J Craniofac Surg.* (2011) 22:1666–70. doi: 10.1097/SCS.0b013e31822f3b1f
- Liu Y, Zhou G, Cao Y. Recent progress in cartilage tissue engineering—Our experience and future directions. *Engineering.* (2017) 3:28–35. doi: 10.1016/J.ENG.2017.01.010
- Tarhan E, Cakmak O, Ozdemir BH, Akdogan V, Suren D. Comparison of AlloDerm, fat, fascia, cartilage, and dermal grafts in rabbits. *Arch Facial Plast Surg.* (2008) 10:187–93. doi: 10.1001/archfaci.10.3.187
- Albrecht T, Wallner F. Options for reconstruction after injuries in the head and neck region. *HNO.* (2023) 71:57–62. doi: 10.1007/s00106-022-01230-5
- Strüder D, Ebert J, Kalle F, Schraven SP, Eichhorst L, Mlynski R, et al. Head and neck cancer: A study on the complex relationship between qoL and swallowing function. *Curr Oncol Tor Ont.* (2023) 30:10336–50. doi: 10.3390/curroncol30120753
- American Society of Clinical Oncology, DG P, SA L, GS W, WM M, DJ A, et al. American Society of Clinical Oncology clinical practice guideline for the use of larynx-
- preservation strategies in the treatment of laryngeal cancer. *J Clin Oncol Off J Am Soc Clin Oncol.* (2006) 24:3693–704. doi: 10.1200/JCO.2006.07.4559
- Wagner MM, Curé JK, Caudell JJ, Spencer SA, Nabell LM, Carroll WR, et al. Prognostic significance of thyroid or cricoid cartilage invasion in laryngeal or hypopharyngeal cancer treated with organ preserving strategies. *Radiat Oncol Lond Engl.* (2012) 7:219. doi: 10.1186/1748-717X-7-219
- Patel UA, Howell LK. Local response to chemoradiation in T4 larynx cancer with cartilage invasion. *Laryngoscope.* (2011) 121:106–10. doi: 10.1002/lary.21181
- Terrell JE, Ronis DL, Fowler KE, Bradford CR, Chepeha DB, Prince ME, et al. Clinical predictors of quality of life in patients with head and neck cancer. *Arch Otolaryngol Head Neck Surg.* (2004) 130:401–8. doi: 10.1001/archotol.130.4.401
- Jose J, Moor JW, Coatesworth AP, Johnston C, MacLennan K. Soft tissue deposits in neck dissections of patients with head and neck squamous cell carcinoma: prospective analysis of prevalence, survival, and its implications. *Arch Otolaryngol Neck Surg.* (2004) 130:157–60. doi: 10.1001/archotol.130.2.157
- Barsouk A, Aluru JS, Rawla P, Saginala K, Barsouk A. Epidemiology, risk factors, and prevention of head and neck squamous cell carcinoma. *Med Sci.* (2023) 11:42. doi: 10.3390/medsci11020042
- Loos E, Meulemans J, Vranckx J, Poorten VV, Delaere P. Tracheal autotransplantation for functional reconstruction of extended hemilaryngectomy defects: A single-center experience in 30 patients. *Ann Surg Oncol.* (2016) 23:1674–83. doi: 10.1245/s10434-015-5033-y
- Piazza C, Paderno A, Nicolai P. Conservative surgery for laryngeal chondrosarcoma: a review of the most recently proposed approaches. *Curr Opin Otolaryngol Head Neck Surg.* (2017) 25:93–100. doi: 10.1097/MOO.0000000000000337
- Navach V, Chu F, Cattaneo A, Zorzi S, Scelsi D, Ansarin M. Cartilage framework reconstruction after resection of thyroid cartilage chondrosarcoma: A case report. *Otolaryngol Case Rep.* (2017) 4:12–4. doi: 10.1016/j.xocr.2017.07.002
- Maletzki C, Freiin Grote V, Kalle F, Kleitke T, Zimpfer A, Becker AS, et al. Establishing safe high hydrostatic pressure devitalization thresholds for autologous head and neck cancer vaccination and reconstruction. *Cell Death Discov.* (2023) 9:1–11. doi: 10.1038/s41420-023-01671-z
- Adkins I, Fucikova J, Garg AD, Agostinis P, Špišek R. Physical modalities inducing immunogenic tumor cell death for cancer immunotherapy. *Oncoimmunology.* (2014) 3:e968434. doi: 10.4161/21624011.2014.968434
- Urbanova L, Hradilova N, Moserova I, Vosahlikova S, Sadilkova L, Hensler M, et al. High hydrostatic pressure affects antigenic pool in tumor cells: Implication for dendritic cell-based cancer immunotherapy. *Immunol Lett.* (2017) 187:27–34. doi: 10.1016/j.imlet.2017.05.005
- Yan S, Liu K, Mu L, Liu J, Tang W, Liu B. Research and application of hydrostatic high pressure in tumor vaccines. *Oncol Rep.* (2021) 45:75. doi: 10.3892/or.2021.8026
- Mikyskova R, Indrova M, Stepanek I, Kanchev I, Bieblova J, Vosahlikova S, et al. Dendritic cells pulsed with tumor cells killed by high hydrostatic pressure inhibit prostate tumor growth in TRAMP mice. *Oncoimmunology.* (2017) 6:e1362528. doi: 10.1080/2162402X.2017.1362528
- Mikysková R, Štěpánek I, Indrová M, Bieblůva J, Šimová J, Truxová I, et al. Dendritic cells pulsed with tumor cells killed by high hydrostatic pressure induce strong immune responses and display therapeutic effects both in murine TC-1 and TRAMP-C2 tumors when combined with docetaxel chemotherapy. *Int J Oncol.* (2016) 48:953–64. doi: 10.3892/ijo.2015.3314
- Hradilova N, Sadilkova L, Palata O, Mysikova D, Mrazkova H, Lischke R, et al. Generation of dendritic cell-based vaccine using high hydrostatic pressure for non-small cell lung cancer immunotherapy. *PLoS One.* (2017) 12:e0171539. doi: 10.1371/journal.pone.0171539
- Fucikova J, Moserova I, Truxova I, Hermanova I, Vancurova I, Partlova S, et al. High hydrostatic pressure induces immunogenic cell death in human tumor cells. *Int J Cancer.* (2014) 135:1165–77. doi: 10.1002/ijc.28766

36. Eisenthal A, Ramakrishna V, Skornick Y, Shinitzky M. Induction of cell-mediated immunity against B16-BL6 melanoma in mice vaccinated with cells modified by hydrostatic pressure and chemical crosslinking. *Cancer Immunol Immunother CII*. (1993) 36:300–6. doi: 10.1007/BF01741168
37. Weiss EM, Meister S, Janko C, Ebel N, Schlücker E, Meyer-Pittroff R, et al. High hydrostatic pressure treatment generates inactivated mammalian tumor cells with immunogenic features. *J Immunotoxicol*. (2010) 7:194–204. doi: 10.3109/15476911003657414
38. Rückert M, Deloch L, Frey B, Schlücker E, Fietkau R, Gaipl US. Combinations of radiotherapy with vaccination and immune checkpoint inhibition differently affect primary and abscopal tumor growth and the tumor microenvironment. *Cancers*. (2021) 13:714. doi: 10.3390/cancers13040714
39. Li K, Zeng X, Liu P, Zeng X, Lv J, Qiu S, et al. The role of inflammation-associated factors in head and neck squamous cell carcinoma. *J Inflammation Res*. (2023) 16:4301–18. doi: 10.2147/JIR.S428358
40. Landskron G, la FMD, Thuwajit P, Thuwajit C, Hermoso MA. Chronic inflammation and cytokines in the tumor microenvironment. *J Immunol Res*. (2014) 2014:149185. doi: 10.1155/2014/149185
41. Briukhovetska D, Dörr J, Endres S, Libby P, Dinarello CA, Kobold S. Interleukins in cancer: from biology to therapy. *Nat Rev Cancer*. (2021) 21:481–99. doi: 10.1038/s41568-021-00363-z
42. Zhao T, Cai Y, Jiang Y, He X, Wei Y, Yu Y, et al. Vaccine adjuvants: mechanisms and platforms. *Signal Transduct Target Ther*. (2023) 8:1–24. doi: 10.1038/s41392-023-01557-7
43. Paston SJ, Brentville VA, Symonds P, Durrant LG. Cancer vaccines, adjuvants, and delivery systems. *Front Immunol*. (2021) 12:627932. doi: 10.3389/fimmu.2021.627932
44. Kurane S, Arca MT, Aruga A, Krinock RA, Krauss JC, Chang AE. Cytokines as an adjuvant to tumor vaccines: efficacy of local methods of delivery. *Ann Surg Oncol*. (1997) 4:579–85. doi: 10.1007/BF02305540
45. Cuzzubbo S, Mangsbo S, Nagarajan D, Habra K, Pockley AG, McArdle SEB. Cancer vaccines: adjuvant potency, importance of age, lifestyle, and treatments. *Front Immunol*. (2020) 11:615240. doi: 10.3389/fimmu.2020.615240
46. Schoenwaelder N, Krause M, Freitag T, Schneider B, Zonnur S, Zimpfer A, et al. Preclinical head and neck squamous cell carcinoma models for combined targeted therapy approaches. *Cancers*. (2022) 14:2484. doi: 10.3390/cancers14102484
47. Cesa CM, Kirchgessner N, Mayer D, Schwarz US, Hoffmann B, Merkel R. Micropatterned silicone elastomer substrates for high resolution analysis of cellular force patterns. *Rev Sci Instrum*. (2007) 78:034301. doi: 10.1063/1.2712870
48. Hiemer B, Genz B, Jonitz-Heincke A, Pasold J, Wree A, Dommerich S, et al. Devitalisation of human cartilage by high hydrostatic pressure treatment: Subsequent cultivation of chondrocytes and mesenchymal stem cells on the devitalised tissue. *Sci Rep*. (2016) 6:33747. doi: 10.1038/srep33747
49. Niklander SE, Murdoch C, Hunter KD. IL-1/IL-1R signaling in head and neck cancer. *Front Oral Health*. (2021) 2:722676. doi: 10.3389/froh.2021.722676
50. Chew V, Toh HC, Abastado JP. Immune microenvironment in tumor progression: characteristics and challenges for therapy. *J Oncol*. (2012) 2012:608406. doi: 10.1155/2012/608406
51. Guo B, Fu S, Zhang J, Liu B, Li Z. Targeting inflammasome/IL-1 pathways for cancer immunotherapy. *Sci Rep*. (2016) 6:36107. doi: 10.1038/srep36107
52. Zhao H, Wu L, Yan G, Chen Y, Zhou M, Wu Y, et al. Inflammation and tumor progression: signaling pathways and targeted intervention. *Signal Transduct Target Ther*. (2021) 6:263. doi: 10.1038/s41392-021-00658-5
53. Wen Y, Zhu Y, Zhang C, Yang X, Gao Y, Li M, et al. Chronic inflammation, cancer development and immunotherapy. *Front Pharmacol*. (2022) 13:1040163. doi: 10.3389/fphar.2022.1040163
54. Kay J, Thadhani E, Samson L, Engelward B. Inflammation-induced DNA damage, mutations and cancer. *DNA Repair*. (2019) 83:102673. doi: 10.1016/j.dnarep.2019.102673
55. Cohen I, Rider P, Carmi Y, Braiman A, Dotan S, White MR, et al. Differential release of chromatin-bound IL-1 α discriminates between necrotic and apoptotic cell death by the ability to induce sterile inflammation. *Proc Natl Acad Sci*. (2010) 107:2574–9. doi: 10.1073/pnas.0915018107
56. Di Paolo NC, Shayakhmetov DM. Interleukin 1 α and the inflammatory process. *Nat Immunol*. (2016) 17:906–13. doi: 10.1038/ni.3503
57. Squarize CH, Castilho RM, Sriuranpong V, Pinto DS, Gutkind JS. Molecular cross-talk between the NF κ B and STAT3 signaling pathways in head and neck squamous cell carcinoma. *Neoplasia N Y N*. (2006) 8:733–46. doi: 10.1593/neo.06274
58. Riebe C, Pries R, Schroeder KN, Wollenberg B. Phosphorylation of STAT3 in Head and Neck Cancer Requires p38 MAPKinase, whereas Phosphorylation of STAT1 Occurs via a Different Signaling Pathway. *Anticancer Res*. (2011) 31(11):3819–25.
59. Chan LP, Liu C, Chiang FY, Wang LF, Lee KW, Chen WT, et al. IL-8 promotes inflammatory mediators and stimulates activation of p38 MAPK/ERK-NF- κ B pathway and reduction of JNK in HNSCC. *Oncotarget*. (2017) 8:56375–88. doi: 10.18632/oncotarget.16914
60. Cohen AN, Veena MS, Srivatsan ES, Wang MB. Suppression of interleukin 6 and 8 production in head and neck cancer cells with curcumin via inhibition of I κ B Kinase. *Arch Otolaryngol Neck Surg*. (2009) 135:190–7. doi: 10.1001/archotol.135.2.190
61. Xu Q, Ma H, Chang H, Feng Z, Zhang C, Yang X. The interaction of interleukin-8 and PTEN inactivation promotes the Malignant progression of head and neck squamous cell carcinoma via the STAT3 pathway. *Cell Death Dis*. (2020) 11:1–14. doi: 10.1038/s41419-020-2627-5
62. Nisar H, Brauny M, Labonté FM, Schmitz C, Konda B, Hellweg CE, et al. and inflammatory response of p53 null H358 non-small cell lung cancer cells to X-ray exposure under chronic hypoxia. *Int J Mol Sci*. (2024) 25:12590. doi: 10.3390/ijms252312590
63. Yoshimura T, Howard OMZ, Ito T, Kuwabara M, Matsukawa A, Chen K, et al. Monocyte chemoattractant protein-1/CCL2 produced by stromal cells promotes lung metastasis of 4T1 murine breast cancer cells. *PLoS One*. (2013) 8:e58791. doi: 10.1371/journal.pone.0058791
64. Wang H, Zhang Q, Kong H, Zeng Y, Hao M, Yu T, et al. Monocyte chemotactic protein-1 expression as a prognostic biomarker in patients with solid tumor: a meta analysis. *Int J Clin Exp Pathol*. (2014) 7:3876–86.
65. Teng F, Tian WY, Wang YM, Zhang YF, Guo F, Zhao J, et al. Cancer-associated fibroblasts promote the progression of endometrial cancer via the SDF-1/CXCR4 axis. *J Hematol Oncol J Hematol Oncol*. (2016) 9:8. doi: 10.1186/s13045-015-0231-4
66. Johansson N, Airola K, Grénman R, Kariniemi AL, Saarialho-Kere U, Kähäri VM. Expression of collagenase-3 (matrix metalloproteinase-13) in squamous cell carcinomas of the head and neck. *Am J Pathol*. (1997) 151:499–508.
67. Jin J, Yoshimura K, Sewastjanow-Silva M, Song S, Ajani JA. Challenges and prospects of patient-derived xenografts for cancer research. *Cancers*. (2023) 15:4352. doi: 10.3390/cancers15174352
68. Idrisova KF, Simon HU, Gomzikova MO. Role of patient-derived models of cancer in translational oncology. *Cancers*. (2022) 15:139. doi: 10.3390/cancers15010139
69. Lepikhova T, Karhemo PR, Louhimo R, Yadav B, Murumägi A, Kuleskiy E, et al. Drug-sensitivity screening and genomic characterization of 45 HPV-negative head and neck carcinoma cell lines for novel biomarkers of drug efficacy. *Mol Cancer Ther*. (2018) 17:2060–71. doi: 10.1158/1535-7163.MCT-17-0733
70. Zhao R, Choi BY, Lee MH, Bode AM, Dong Z. Implications of genetic and epigenetic alterations of CDKN2A (p16(INK4a)) in cancer. *EBioMedicine*. (2016) 8:30–9. doi: 10.1016/j.ebiom.2016.04.017
71. Ostrand-Rosenberg S. Immune surveillance: a balance between protumor and antitumor immunity. *Curr Opin Genet Dev*. (2008) 18:11–8. doi: 10.1016/j.gde.2007.12.007
72. Galli F, Aguilera JV, Palermo B, Markovic SN, Nisticò P, Signore A. Relevance of immune cell and tumor microenvironment imaging in the new era of immunotherapy. *J Exp Clin Cancer Res*. (2020) 39:89. doi: 10.1186/s13046-020-01586-y
73. Tang T, Huang X, Zhang G, Hong Z, Bai X, Liang T. Advantages of targeting the tumor immune microenvironment over blocking immune checkpoint in cancer immunotherapy. *Signal Transduct Target Ther*. (2021) 6:1–13. doi: 10.1038/s41392-020-00449-4
74. Kartikasari AER, Huertas CS, Mitchell A, Plebanski M. Tumor-induced inflammatory cytokines and the emerging diagnostic devices for cancer detection and prognosis. *Front Oncol*. (2021) 11:692142. doi: 10.3389/fonc.2021.692142
75. Yi M, Li T, Niu M, Zhang H, Wu Y, Wu K, et al. Targeting cytokine and chemokine signaling pathways for cancer therapy. *Signal Transduct Target Ther*. (2024) 9:1–48. doi: 10.1038/s41392-024-01868-3
76. Lin WW, Karin M. A cytokine-mediated link between innate immunity, inflammation, and cancer. *J Clin Invest*. (2007) 117:1175–83. doi: 10.1172/JCI15137
77. Hazuda DJ, Lee JC, Young PR. The kinetics of interleukin 1 secretion from activated monocytes. Differences between interleukin 1 alpha and interleukin 1 beta. *J Biol Chem*. (1988) 263:8473–9. doi: 10.1016/S0021-9258(18)68502-3
78. Wirtz DC, Heller KD, Miltner O, Zilkens KW, Wolff JM. Interleukin-6: a potential inflammatory marker after total joint replacement. *Int Orthop*. (2000) 24:194–6. doi: 10.1007/s002640000136
79. Wang E, Long B, Wang K, Wang J, He Y, Wang X, et al. Interleukin-8 holds promise to serve as a molecular adjuvant in DNA vaccination model against *Streptococcus iniae* infection in fish. *Oncotarget*. (2016) 7:83938–50. doi: 10.18632/oncotarget.13728
80. Wang E, Wang J, Long B, Wang K, He Y, Yang Q, et al. Molecular cloning, expression and the adjuvant effects of interleukin-8 of channel catfish (*Ictalurus punctatus*) against *Streptococcus iniae*. *Sci Rep*. (2016) 6:29310. doi: 10.1038/srep29310
81. Van Den Eeckhout B, Huyghe L, Van Lint S, Burg E, Plaisance S, Peelman F, et al. Selective IL-1 activity on CD8 $^{+}$ T cells empowers antitumor immunity and synergizes with neovascularization-targeted TNF for full tumor eradication. *J Immunother Cancer*. (2021) 9:e03293. doi: 10.1136/jitc-2021-003293
82. Staats HF, Ennis FA Jr. IL-1 is an effective adjuvant for mucosal and systemic immune responses when coadministered with protein immunogens. *J Immunol*. (1999) 162:6141–7. doi: 10.4049/jimmunol.162.10.6141
83. Wright K, Ly T, Kriet M, Czirok A, Thomas SM. Cancer-associated fibroblasts: master tumor microenvironment modifiers. *Cancers*. (2023) 15:1899. doi: 10.3390/cancers15061899
84. Mao X, Xu J, Wang W, Liang C, Hua J, Liu J, et al. Crosstalk between cancer-associated fibroblasts and immune cells in the tumor microenvironment: new findings and future perspectives. *Mol Cancer*. (2021) 20:131. doi: 10.1186/s12943-021-01428-1

