**Mechanism of Enhancing Chemotherapy Efficacy in Pancreatic Ductal Adenocarcinoma with Paricalcitol and Hydroxychloroquine: A Single Cell RNA Sequencing**

**Ganji Purnachandra Nagaraju1, Madhu Sudhana Saddala2, Jeremy B Foote3, Ateeq M Khaliq4, Ashiq Masood4, Yuvasri Golivi1, Dhana Sekhar Reddy Bandi1, Sujith Sarvesh1, Sudhir Putty Reddy5, Jeffrey Switchenko6, Julienne L Carstens1, Mehmet Akce1, Cameron Herting7, Olatunji B. Alese7, Karina J. Yoon8, Upender Manne9, Manoj K Bhasin6,10, Gregory B Lesinski7, Vikas P Sukhatme7,11, Bassel F. El-Rayes1\***

1. Department of Hematology and Oncology, University of Alabama at Birmingham, Birmingham, AL 35233, USA.
2. Bioinformatics, Genomics and Proteomics, University of California, Irvine, Los Angeles- 92697, USA.
3. Department of Microbiology, University of Alabama at Birmingham, Birmingham, AL 35233, USA.
4. Indiana University School of Medicine, Indianapolis, IN-46202, USA.
5. Department of Protein Sciences & Mass Spectrometry, Translational Medicine, Bristol Myers Squibb, Princeton, NJ-08543, USA.
6. Department of Biomedical Informatics, Emory University School of Medicine, Atlanta, GA-30322, USA.
7. Department of Hematology and Medical Oncology, Winship Cancer Institute, Emory University, Atlanta, GA-30322, USA.
8. Department of Pharmacology and Toxicology, University of Alabama at Birmingham, Birmingham, AL 35233, USA.
9. Department of Pathology, University of Alabama at Birmingham, AL-35233, USA.
10. Department of Pediatrics, Emory University, Atlanta, GA-30322, USA.
11. Department of Medicine, Emory University, Atlanta, GA-30322, USA.

**Running title:**

Enhancing Chemotherapy in Pancreatic Cancer

**Corresponding Author**

***Bassel F. El-Rayes, MD***

*Professor, Deputy Director of the UAB O’Neal Comprehensive Cancer Center*

*Division of Hematology & Oncology – Heersink School of Medicine*

*The University of Alabama at Birmingham*

*Birmingham, AL 35233-3300*

*E-mail: belrayes@uabmc.edu*

**Declaration of interests**

The authors declare no competing interests.

**ABSTRACT**

Pancreatic ductal adenocarcinoma (PDAC) boasts a dismal five-year survival rate of less than 15%, mainly due to therapy resistance. Recent studies have highlighted hydroxychloroquine (H) and paricalcitol (P), reducing stromal density and enhancing PDAC sensitivity to chemotherapy. This investigation aimed to elucidate the molecular impacts of combining H and P, focusing on their ability to sensitize PDAC to chemotherapy. *In vitro* and *in vivo* experiments demonstrated that the HP combination significantly (p<0.001) enhanced gemcitabine (G) effects, validated in orthotopic mouse models and patient-derived xenografts (PDX). Mechanistically, GPH induced cell death via the vitamin D receptor pathway upregulated autophagy and ER stress-related transcripts and suppressed mTOR signaling. Single-cell (sc) RNAseq analyses showed GPH increased quiescent cancer associated fibroblasts and reduced autophagy related transcripts. GPH treatment modulated T-cell populations favoring antitumor immunity. Findings from clinical trial patient biopsies underscored these effects, highlighting GPH's potential as a therapeutic adjunct in PDAC management (NCT04524702).

**INTRODUCTION**

Pancreatic ductal adenocarcinoma (PDAC) is expected to be the second leading cause of cancer mortality in the United States before 2040 (1). A significant clinical challenge in PDAC is the early occurrence of metastasis, and thus, effective systemic therapy is critical for improving survival (2,3). Unfortunately, the standard cytotoxic chemotherapy treatment regimens have only demonstrated a modest impact on survival (4). Mechanisms contributing to drug resistance include dense inflammatory stroma in the tumor microenvironment (TME) and activation of autophagy (5). The TME is characterized by low vasculature, absent or poorly activated immune cells, and dense extracellular matrix (ECM) (5). Cancer-associated fibroblasts (CAFs) are central to supporting PDAC and its TME through the production of ECM proteins, along with the secretion of cytokines and growth factors (6). The ECM, in turn, prevents tumor blood vessel formation and impairs chemotherapy transport. Thus, identifying more effective ways to target the TME in PDAC could complement existing chemotherapy or immune-based therapies and benefit patients.

Autophagy has gained attention as a biologically relevant process that can be targeted in tumors. This mechanism maintains tumor cell homeostasis by degrading cargo (pathogens, organelles, proteins, etc.) (7). Although autophagy primarily promotes cell survival under stress, it can induce apoptosis through autophagic cell death (7). Autophagy is regulated by 18 known autophagy-related (ATG) genes (7). Various environmental factors, such as stress, can trigger this cellular process by activating AMPK and blocking the mTOR pathway (8). Autophagy initiation is controlled by Beclin-1 and the ULK kinase complex, which then translocate to ATG9, an initiation site (9). PDAC cell lines and patient tumors show elevated autophagy under baseline conditions. Importantly, autophagy also plays a central role in the immune system. It indirectly impacts T-cell triggering and maturation by being involved in LC3-mediated phagocytosis (10) and contributes to CD4+ T-cell stimulation by promoting antigen presentation via MHC class I and II molecules (11). These results propose that autophagy-mediated proteins could be involved in the communication between cancer and immune cells.

Several efforts are ongoing to identify unique vulnerabilities in these cells that can enable more precise targeting. Aligned with this, previous reports demonstrate that CAFs have highly expressed vitamin D receptors (VDR) compared to other cells within the TME (12,13). Vitamin D analogs can reduce activated CAF proportions in PDAC-TME (14,15). Activating VDR reprograms CAF phenotypes, improving antitumor immune response when combined with anti-PD-1 and with chemotherapy in PDAC (16). Recent studies also revealed that VDR agonists induced autophagy in various cancers by binding to the VDR-RXR complex and regulating genes essential for cancer growth and metastasis (17,18). Paricalcitol (P), a vitamin D analog administered with G, reduced stromal activation and tumor size and increased overall survival in orthotopic mouse models of PDAC (19,20). Previous studies have also presented that elevated vitamin D levels correlate with reduced risk of metastasis and death in PDAC patients (21). Chloroquine (CQ) and its derived hydroxychloroquine (H) are quinolone-based drugs that block autophagy indirectly by preventing the fusion of autophagosomes with lysosomes (22). CQ can also have immune modulatory effects as it promotes M1 macrophages (23), while H has been shown to affect CAF activation and decrease TGFβ secretion, which delays tumor development (24). As a monotherapy, H effectively impacted tumor progression; however, when combined with gemcitabine (G) in PDAC patients, decreased CA19.9, increased LC3-II in PDAC tumor biopsy tissues, and improved progression-free survival (PFS) were noted (25,26). Finally, combining H G/nab‐paclitaxel (neoadjuvant chemotherapy) enhanced complete response rates in 41% of patients and increased biochemical and histopathologic response in PDAC patients (27).