Supplementary figures

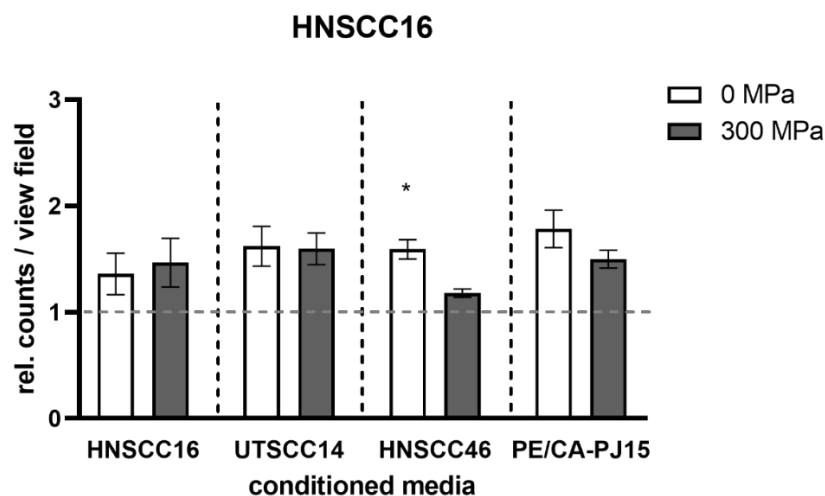


Supplementary Figure 1: Identification of relevant cytokines associated with high hydrostatic pressure-treated tumor cells. HNSCC16 cells were treated with 0 (A), 200 (B), 300 MPa (C). Conditioned medium was analyzed in a dot blot assay with correlation of the dot intensity to the respective cytokine concentration. Dot blot assay was performed once for qualitative cytokine screening to identify relevant targets for subsequent quantitative analysis.



Supplementary Figure 2: Spheroid growth analysis of HNSCC16 cells exposed to conditioned medium (CM). HNSCC16 was cultured for 48 h in CM (0 and 300 MPa) and control medium, respectively. The conditioned cells were seeded into in house fabricated μ -well culture inserts. Spheroid growth was observed over five days and progress was documented for 24, 48 and 120 h. Spheroid growth was determined by measuring the diameter (A) and area (B) of each spheroid. Representative images of spheroids are shown (C). Results are shown as means

and standard deviations; n=62. Statistical analysis: one-way ANOVA, Tukey's multiple comparison post hoc test; *p<0.05; **p<0.01, ***p<0.001, ****p<0.0001; scale bar: 100 μ m.



Supplementary Figure 3: Invasion rates of HNSCC16 cells exposed to conditioned medium (CM) for prolonged incubation time (24 h). The HNSCC cell line HNSCC16 was examined using a Boyden chamber with a pore size of 8 μ m. The lower compartment contains the CM from their own or another HNSCC cell line after high hydrostatic pressure-treatment (0 and 300 MPa). Serum-free culture medium served as reference and was set to 1 (dashed line). The number of HNSCC16 cells that invaded through the membrane was determined and normalized to the serum-free medium control. Data are presented as means and standard deviations; n=5-6 (from three independent experiments). Statistical analysis: Kruskal-Wallis test with Dunn's multiple comparison test; *p<0.05.



Article

Oncological Safety of High Hydrostatic Pressure Treatment: Effects on Cancer-Associated Fibroblast-like Transdifferentiation of Adipose Stromal Cells

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Abstract

Oncological safety is essential for autologous reconstruction after resection of cartilage-infiltrating head and neck tumors. High hydrostatic pressure (HHP) enables complete devitalization of tumor-infiltrated tissue while preserving extracellular matrix integrity. However, residual soluble tumor-derived products may influence infiltrating stromal cells. This study examined whether conditioned media (CM) from HHP-treated head and neck squamous cell carcinoma (HNSCC) cells induce cancer-associated fibroblast (CAF)-like transdifferentiation of human adipose stromal cells (hASCs). hASCs were exposed to CM from untreated or HHP-treated (300 MPa) HNSCC cells, tumor-CM (TCM), or TGF- β 1. Morphological changes in hASCs were evaluated, and CAF marker expression was analyzed by qRT-PCR, immunofluorescence, Western blot, and ELISA. Cytokines were quantified via multiplex analysis. TGF- β 1 induced a CAF-like phenotype with α -SMA upregulation, whereas TCM and 0 MPa-CM caused only modest increases in selected markers. Although 300 MPa-CM did not induce CAF-associated molecular signatures, hASCs exhibited morphological alterations, underscoring that morphology alone is insufficient to define CAF transdifferentiation. Cytokine secretion was elevated in response to all CM conditions. These findings indicate that HHP treatment at 300 MPa abolishes the paracrine CAF-inducing potential of tumor-derived mediators in vitro, supporting the oncological safety of HHP-treated tissues under these experimental condition, although further in vivo validation is warranted

Keywords: high hydrostatic pressure; head and neck cancer; squamous cell carcinoma; adipose stromal cells; cancer associated fibroblasts; tumor secretome; cytokine effects



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1. Introduction

High hydrostatic pressure (HHP) treatment is an emerging technique for tissue devitalization that applies pressures of up to 600 MPa to disrupt cellular membranes while preserving the extracellular matrix and the mechanical properties of the graft [1–7]. Its uniform pressure distribution according to Pascal's law enables standardized processing of complex tissues, including tumor-infiltrated cartilage [2,5,8]. Beyond safe devitalization, HHP-treated tumor cells show an immunogenic cell death and retain antigenicity, making this approach suitable not only for reconstructive purposes but also for potential cancer vaccination strategies [9–11]. Previous work from our group demonstrated that HHP ≥ 300 MPa for 10 min effectively devitalizes head and neck squamous cell carcinoma (HNSCC) cells while preserving the structural integrity of cartilage grafts [11,12].

The composition of soluble factors is an important determinant of the biological response to HHP-treated tissue, as these mediators may critically influence surrounding stromal and residual tumor cells. In earlier studies, we observed that HHP treatment of HNSCC cells causes the release of intracellular cytokines into the extracellular milieu [13]. Specifically, interleukin (IL)-1 α and IL-1 β are released in a pressure-dependent manner and remain present at high concentrations after treatment [13]. Additionally, IL-6 and IL-8 have been detected in the conditioned medium (CM) of HHP-treated HNSCC cells but at lower levels than in untreated controls [13]. Functional assays have revealed that the cytokines present in the supernatants promote proliferation, migration, and invasion of viable tumor cells [13]. However, a direct link between cytokine release and pressure treatment has not been established. As a further step in evaluating the oncological safety of HHP-treated tissues for autologous reimplantation, it is crucial to determine whether residual cytokines in the post-treatment tumor secretome affect non-malignant cells in the tumor microenvironment (TME).

Within the TME, stromal cells, particularly cancer-associated fibroblasts (CAFs), play a pivotal role in tumor progression. CAFs enhance invasion, angiogenesis, and immune evasion through extracellular matrix remodeling and secretion of tumor-promoting cytokines and chemokines, epithelial-to-mesenchymal transition (EMT), and therapy resistance, ultimately fostering a permissive microenvironment for tumor growth [14–16]. Moreover, several studies have demonstrated that a high abundance of CAFs, often reflected by increased stromal myofibroblast density, is associated not only with poor overall survival but also with a higher likelihood of disease recurrence [17,18]. Importantly, CAFs represent a highly heterogeneous cell population, encompassing multiple functional subtypes and activation states rather than a uniform phenotype. Based on transcriptomic and functional profiling, CAFs are commonly classified into three major subtypes: myofibroblastic CAFs (myCAFs), characterized by high α -SMA expression and extracellular matrix remodeling; inflammatory CAFs (iCAFs), which secrete high levels of pro-inflammatory cytokines and chemokines; and antigen-presenting CAFs (apCAFs), which express major histocompatibility complex class II molecules and may modulate immune responses [19–23]. Depending on the nature, strength, and duration of tumor-derived stimuli, stromal cells may acquire partial, transient, or alternative activation states that do not necessarily fulfill all molecular or functional criteria of fully differentiated CAFs [24]. The origin of CAFs remains controversial since multiple cellular origins have been proposed, including resident fibroblasts, smooth muscle cells, pericytes, and mesenchymal stromal cells (MSCs), which can acquire a CAF-like phenotype upon exposure to tumor-derived signals [23,25–27]. Among MSCs, human adipose stromal cells (hASCs) are of particular relevance in regenerative medicine due to their abundance, plasticity, and multipotency [28,29]. Known inducers of MSC-to-CAF transdifferentiation include transforming growth factor beta 1 (TGF- β 1), interleukins such as IL-6 and IL-8, tumor necrosis factor alpha (TNF- α), the CXCR4/CXCL12 axis, and

extracellular vesicles carrying microRNAs and other bioactive molecules [30–33]. These stimuli converge on key signaling pathways such as SMAD, signal transducer and activator of transcription 3 (STAT3), nuclear factor kappa B (NF- κ B), and Notch, ultimately leading to the upregulation of CAF-associated markers, which are commonly used but not exclusive indicators of CAF-like phenotypes, including alpha-smooth muscle actin (α -SMA), fibroblast activation protein (FAP), tenascin-C (TNC), secreted protein acidic and rich in cysteine (SPARC), vimentin (VIM), and tissue inhibitor of metalloproteinases 1 (TIMP1) [27,34–37].

Given that pro-inflammatory cytokines such as IL-1 α , IL-1 β , IL-6, and IL-8 are abundant in untreated HNSCC supernatants and remain detectable after HHP, it is important to assess whether these residuals can induce a CAF-like phenotype in surrounding stromal cells. Such an effect would represent a potential oncological risk for the reimplantation of HHP-treated grafts. In the present study, we examined whether hASCs exposed to conditioned media from untreated (0 MPa) or HHP-treated (300 MPa) HNSCC cell lines undergo a phenotypic and functional shift toward CAF-like cells. In addition, tumor-conditioned medium (TCM) with an extended conditioning period was included as a supplementary group to assess whether prolonged exposure to tumor-derived factors could enhance CAF conversion. TGF- β 1 stimulation, a well-established inducer of CAF activation, served as a positive control in our study. Using a combination of morphological analysis, mRNA and protein quantification of CAF markers, and cytokine profiling, we aimed to determine whether HHP-treated tumor supernatants possess the capacity to induce a tumor-promoting stromal phenotype. This approach provides important insights into the oncological safety of HHP for the autologous reimplantation of tumor-infiltrated cartilage and advances our understanding of MSC-to-CAF conversion under tumor-derived stimuli.

2. Materials and Methods

2.1. Isolation and Cultivation of hASC

Subcutaneous adipose tissue was obtained from eight healthy donors undergoing elective procedures such as abdominoplasty, autologous fat transfer, or liposuction at the department for plastic surgery, University Hospital RWTH Aachen. Importantly, none of the samples were derived from tumor-associated tissue, all specimens originated from non-pathological surgical indications. All donors were informed about the use of their tissue for research purposes and provided written consent. The study was approved by the local Ethics Committee (EK163/07).

Adipose tissue was processed for isolation of hASCs as previously described [38]. Briefly, adipose tissue was minced and digested in a collagenase solution (0.2% collagenase I in PBS) for 45 min at 37 °C. The digested suspension was filtered through a 250 μ m nylon mesh (Neolab, Heidelberg, Germany). Mature adipocytes, oil, and cell debris were separated from the stromal vascular fraction by centrifugation at 400 $\times$ g for 10 min. The cell sediment was resuspended in Dulbecco's modified Eagle medium (DMEM)/Ham's F12 (1:1) (Pan Biotech, Aidenbach, Germany) supplemented with penicillin and streptomycin (P/S; Sigma-Aldrich, St. Louis, MO, USA; each at 100 U/mL), 0.1% bFGF (Peprotech, Cranbury, NJ, USA), 200 μ M ascorbic acid, 10% fetal bovine serum (FBS, Pan Biotech, Aidenbach, Germany). Primary cells were expanded until they reached approximately 90% confluence. From passage 1 onward, the cells were cultured in DMEM/Ham's F12 (1:1) supplemented with 10% FBS only. Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO₂, and passages two to five were used for subsequent experiments.

HASCs were isolated from eight independent healthy donors; however, due to limited cell availability and assay-specific cell number requirements, not all experiments could be performed with hASCs from all donors. Subsets of donors were therefore used, while key findings were consistently reproduced across multiple independent donors.

2.2. HNSCC Cell Line Cultivation

The head and neck squamous cell carcinoma cell lines HNSCC16 (larynx P1 M1) and UTSCC14 (tongue, RRID:CVCL_7810) were utilized in this study. The primary tumor cell line HNSCC16 was established by the research group Schoenwalder et al. [39] and authenticated by STR profiling (against the respective patient tumor and patient-derived xenograft (PDX)). Both cell lines were human papillomavirus (HPV) negative, and all experiments were performed with mycoplasma-free cells.

Cancer cells were grown in DMEM/Ham's F12 (1:1) (Pan Biotech, Aidenbach, Germany) supplemented with 10% FBS (Pan Biotech, Aidenbach, Germany) and 1% P/S (Sigma-Aldrich, St. Louis, MO, USA) and cultured in 75-cm² culture flasks at 37 °C with 5% CO₂ in a humidified incubator. The medium was replaced every 2–3 days. Upon reaching a confluence of 80–90%, cells were washed with Dulbecco's Phosphate-Buffered Saline (DPBS, Gibco, Waltham, MA, USA), detached with 0.25% Trypsin-ethylenediaminetetraacetic acid (EDTA, Pan Biotech, Aidenbach, Germany), and seeded in new flasks or treatment wells.

For the experiments DMEM/Ham's F12 (1:1) supplemented with 1% Insulin-Transferrin-Selenium (ITS, Gibco, Waltham, MA, USA) and 1% P/S was used to eliminate interfering effects of FBS and is labeled as serum-free medium or control (CTRL) medium in this study.

2.3. High Hydrostatic Pressure Treatment and Preparation of Conditioned Medium

CM was prepared from HNSCC cells and subsequently used for hASC stimulation experiments. Two different types of CM were generated: (i) high hydrostatic pressure-treated conditioned medium (HHP-CM) and (ii) tumor-conditioned medium (TCM).