Independently, H and P have overlapping mechanisms of action in PDAC. Both agents can impact critical resistance mechanisms, including CAFs, autophagy (H inhibiting and P inducing), and immune-suppressive TME. Preliminary preclinical and clinical data support that both P and H independently can potentiate the effects of systemic chemotherapy. Given the limited and nonoverlapping toxicities of P and H, we hypothesized the combination of P and H would dramatically impact the biology of PDAC, CAFs, and immune activation and enhance the activity of standard cytotoxic chemotherapy.

**RESULTS**

**The combination of paricalcitol and hydroxychloroquine potentiates gemcitabine's growth inhibition effects in mouse PDAC models.**

The effect of PH, G, and GPH on the short-term viability of PDAC cells was evaluated using an XTT cell proliferation assay. Human (Mia PaCa2, PANC1 **Fig. S1A and S1B**) and mouse PDAC lines (KPC-Luc and 5363, **Fig. S1C and S1D**) were treated with PH, with or without G, for 36 h, and the cell viability was assessed. The combination GPH treatment significantly (\*\*p<.001; and \*\*\*\*p<0.00001) inhibits the growth of the PDAC cell lines compared to sham or G or PH **(Fig. S1A-S1D)** in all cell lines examined. The combination of GPH inhibited the colony formation of all PDAC cell lines compared to sham or G or PH-treated PDAC cell lines (PANC-1, MIA PaCa2, KPC-Luc and 5363; **Fig. S1E-S1H**).

Next, we evaluated the impact of this combination on tumor growth *in vivo*. GPH-treated mice had a significantly lower change rate in bioluminescent signal over time than G, PH, or sham (**Fig. 1A and 1B**). The body weight of all mouse groups was stable, suggesting all treatment regimens were well-tolerated (**Fig. S2A**). End study KPC-Luc tumor weight also confirmed the potentiation of G by PH (**Fig. S2B)**. Similar findings were reported in another KPC-Luc cell line, 5363, where GPH-treated mice showed significantly (p<0.001) lower tumor weights and sizes than other treatment groups (**Fig. S2C**). We also tested the clinically relevant regimens of gemcitabine and paclitaxel (GnP) combined with P, H, or both on KPC-Luc subcutaneous xenografts. GnP-PH treatment significantly reduced the tumor volume and weight compared to GnP-H or, GnP-P or sham (**Fig. 1C and 1D; Fig. S2D**). The body weight of all mouse groups was stable, suggesting all treatment regimens were well-tolerated (**Fig. S2E**).

The efficacy of GPH treatment was assessed in a pancreaticobiliary patient-derived xenograft (PDX). GPH treatment significantly reduced the PDX tumor size and weight (**Fig. 1E and 1F**) to a greater extent than G or the combination of P and H. Similar results were also observed in other KRAS mutant G12D PDX subcutaneous models (**Fig. 1G and 1H; Fig. S2F)**. No significant variation was perceived in the body weight of each group of animals, again indicating that this combination was well tolerated (**Fig. 2G and 2H)**. Finally, we evaluate the effects of GPH on the survival of mice implanted orthotopically with the KPC-Luc cell line. A significant improvement in survival was observed in favor of GPH compared to sham, G, GH, or GP-treated animals (**Fig. 1I and** **Fig. S3A-S3E)**. No significant variation was perceived in the body weight of each group of animals, again indicating that this combination was well tolerated.

**Combination therapy with GPH increases autophagy in mouse and human PDAC cell lines.**

Next, we evaluated the effect of the combination (GPH) compared to PH vs. single agent G treatment on autophagy in human and mouse PDAC cell lines. *In vitro* treatment with this combination in mouse (KPC-Luc) and human PDAC cell lines (MIA PaCa2) induced upregulation of the autophagy-related proteins BECN-1, Atg 7, LC3A/B, and vitamin D receptor (**Fig. 2A**). Similar effects were seen in the *in vivo* assessment of autophagy in the KPC-Luc PDAC model by western blotting for autophagy-related proteins LC3A/B, ATG5, and ATG7. The combination of GPH enhanced the expression of these autophagy proteins (**Fig. 2B**). Furthermore, autophagosomes identified by the expression of autophagy marker LC3A/B were noted using confocal microscopy in mouse and human PDAC cell lines (**Fig. S4A-S4B**) and by transmission electron microscopy (TEM) where autophagosomes exhibited a double-layer organelle having cargo (**Fig. 2C-2H**). Protein expression and imaging revealed that PH treatment also induced autophagosomes compared to sham and G. Still, this process was considerably enhanced with the combination of all three treatments (**Fig. 2**). Overall, GPH treatment acted synergistically compared to G or PH in promoting the formation of autophagosomes.

**The combination of GPH induces upregulation of autophagy and ER stress-related pathways in tumor cells through VDR-dependent pathways.**

The above findings indicate that the combination of GPH significantly delays tumor growth via the stimulation of autophagy. To better understand this process, KPC-Luc orthotopic tumors were enzymatically disassociated into single cells, using a 10X Genomics platform for single-cell RNA sequencing (scRNAseq). After quality control, cells were identified using known cell-type biomarkers from the literature (**Fig. 3A and 3B**). Using the t-SNE plot, we identified 0-26 clusters and annotated cell types. The seven most notable cell types were PDAC, B cells, endothelial cells, T cells, fibroblast cells, macrophages, and NK cells (**Fig. 3A and 3B**). t-SNE plot analysis revealed that the proportions and numbers (**Fig. 3C**) of PDAC and other immune cells decreased significantly in GPH-treated tumors compared to other treatments (**Fig. 3D).**

Given the robust impact on autophagy-related pathways in PDAC cells *in vitro* and *in vivo*, we first explored the effect of GPH on autophagy-related pathway gene expression in PDAC tumor cells by single-cell RNAseq (scRNAseq). Interestingly, gene expression analysis demonstrated that PDAC clusters (all PDAC, the eighth and ninth clusters individually) also showed higher expression of autophagy gene markers (Ambra1, Ulk1, Rb1cc1, Atgs, Pik3C3, Pik3r4, Becn1, Uvrag, Nrbf2, Nbr1, Sqstm1, Ins1 & 2, Casp8, Prkaa1, Fadd, Senp3, Ulk3, Clip1, Cd74), and reduced expression of Gabarap, Gabarapl2, and Pik3cg in GPH treated KPC-Luc tumor-bearing mice compared to PH, G, and sham (**Fig. 4A to 4C**). Quantitative proteomics and heatmap analysis data reveal that most of the autophagy markers (Atg 3, 4, 5, 7, Becn1, Ins1, 2, GABARAPL1, FADD, SENP3, MHC class2) upregulated GPH treated compared to sham or G or PH groups (**Fig. 4D**). *In vitro* experiments (western blot, Electron microscopy, and immunofluorescence data) also confirmed these results. As indicated in **Fig. 2**, treating KPC-Luc and MIA PaCa-2 with G alone did not enhance autophagy; these observations are consistent with proteomics and *in vitro* studies **(Fig. 3D, 3G, 3H, and 3I)**.

Insulin receptor expression on PDAC tumors modulates cellular growth (28) through MAPK, PI3K, and mTOR signaling pathways. In our study, GPH treatment caused an increased abundance of ER stress-related mRNAs (**Fig. 4E**), in addition to Ins1, Ins2, and AMPK (PRKAA1) mRNA and protein (**Fig. 4C and 4D**) when compared to sham, G, and the combination of PH. We discovered that a high-confidence interaction of gene–a gene-gene (nodes) network of 32 genes is associated with autophagy (**Fig. 4F**). Nodes are connected via edges, indicating functional association with this autophagy and its associated genes. The VDR-RXR complex regulates autophagy markers at the transcripts level and mediates autophagy through insulin signaling. Furthermore, VDR is connected to AMPK (PRKAA1) through insulin genes. Insulin genes are the connecting rod between VDR and autophagy genes. Yellow node borders indicate the gene differentially expressed in KPC-Luc -tumors.

The above findings indicate a role for paricalcitol (P)-VDR signaling in amplifying both the autophagocytotic process and ER stress, leading to PDAC cell death *in vivo*. To confirm the mechanistic importance of P-VDR signaling in amplifying autophagy, we performed genetic knockdown of VDR in PDAC cells and assessed the effects of GPH in PANC-1, MIA PaCa2, and murine KPC-Luc cell lines. siRNA knockdown of VDR expression revealed that downregulating VDR abrogated the autophagic impact of the combination of GPH on PDAC cell lines (**Fig. 4G and 4I**). These observations suggest that autophagy is mediated through VDR signalling.

**The combination of GPH modulates the abundance and phenotypes of CAFs**

CAFs, known for their pivotal role in tumor progression and chemoresistance, were the focus of our study. scRNA-Seq data analysis revealed five distinct subclusters of CAFs, including quiescent (qCAF), myofibroblastic (myCAF), inflammatory (iCAF), proliferative (pCAF), and antigen-present (apCAF) CAF subsets (**Fig. 5B; Table S1**). GPH treatment was found to decrease the percentage of iCAF, myCAF, apCAF, and pCAF, and increase the percentage of qCAF (an inactive form of CAF) (**Fig. 5C and 5D**). GPH treatment also led to a decrease of more than 90% of canonical mRNA markers used to subset CAF-associated markers compared to G, PH, or sham (**Fig. 5E**). Immunohistochemistry analysis suggested that GPH treatment reduced α-SMA in KPC-Luc tumor tissue compared to sham, G, and PH treated animals (**Fig. 5F**).

qCAFs (inactive form; **Fig. 5A**) showed low expression of ER stress and autophagy markers. Furthermore, GPH treatment also inhibited the expression of ER stress and autophagy-associated markers compared to G, PH, or sham (**Fig. 5G and 5H; Fig. S5B-S5F**). scRNAseq data indicate that GPH treatment alters the phenotypes and abundance of CAFs in the KPC-Luc-TME, which may increase the diffusion of chemotherapeutics into the tumor.

**The combination of GPH alters innate immune cells' proportion and activation status in the KPC-Luc-TME.**

scRNA sequence analysis of the myeloid compartment revealed the presence of macrophage, monocytes, granulocytes, and dendritic cell subsets (**Fig. 6A and 6B**). GPH treatment increased the percentage of monocytes, granulocytes, and M1 polarization (**Fig. 6C and 6D**). To correlate single cell mRNA expression patterns with protein and cellular abundance, KPC-Luc tumors were digested to generate single cell suspensions, and multiparameter flow cytometry was executed to identify and enumerate the relative abundance of myeloid cell residents in the KPC-Luc TME. Multiparameter flow cytometry studies revealed that G or GPH increased the number of macrophages (Gated on Live/Dead Aqualo CD45+ CD11b+ F4/80+) and decreased the abundance of granulocytes (Gated on Live/Dead Aqualo CD45+ CD11b+ Ly6C+ Ly6G+) and cDC1 (Gated on Live/Dead Aqualo CD45+ CD11b- MHCII+ XCR1+) subsets in the KPC-Luc-TME using an established gating methodology (**Fig. 6E; Fig. S6A-S6H; Table S2**). Furthermore, each treatment had a different impact on myeloid cell phenotypes with G or PH or GPH inducing upregulation of mRNA for M1 phenotypic markers (MHCII, CD80, and CD86) and downregulation of mRNA for M2 phenotypic markers (Arg1, C1q, Mrc1) suggestive of M1 polarization (**Fig. 6F**). Conversely, in sham and PH treatments, there was a predominant expression of mRNAs (Arg1, C1q, Mrc1) associated with an M2 phenotype (**Fig. 6F**) and comparable upregulation of MHCII and downregulation of CD206 on other myeloid subsets (**Fig. 6G**). This is consistent with proteomic analyses indicating an upregulation of MHCII proteins and downregulation of CD206 protein expression on myeloid cell subsets isolated from the KPC-Luc TME (**Fig. 6G**) as assessed by proteomic and flow cytometric expression. Overall, these findings suggested that combining GPH induces the repolarization of myeloid cells to a pro-inflammatory phenotype (M1, N1, mature DC).

**The combination of GPH modulates the proportions and activation status of T cells in the KPC-Luc-TME.**

Next, using established gating schemes, we assessed the proportions of T and NK cell subsets by multiparameter flow cytometry (Figures S7 and S8). The combination of GPH-activated myeloid cell subsets to a pro-inflammatory phenotype capable of supporting cytotoxic T and NK cell responses in KPC-Luc-TME. T cell sub-clustering revealed diverse CD4, CD8, and NK cell populations (**Fig. 6H-6J**). GPH treatment increased the percentage of CD4+ and CD8+ T-cells and reduced the CD4+ and CD8+ Tregs (**Fig. 6J**). Multiparameter flow cytometric analysis revealed an increase in proportions of CD4+ Foxp3+, CD4+ Foxp3-, CD3+ NK1.1+, CD3- NK1.1+(NK), CD3+ TCRγδ+ and CD3+ CD4/CD8/NK1.1/ TCR γδ- (DN T) cell subsets in G and GPH treated KPC-Luc tumors (**Fig.** **6K**). The scRNA sequence analysis demonstrated that GPH treatment increased CD4, CTLA4, and CD3e and decreased FoxP3, Lag3, Granzyme B, and Tnfrsf4 (OX40) mRNA (**Fig. 6L and 6M**). GPH treatment had no significant effect on CD8. The proportions of CD3+CD8+ T cells did not significantly change in G- and GPH-treated KPC-Luc-tumors (**Fig. 6L**). These findings agree with the scRNAseq data concerning CD4 and innate-like NK and NKT cells, where mRNA for signature proteins that identify these subsets was increased. Furthermore, in agreement with scRNAseq, expression of the protein CTLA-4 was increased in CD4+ and CD8+ T cell subsets (**Fig. 6N and 6O**) whereas, protein expression of PD-1 was upregulated in CD4+Foxp3+, CD4+Foxp3- and CD8+ T cells with the combination of G with PH (**Fig. 6N and 6O**). Likewise, the PD-L1 expression increased in the macrophage population (**Fig. 6G**). Evaluation of CD4+ and CD8+ T cell activation through expression of CD44, CD62L, and CD69 revealed an increase in CD44+CD62L- effector memory and a corresponding increase in CD44-CD62L- pre-effector-like T cells (**Fig. S9**), which have been demonstrated to be an essential pool of T cells that are capable of replenishing effector memory T cells and capable of responses to immune checkpoint blockade therapy (31). Interestingly, there was a small but statistically insignificant increase in CD44hiCD62Lhi central memory T cells in CD4+ and CD8+ T cell populations in mice treated with the combination of GPH (**Fig. S9**), suggesting that this combination may improve memory T cell responses.