For HHP-CM, 1×10^6 cells (HNSCC16 or UTSCC14) were suspended in serum-free medium (DMEM/F12 supplemented with 1% ITS and 1% P/S) and transferred into 1.8 mL cryotubes (Thermo Fisher Scientific, Waltham, MA, USA). Cryotubes were centrifuged at $120 \times g$ for 8 min, completely filled with serum-free medium, and carefully sealed to avoid air bubbles. To prevent leakage, tubes were wrapped with Parafilm M (Pechiney Plastic Packaging Inc., Stamford, CT, USA) and placed into water-filled, air-free centrifuge tubes, which were then positioned in the glycol-filled chamber of the high hydrostatic pressure device (Dustec Hochdrucktechnik GmbH, Wismar, Germany). Cells were subjected to HHP treatment at 300 MPa for 10 min at 20 °C or left untreated for reference samples (0 MPa). Following treatment, cryotubes were centrifuged again and incubated for 1 h at 37 °C to allow additional cytokine release from pressure-damaged cells and cytokine secretion from viable control cells (0 MPa). The resulting supernatants (conditioned media) were collected, transferred into new cryotubes, and stored at –80 °C until further use. Before application to hASC cultures, all HHP-CM samples were sterilized by filtration through 0.2 µm syringe filters (Corning, Inc., Corning, NY, USA).

For TCM, ~80% confluent T75 flasks of HNSCC16 or UTSCC14 cells were cultured in 10 mL serum-free medium for 24 h. The supernatant was collected, centrifuged at $3000 \times g$ to remove cell debris, sterilized through a 0.2 µm filter, and stored at –80 °C until use. The longer incubation period with a higher number of viable cells was chosen to allow sufficient accumulation of tumor-derived soluble factors and cytokines, providing a representative model of tumor-conditioned medium.

2.4. Exposure of hASC to Conditioned Medium

ASC were seeded either in a 6-well round bottom plate (Avantor, Inc., Radnor, PA, USA) at a density of 1×10^5 cells for gene expression analysis and Western blot analysis, or in a 12-well glass bottom fibronectin-coated plate (Corning, Corning, NY, USA) at a density

of 0.2×10^5 cells for immunostaining. Cells were seeded in DMEM/F12 supplemented with 10% FBS and allowed to attach overnight. Culture medium was then replaced by serum-free medium for 24 h. Finally, hASCs were exposed to CM or control medium for 96 h, including a medium change at 48 h. Serum-free medium served as a negative control and serum-free medium supplemented with TGF- β 1 (10 ng/mL; Peprotech, Cranbury, NJ, USA) was used as a positive control. On a 6-well plate, 2 mL of CM were added per well, whereas on a 12-well plate, 0.75 mL were used per well. All approaches were supplemented with 100 nM Dexamethasone (Peprotech, Cranbury, NJ, USA) throughout the incubation period to maintain ASC viability and phenotypic stability during prolonged serum-free culture. Low-dose dexamethasone preserves MSC stemness and reduces culture-induced drift [40]. Importantly, dexamethasone was applied uniformly across all conditions to avoid systematic bias and enable relative comparison of CAF-related responses to tumor-derived conditioned media. Finally, after 96 h of incubation, the spent medium was collected and frozen at -80°C for subsequent cytokine analysis.

For subsequent use, cells were washed and either harvested for quantitative real time (qRT)-PCR, Western blot or ELISA analysis, or fixed for immunostaining.

2.5. Quantitative Real-Time PCR Analysis

To analyze the gene expression profile of CAF-associated markers, qRT-PCR was performed. Following exposure of hASCs to CM, total RNA was isolated using the RNeasy Micro Kit (Qiagen GmbH, Hilden, Germany) including on-column DNase digestion according to the manufacturer's instructions. RNA concentration and purity were assessed with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA (cDNA) was synthesized from the isolated RNA using the Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA).

The qRT-PCR was conducted using SYBRTM Green chemistry (Thermo Fisher Scientific, Waltham, MA, USA) with 10 ng cDNA template per reaction. Primer sets by Sigma-Aldrich were used for CAF-associated genes including α -SMA, FAP, TNC, SPARC, TIMP1, and VIM. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as the internal reference gene. The qRT-PCR system-integrated StepOneTM Software (version 2.3, Life Technologies, Carlsbad, NJ, USA) was used for evaluation. Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method.

2.6. Immunofluorescence Staining

Indirect immunostaining was performed to evaluate the expression of CAF-associated proteins. HASCs were cultured for 96 h with CM (HHP-CM or TCM) or control medium (serum-free medium or with TGF- β 1). Cells were seeded at a density of 2×10^4 cells on UV-sterilized glass coverslips coated with fibronectin and placed in 12-well plates. After incubation, cells were fixed with 4% paraformaldehyde (PFA; Otto Fischar GmbH & Co. KG, Saarbrücken, Germany) for 10 min at 37°C , washed with DPBS, and stored in DPBS containing 1% P/S until further processing.

Blocking was performed with 5% bovine serum albumin (BSA, Merck KGaA, Darmstadt Germany) and 1% goat serum (Invitrogen, Carlsbad, NJ, USA) in PBS for 45 min at room temperature (RT). Primary antibodies were diluted in 1% BSA in PBS-T (PBS with 0.1% Tween-20 (Merck KGaA, Darmstadt, Germany)) and applied as follows: α -SMA (mouse monoclonal, 1:200, 1 h at RT; Abcam, Cambridge, UK), FAP (rabbit monoclonal, 1:200, overnight at 4°C ; Cell Signaling Technology, Danvers, MA, USA), and TNC (mouse monoclonal, 1:200, 1 h at RT; Invitrogen, Carlsbad, NJ, USA). After washing, secondary antibodies diluted in 1% BSA in PBS-T were applied for 1 h at RT: Alexa Fluor 594 goat anti-mouse IgG (1:400; Invitrogen, Carlsbad, NJ, USA) and Alexa Fluor 488 goat anti-rabbit IgG (1:400; Invitrogen, Carlsbad, NJ, USA).

USA). Nuclei were counterstained with Hoechst reagent (0.5 µg/mL; Sigma-Aldrich, St. Louis, MO, USA) for 5 min at RT. Following final washes, wells were covered with PBS containing 1% P/S and imaged using a fluorescence microscope (Carl Zeiss AG, Oberkochen, Germany) at 10× or 20× magnification, respectively.

For α-SMA and FAP, the mean fluorescence intensity (MFI) was quantified on a per-cell basis by segmenting individual cells and determining the MFI within each single-cell mask. The MFI of the unstimulated control, including its standard deviation (SD) served as reference threshold for determination of positively marked cells. The proportion of positive cells was then calculated as the percentage of stained cells relative to the total number of nuclei in ten independent fields per condition, and values were normalized to the respective negative control. At least 200 cells per condition and per donor were analyzed. For TNC, mean fluorescence intensity per cell was quantified and normalized to the corresponding negative control. Image analysis was performed with ImageJ (version 1.54f, National Institutes of Health, Bethesda, MD, USA). Each condition was analyzed in duplicate for each marker.

2.7. Microscopy and Analysis of Cell Numbers

Morphological changes in hASCs were documented after 96 h of incubation using a Leica light microscope (Leica, Wetzlar, Germany). Immunofluorescence images were acquired with a Zeiss Axio Observer microscope equipped with a fluorescence Colibri5 light source providing different excitation wave lengths, an LED light source, an Axiocam 503 Mono, and the corresponding Zen 3.8 software (Carl Zeiss AG, Oberkochen, Germany) at 10× or 20× magnification following immunofluorescence staining. Using the optimally adjusted setups for the dyes employed in this study, all experimental conditions within and between donors were imaged under identical settings, including exposure times, to ensure comparable data acquisition. A negative control containing only the secondary antibody was included in each staining. Merged images are presented in the main figures, while the individual fluorescence channels are shown in the Supplementary Materials (Supplementary Figures S1 and S2).

Proliferation data were derived from the DAPI channel of the immunofluorescence images. For quantification, nuclei were counted in ten randomly selected fields per condition (10× magnification). For morphological analysis, cell area and aspect ratio were quantified using ImageJ software. Individual cells were manually segmented based on cytoplasmic staining, and cell area was measured following spatial calibration. Cell elongation was assessed by calculating the aspect ratio as the ratio of the major to the minor cell axis. For each condition, measurements were performed on cells from three randomly selected fields per donor (20× magnification).

2.8. Western Blot Analysis

For protein isolation, hASCs cultured under the respective experimental conditions were washed with cold DPBS and lysed directly on the culture plates using RIPA buffer supplemented with protease-inhibitor (Roche, Basel, Switzerland). Cells were detached by scraping, and the lysates were collected and incubated on ice for 30 min with intermittent mixing. Subsequently, samples were centrifuged at 14,000× g for 15 min at 4 °C to remove cell debris. The supernatant was transferred to fresh tubes, and protein concentrations were determined using a BCA assay (Thermo Fisher Scientific, Waltham, MA, USA). All samples were adjusted to equal protein concentrations prior to further processing.

For SDS-PAGE, 20 µg of total protein per condition were loaded onto a 10-well TGX Stain-Free™ gel (Bio-Rad Laboratories, Hercules, UK). Each protein sample (15 µL) was mixed with 4× Laemmli loading buffer (Bio-Rad Laboratories, Hercules, UK) supplemented with

β -mercaptoethanol (Merck KGaA, Darmstadt, Germany; 1:9, β -mercaptoethanol:loading buffer) in a ratio of 3:1 (sample:loading dye) and loaded onto the gel. Precision Plus Protein™ All Blue Prestained Standards (Bio-Rad Laboratories, Hercules, UK) served as molecular weight reference. Electrophoresis was carried out at 120 V for approximately 45 min.

Proteins were transferred onto LF-PVDF membranes using the Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, Hercules, UK) with the Mixed MW (Turbo) program (7 min, 1.3 A, 25 V). Membranes were blocked for 1 h at RT in PBS containing 5% (*w/w*) BSA and 0.05% (*v/v*) Tween-20. α -SMA antibody (1:1000, mouse monoclonal, Abcam, Cambridge, UK) was diluted in TBS-T and incubated for 1 h at RT. After three washes in TBS containing 0.05% Tween-20, membranes were incubated for 1 h at RT with fluorophore-conjugated secondary antibodies (StarBright® goat anti-mouse IgG, 1:5000, Bio-Rad Laboratories, Hercules, UK).

Fluorescent signals were acquired using the ChemiDoc MP Imaging System (Bio-Rad Laboratories, Hercules, UK) with the appropriate emission channels. Stain-Free technology was applied to normalize for total protein transfer, serving as a loading control. Band intensities were quantified using Image Lab software (Version 6.1.0 build 7, Bio-Rad Laboratories, Hercules, UK). Uncropped Western blot images and stain free images are provided in the Supplementary Materials (Supplementary Figure S3).

2.9. Enzyme-Linked Immunosorbent Assay

Determination of FAP concentration was performed on cell lysates using the DuoSet Human FAP enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. For each sample, 1 μ g of total protein was diluted in the sample buffer supplied with the kit, and both samples and standards were pipetted in duplicate into pre-coated wells. Following incubation with the detection antibody and subsequent addition of the streptavidin–HRP conjugate, color development was achieved using the substrate solution provided in the kit.

Absorbance was measured at 450 nm using a microplate reader (Molecular Devices, San Jose, CA, USA). FAP concentrations were determined from a standard curve generated with recombinant FAP standards using a four-parameter logistic (4-PL) curve fit.

2.10. Cytokine Analyses

The Human Cytokine Array C3 (RayBiotech Inc., Norcross, GA, USA) Dot blot assay was used as an initial screening method to analyze the secretion profile of 42 cytokines, including key pro-inflammatory cytokines, chemokines, and growth factors. The assay was performed on thawed HHP-CM, TCM or control medium after incubation with hASCs. The assay was conducted according to the manufacturer's instructions. Labeled proteins were visualized via enhanced chemiluminescence using detection buffer and X-ray film exposure. Cytokines appeared as dots of varying intensity and size.

For quantitative analysis, selected cytokines identified in the screening assay, MCP-1, IL-6, IL-8, MCP-3, CCL5, and CXCL5 were measured using the LEGENDplex™ Human Custom Panel (BioLegend, San Diego, CA, USA). HHP-CM, TCM and control medium were analyzed both prior to their addition to hASCs and after 48 h of ASC cultivation. Briefly, CM was incubated with capture beads overnight at 4 °C on an orbital shaker. Detection antibodies were then added, and samples were incubated for 1 h at RT. After washing, bead fluorescence was measured on a FACSCanto™ flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Cytokine concentrations were calculated from standard curves using the LEGENDplex data analysis software (version 2023-02-15, BioLegend, San Diego, CA, USA). To account for baseline cytokine levels, concentrations measured in CM before ASC incubation were subtracted from the corresponding post-incubation values. The cytokine concentration

then was normalized to the cell number determined before in the proliferation analysis (see Section 2.7). All measurements were performed in duplicate.

2.11. Statistical Analysis

Statistical analysis was performed using GraphPad PRISM software, version 8.0.1 (GraphPad Software, Boston, MA, USA). A p -value < 0.05 was considered statistically significant. Each individual hASC donor and each experimental run were considered independent biological replicates for statistical analysis. As different hASC donors were assigned to independent treatment conditions, comparisons across more than two groups were performed using ordinary one-way ANOVA for normally distributed data. In cases of non-normal distribution, the Kruskal–Wallis test was applied.

Predefined post hoc pairwise comparisons were conducted to assess (i) induction relative to baseline by comparing the negative control with all other experimental conditions (TGF- β 1, TCM, 0 MPa-CM, and 300 MPa-CM) and (ii) the effect of HHP treatment by comparing TCM and 0 MPa-CM to 300 MPa-CM. For parametric analyses, multiple comparisons were corrected using the Holm–Šidák method, whereas for non-parametric analyses, Dunn’s multiple comparisons test was applied.

3. Results

3.1. Morphological Characterization of hASCs After Incubation with Conditioned Media

Morphological alterations of hASCs were analyzed after incubation with the respective conditioned media to identify potential phenotypic changes indicative for CAF-like transdifferentiation.

After 96 h of stimulation with the respective conditioned media, cells were examined by light microscopy to assess morphological alterations (Figure 1A). HASCs cultured in serum-free control medium predominantly displayed a spread, polygonal morphology typical for undifferentiated hASCs, although some cells appeared slightly elongated and spindle-shaped. Cells treated with TGF- β 1 also exhibited mainly spread cell bodies without marked elongation. In contrast, hASCs incubated with TCM or 0 MPa-CM of HNSCC16 and UTSCC14 showed a predominantly spindle-shaped morphology. Cells exposed to 300 MPa-CM derived from HNSCC16 or UTSCC14 displayed elongated, fibroblast-like shapes forming strand-like growth patterns.

After 96 h, exposure to 300 MPa-CM resulted in a significant increase in hASC cell numbers in both HNSCC16- and UTSCC14-derived conditions compared to the control, TCM, and 0 MPa-CM groups (Figure 1B; 2.5–3.8-fold for HNSCC16 and 2.4–4.2-fold for UTSCC14).

Quantification of cell area (Figure 1B) revealed comparable values in control and TGF- β 1-treated hASCs. In both HNSCC16- and UTSCC14-derived conditions, cell area was significantly reduced following exposure to 300 MPa-CM compared to the control, TCM, and 0 MPa-CM conditions. The aspect ratio (Figure 1B), defined as the ratio of the major to the minor cell axis, was used to quantify cell elongation. Control and TGF- β 1-treated hASCs showed comparable aspect ratio values. In both HNSCC16- and UTSCC14-derived conditions, aspect ratio values were significantly increased following exposure to TCM and 300 MPa-CM compared to the control. In HNSCC16-derived conditions, 300 MPa-CM-treated hASCs exhibited a significantly higher aspect ratio than the corresponding 0 MPa-CM condition, whereas in UTSCC14-derived conditions, TCM-treated cells showed significantly higher aspect ratio values compared to 300 MPa-CM.