The above information indicates that G or the combination of GPH activates T cells in the KPC-Luc-TME. To determine whether this combination enhanced T cell function, single cells from tumors were stimulated *in vitro* with PMA and ionomycin in the presence of brefeldin A for 5 hours. Then, intracellular expression of granzyme B, IL-2, IL-17, IFNγ, and TNFα was assessed in CD4+ and CD8+ T cells (**Fig. S8A-S8D).** Cytokine production increased in CD4+ (**Fig. S10A-S10E**) and CD8+ T cells (**Fig. S10F-S10J**) taken from G or GPH-treated KPC-Luc tumors. In our study, GPH treatment caused an increased abundance of mRNAs Tbx21, Prf1 and IFNγ compared to sham, G, and the combination of PH (**Fig. 6L**). These scRNA and FLOW findings indicate that this combination enhances cytotoxic T-cell activities.

**PDAC patients**

The study was activated in September 2020. The COVID-19 pandemic, however, prompted a lower-than-expected enrollment rate, and the study was terminated prematurely. Twelve patients with metastatic pancreatic cancer were enrolled between October 2020 and November 2022. Patient characteristics are summarized in **table S3**. Two patients did not receive study medications and were withdrawn from the study. Three additional patients had clinical progression of disease after receiving one dose of study drug but before the radiographic assessment for treatment response. Among the 7 patients evaluated, 3 demonstrated partial radiographic responses in their measured target lesions, while 4 patients achieved stable disease. The calculated objective ORR was 43% (90% CI, 0.1 - 0.82). **Fig. 7A** illustrates the maximum percentage change in the size of target lesions from baseline. Median PFS was 6 months (95% CI, 1.4 to 6.7 months), as depicted in **Fig. 7B**. The 3- and 6-month progression-free survival rates were 80.0% (95% CI, 40.9% to 94.6%) and 50.0% (95% CI, 18.4% to 75.3%), respectively. Overall survival (OS) had a median of 6.8 months (95% CI, 1.8 to 9.9 months), detailed in **Fig. 7B** and **7C**. The 3- and 6-month overall survival rates were 70.0% (95% CI, 32.9% to 89.2%) and 20.0% (95% CI, 3.1% to 47.5%), respectively.

Regarding safety, drug-related grade 3 AEs and SAEs occurred in 60% (6 out of 10) of patients included in the safety analysis, as outlined in **table S4**. The most frequently reported adverse effects, in descending order of occurrence, included nausea, anemia, thrombocytopenia, vomiting, fatigue, constipation, hypercalcemia, dyspnea, neutropenia, leukopenia, and diarrhea.

**scRNA-seq in PDAC patients**

Core biopsies of metastasized PDAC in the liver were dissociated using GentleMACs dissociation kit (Miltenyi Biotec) according to manufacturer instructions and viably frozen. For preliminary analysis scRNA-Seq library was prepared using NextGem 3’V3.1 kit. The single-cell transcriptome profiles generated from pre-treatment (1537 cells) and post-treatment (367 cells) human liver biopsy samples from one patient identified PDAC clusters as well as T cells and endothelial cells (**Fig. S11A**). The top genes in PDAC clusters (P1-P4, P5), T cells and endothelial cells are shown in the heat map (**Fig. S11B**). The post-treatment sample showed a 50% reduction in PDAC cells (Figure 7D), with most PDAC clusters (P1-P3, P5) decreasing. Cluster P4 comprises most PDAC cells in the post-treatment needle biopsy sample. Of the two T cell clusters, T cell-2 is primarily present in the post-treatment sample and is a CD8+ cytotoxic T cell expressing various cytokines genes, including *Ifnɣ* and *Ccl20* (**Fig. 7F**). Further analysis of the T cell clusters showed that T cell-1 is a mix of CD4+ and CD8+ T cells with more expression of T cell activation marker genes, *Cd69* and *CD44* (**Fig. 7E**). T cell-2 also showed enhanced expression of *Cd69* and *Cd44* and T cell activation maintenance marker genes, *Tnfrsf4/9,* in treated samples. Analysis of exhaustion marker genes showed enhanced expression of various exhaustion marker genes in T cell-2 (*Lag3, Pdcd1, Cd160*) and T cell-1 (*Vsir, Icos, Lag3*) in the treated samples indicative of active T cell response in the metastasized TME, post-treatment.

Cells were annotated as done previously for mouse and human tumor samples. Differences in immune response following treatment between systemic sites and TME have been reported. Biopsy samples exhibited more significant expression of T cell activation marker genes (*Cd69*) and inflammatory cytokines genes (*Ifng, Ccl5*), especially the T cell-2 cluster that is present in the post-treatment biopsy sample (**Fig. 7F**).

**PDAC spatial analysis**

Tissue sections from liver biopsies were taken and assessed using an AKOYA bioscience immunofluorescence assay before and after receiving GPH therapy. Paired samples were available from two patients (labeled A and D), while a third patient (labeled C) could only be assessed at baseline.

In H&E-stained samples, PDAC metastases are visibly distinct from normal tissue by duct-like formations in the tissue and cells with enlarged nuclei (**Data not shown**). Desmoplastic stroma surrounds the metastases, denoted by decellularized fibrotic regions. Normal, fibrotic, and metastatic regions form distinct areas in samples A and D, while sample C primarily consists of desmoplastic stroma with some ductal formations.

Immunofluorescence imaging confirms the fibrotic remodeling around PDAC metastases. The ductal formations strongly stain for pan-cytokeratin (PCK) and E-cadherin, consistent with PDAC (**Fig. 7G**). Fibrotic regions lack E-cadherin expression entirely, unlike normal and metastatic tissue, but stain strongly for Collagen-IV. Assessing immune markers found that these fibrotic regions contained large T cells (**Fig. 7G**), including CD4+ and CD8+ T cells. T cells show poor infiltration into the dense, PCK-high regions, primarily accumulating in this desmoplastic stroma.

Individual cells were segmented based on their nuclear stain and annotated based on marker expression into ‘Normal,’ ‘Fibrotic,’ ‘Tumor,’ ‘CD8+ T cell’, or ‘CD4+ T cell’ (**Fig. 7H**). Neighborhood spatial analysis was performed on these populations to assess the likelihood of cells of a given type being found near one another. Tumor regions show little association with all populations besides other tumor cells, indicating the tendency for the metastases to form dense tumor nests (Figure 7I). CD4 and CD8 T cells positively associate with other T cells and fibrotic regions while displaying shallow associations with normal tissue and tumor, confirming their tendency to accumulate in the desmoplastic stroma (**Fig. 7I**).