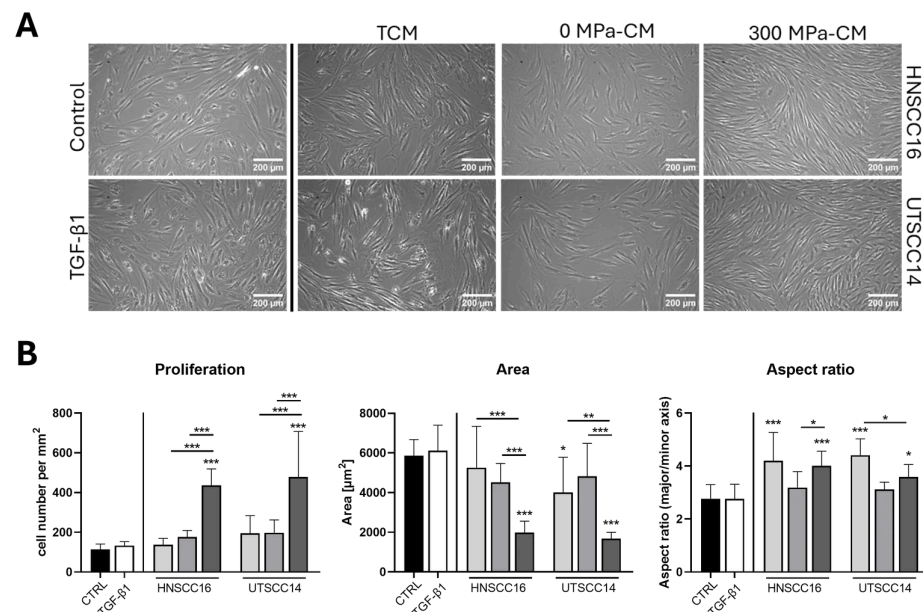


Figure 1. Morphology and proliferation of human adipose stromal cells (hASCs) after 96 h incubation with conditioned media (CM). **(A)** hASCs were incubated for 96 h with tumor-conditioned medium (TCM), 0 MPa-CM, or 300 MPa-CM derived from HNSCC16 or UTSCC14 cells, TGF-β1 (10 ng/mL) or serum-free control medium, respectively. Representative phase-contrast images of one out of eight donors are shown. Scale bar: 200 μm. **(B)** Proliferation and morphological quantification analysis of hASC after cultivation with CM or control medium for 96 h. For proliferation analysis, cell numbers were quantified via Hoechst fluorescence from three different donors (mean ± SD, n = 3). Morphological analysis was performed using ImageJ. Statistical significance was assessed using predefined post hoc comparisons following one-way ANOVA. Differences were evaluated relative to the control group or between specific treatment conditions as indicated in the figures. Statistically significant differences are indicated by asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). CTRL = control.

3.2. CAF Marker Gene Expression in hASCs After Incubation with Conditioned Media

To investigate a potential CAF-like transdifferentiation of hASCs under the influence of different conditioned media, the relative mRNA expression levels of several representative markers were quantified by qRT-PCR (Figure 2). Since no single marker exclusively defines CAFs, a panel including α-SMA, FAP, TNC, TIMP1, VIM, and SPARC was selected to capture distinct aspects of fibroblast activation and matrix remodeling. For all analyses, expression values of CM-treated hASCs were normalized to the serum-free medium control. This control was defined as 1, and all other conditions were reported as fold changes relative to it.

Expression of the activation marker α-SMA remained low in hASCs exposed to all conditioned media, except for 0 MPa-CM from HNSCC16, which showed a slight upregulation (1.6-fold). TGF-β1 stimulation led to a significant upregulation of α-SMA compared to the control (4.4-fold). FAP expression remained at baseline levels across all CM conditions, although a modest increase (1.5-fold) was detected in the 0 MPa-CM condition of HNSCC16. In contrast, TGF-β1 treatment resulted in only a minor, non-significant increase in FAP expression (1.8-fold). TNC expression was upregulated following stimulation with TCM from both cell lines (1.8- and 2.5-fold, respectively) and was downregulated in response to 0 MPa- and 300 MPa-CM compared to the control. For both HNSCC16 and UTSCC14, TNC expression in the 300 MPa-CM condition was significantly lower than in the corresponding TCM. TGF-β1 stimulation resulted in an approximately twofold increase in TNC expression. VIM was constitutively expressed at slightly lower levels than the control across most conditions. Only TCM from UTSCC14 induced a minor, non-significant increase in VIM

expression (1.5-fold) with high variability across donors. TIMP1 expression was upregulated in response to TCM from both cell lines (16–1.8-fold). In contrast, 0 MPa-CM and 300 MPa-CM resulted in expression levels lower than the control. Accordingly, comparison of TCM to 300 MPa-CM revealed a significant downregulation of TIMP1 expression in both cell lines in response to the HHP-treated CM. SPARC expression was decreased in response to all conditioned media conditions, with a statistically significant reduction observed in both cell lines in the 300 MPa-CM condition relative to the control. In contrast, TGF- β 1 stimulation led to a significantly higher SPARC expression than in the control (1.9-fold).

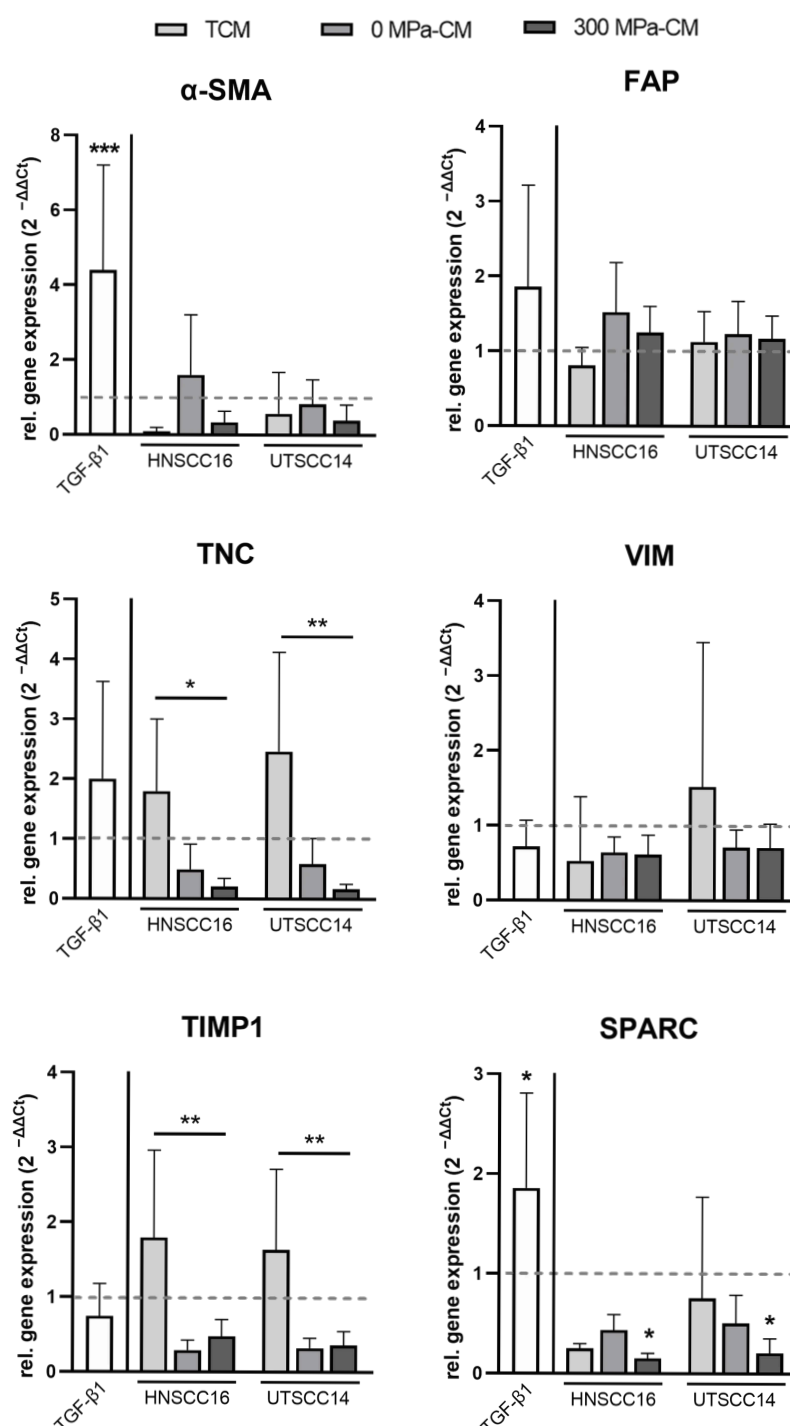


Figure 2. Relative mRNA expression of cancer-associated fibroblast (CAF) markers in human adipose stromal cells (hASCs) after stimulation with conditioned media (CM). HASCs were incubated for 96 h with tumor-conditioned medium (TCM), 0 MPa-CM, or 300 MPa-CM derived from HNSCC16

or UTSCC14 cells, TGF- β 1 (10 ng/mL) or serum-free control medium, respectively. The expression of α -SMA, FAP, TNC, VIM, TIMP1, and SPARC was analyzed by quantitative real time-PCR. Evaluation was performed using the $2^{-\Delta\Delta C_t}$ method. Data are presented as relative expression normalized to Glyceraldehyde-3-phosphate dehydrogenase (GAPDH; mean $\pm$ SD, $n = 7$ –8 independent donors) and shown as fold change compared to the control (set to 1; illustrated as dashed lines). Statistical significance was assessed using predefined post hoc comparisons following one-way ANOVA or Kruskal–Wallis testing. Differences were evaluated relative to the control group or between specific treatment conditions as indicated in the figures. Statistically significant differences are indicated by asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.3. Protein Analysis of CAF-Associated Protein Expression in hASCs After Incubation with Conditioned Media

Among CAF markers, α -SMA and FAP are regarded as the most prominent CAF-associated proteins and were therefore analyzed by immunostaining. In addition, the extracellular matrix glycoprotein TNC, which is closely linked to CAF conversion and tissue remodeling, was included to provide a more comprehensive phenotypic characterization.

Staining of α -SMA (Figure 3A) revealed that hASCs treated with 300 MPa-CM displayed markedly lower signal intensities compared to all other experimental groups. Quantitative analysis (Figure 3B) confirmed significantly higher α -SMA intensities in the TGF- β 1-treated group (5.3-fold) compared to the control. In contrast, 0 MPa-CM from both cell lines induced only a slight increase in α -SMA intensity (1.5–1.8-fold). Notably, α -SMA expression in the 300 MPa-CM condition was markedly lower than in the control and all other conditions. Staining of FAP (Figure 3A,C) revealed a 2.9-fold increase in protein expression following TGF- β 1 stimulation. In hASCs treated with CM from HNSCC16, both TCM and 0 MPa-CM induced 1.7–2.1-fold higher mean fluorescence intensities compared to the control, whereas 300 MPa-CM resulted in a 3.3-fold increase in FAP expression, although accompanied by high variability. In contrast, 300 MPa-CM from UTSCC14 induced only a minor increase in FAP expression, while TCM and 0 MPa-CM from UTSCC14 remained at baseline levels. Notably, no statistically significant differences were detected between the CM-treated groups. TNC staining (Figure 3D,E) showed a slight increase in signal intensity in hASCs incubated with TCM from both HNSCC cell lines (1.3–1.4-fold) compared to the control, whereas TGF- β 1 stimulation did not induce TNC expression. Cells exposed to 0 MPa-CM exhibited a slight reduction in TNC signal, whereas treatment with 300 MPa-CM resulted in a marked and statistically significant decrease in TNC expression compared to the control. Moreover, TNC levels were significantly reduced in both cell lines following 300 MPa-CM treatment compared to the respective TCM and 0 MPa-CM conditions.

In addition to the immunofluorescence analyses, α -SMA expression was examined by Western blot, and FAP levels were quantified using ELISA (Figure 4). Quantitative densitometric analysis of the Western blot, normalized to total protein, confirmed an approximately two-fold and statistically significant increase in α -SMA expression exclusively in the TGF- β 1-treated samples compared to the control, whereas all other conditions remained slightly below baseline levels.

Quantification of FAP concentrations by ELISA revealed only minor variations across treatment groups. TGF- β 1 induced a slight increase in FAP levels (1.2-fold) compared to the control. HASCs exposed to TCM from both HNSCC16 and UTSCC14 showed moderately elevated FAP concentrations (1.4–1.6-fold), comparable to those observed with 0 MPa-CM (1.4–1.5-fold). Treatment with 300 MPa-CM resulted in a 1.6–1.8-fold increase in FAP levels. Upregulation reached statistical significance in the TCM group of UTSCC14 and in the 300 MPa-CM conditions from both cell lines compared to the control; however, no statistically significant differences were observed between the treatment groups.

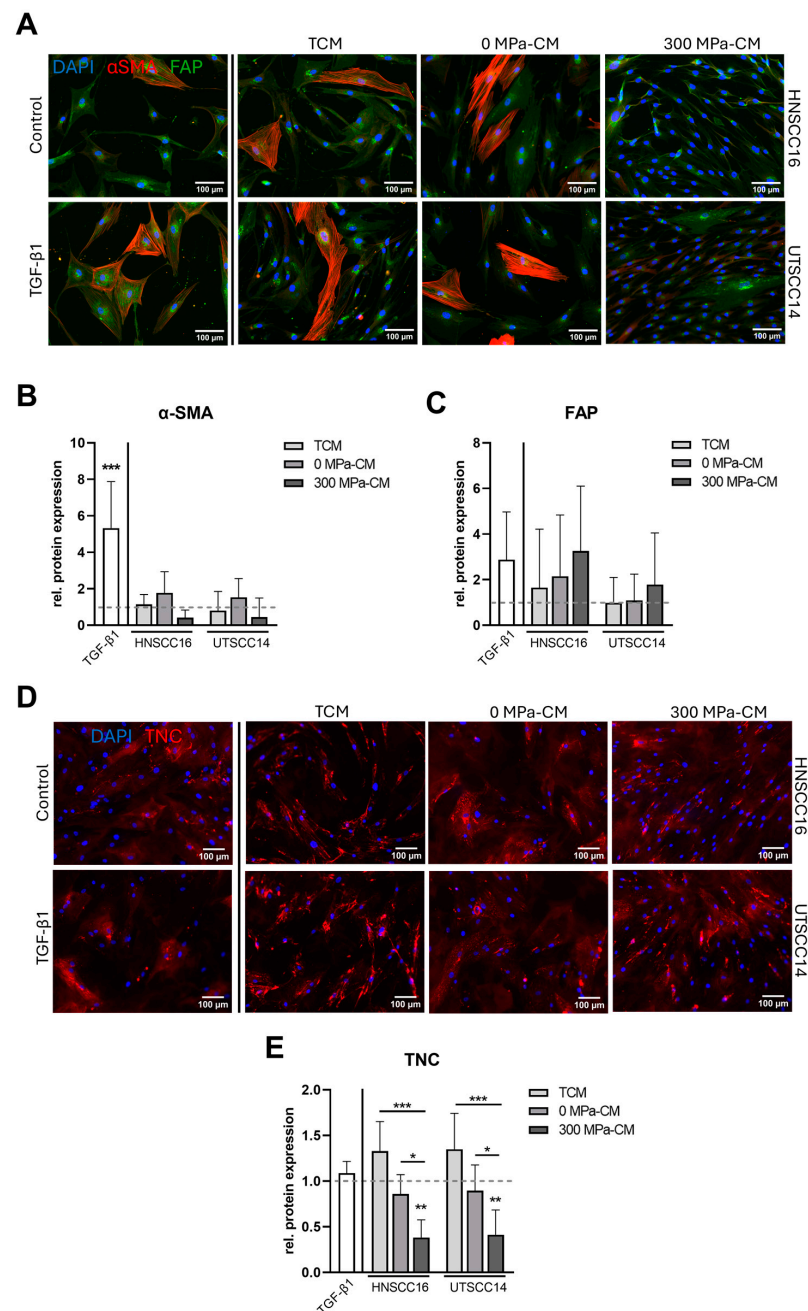


Figure 3. Immunofluorescence analysis of cancer-associated fibroblast (CAF) markers in stimulated human adipose stromal cells (hASCs). HASCs were incubated for 96 h with tumor-conditioned medium (TCM), 0 MPa-CM, or 300 MPa-CM derived from HNSCC16 or UTSCC14 cells, TGF-β1 (10 ng/mL), or serum-free control medium, respectively. (A) Representative immunofluorescence images showing staining of CAF markers α-SMA (red, intracellular) and FAP (green, membrane-associated) with nuclear counterstaining using Hoechst (blue). Scale bar: 100 μm. (B,C) Quantification of relative α-SMA (B) and FAP (C) fluorescence intensity across all samples (mean ± SD, n = 4 independent donors, each analyzed in duplicate from two independent experiments). Data are presented as relative protein expression normalized to the serum-free control (set to 1; illustrated as dashed lines). (D) Representative immunostaining of TNC (red) and Hoechst (blue) after 96 h of stimulation. Scale bar: 100 μm. (E) Quantification of TNC fluorescence intensity (mean ± SD, n = 3 independent donors, each analyzed in duplicate from two independent experiments). Results are shown as relative expression normalized to the serum-free control (set to 1). Statistical significance was assessed using predefined post hoc comparisons following one-way ANOVA or Kruskal–Wallis testing. Differences were evaluated relative to the control group or between specific treatment conditions as indicated in the figures. Statistically significant differences are indicated by asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

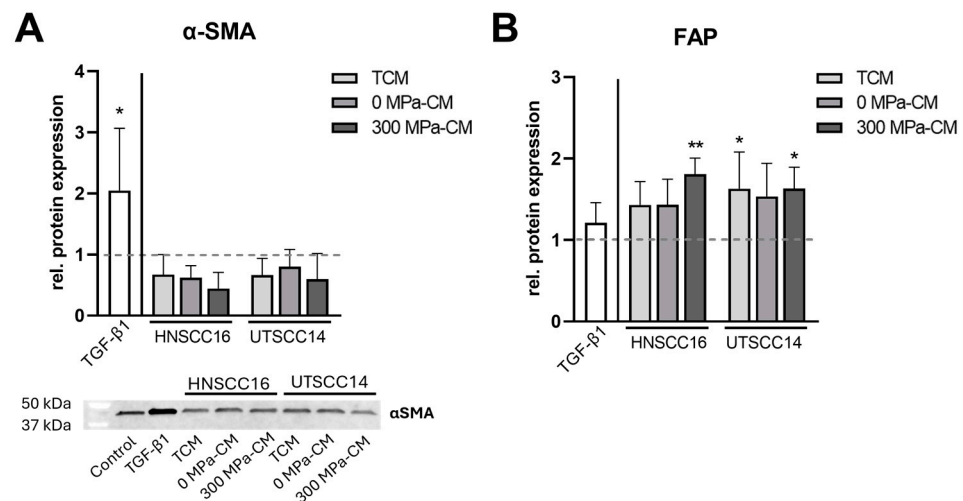


Figure 4. Protein expression analysis of cancer-associated fibroblast (CAF) markers in stimulated human adipose stromal cells (hASCs). hASCs were incubated for 96 h with tumor-conditioned medium (TCM), 0 MPa-CM, or 300 MPa-CM derived from HNSCC16 or UTSCC14 cells, TGF-β1 (10 ng/mL), or serum-free control medium, respectively. **(A)** α-SMA protein expression was determined by Western blot analysis. Representative Western blot membrane with protein bands of α-SMA following incubation with primary and secondary antibody is shown. Precision Plus Protein™ All Blue prestained standard served as molecular weight marker. Densitometric quantification of α-SMA protein expression was evaluated and total protein normalization was performed using Bio-Rad Stain-Free™ technology. Data represents $n = 4$ independent donors (mean $\pm$ SD). Results are presented as relative protein expression normalized to the serum-free control (set to 1; illustrated as dashed lines). **(B)** FAP levels were determined by ELISA in hASCs from six independent donors after 96 h of stimulation with the respective conditioned media. Results are presented as relative protein expression normalized to the serum-free control (set to 1; illustrated as dashed lines). Statistical significance was assessed using predefined post hoc comparisons following one-way ANOVA or Kruskal–Wallis testing. Differences were evaluated relative to the control group or between specific treatment conditions as indicated in the figures. Statistically significant differences are indicated by asterisks (* $p < 0.05$, ** $p < 0.01$).