Following GPH therapy, a few notable ductal formations appeared in H&E stained samples A and C. However, some appear in the upper portion of sample D. Sample A shows some fibrotic remodeling towards the lower portion of the sample but otherwise seems to be primarily normal tissue. Sample C still shows extensive desmoplasia, similar to the baseline. Sample D does not display transparent regions of desmoplastic stroma.

Immunofluorescence imaging did identify some small PCK+ regions in sample A and the lower portion of sample D following therapy despite the lack of precise ductal formations (**Fig. 7J**). These tumor regions are surrounded by fibrotic stroma with T cell infiltration, as seen at baseline (**Fig. 7J**). However, post-treated samples A and D generally have less fibrotic remodeling than their baseline (**Fig. 7K**). Adjacency analysis of annotated cell regions post-therapy confirms again the tendency for T cells to accumulate in these fibrotic regions and a generally higher likelihood that T cells will be found adjacency to PCK+ regions, possibly indicating improved tumor accessibility due to the reduced desmoplastic remodeling, and typically reduced tumor presence (**Fig. 8L, M**).

**DISCUSSION**

This study provides unique mechanistic insight into the collective effects of vitamin D analog paricalcitol (P), hydroxychloroquine (H), and the chemotherapeutic agent gemcitabine (G) in accentuating VDR signaling, leading to ER stress-related autophagic tumor cell death. In this study, adding PH treatment gemcitabine or gemcitabine and paclitaxel significantly decreased PDAC proliferation in multiple *in vitro* and in vivo PDAC models compared to chemotherapy or the doublet (HP) treatments.

The efficacy of this regimen in the clinical trial is hard to establish due to early termination of the trial. The overall response rates from the small number of patients enrolled appear at least comparable to historic rates reported with chemotherapy. It is reassuring that no added toxicities were associated with adding PH to gemcitabine and nab-paclitaxel. Adding PH to gemcitabine resulted in several effects in the preclinical models, including increased autophagy in tumor cells. This led to a global reduction in CAF populations with the remaining CAFs adopting an iCAF phenotype. Our data highlight a mechanism of promoting autophagy by GPH involving AMPK (PRKAA1), a key checkpoint for the cell's energy source, which stimulates autophagy through phosphorylation of the ULK1 (ATG1; autophagy activating kinase 1) (32). GPH treatment activates AMPK phosphorylation in PDAC. AMPK also inhibits cell growth and intermediary metabolism by inhibiting the mTOR signalling (over TSC2 activation) (33). The ULK1 complex has an autophagy-initiating role. Upon activation for autophagy initiation, ATG1 binds with the ATG17, a FAK family kinase, and Atg 101 (34). This complex activates and increases the expression of class III PtdIns3K complex (AMBRA1, PIK3C3/VPS34, PIK3R4/VPS15, BECN1, ATG14 (UVRAG), and NRBF2 (35). The ATG16L1-ATG12 increase the phagophore–ATG5 complex, and Atg8-class proteins, GABARAP and LC3 subclasses, are allied to PE employing them to the phagophore membrane. The coupling of LC3-I to PE affects the production of LC3-II in a manner that contributes to ATG4B and its assistance with ATG3 and ATG7. LC3-II relates to the complete autophagosome (35). NBR1 and SQSTM1/p62 are receptors for cargo, and they interact with ATG8-class proteins on the phagophore membrane (35). Our data proves that GPH elevated the ATG1, ATG17, and Atg 101 expressions and increased the Beclin-1 expression and activation of the class-III PI3K complex. Atg5-Atg12-Atg16 complex lipidated LC3 helps the generation of autophagosomes, which engulf the protein aggregates and dysfunctional organelles (34). Autophagosomes merge with the lysosomes to generate autophagolysosomes. In our study, GPH treatment showed elevated expression of LC3A/B and Atg5 and increased autophagic puncta (autophagosomes and their fusion with lysosomes). Further, our results also showed that GPH significantly enhanced the fluorescence of LC3A/B, as assessed by confocal microscopy.

Autophagy provides a complementary mechanism for cell death in PDAC with defective apoptosis (36) as autophagic pathways are critical in retaining the β cells necessary for insulin production and glucose homeostasis. Indeed, studies indicate impairments in autophagy correlate with the reduction of β cells developing an insulin deficit, thereby causing diabetes (37). Interestingly, our findings demonstrate that GPH increased Ins1 and 2 in PDAC tumors. Overall, these novel findings demonstrate the critical role of insulin-driven energy metabolism in both β cell and PDAC cell survival, ensuring the ability to support the acquisition of glucose necessary for tumor cell survival. Autophagy behaves as a dual function (double-edged sword), and targeting it may affect the metabolic regulation of insulin-glucose levels in patients with PDAC.

Autophagy is also essential in regulating the biology of complex CAF populations in PDAC. CAFs are heterogeneous and can be classified into distinct sub-populations such as quiescent CAFs (qCAFs), myofibroblastic CAFs (myCAFs), proliferative CAFs (pCAFs) inflammatory CAFs (iCAFs), and antigen-presenting CAFs (apCAFs), among others (29,30). CAFs in the TME produce pro-inflammatory factors, growth factors, ECM components, and high-energy nutrients essential for survival, metastasis, resistance, and drug delivery interference cues to PDAC cells (38,39). Autophagy in the CAFs fuels tumor growth and metastasis (40). Hydroxychloroquine or other lysosome inhibitors may restore the myCAFs by inhibiting their hyper-autophagy state (41). In PDAC preclinical models, inhibiting VDR by P shifts CAFs toward a quiescent phenotype with decreased tumor progression and enhances chemotherapy delivery (42). Our gene expression data suggest that GPH treatment decreased the apCAF, pCAFs, iCAF, and myCAFs-associated markers (Hilpda, VEGFa, Higd1b, Cenpf, Pttg1, Stmn1, Cfd, and Postn, etc.,) and autophagy associated markers (ATGs, Becn1, etc.,) compared to G, PH, or sham.

The immune TME of PDAC is predominantly composed of myeloid cell subsets and smaller proportions of regulatory T and B cells, which exclude and suppress cytotoxic T cell responses to tumor cells (43). In particular, M2-polarized macrophage and myeloid-derived suppressor cells comprise a considerable fraction of the intratumoral leukocytes in animal models and patients with PDAC (44,45). Recent findings indicate that neo-adjuvant chemotherapies involving G with nab-paclitaxel or FOLFIRINOX promote anti-tumor immunity in part through M1 macrophage polarization and enhancing infiltration of T cells into the PDAC TME (46,47). Of importance to this study is that VDR signaling also inhibits B cell activation (48)VDR signaling has positive and negative effects on innate cell activation, including inhibiting neutrophil activation but promoting NETosis (49,50). Our data indicate the combination of GPH increased M1 macrophage polarization and T-cell Infiltration into the KPC-Luc-TME but did not significantly affect MHCII expression. Aligned with these macrophage changes, we also assessed integrin-associated protein (CD47), an immune checkpoint for macrophages that plays an essential function in immune avoidance in many malignancies, including PDAC (51,52). Low expression of CD47 was observed in GPH-treated PDAC tissue compared to all other treatments. CD14/TLR4 priming possibly recalibrates and shows an anti-tumor effect in TAM (53). GPH treatment increased the expression of CD14 compared to sham and PH. Similar effects were observed in the paired biopsy samples from our patients on the trial, with an increase in M1 macrophages and a decrease in M2.