3.4. Cytokine Secretion Profile of hASCs After Incubation with Conditioned Media

To identify cytokines secreted by hASCs in response to conditioned media, a human cytokine antibody array was applied on culture supernatants collected after 96 h of incubation (Supplementary Figure S4A,B). This assay enabled the simultaneous detection of 42 cytokines, including key pro-inflammatory mediators, chemokines, and growth factors. Based on the resulting cytokine profiles, the most prominently regulated factors (MCP-1, IL-6, IL-8, CCL5, CXCL5, and MCP-3) were selected for further quantitative analysis using a bead-based multiplex assay.

The Dot blot membranes (Supplementary Figure S4A,B) revealed distinct cytokine secretion patterns depending on the applied conditioned medium. In the serum-free control, signals were mainly detected for MCP-1 and IL-6. In contrast, stimulation with TCM from HNSCC16 markedly enhanced the secretion of several cytokines, most prominently MCP-1, IL-6, IL-8, and CCL5. Additional signals for MCP-3 and CXCL5 were observed at 0 MPa-CM and 300 MPa-CM.

Quantitative analysis of selected cytokines (Figure 5) was then performed to validate and compare the secretion profiles of hASCs under the different experimental conditions. Cytokine levels were measured both in the respective conditioned media prior to hASC incubation and in the supernatants collected after cultivation, thereby capturing the hASC response to the applied stimuli. For each condition, cytokine concentrations detected in the original CM were subtracted from the values obtained after hASC cultivation to isolate hASC-derived secretion. Finally, all cytokine concentrations were normalized to

the total cell number determined from Hoechst-stained images to account for the varying proliferation rates observed between treatments.

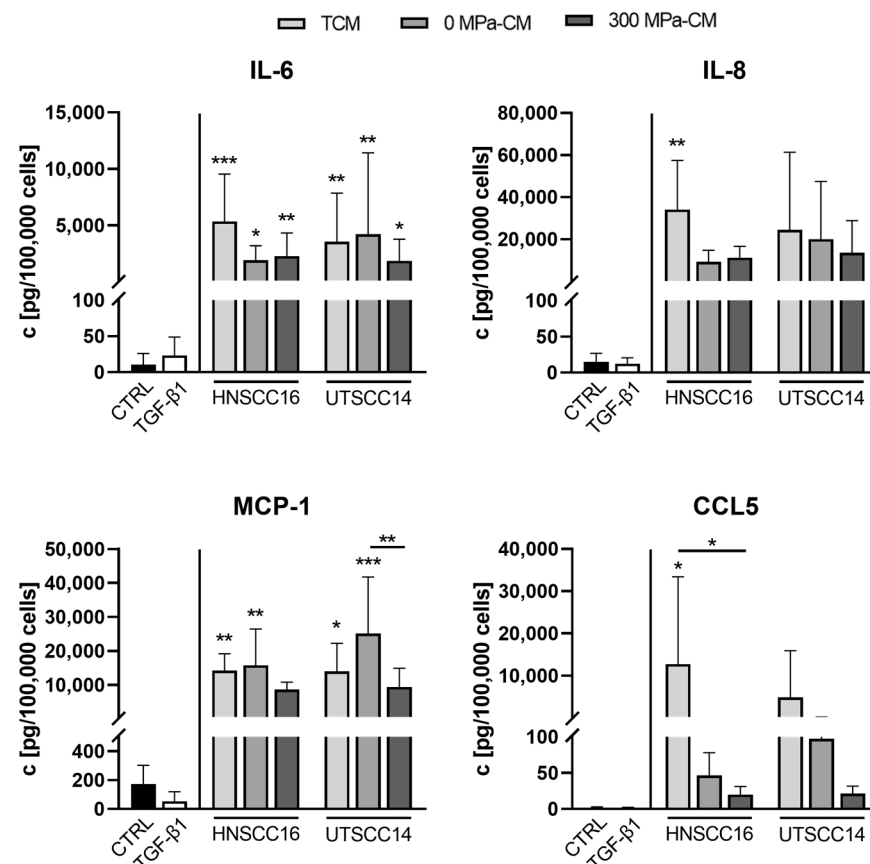


Figure 5. Quantification of cytokines secreted by human adipose stromal cells (hASCs) after incubation with conditioned media (CM). HASCs were incubated for 96 h with tumor-conditioned medium (TCM), 0 MPa-CM, or 300 MPa-CM derived from HNSCC16 or UTSCC14 cells, TGF-β1 (10 ng/mL), or serum-free control medium, respectively. After 48 h, the medium was replaced with fresh CM, and the supernatants were collected for cytokine analysis. Quantification of selected cytokines (IL-6, IL-8, MCP-1, CCL5) in hASC supernatants was determined by multiplex bead-based assay. Values represent the mean $\pm$ SD of $n = 8$ independent donors. Cytokine concentrations measured before and after incubation with hASCs were measured, and the difference (post-pre) normalized to 100,000 cells is shown. Statistical significance was assessed using predefined post hoc comparisons following one-way ANOVA or Kruskal–Wallis testing. Differences were evaluated relative to the control group or between specific treatment conditions as indicated in the figures. Statistically significant differences are indicated by asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). c = concentration; CTRL = control.

Overall, both the negative control and the TGF-β1-treated condition showed only minimal cytokine secretion, whereas hASCs exposed to TCM, 0 MPa-CM, and 300 MPa-CM from both tumor cell lines exhibited markedly elevated cytokine levels.

IL-6 concentrations were significantly increased in all conditioned media groups compared to the control; however, no significant differences in IL-6 secretion were observed between the CM-treated groups.

IL-8 levels in all CM conditions from both cell lines were higher compared to the control and TGF-β1 treatment; however, a statistically significant upregulation was observed only in the TCM condition of HNSCC16.

For MCP-1, hASCs stimulated with TCM and 0 MPa-CM from both HNSCC16 and UTSCC14 showed significantly higher MCP-1 concentrations compared to the control.

Additionally, for UTSCC14, MCP-1 secretion following exposure to 300 MPa-CM was significantly reduced compared to the corresponding 0 MPa condition.

CCL5 secretion in the serum-free control and after TGF- β 1 stimulation was nearly undetectable. In contrast, CCL5 levels were significantly increased after treatment with TCM from HNSCC16 and were significantly lower following exposure to 300 MPa-CM from the same cell line.

CXCL5 concentrations (Supplementary Figure S4C) were elevated in all CM groups compared to the serum-free control and TGF- β 1 stimulation. Notably, CXCL5 levels in the 300 MPa-CM condition from UTSCC14 were significantly increased compared to the control. For MCP-3 (Supplementary Figure S4C), cytokine levels were higher in response to TCM, 0 MPa-CM, and 300 MPa-CM derived from HNSCC16 compared to the control.

4. Discussion

Oncological safety is crucial for autologous reconstruction following resection of cartilage-infiltrating head and neck tumors. Potential residual cytokines remaining after HHP treatment must be carefully evaluated, as they could affect stromal or immune cells, including mesenchymal stem cells within the tumor microenvironment. Since MSCs can differentiate into CAF-like cells under the influence of tumor-derived soluble factors, it is essential to exclude that cytokines released during HHP treatment could trigger a transformation into CAFs [41,42]. In this study, hASCs were chosen as a well-established model for mesenchymal stem cells involved in wound healing [43–46]. During tissue repair, these cells migrate into the reconstruction site and are exposed to local cytokines and growth factors, making them a relevant *in vitro* system to assess potential effects of HHP-treated tumor-derived mediators on stromal cell activation [47,48]. Accordingly, we examined whether conditioned media from HHP-treated HNSCC cells could induce a CAF-like phenotype in hASCs. By combining gene and protein expression, with cytokine analyses and morphological assessment, we aimed to evaluate the biological safety of HHP treatment for potential clinical applications. Fibroblasts can acquire a myofibroblast-like phenotype upon exposure to TGF- β 1, and several studies further demonstrated that MSCs likewise undergo myofibroblast transdifferentiation with increased α -SMA production in response to TGF- β 1 stimulation [49–52]. Accordingly, inhibition of the TGF- β 1 pathway in hASCs has been proposed as a potential strategy to prevent acquiring a tumor-supportive phenotype and contributing to the tumor microenvironment [50]. In our present study, stimulation of hASCs with TGF- β 1 also consistently induced a CAF-like phenotype, characterized by a pronounced upregulation of α -SMA and a moderate increase in FAP and TNC expression at mRNA and protein levels. This finding confirms the established role of TGF- β 1 as a central regulator of fibroblast activation and myofibroblast transdifferentiation and validates the reliability of the experimental system applied in this study. In comparison, TCM and 0 MPa-CM from untreated HNSCC cells induced only isolated, modest changes in CAF marker expression, without indicating a consistent activation pattern. This mild response contrasts with findings by Werner et al., who reported a marked elevation of α -SMA expression at both the mRNA and protein levels in MSCs from human bone marrow after 24 and 48 h incubation with TCM from HCT8 colorectal cancer cells [53]. Remarkably, in their study, the TCM exerted an almost comparable effect on α -SMA induction as direct TGF- β 1 stimulation, suggesting that tumor-derived soluble factors can strongly promote myofibroblast-like transdifferentiation of MSCs depending on the tumor type and its specific secretome composition [53]. The weaker effect observed in our HNSCC model may therefore reflect differences in cytokine abundance and composition among tumor entities and/or a comparatively lower TGF- β 1 activity within HNSCC-derived CM, in line with our previous findings showing no relevant TGF- β 1 abundance in any of the tested CM samples [13]. Additionally, we observed large interindi-

vidual variance across hASC donors that contributed to the modest overall effect sizes. Since primary cells exhibit donor-specific differences in cytokine responsiveness, proliferation, and differentiation potential, this biological heterogeneity must be considered when interpreting CAF marker expression and cytokine secretion data [54]. CM derived from HHP-treated HNSCC cells did not trigger any substantial CAF-associated response. Although hASCs treated with 300 MPa-CM showed an altered morphology with elongated, spindle-shaped cell bodies and reduced cell area, no further indications of CAF-like transdifferentiation were observed. Instead, most markers in gene and protein expression analyses showed a tendency toward downregulation, with significantly lower effects observed for 300 MPa-CM compared to either TCM or 0 MPa-CM. An exception was a modest increase in FAP levels detected by ELISA. As FAP is not an exclusive CAF marker and no concomitant upregulation of other CAF markers, particularly α -SMA, was observed, this does not indicate stable CAF-like transdifferentiation.

Notably, both HNSCC cell lines exhibited largely consistent response patterns across molecular, cytokine, and morphological readouts, indicating that the observed effects were not driven by cell line-specific properties but rather reflect a general response to HHP-treated versus untreated tumor-derived conditioned media.

Morphological changes observed in hASCs after exposure to 300 MPa-CM were accompanied by significantly increased proliferation rates. Importantly, morphological alteration of MSCs is not specific to CAF commitment and can result from transient cytoskeletal remodeling in response to non-differentiating stimuli, including changes in cell density, cytokine exposure, or culture conditions [55,56]. Factors that may be released from pressure-damaged cells and are potentially present in 300 MPa-CM, such as inflammatory cytokines, stress-associated mediators, or damage-associated molecular patterns (DAMPs), could modulate cell adhesion and actin dynamics via pathways regulating cytoskeletal organization without activating a CAF-associated transcriptional program [57,58]. Consistent with this notion, no concomitant upregulation of canonical CAF markers at the mRNA or protein level was observed, arguing against stable CAF-like transdifferentiation and suggesting that the morphological alterations reflect a transient activation or adaptive response rather than durable pro-fibrotic stromal reprogramming. In a previous study investigating the effects of HHP-CM on vital tumor cells, we observed that HNSCC16 cells treated with 0 MPa-CM exhibited significantly increased proliferation compared to serum-free controls, whereas treatment with 300 MPa-CM did not affect cell numbers [13]. This apparent discrepancy between tumor and stromal cell responses can likely be attributed to cell type-specific responses to the altered cytokine milieu following HHP treatment. Such proliferative responses are characteristic of early wound-healing-like activation states in MSCs, in which stromal progenitor cells transiently expand in response to tissue damage and inflammatory cues to support regeneration and repair [47,48,59].

Cytokine analyses provided further mechanistic insights into the response of hASCs to the different conditioned media. In this study, cytokine concentrations were measured both in the conditioned media before hASC stimulation and in the supernatants collected afterward. By subtracting the cytokine levels present in the original CM from those detected after cultivation, the values presented here represent the isolated cytokine secretion derived from hASCs in response to the respective stimuli. HASCs exposed to TCM secreted markedly higher levels of IL-6, IL-8, MCP-1, and CCL5 compared to both the negative and the TGF- β 1 controls. IL-6 and IL-8 promote fibroblast activation, inflammation, proliferation, and secretion of matrix-remodeling enzymes, while MCP-1 recruits monocytes and macrophages that may further amplify pro-tumorigenic signaling through feedback cytokine loops [60,61]. CCL5, in particular, has been associated with myofibroblast-like transdifferentiation of tumor-activated hASCs and contributes to a self-reinforcing stromal

activation cycle. Escobar et al. (2015) demonstrated that MSCs secreted higher levels of chemokines, including CCL5, when exposed to TCM from aggressive breast cancer cells compared to less aggressive ones, highlighting that the tumor's cytokine profile critically shapes stromal behavior [62]. In line with these findings, CCL5 secretion in our study was only detectable in the TCM from both HNSCC cell lines, whereas it was absent in all HHP-treated CM. In response to 300 MPa-CM, significant lower cytokine levels of MCP-1, IL-8, and CCL5 were observed compared to TCM. This observation indicates that while HHP-treated CM can still induce a limited inflammatory response in hASCs, the magnitude of this effect is reduced compared to untreated tumor CM. Supernatants from HHP-treated tumor cells have previously been shown to contain high levels of IL-1 α , IL-1 β , IL-6 and IL-8, potent mediators of inflammatory responses [13]. However, despite their abundance, no corresponding activation of CAF markers in hASCs was observed. This apparent dissociation between increased cytokine exposure and the absence of CAF-like transdifferentiation suggests that elevated inflammatory signaling alone may be insufficient to drive stable CAF commitment. Rather, CAF conversion likely depends on the presence, combination, and temporal dynamics of specific tumor-derived factors, which appear to be lacking or functionally altered in HHP-treated conditioned media. In this context, sustained profibrotic signaling pathways, including but not limited to TGF- β -associated mechanisms, may play a contributory role but were not dominantly activated under the applied experimental conditions. Thus, the observed cytokine secretion is more consistent with a transient inflammatory activation than with durable pro-fibrotic stromal reprogramming.

Interestingly, Al-toub et al. reported that stimulation of MSCs with IL-1 β (50 ng/mL), either alone or in combination with IL-6 (50 ng/mL each), strongly upregulated pro-inflammatory cytokines such as IL-1 β , CXCL5, IL-6, and CXCL6 at the transcriptional level, whereas IL-6 alone induced only a weak response [63]. In our study, IL-1 β concentrations in the conditioned media ranged from 20 to 115 pg/mL in 0 MPa-CM and from 480 to 1220 pg/mL in 300 MPa-CM, corresponding to levels approximately 40- to 2500-fold lower than those used in the Al-toub et al. study. This substantial difference in cytokine concentration likely accounts for the absence of a robust pro-inflammatory or CAF-like conversion in hASCs in our model. A study employing a comparable experimental setup in breast cancer demonstrated that hASCs incubated with TCM from two different breast cancer cell lines for 96 h secreted significantly higher amounts of stromal derived factor 1 (SDF-1) and TGF- β 1 than unstimulated cells [50]. This paracrine feedback was accompanied by a more than ten-fold increase in α -SMA-positive cells compared to untreated controls, highlighting a strong reciprocal activation between tumor-derived and stromal cytokines [50]. In contrast, our semi-quantitative cytokine screening did not reveal enhanced secretion of either SDF-1 or TGF- β 1 after CM exposure. The corresponding array spots were barely visible in both control and CM-treated samples, suggesting that hASCs did not produce relevant amounts of these cytokines under the respective types of stimulation.

From a translational perspective, these findings are highly relevant for a potential clinical application of HHP-treated tissues in reconstructive surgery following tumor resection. The absence of any indications of hASC-to-CAF transdifferentiation in response to HHP-treated tumor CM suggests that devitalized grafts are unlikely to contribute to a tumor-promoting microenvironment upon re-implantation, even in the presence of residual cytokines within the devitalized cartilage. This directly addresses a key oncological safety concern in the use of HHP for the preparation of autologous cartilage grafts, as residual tumor-derived factors must not stimulate stromal cells in a pro-oncogenic manner. Together with previous evidence demonstrating that HHP treatment preserves tissue integrity and antigenicity while ensuring complete devitalization, and that a tumor-promoting influence on other viable tumor cells can likewise be excluded [11–13], our data further strengthen

the rationale for its clinically safe implementation. Nevertheless, several limitations of our present in vitro study must be acknowledged. The experiments were performed in a two-dimensional monoculture system that does not fully recapitulate the complexity of the tumor microenvironment. Mechanical cues, ECM stiffness, and the presence of immune cells critically modulate fibroblast activation and CAF plasticity. Furthermore, the incubation period of 96 h may not capture delayed transcriptional or epigenetic reprogramming events. In addition, although the conditioned media were filtered through a 0.2 µm membrane, expected to remove apoptotic bodies and larger vesicular structures, smaller extracellular vesicles (EVs) may persist and contribute to the observed biological effects. Importantly, EV-mediated signaling can modulate cell behavior without necessarily inducing stable CAF-like differentiation; however, the specific contribution of EVs following HHP treatment was not addressed in the present study and warrants further investigation. Future studies should incorporate three-dimensional collagen or hydrogel matrices, longer stimulation periods, and co-cultures with immune cells to better approximate in vivo conditions. In addition, the use of primary HNSCC cells instead of established cell lines would provide a more physiologically relevant cytokine milieu and better reflect the intertumoral heterogeneity observed in patient tumors. The combination of such approaches with HNSCC organoid models or animal implantation studies could further validate the oncological safety of HHP treatment under more representative conditions.

In conclusion, this study demonstrates that while hASCs retain the capacity to adopt a CAF-like phenotype under strong stimuli such as TGF-β1, HHP-treated tumor supernatants fail to induce such changes under the applied in vitro conditions. HHP devitalization substantially reduces the paracrine tumor-promoting potential of malignant cells while preserving extracellular matrix integrity. Collectively, these findings provide preclinical in vitro evidence supporting the oncological safety of HHP-treated tissues. Accordingly, HHP treatment may represent a promising tissue-preserving strategy; however, further in vitro and in vivo investigations are warranted to confirm these observations under physiologically relevant conditions and to assess the translational potential of HHP-treated tissues in clinical oncology.

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Abbreviations

The following abbreviations are used in this manuscript:

HHP	High hydrostatic pressure
HNSCC	head and neck squamous cell carcinoma
IL	Interleukin
TME	tumor microenvironment
CAF	cancer-associated fibroblast
EMT	epithelial-to-mesenchymal transition
MSC	mesenchymal stromal cells
hASC	adipose stromal cells
TGF- β 1	transforming growth factor beta 1
TNF- α	tumor necrosis factor alpha
CXCR4	C-X-C chemokine receptor 4
CXCL5/12	C-X-C motif chemokine ligand 5/12
STAT3	signal transducer and activator of transcription 3
NF- κ B	nuclear factor kappa B
α SMA	alpha-smooth muscle actin
FAP	fibroblast activation protein
TNC	tenascin-C
SPARC	secreted protein acidic and rich in cysteine
VIM	vimentin
TIMP1	tissue inhibitor of metalloproteinases 1
DMEM	Dulbecco's modified Eagle medium
PDX	patient-derived xenograft
HPV	Human papillomavirus
P/S	Penicillin/Streptomycin
EDTA	ethylenediaminetetraacetic acid
ITS	Insulin-Transferrin-Selenium
CTRL	control
CM	Conditioned medium
HHP-CM	High hydrostatic pressure-treated conditioned medium
TCM	tumor-conditioned medium
qRT-PCR	quantitative real-time PCR
cDNA	cComplementary DNA
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
PFA	paraformaldehyde
BSA	bovine serum albumin
RT	room temperature
MFI	mean fluorescence intensity
SD	standard deviation
ELISA	Enzyme-linked immunosorbent assay
4-PL	Four-parameter logistic
MCP-1	Monocyte Chemoattractant Protein-1

CCL5	CC-chemokine ligand 5
SDF-1	stromal derived factor 1
EV	extracellular vesicle

References

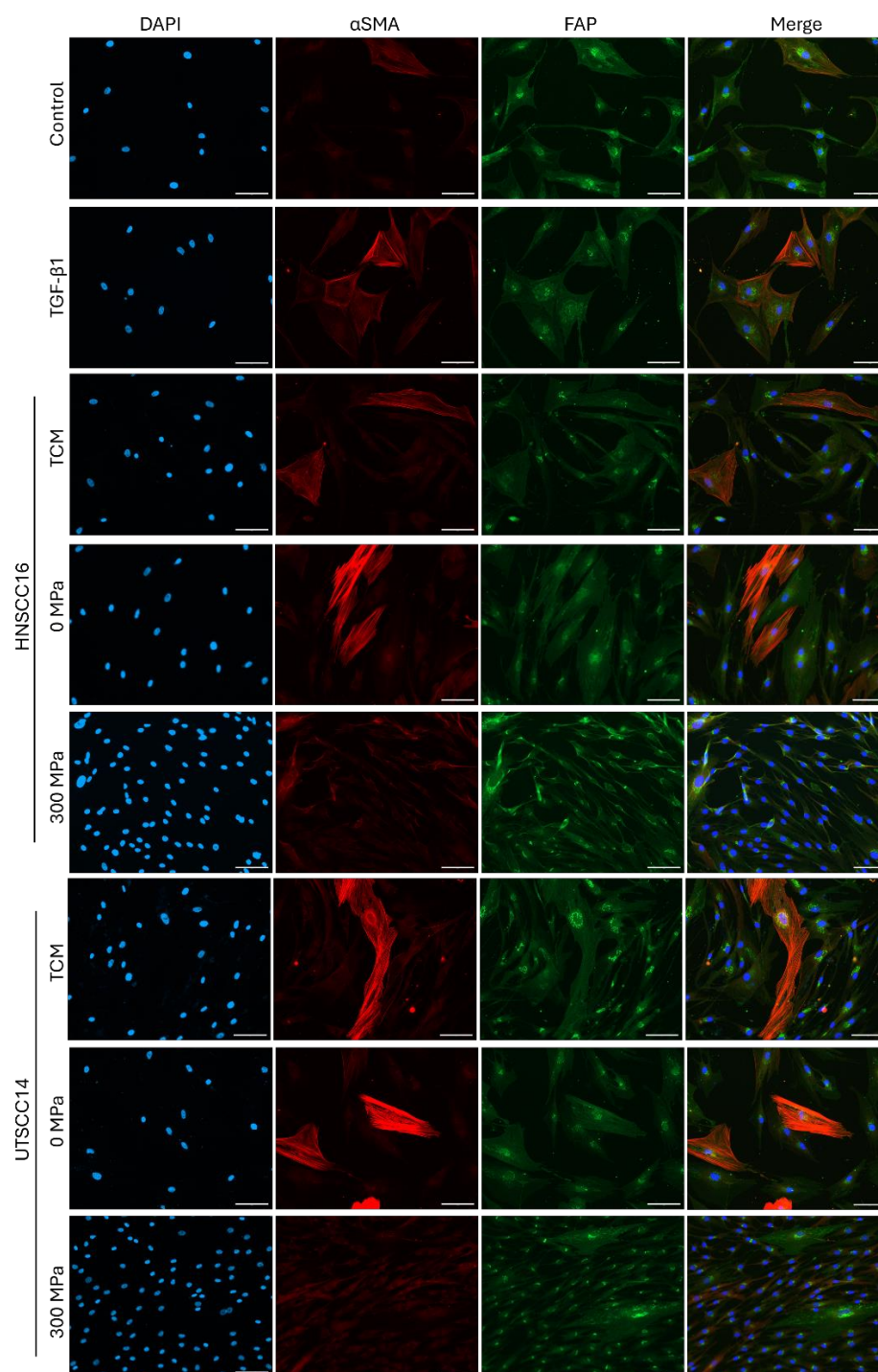
- Diehl, P.; Schmitt, M.; Schauwecker, J.; Eichelberg, K.; Gollwitzer, H.; Gradinger, R.; Goebel, M.; Preissner, K.T.; Mittelmeier, W.; Magdolen, U. Effect of High Hydrostatic Pressure on Biological Properties of Extracellular Bone Matrix Proteins. *Int. J. Mol. Med.* **2005**, *16*, 285–289. [\[CrossRef\]](#)
- Weiss, E.-M.; Frey, B.; Rödel, F.; Herrmann, M.; Schlücker, E.; Voll, R.E.; Fietkau, R.; Gaipl, U.S. Ex Vivo- and in Vivo-Induced Dead Tumor Cells as Modulators of Antitumor Responses. *Ann. N. Y. Acad. Sci.* **2010**, *1209*, 109–117. [\[CrossRef\]](#) [\[PubMed\]](#)
- Waletzko, J.; Dau, M.; Seyfarth, A.; Springer, A.; Frank, M.; Bader, R.; Jonitz-Heincke, A. Devitalizing Effect of High Hydrostatic Pressure on Human Cells—Influence on Cell Death in Osteoblasts and Chondrocytes. *Int. J. Mol. Sci.* **2020**, *21*, 3836. [\[CrossRef\]](#) [\[PubMed\]](#)
- Waletzko-Hellwig, J.; Pohl, C.; Riese, J.; Schlosser, M.; Dau, M.; Engel, N.; Springer, A.; Bader, R. Effect of High Hydrostatic Pressure on Human Trabecular Bone Regarding Cell Death and Matrix Integrity. *Front. Bioeng. Biotechnol.* **2021**, *9*, 730266. [\[CrossRef\]](#) [\[PubMed\]](#)
- Rivalain, N.; Roquain, J.; Demazeau, G. Development of High Hydrostatic Pressure in Biosciences: Pressure Effect on Biological Structures and Potential Applications in Biotechnologies. *Biotechnol. Adv.* **2010**, *28*, 659–672. [\[CrossRef\]](#) [\[PubMed\]](#)
- van de Sande, M.A.J.; Bovée, J.V.M.G.; van Domselaar, M.; van Wijk, M.J.; Sanders, I.; Kuijper, E. Successful Disinfection of Femoral Head Bone Graft Using High Hydrostatic Pressure. *Cell Tissue Bank.* **2018**, *19*, 333–340. [\[CrossRef\]](#)
- Frey, B.; Janko, C.; Ebel, N.; Meister, S.; Schlücker, E.; Meyer-Pittroff, R.; Fietkau, R.; Herrmann, M.; Gaipl, U.S. Cells under Pressure—Treatment of Eukaryotic Cells with High Hydrostatic Pressure, from Physiologic Aspects to Pressure Induced Cell Death. *Curr. Med. Chem.* **2008**, *15*, 2329–2336. [\[CrossRef\]](#) [\[PubMed\]](#)
- Seitz, C.; Rückert, M.; Deloch, L.; Weiss, E.-M.; Utz, S.; Izydor, M.; Ebel, N.; Schlücker, E.; Fietkau, R.; Gaipl, U.S.; et al. Tumor Cell-Based Vaccine Generated with High Hydrostatic Pressure Synergizes with Radiotherapy by Generating a Favorable Anti-Tumor Immune Microenvironment. *Front. Oncol.* **2019**, *9*, 805. [\[CrossRef\]](#)
- Yan, S.; Liu, K.; Mu, L.; Liu, J.; Tang, W.; Liu, B. Research and Application of Hydrostatic High Pressure in Tumor Vaccines. *Oncol. Rep.* **2021**, *45*, 75. [\[CrossRef\]](#)
- Urbanova, L.; Hradilova, N.; Moserova, I.; Vosahlikova, S.; Sadilkova, L.; Hensler, M.; Spisek, R.; Adkins, I. High Hydrostatic Pressure Affects Antigenic Pool in Tumor Cells: Implication for Dendritic Cell-Based Cancer Immunotherapy. *Immunol. Lett.* **2017**, *187*, 27–34. [\[CrossRef\]](#)
- Maletzki, C.; Frein Grote, V.; Kalle, F.; Kleitke, T.; Zimpfer, A.; Becker, A.-S.; Bergmann-Ewert, W.; Jonitz-Heincke, A.; Bader, R.; Vollmar, B.; et al. Establishing Safe High Hydrostatic Pressure Devitalization Thresholds for Autologous Head and Neck Cancer Vaccination and Reconstruction. *Cell Death Discov.* **2023**, *9*, 390, Correction in *Cell Death Discov.* **2024**, *15*, 437. <https://doi.org/10.1038/s41420-024-02172-3>. [\[CrossRef\]](#)
- Hiemer, B.; Genz, B.; Jonitz-Heincke, A.; Pasold, J.; Wree, A.; Dommerich, S.; Bader, R. Devitalisation of Human Cartilage by High Hydrostatic Pressure Treatment: Subsequent Cultivation of Chondrocytes and Mesenchymal Stem Cells on the Devitalised Tissue. *Sci. Rep.* **2016**, *6*, 33747. [\[CrossRef\]](#) [\[PubMed\]](#)
- Brach, J.K.; Frein Grote, V.; Strüder, D.; Kalle, F.; Jonitz-Heincke, A.; Bader, R.; Hoffmann, M.; Scherzad, A.; Wirth, M.; Hackenberg, S. Impact of High Hydrostatic Pressure on the Cytokine Profile and Head and Neck Cancer Cell Behavior: Implications for Oncological Safety. *Front. Immunol.* **2025**, *16*, 1581014. [\[CrossRef\]](#)
- Nedaeinia, R.; Najafgholian, S.; Salehi, R.; Goli, M.; Ranjbar, M.; Nickho, H.; Haghjooy Javanmard, S.; Ferns, G.A.; Manian, M. The Role of Cancer-Associated Fibroblasts and Exosomal miRNAs-Mediated Intercellular Communication in the Tumor Microenvironment and the Biology of Carcinogenesis: A Systematic Review. *Cell Death Discov.* **2024**, *10*, 380. [\[CrossRef\]](#)
- Wright, K.; Ly, T.; Kriet, M.; Czirok, A.; Thomas, S.M. Cancer-Associated Fibroblasts: Master Tumor Microenvironment Modifiers. *Cancers* **2023**, *15*, 1899. [\[CrossRef\]](#)
- Jia, H.; Chen, X.; Zhang, L.; Chen, M. Cancer Associated Fibroblasts in Cancer Development and Therapy. *J. Hematol. Oncol.* **2025**, *18*, 36. [\[CrossRef\]](#)
- Li, K.; Wang, R.; Liu, G.-W.; Peng, Z.-Y.; Wang, J.-C.; Xiao, G.-D.; Tang, S.-C.; Du, N.; Zhang, J.; Zhang, J.; et al. Refining the Optimal CAF Cluster Marker for Predicting TME-Dependent Survival Expectancy and Treatment Benefits in NSCLC Patients. *Sci. Rep.* **2024**, *14*, 16766. [\[CrossRef\]](#)
- Tsujino, T.; Seshimo, I.; Yamamoto, H.; Ngan, C.Y.; Ezumi, K.; Takemasa, I.; Ikeda, M.; Sekimoto, M.; Matsuura, N.; Monden, M. Stromal Myofibroblasts Predict Disease Recurrence for Colorectal Cancer. *Clin. Cancer Res.* **2007**, *13*, 2082–2090. [\[CrossRef\]](#)