Several cellular activities are controlled by neutrophil (CD177 marker) recruitment and are involved in tumor lesions and tumorigenesis (54). CD177 is a neutrophil marker and was found to be decreased in GPH-treated tumors. CD276 (B7-H3) is an immune checkpoint that is over-expressed in malignant cells and stimulates cancer-infiltrating immune cells (55). CD276 also enhances malignant cells to avoid the NK and cytotoxic T-cells (55). CD276 was reduced in GPH-treated tissues compared to PH and sham groups. It elevated IL-35-producing B-regulatory cells with increased CD8+ T cell infiltration. In PDAC, B-cell-mediated production of IL-35 induces tumor progression by reducing the CD8+ T cell infiltration via downregulating the chemotactic receptors (CXCR3 and CCR5) and reducing the secretion of the effector cytokine IFN-g (56). However, GPH treatment increases the expression of PD-1 and CTLA4 on T cells, suggesting robust activation of CD4 and CD8 T cells in the KPC-Luc murine model. Paired biopsies from the PDAC patients on trial and animal models show that combination therapy increases T-cells and exhaustion markers such as PD-1, Lag3, and CTLA-4. These data from preclinical and clinical samples suggest that proper sequencing of the GPH treatment with anti-PD-1, anti-CTLA-4, or both ICB may enhance the therapeutic outcomes of this regimen.

Considerable evidence suggests the beneficial translational impact of either P or H with standard-of-care chemotherapy (25,57,58). This study demonstrates the benefit of combining PH with chemotherapy in PDAC. The underlying mechanisms include the upregulation of autophagy and cellular stress pathways that culminate with the death of tumor cells. A significant impact is demonstrated by the combination of GPH on the TME, including a favorable change in the phenotype of CAF and macrophages. We observed, in animal models and patient samples, an increase in T cells and shift in macrophage phenotypes, indicating the potential of this regimen to convert these immunologically “cold” tumors to hot. A remaining challenge is the increase in the expression of exhaustion makers, which suggests the need to combine this regimen with immune checkpoint inhibitors. Our results support exploring this combination in pre-clinical and clinical studies of GPH with immune therapy.

**METHODS**

**Cell line authentication and pathogen testing**

Purchased and gifted cell lines were routinely authenticated according to ATCC guidelines. Additionally, all cell lines were tested for a comprehensive panel of pathogens, including mycoplasma, prior to in vivo investigations, in accordance with the University of Alabama IACUC and ARP procedures.

**Cell proliferation assay**

Cell viability under both treated and untreated conditions was quantified using the XTT assay (Cat # 30-1011K, ATCC, Manassas, VA, USA). PDAC human (Panc-1, RRID:CVCL\_0480, and MIA PaCa2, RRID:CVCL\_0428, ATCC, Manassas, VA, USA) and mouse cell lines (KPC-Luc, RRID:CVCL\_UY60, a gift from Dr Craig Logsdon, MD Anderson Cancer Center; 5363, a gift from Chris Klug’s lab, University of Alabama) were seeded into a 96-well plate at a 7.5x103 cells/100μl density in each well. Cell confluence of 70% was attained after 16 hours, followed by cell line treatment with drugs hydroxychloroquine (H; Cat. No. H1126, Portland, OR, USA), paricalcitol (P, Cat. No. 1499414 USP, Millipore Sigma, Burlington, MA, United States), and gemcitabine (G; Cat. No. A433642-5g, AM Beed, Arlington Hts, IL, USA) to a final concentration of 25μM, 300nM, and 7.5nM, respectively and incubated at 370C incubator for 36hrs. Subsequently, the cells were given 40ul of XTT reagent in each well and incubated at 370C for 2 hours. Using a Bio-Tek 800TS absorbance microplate reader, the optical density (OD) was determined at 450 and 660 nanometers. The OD at 660nm was subtracted from the OD at 450nm, and the average at 450nm was calculated. The experiment was performed in triplicates, and the viability percentage is shown relative to sham cells.

**Clonogenic assay**

The colony-developing ability of the cells was assessed by the clonogenic test in both the treated and untreated PDAC cell lines. Initially, 500 cells (KPC-Luc, 5363, PANC, and MIA PaCa2) were plated in triplicate wells of a 6-well plate and kept alive at 37 **°**C in 5% CO2 overnight. H (25μM) plus P (300nM), without G (7.5nM), were administered in each cell line for 36 h. After 36 h, old media was substituted with fresh complete media every three days, and cells were grown for about two weeks in the incubator. The colonies were fixed and stained with 0.5% crystal violet in 20% methanol. Each colony (containing <50 cells) was considered and counted manually for each cell line. The experiment was performed in triplicate, and the survival percentage is shown relative to sham cells. The average number of colonies in sham triplicate wells was 1%.

**Immunocytochemistry**

KPC-Luc, 5363, and PANC-1 (1 X 104) cells were seeded and allowed to grow overnight on an 8-well chamber plate. The cell lines were exposed to final concentrations of P (300nM), H (25μM), and G (7.5nM) for 36h. The cells were fixed with 4% buffered formalin for 30 mins in RT, followed by permeabilization using 0.25% Triton-X-100 for 15 mins. The cells were blocked with 1% BSA for one hour at RT to obstruct non-specific binding. The cells were exposed to rabbit anti-LC3A/B (1:1000, Cat # 12741 (D3U4C) Cell Signaling) for 2 hours at RT, followed by washing with 1x PBST (3X). The cell lines were then exposed to goat anti–rabbit (Alexa Fluor 568; RRID:AB\_10563566, 1:1500, Invitrogen, Carlsbad, CA) secondary antibody for one hour at RT, followed by washing with 1x PBST (3X). Next, the cells were mounted using Prolong Gold antifade with DAPI (Invitrogen) and visualized using ECHO Revolution. Data was analyzed using a Java-based image processing program (NIH, LOCI, University of Wisconsin).

**Electron microscopy**

Hydroxychloroquine (H; 25μM) plus paricalcitol (P, 300nM), with or without gemcitabine (G, 7.5nM), were administered to each cell line for 36 h. After 36 h, cell lines were collected using trypsin and washed (2X) with 1×PBS (ice-cold). Cell lines (MIA PaCa2, PANC-1, and KPC-Luc) were resuspended in 1XPBS: fixative agent (2.5% glutaraldehyde plus 2% paraformaldehyde in 100mM cacodylate solution; pH 7.0). After washing with PBS, the cells post-fixed in osmium tetroxide plus potassium ferrocyanide (2%; Sigma-Aldrich) for 60 min at 4°C. Then, the fixed cells were placed in an ethanol gradient (50 to 100% Sigma-Aldrich) for 5 minutes. The cells were mounted in an embedding medium (Embed-812). Sections (60 nm) were obtained horizontally by ultramicrotome (Leica, Germany) and mounted on slot grids with a sample support film. The mounted slides were dual stained with uranyl acetate (2% Sigma-Aldrich) for 15 minutes and then citrated for 5 minutes. The specimens were examined using a Hitachi H-7500 electron microscope operated at 75 kV at Robert P. Apkarian Integrated Electron Microscopy Core facility in Emory University.