19. Liu, Y.; Sinjab, A.; Min, J.; Han, G.; Paradiso, F.; Zhang, Y.; Wang, R.; Pei, G.; Dai, Y.; Liu, Y.; et al. Conserved Spatial Subtypes and Cellular Neighborhoods of Cancer-Associated Fibroblasts Revealed by Single-Cell Spatial Multi-Omics. *Cancer Cell* **2025**, *43*, 905–924.e6. [[CrossRef](#)] [[PubMed](#)]
20. Ren, Y.; Lu, D.; Wang, F.; Wang, Z.; Li, J.; Huang, R.; Lu, Y.; Duan, A.; Shou, R.; Liu, J.; et al. A Multi-Omics Atlas of CAF Subtypes Reveals apCAF–M2 Macrophage Interactions Driving Immune Resistance in Glioma. *PLoS ONE* **2025**, *20*, e0329801. [[CrossRef](#)] [[PubMed](#)]
21. Cords, L.; Tietscher, S.; Anzeneder, T.; Langwieder, C.; Rees, M.; de Souza, N.; Bodenmiller, B. Cancer-Associated Fibroblast Classification in Single-Cell and Spatial Proteomics Data. *Nat. Commun.* **2023**, *14*, 4294. [[CrossRef](#)] [[PubMed](#)]
22. Chen, B.; Chan, W.N.; Xie, F.; Mui, C.W.; Liu, X.; Cheung, A.H.K.; Lung, R.W.M.; Chow, C.; Zhang, Z.; Fang, C.; et al. The Molecular Classification of Cancer-Associated Fibroblasts on a Pan-Cancer Single-Cell Transcriptional Atlas. *Clin. Transl. Med.* **2023**, *13*, e1516. [[CrossRef](#)]
23. Hu, C.; Zhang, Y.; Wu, C.; Huang, Q. Heterogeneity of Cancer-Associated Fibroblasts in Head and Neck Squamous Cell Carcinoma: Opportunities and Challenges. *Cell Death Discov.* **2023**, *9*, 124. [[CrossRef](#)] [[PubMed](#)]
24. de Visser, K.E.; Joyce, J.A. The Evolving Tumor Microenvironment: From Cancer Initiation to Metastatic Outgrowth. *Cancer Cell* **2023**, *41*, 374–403. [[CrossRef](#)]
25. Haviv, I.; Polyak, K.; Qiu, W.; Hu, M.; Campbell, I. Origin of Carcinoma Associated Fibroblasts. *Cell Cycle* **2009**, *8*, 589–595. [[CrossRef](#)] [[PubMed](#)]
26. Fotsitzoudis, C.; Koulouridi, A.; Messaritakis, I.; Konstantinidis, T.; Gouvas, N.; Tsiaoussis, J.; Souglakos, J. Cancer-Associated Fibroblasts: The Origin, Biological Characteristics and Role in Cancer—A Glance on Colorectal Cancer. *Cancers* **2022**, *14*, 4394. [[CrossRef](#)]
27. Yang, D.; Liu, J.; Qian, H.; Zhuang, Q. Cancer-Associated Fibroblasts: From Basic Science to Anticancer Therapy. *Exp. Mol. Med.* **2023**, *55*, 1322–1332. [[CrossRef](#)]
28. Tabatabaei Qomi, R.; Sheykhasan, M. Adipose-Derived Stromal Cell in Regenerative Medicine: A Review. *World J. Stem Cells* **2017**, *9*, 107–117. [[CrossRef](#)]
29. Al-Ghadban, S.; Artilles, M.; Bunnell, B.A. Adipose Stem Cells in Regenerative Medicine: Looking Forward. *Front. Bioeng. Biotechnol.* **2022**, *9*, 837464. [[CrossRef](#)]
30. Burger, J.A.; Kipps, T.J. CXCR4: A Key Receptor in the Crosstalk between Tumor Cells and Their Microenvironment. *Blood* **2006**, *107*, 1761–1767. [[CrossRef](#)]
31. Miyazaki, Y.; Oda, T.; Mori, N.; Kida, Y.S. Adipose-Derived Mesenchymal Stem Cells Differentiate into Pancreatic Cancer-Associated Fibroblasts in Vitro. *FEBS Open Bio* **2020**, *10*, 2268–2281. [[CrossRef](#)]
32. Pan, C.; Fang, Q.; Liu, P.; Ma, D.; Cao, S.; Zhang, L.; Chen, Q.; Hu, T.; Wang, J. Mesenchymal Stem Cells with Cancer-Associated Fibroblast-Like Phenotype Stimulate SDF-1/CXCR4 Axis to Enhance the Growth and Invasion of B-Cell Acute Lymphoblastic Leukemia Cells Through Cell-to-Cell Communication. *Front. Cell Dev. Biol.* **2021**, *9*, 708513. [[CrossRef](#)]
33. Li, C.; Yang, C.; Yang, Q. The Role of miRNAs in the Extracellular Vesicle-Mediated Interplay Between Breast Tumor Cells and Cancer-Associated Fibroblasts. *J. Cancer Metastasis Treat.* **2024**, *10*. [[CrossRef](#)]
34. Wu, F.; Yang, J.; Liu, J.; Wang, Y.; Mu, J.; Zeng, Q.; Deng, S.; Zhou, H. Signaling Pathways in Cancer-Associated Fibroblasts and Targeted Therapy for Cancer. *Signal Transduct. Target. Ther.* **2021**, *6*, 218. [[CrossRef](#)]
35. Alcaraz, L.B.; Mallavialle, A.; Mollevi, C.; Boissière-Michot, F.; Mansouri, H.; Simony-Lafontaine, J.; Laurent-Matha, V.; Chardès, T.; Jacot, W.; Turtoi, A.; et al. SPARC in Cancer-Associated Fibroblasts Is an Independent Poor Prognostic Factor in Non-Metastatic Triple-Negative Breast Cancer and Exhibits pro-Tumor Activity. *Int. J. Cancer* **2023**, *152*, 1243–1258. [[CrossRef](#)] [[PubMed](#)]
36. Drev, D.; Harpain, F.; Beer, A.; Stift, A.; Gruber, E.S.; Klimpfinger, M.; Thalhammer, S.; Reti, A.; Kenner, L.; Bergmann, M.; et al. Impact of Fibroblast-Derived SPARC on Invasiveness of Colorectal Cancer Cells. *Cancers* **2019**, *11*, 1421. [[CrossRef](#)]
37. Dwivedi, N.; Shukla, N.; Prathima, K.M.; Das, M.; Dhar, S.K. Novel CAF-Identifiers via Transcriptomic and Protein Level Analysis in HNSC Patients. *Sci. Rep.* **2023**, *13*, 13899. [[CrossRef](#)] [[PubMed](#)]
38. Ruhl, T.; Storti, G.; Pallua, N. Proliferation, Metabolic Activity, and Adipogenic Differentiation of Human Preadipocytes Exposed to 2 Surfactants In Vitro. *J. Pharm. Sci.* **2018**, *107*, 1408–1415. [[CrossRef](#)]
39. Schoenwaelder, N.; Krause, M.; Freitag, T.; Schneider, B.; Zonnur, S.; Zimpfer, A.; Becker, A.S.; Salewski, I.; Strüder, D.F.; Lemcke, H.; et al. Preclinical Head and Neck Squamous Cell Carcinoma Models for Combined Targeted Therapy Approaches. *Cancers* **2022**, *14*, 2484. [[CrossRef](#)] [[PubMed](#)]
40. Xiao, Y.; Peperzak, V.; van Rijn, L.; Borst, J.; de Bruijn, J.D. Dexamethasone Treatment during the Expansion Phase Maintains Stemness of Bone Marrow Mesenchymal Stem Cells. *J. Tissue Eng. Regen. Med.* **2010**, *4*, 374–386. [[CrossRef](#)] [[PubMed](#)]
41. Quante, M.; Tu, S.P.; Tomita, H.; Gonda, T.; Wang, S.S.W.; Takashi, S.; Baik, G.H.; Shibata, W.; DiPrete, B.; Betz, K.S.; et al. Bone Marrow-Derived Myofibroblasts Contribute to the Mesenchymal Stem Cell Niche and Promote Tumor Growth. *Cancer Cell* **2011**, *19*, 257–272. [[CrossRef](#)]

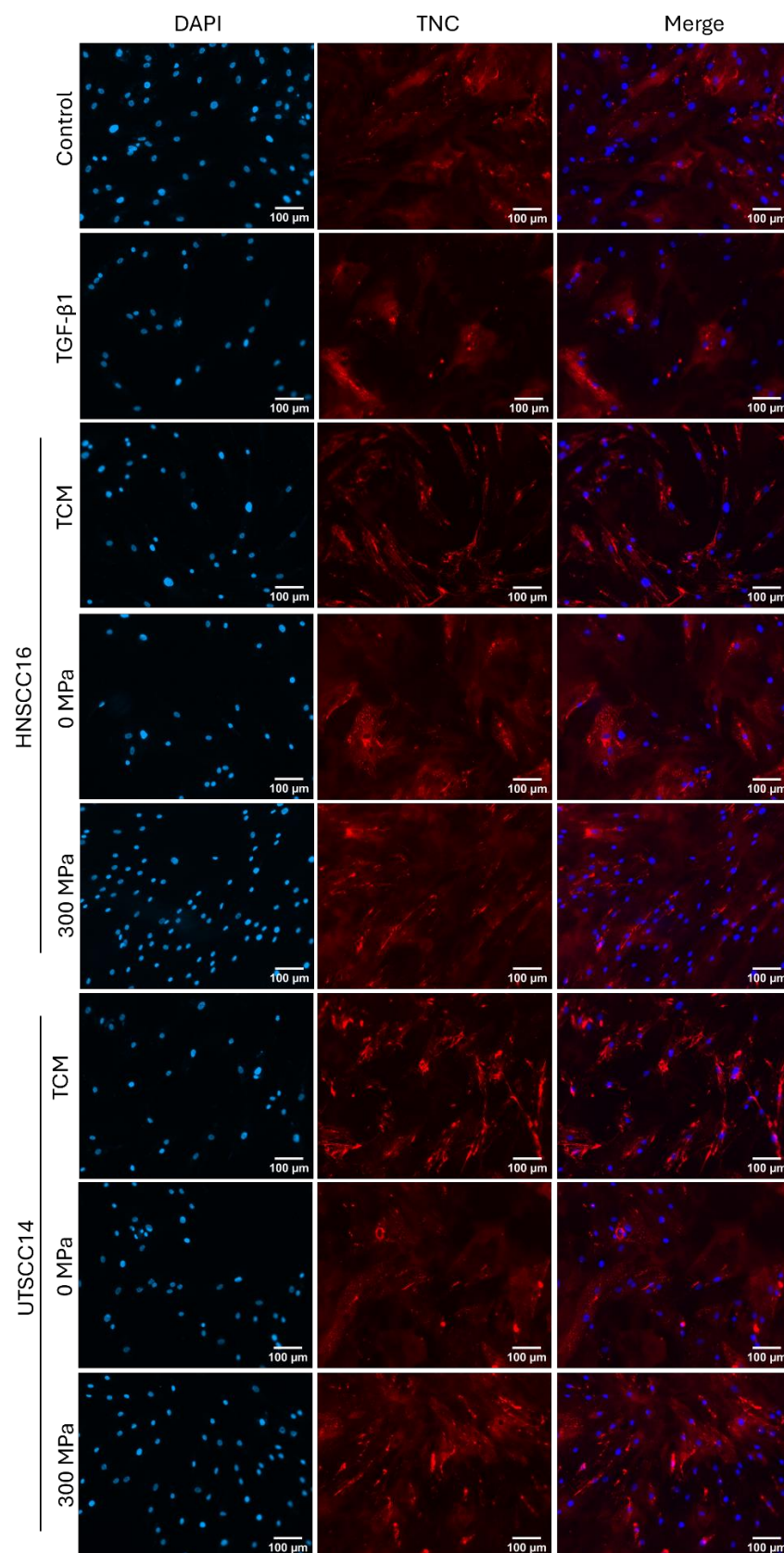
42. Mishra, P.J.; Mishra, P.J.; Humeniuk, R.; Medina, D.J.; Alexe, G.; Mesirov, J.P.; Ganesan, S.; Glod, J.W.; Banerjee, D. Carcinoma Associated Fibroblast Like Differentiation of Human Mesenchymal Stem Cells. *Cancer Res.* **2008**, *68*, 4331–4339. [[CrossRef](#)]
43. Mieczkowska, A.; Schumacher, A.; Filipowicz, N.; Wardowska, A.; Zieliński, M.; Madanecki, P.; Nowicka, E.; Langa, P.; Deptuła, M.; Zieliński, J.; et al. Immunophenotyping and Transcriptional Profiling of in Vitro Cultured Human Adipose Tissue Derived Stem Cells. *Sci. Rep.* **2018**, *8*, 11339. [[CrossRef](#)] [[PubMed](#)]
44. Mazini, L.; Rochette, L.; Amine, M.; Malka, G. Regenerative Capacity of Adipose Derived Stem Cells (ADSCs), Comparison with Mesenchymal Stem Cells (MSCs). *Int. J. Mol. Sci.* **2019**, *20*, 2523. [[CrossRef](#)] [[PubMed](#)]
45. Hassan, W.U.; Greiser, U.; Wang, W. Role of Adipose-Derived Stem Cells in Wound Healing. *Wound Repair Regen.* **2014**, *22*, 313–325. [[CrossRef](#)] [[PubMed](#)]
46. Milan, M.; Takla, J.; Hayek, F.; Emmanuel, N.; Nicolas, G.; Menendez, J.P. Regenerative Potential of Adipose Derived Stem Cells in Wound Healing and Scar Management: A Review. *Curr. Dermatol. Rep.* **2024**, *13*, 351–360. [[CrossRef](#)]
47. DiMarino, A.M.; Caplan, A.I.; Bonfield, T.L. Mesenchymal Stem Cells in Tissue Repair. *Front. Immunol.* **2013**, *4*, 201. [[CrossRef](#)] [[PubMed](#)]
48. Fu, X.; Liu, G.; Halim, A.; Ju, Y.; Luo, Q.; Song, G. Mesenchymal Stem Cell Migration and Tissue Repair. *Cells* **2019**, *8*, 784. [[CrossRef](#)]
49. Wang, D.; Park, J.S.; Chu, J.S.F.; Krakowski, A.; Luo, K.; Chen, D.J.; Li, S. Proteomic Profiling of Bone Marrow Mesenchymal Stem Cells upon Transforming Growth Factor Beta1 Stimulation. *J. Biol. Chem.* **2004**, *279*, 43725–43734. [[CrossRef](#)]
50. Jotzu, C.; Alt, E.; Welte, G.; Li, J.; Hennessy, B.T.; Devarajan, E.; Krishnappa, S.; Pinilla, S.; Droll, L.; Song, Y.-H. Adipose Tissue Derived Stem Cells Differentiate into Carcinoma-Associated Fibroblast-like Cells under the Influence of Tumor Derived Factors. *Cell. Oncol.* **2011**, *34*, 55–67. [[CrossRef](#)]
51. Tuxhorn, J.A.; Ayala, G.E.; Rowley, D.R. Reactive Stroma in Prostate Cancer Progression. *J. Urol.* **2001**, *166*, 2472–2483. [[CrossRef](#)]
52. Rønnev-Jessen, L.; Petersen, O.W. Induction of Alpha-Smooth Muscle Actin by Transforming Growth Factor-Beta 1 in Quiescent Human Breast Gland Fibroblasts. Implications for Myofibroblast Generation in Breast Neoplasia. *Lab. Investig. J. Tech. Methods Pathol.* **1993**, *68*, 696–707.
53. Werner, S.; Lützkendorf, J.; Müller, T.; Müller, L.P.; Posern, G. MRTF-A Controls Myofibroblastic Differentiation of Human Multipotent Stromal Cells and Their Tumour-Supporting Function in Xenograft Models. *Sci. Rep.* **2019**, *9*, 11725. [[CrossRef](#)]
54. Li, J.; Wu, Z.; Zhao, L.; Liu, Y.; Su, Y.; Gong, X.; Liu, F.; Zhang, L. The Heterogeneity of Mesenchymal Stem Cells: An Important Issue to Be Addressed in Cell Therapy. *Stem Cell Res. Ther.* **2023**, *14*, 381. [[CrossRef](#)]
55. Maloney, J.M.; Nikova, D.; Lautenschläger, F.; Clarke, E.; Langer, R.; Guck, J.; Van Vliet, K.J. Mesenchymal Stem Cell Mechanics from the Attached to the Suspended State. *Biophys. J.* **2010**, *99*, 2479–2487. [[CrossRef](#)] [[PubMed](#)]
56. Yourek, G.; Hussain, M.A.; Mao, J.J. Cytoskeletal Changes of Mesenchymal Stem Cells During Differentiation. *ASAIO J. Am. Soc. Artif. Intern. Organs* **2007**, *53*, 219–228. [[CrossRef](#)]
57. Lotfi, R.; Eisenbacher, J.; Solgi, G.; Fuchs, K.; Yildiz, T.; Nienhaus, C.; Rojewski, M.T.; Schrezenmeier, H. Human Mesenchymal Stem Cells Respond to Native but Not Oxidized Damage Associated Molecular Pattern Molecules from Necrotic (Tumor) Material. *Eur. J. Immunol.* **2011**, *41*, 2021–2028. [[CrossRef](#)] [[PubMed](#)]
58. Pistoia, V.; Raffaghello, L. Damage-Associated Molecular Patterns (DAMPs) and Mesenchymal Stem Cells: A Matter of Attraction and Excitement. *Eur. J. Immunol.* **2011**, *41*, 1828–1831. [[CrossRef](#)] [[PubMed](#)]
59. Maxson, S.; Lopez, E.A.; Yoo, D.; Danilkovitch-Miagkova, A.; LeRoux, M.A. Concise Review: Role of Mesenchymal Stem Cells in Wound Repair. *Stem Cells Transl. Med.* **2012**, *1*, 142–149. [[CrossRef](#)] [[PubMed](#)]
60. Karakasheva, T.A.; Lin, E.W.; Tang, Q.; Qiao, E.; Waldron, T.J.; Soni, M.; Klein-Szanto, A.J.; Sahu, V.; Basu, D.; Ohashi, S.; et al. IL-6 Mediates Cross-Talk between Tumor Cells and Activated Fibroblasts in the Tumor Microenvironment. *Cancer Res.* **2018**, *78*, 4957–4970. [[CrossRef](#)] [[PubMed](#)]
61. Ji, Z.; Tian, W.; Gao, W.; Zang, R.; Wang, H.; Yang, G. Cancer-Associated Fibroblast-Derived Interleukin-8 Promotes Ovarian Cancer Cell Stemness and Malignancy Through the Notch3-Mediated Signaling. *Front. Cell Dev. Biol.* **2021**, *9*, 684505. [[CrossRef](#)] [[PubMed](#)]
62. Escobar, P.; Bouclier, C.; Serret, J.; Bièche, I.; Brigitte, M.; Caicedo, A.; Sanchez, E.; Vacher, S.; Vignais, M.-L.; Bourin, P.; et al. IL-1 β Produced by Aggressive Breast Cancer Cells Is One of the Factors That Dictate Their Interactions with Mesenchymal Stem Cells through Chemokine Production. *Oncotarget* **2015**, *6*, 29034–29047. [[CrossRef](#)] [[PubMed](#)]
63. Al-toub, M.; Almusa, A.; Almajed, M.; Al-Nbaheen, M.; Kassem, M.; Aldahmash, A.; Alajez, N.M. Pleiotropic Effects of Cancer Cells' Secreted Factors on Human Stromal (Mesenchymal) Stem Cells. *Stem Cell Res. Ther.* **2013**, *4*, 114. [[CrossRef](#)] [[PubMed](#)]