**Stable cell line preparations**

ShRNAs targeting Vitamin D receptor (VDR) and NS shRNAs (pLKO.1; RRID:Addgene\_10878) of the mouse (TRC ID # N0000027104) and human (TRC ID # N0000019504) specific shRNAs were obtained from Sigma Aldrich. Lentiviral particles transmitting shRNA were produced by co-transfecting packaging plasmids pMD2.G (RRID:Addgene\_12259) and pSPAX2 (RRID:Addgene\_12260) with shRNA plasmids into HEK293T cells (RRID:CVCL\_0063) by effectene (Cat. # 301425, Qiagen). The culture medium was filtered with a 0.45µm sterile filter to remove dead/live cells from lentiviral particles. Stable cells were made by infecting PDAC cell lines (KPC-Luc, PANC-1, and MIA PaCa2) by adding puromycin to a final concentration of 0.5μg/ml (KPC-Luc, MIA PaCa2), 0.75μg/ml (PANC-1) and 3.5μg/ml (5363).

**Immunoblot analysis**

Treated and non-treated cell lines were lysed using RIPA (containing phosphor-protease inhibitors) buffer. 30 µg of the protein sample was resolved with 4-20% of precast gels (BIO-RAD, Cat # 4568096) for polyacrylamide gel electrophoresis (PAGE) and blotted to Polyvinyl difluoride (PVDF) membrane (iBlot 3 PVDF Regular stacks Cat No: IB34001) using iBlot dry transfer technology (Invitrogen). The membrane was fixed with methanol and 2% BSA buffer blocking. The membrane was probed with primary antibody (Atg5 Rabbit mAb RRID:AB\_2630393 (D5F5U); Atg7 Rabbit mAb RRID:AB\_10831194 (D12B11); Beclin-1 Rabbit mAb RRID:AB\_1903911 (D40C5); LC3A/B Rabbit mAb RRID:AB\_2728823 (D3U4C) and Vitamin D3 Receptor Rabbit mAb RRID:AB\_2637002 (D2K6W) (1:1000 dilution) incubated, followed by HRP-conjugated secondary antibody (Anti-mouse, RRID:AB\_330924; Anti-rabbit, RRID:AB\_2099233) and incubation. Later, the membrane was developed with a chemiluminescent substrate (Cat # 34579). The results of the bands were evaluated using LI-COR Odyssey XF. The image J (RRID:SCR\_003070) program quantified the band intensity.

***In vivo* experiments**

The University of Alabama Institutional Animal Care and Use Committee (IACUC) approved all animal studies in this manuscript. For tumor efficacy in 5–6-week-old C57BL/6J (Strain #:000664; RRID: IMSR\_JAX:000664; Jackson Labs) male mice were used. 2×105 KPC-Luc cells (RRID:CVCL\_UY60) (59) in 30ul 1:1 1XPBS and Matrigel (Cat # 354263, Discovery Labware, Inc., Bedford, MA, USA) were injected into the central section of the pancreas (orthotropic) and separated into four groups (each group n=5). Five days after injection, establishment of KPC-Luc tumors was verified via bioluminescent images in mice, and treatment was initiated. The first group received PBS and served as a sham; the second group received G (60mg/kg; weekly twice); the third group received P (0.3μg/kg; weekly twice) plus H (60 mg/kg; oral daily for fourteen days), and the fourth group received GPH. On days 5 and 20 following treatment, tumor development was evaluated by the IVIS system (bioluminescent imaging). The experiment ended on day 21 when mice were exposed to CO2 for euthanization as per IACUC instructions. KPC-Luc tumors were collected, weighed, preserved, and processed for further biomarker, proteomics, and single-cell RNA analysis.

The second set of experiments were performed in subcutaneous tumor models. The KPC-Luc cells (0.5×106) were injected subcutaneously into the C57BL/6J male mice. The first group received PBS and served as a sham; the second group received GnP-H (G, 60mg/kg (I.P.); nab-paclitaxel (nP), 10mg/kg (i.v) weekly once; H (60 mg/kg; oral daily); the third group received GnP-P (P, 0.3μg/kg; weekly twice), and the fourth group received GnP-PH for four weeks. Tumour volumes were measured weekly, once with the help of digital callipers. Tumor volume was determined using the formula v=LXW2/2. Terminal tumor weight of treated and untreated mice was noted.

The third set of experiments was performed using orthotopic pancreatic tumor models similar to that described above in the first set of experiments to assess overall survival as the endpoint. This study's treatment was given until the final observation week, excluding death or morbidity. This study was terminated at day 53 and survival estimates were calculated via Kaplan-Meier (using Logrank test (Mantel-Cox) survival curves from the time of implanting KPC-Luc cells in the pancreas for each group.

The fourth set of experiments was performed used a PDX model. For PDX tumor efficacy in 6–7-week-old NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (Strain # 005557; RRID: IMSR\_JAX:005557; Jackson Labs) male mice were used. G12V mutated pancreaticobiliary-PDX (freshly acquired from an 81-year male patient with a previous history of PDAC; MPDX0028; Gly12Val; (60)) cell suspension (equal to 10 mg) was injected into the pancreas of NSG mice. After two weeks, the animals were randomized into four groups (each group n=6). Treatment was given for two weeks, as indicated above. The experiment ended on day 19, when mice were exposed to CO2 for euthanization as per IACUC instructions. Tumors were collected and weighed, and body weight was measured.

For the fifth set of experiments, 10 mg PDAC-PDX fragments (KrasGly12D; (61)) immersed lightly in Matrigel were implanted subcutaneously and separated into four groups (each group n=6) when the tumor reached nearly 100 mm3. Treatment was given the above-indicated doses for 33 days, and the digital calipers evaluated tumor development. Tumor volumes were measured weekly once with digital calipers, and PDX volume was determined using the formula v=LXW2/2. The experiment ended on day 33, when mice were exposed to CO2 for euthanization as per IACUC instructions. Tumors were collected and weighed, and body weight was measured.

**Single-cell tumor processing**

Portions of the tumor tissues (~1g of tumor obtained in total from three animals of each group) were collected in a labeled C-tube with 5 ml of digesting media to isolate single cells from the tumor tissues. Digestion media is DMEM with 1% FBS, 1.5mg/ml collagenase D, 2000 U DNase I, and 25μg/ml of liberase. Tumors were manually excised into small pieces with sterile scissors into a C-tube containing digestion media. Tumors were dissociated twice on each tube using a gentle MACS dissociator followed by incubation at 370C for 15 mins while constantly shaking. The cell suspension (Miltenyi Biotec) was passed through a 100µm cell strainer into a 50 ml conical tube. The cell suspension was washed with DPBS and spun at 1700rpm (575g) for 5 mins. After several washes with DPBS, the cells were resuspended in an appropriate media for downstream analysis.