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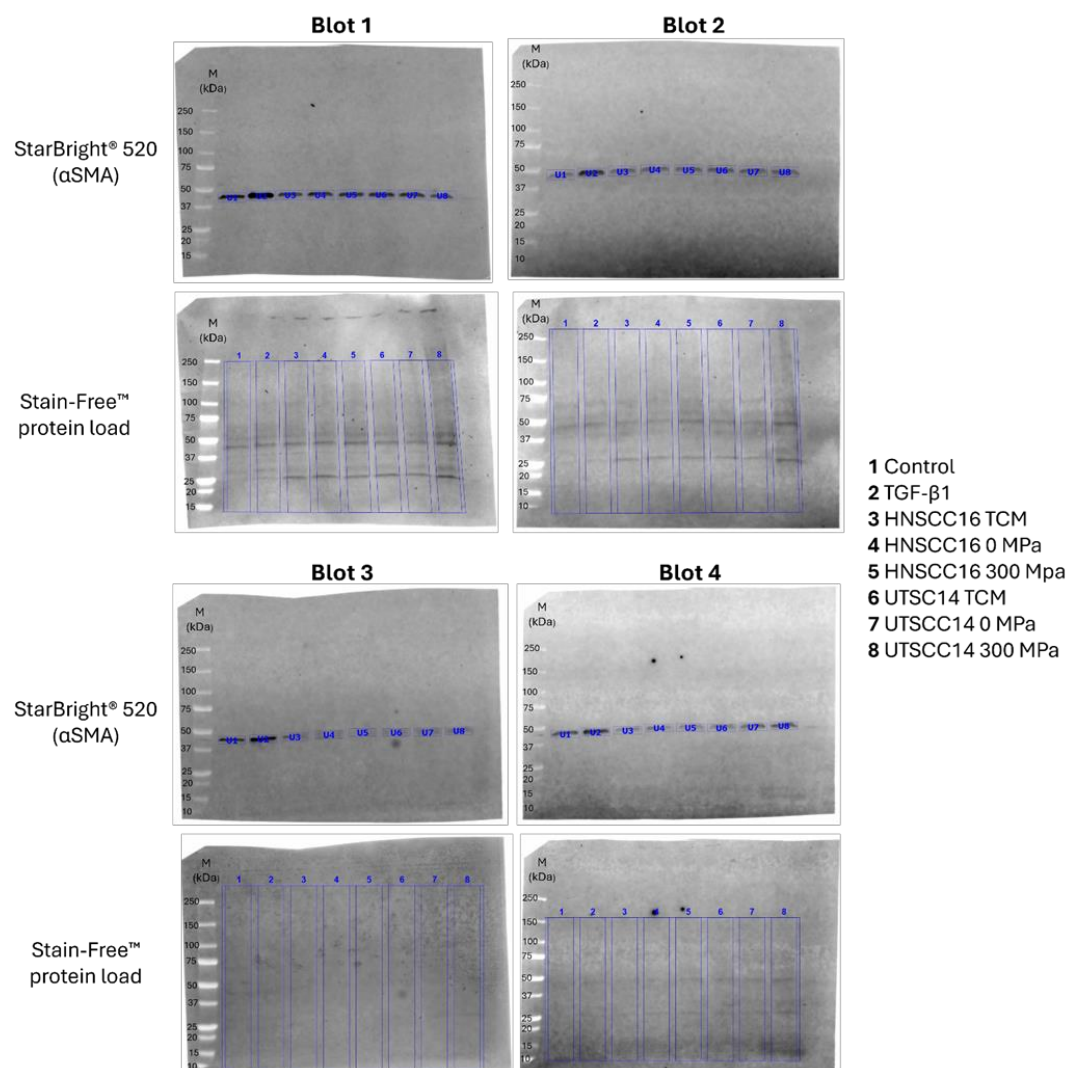
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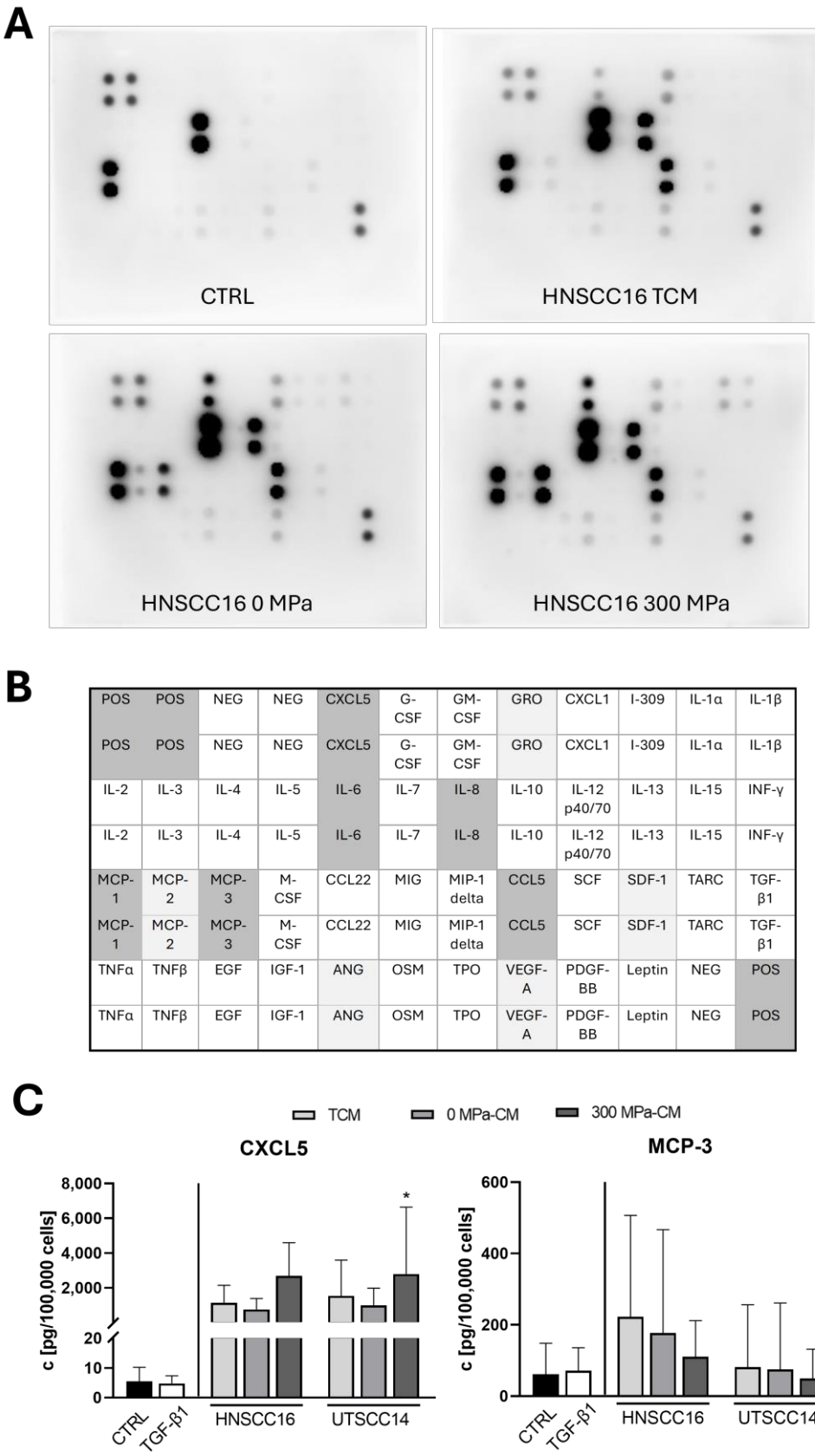
Supplementary Figure S1. Immunofluorescence analysis of α -SMA and FAP in stimulated human adipose stromal cells (hASCs). hASCs were incubated for 96 h with tumor-conditioned medium (TCM), 0 MPa-treated medium, or 300 MPa-treated medium derived from HNSCC16 or UTSCC14 cells, TGF- β 1 (10 ng/mL) or serum-free control medium respectively. Representative immunofluorescence images showing staining of cancer-associated fibroblast (CAF) markers α -SMA (red, intracellular) and FAP (green, membrane-associated) with nuclear counterstaining using Hoechst (blue). Scale bar: 100 μ m.



Supplementary Figure S2. Immunofluorescence analysis of TNC in stimulated human adipose stromal (hASCs). hASCs were incubated for 96 h with tumor-conditioned medium (TCM), 0 MPa-treated medium, or 300 MPa-treated medium derived from HNSCC16 or UTSCC14 cells, TGF-β1 (10 ng/mL) or serum-free control medium respectively. Representative immunofluorescence staining of TNC (red) and Hoechst (blue) after 96 h of stimulation. Scale bar: 100 μm.



Supplementary Figure S3. Protein quantification of α -SMA by Western blot analysis. α -SMA protein expression was determined by Western blot analysis. Western blot membranes of four donors were analyzed. Precision Plus Protein™ All Blue prestained standards served as molecular weight markers (M). Densitometric quantification of α -SMA protein expression was performed and total protein normalization was performed using Bio-Rad Stain-Free™ technology.



Supplementary Figure S4. Identification of cytokines secreted by human adipose stromal (hASCs) after incubation with conditioned media. hASCs were incubated for 96 h with tumor-conditioned medium (TCM), 0 MPa-treated medium, or 300 MPa-treated medium derived from HNSCC16 or

UTSCC14 cells, TGF- β 1 (10 ng/mL), or control medium respectively. After 48 h, the medium was replaced with fresh CM, and the supernatants from the second 48 h incubation period were collected for cytokine analysis. **A** Cytokine array (Dot blot) showing cytokine profiles of serum-free control medium, tumor-conditioned medium (TCM, HNSCC16), 0 MPa-treated CM (HNSCC16), and 300 MPa-treated CM (HNSCC16) after incubation with hASCs. **B** Reference map identifying cytokine positions on the array. **C** Quantification of selected cytokines (MCP-1, IL-6, IL-8, CCL5) in hASC supernatants was determined by multiplex bead-based assay. Values represent the mean $\pm$ SD of $n = 8$ independent donors. Cytokine concentrations measured before and after incubation with hASCs were compared, and the difference (post-pre) normalized to 100,000 cells is shown. Statistical significance was assessed using predefined post hoc comparisons following one-way ANOVA or Kruskal-Wallis testing. Differences were evaluated relative to the control group or between specific treatment conditions as indicated in the figures. Statistically significant differences are indicated by asterisks (* $p < 0.05$).

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Erklärung § 5 Abs. 1 zur Datenaufbewahrung

Hiermit erkläre ich, dass die dieser Dissertation zu Grunde liegenden Originaldaten bei mir, Julia Kristin Brach, Guaitastr. 18, 52064 Aachen, hinterlegt sind.

Erklärung gemäß § 5 Abs. (1) und (2), und § 11 Abs. (3) 12. der Promotionsordnung

Hiermit erkläre ich, **Julia Kristin Brach**, an Eides statt, dass ich den wesentlichen Anteil an der Publikation geleistet habe:

Brach, J.K., Freiin Grote, V., Strüder, D., Kalle, F., Jonitz-Heincke, A., Bader, R., Hoffmann, M., Scherzad, A., Wirth, M., Hackenberg, S.: Impact of high hydrostatic pressure on the cytokine profile and head and neck cancer cell behavior: implications for oncological safety. *Frontiers in Immunology.*; 2025, 16:1581014.

Die Anteile an der Arbeit waren wie folgt:

	Julia Kristin Brach	Vivica Freiin Grote	Daniel Strüder	Friederike Kalle	Anika Jonitz-Heincke	Rainer Bader	Marco Hoffmann	Agmal Scherzad	Markus Wirth	Stephan Hackenberg	Summe (%)
Studienüberwachung			10				30			60	100
Studien-design/ Konzeption	60						10	10		20	100
Datenerhebung	100										100
Datenauswertung	100										100
Durchführung der Experimente	100										100
Durchführung der Hochdruckbehandlung		80		20							100
Statistische Auswertung	90				10						100
Bereitstellung von Materialien			20			20			30	30	100
Interpretation der Datenauswertung	30		30				10			30	100
Verfassung des Manuskripts	100										100
Korrektur des Manuskripts		10	20	10	20		10	10		20	100

Ergänzend wurde zur sprachlichen Überarbeitung des Manuskripts das KI-basierte Sprachmodell ChatGPT-4 (OpenAI, L.L.C., San Francisco, CA, USA) verwendet.

Aus diesem wesentlichen Anteil ergibt sich selbstverständlich die Stellung als Erstautorin.

Julia Kristin Brach (Doktorandin)

Als Doktorvater und Koautor bestätige ich die Angaben von Julia Kristin Brach.

Stephan Hackenberg (Doktorvater)

Ich schließe mich der Erklärung von Stephan Hackenberg als Koautor an.

Vivica Freiin Grote

Daniel Strüder

Friederike Kalle

Anika Jonitz-Heincke

Rainer Bader

Marco Hoffmann

Agmal Scherzad

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Die Anteile an der Arbeit waren wie folgt:

	Julia Kristin Brach	Vivica Freiin Grote	Anika Jonitz-Heincke	Rainer Bader	Daniel Strüder	Marco Hoffmann	Sven Gerlach	Tim Ruhl	Justus P. Beier	Petra Fischer	Markus Wirth	Agmal Scherzad	Stephan Hackenberg	Summe (%)
Studienüberwachung						40							60	100
Studien-design/Konzeption	50											25	25	100
Datenerhebung	90									10				100
Datenauswertung	90	10												100
Durchführung der Experimente	90									10				100
Durchführung der Hochdruckbehandlung		100												100
Mikroskopie	80						20							100
Statistische Auswertung	80							20						100
Bereitstellung von Materialien				20	10			20	20		20		10	100
Interpretation der Datenauswertung	50					20		10					20	100
Verfassung des Manuskripts	100													100
Korrektur des Manuskripts		10	10	10	10	20		20					20	100

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