Viable tumor single cells were employed to produce scRNA-Seq libraries using a standard 10X genomics approach. After the filter, single-cell transcriptome results of 9705 cells from sham, 14840 cells from PH, 12715 cells from G, and 2520 cells from GPH-treated mice were obtained.

**Preprocessing, clustering, sub-clustering, and cell type annotation**

scRNA-seq raw data was demultiplexed, aligned to the mouse reference genome (mm10) for further quantification of gene expression, and preprocessed by cell ranger 6.1 with default setups. The cell ranger output count matrix files were further analyzed and visualized with Seurat R package v.4.3.0 (RRID: SCR\_016341). We filtered as follows as a criteria nFeature\_RNA (nGene) >= 250 & nCount\_RNA (nUMI) <30000 and percent.mt <15 (15% mitochondrial genes). A global scaling method for single-cell normalization was used after filtering unwanted cells and mitochondrial genes to normalize data by Log Normalize. Then we calculated 4000 highly variable features for further analysis, such as scaling the data, linear dimensional reduction (LDR), non-LDR, and clustering the cells by PCA, t-SNE and UMAP with default parameters.

We used the first 30 PCs (principal components) with a 0.5 resolution to create 27 clusters using the FindClusters method. These created clusters were further annotated with the default method of FindAllMarkers used for each cluster's differentially expressed genes (DEGs) with positive and negative markers and Wilcoxon rank-sum test. Another way we used to confirm the clusters with well-known canonical gene markers such as T cells (Cd3d, Cd3e, Cd3g), B cells (Cd79b, Ms4a1, Mzb1), Natural killer (NK) cells (Nkg7, Il2rb), Macrophage (Cd14, Ms4a7, Cd68, C1qc), Endothelial cells (Cdh5, Cldn5, Pecam1), Fibroblasts (Col1a1, Lum, Pdgfra), and PDAC (Krt18, Krt19, Mmp7). Finally, we assigned each cluster with their respective cell type names properly. Further, we were interested in a subset of the T cells, macrophages, and fibroblast cells, respectively. The cell types of interest were subsetted and then normalized, highly variable genes were identified, the data was scaled, and dimensional reduction analysis was applied using the Seurat R package. As mentioned above, we used well-known canonical gene markers for T cells, macrophages, and fibroblast subtypes. Using the Cytoscape tool, we constructed the gene-gene interaction network for autophagy genes with default parameters (<https://cytoscape.org>; RRID # SCR\_003032).

**Flow cytometry analysis**

ACK lysis buffer was used to remove red blood cells from the media containing single cells. Cells were then washed with FACS buffer and blocked using an Fc blocking agent. Tumor single cells were labeled with primary fluorophore-conjugated antibodies and a live/dead stain (**Table S1**) for 60 min on ice, washed with PBS, and re-suspended in 2% BSA in PBS. After the wash, a single cell was stained for surface antigens in the dark at room temperature (RT). Leukocytes in the cell suspension were stimulated with BD leukocyte activation cocktail and BD GolgiPlug (Catalog no. BDB550583). Intracellular staining was performed following permeabilization using an eBioscience™ Foxp3 kit (catalog no. 00-5523-00) in the dark. Cells were then fixed using 2% Paraformaldehyde and filtered to remove debris before data acquisition. Flow cytometry (Symphony A5) was analyzed using the BD FACSymphony A5 cell Analyzer and FLOWJO software (version 10.7.2.; RRID: SCR\_008520; Becton Dickinson).

**Proteomics and sample preparation**

Each sample was homogenized with a lysis buffer containing protease and phosphatase inhibitor cocktail (Pierce). The BCA (bicinchoninic acid) method quantified the protein concentration. Dithiothreitol (DTT; 5 mM) and iodoacetamide (IAA; 10 mM) were added to each lysate (100ug) for 30 minutes. Followed by the lysates were digested with lysyl endopeptidase (1:25 (w/w); Wako) for 16 h. The lysates were further digested with 50 mM NH4HCO3 (<2M urea) and trypsin (1:25 (w/w); Thermofisher Scientific) at 25°C for 16h. Then, the peptide samples were desalinized with a Sep-Pak C18 column (Waters) and vacuumed to remove wetness.

**Tandem Mass Tag (TMT) labeling**

Peptide labeling utilized the TMTPro isobaric tags method (Thermofisher Scientific, A44520 Lot# UK297033). The labeling reagents were brought to room temperature, and 200 μL of acetonitrile was added to each channel, followed by vortexing for 3 minutes. Next, 20 μL of each reagent was added to the peptide samples and incubated at room temperature for 60 minutes. The reaction was stopped using 5% hydroxylamine (Pierce). Subsequently, all 16 channels were dried using a SpeedVac concentrator and then diluted with 1 mL of trifluoroacetic acid (TFA). The samples were acidified to 1% formic acid (FA) and 0.1% TFA. Desalination was performed using a 60 mg HLB plate (Waters), and the eluates were evaporated to dryness.

**Fractionation in high pH conditions**

Dried samples were reconstituted in a loading buffer (0.07% NH4OH, 0.045% FA, 2% CAN; high pH) and filled onto a Water’s BEH (2.1mm x 150 mm with 1.7 µm beads), and fractionation carried out by a UPLC system (Thermo Vanquish). Each eluted fraction was collected in a gradient manner for 25 min (0.6 mL/min flow rate; 48 fractions) and dried with vacuum centrifugation.

**Liquid chromatography-tandem mass spectrometry**

All dried fractions were evaluated in a gradient manner (30 fractions per day) on the Evosep One system with a capillary column (15 cm, 75 μm i.d.) with C18 beads (1.9 μm; Dr. Maisch, Ammerbuch, Germany). Mass spectral analysis was conducted via Q-Exactive Plus (events:10 MS/MS per run limit). Each run contained one complete MS scan tracked by many events (10 MS/MS). Each MS scan was collected at 70,000 resolutions. Each HCD-MS/MS spectrum was obtained at 35,000 resolutions.

**Data processing**

Sequest HT obtained all raw data files with Proteome Discoverer software (Thermo's, version:2.4.1.15; RRID # SCR\_014477). Each spectrum was examined using a database (mouse UniProt; August 2020; 91413 target sequences). Razor (i.e., parsimonious) and unique peptides were counted for protein quantification. We analyzed gene ontology by g: Profiler ([https://biit.cs.ut.ee/gprofiler](about:blank); RRID:SCR\_006809). The tool offered a structured query option that incorporates ranking during enrichment tests. The process involves iterative testing, beginning with the first gene and progressively adding genes in sequence. For each term, it reports the smallest enrichment p-value alongside the size of the corresponding gene list.

**Bias reduction strategies**

Blinding in this context means that the personnel handling the downstream analyses were not aware of certain key details that could influence their interpretation of the results. This is done to minimize bias and ensure that the analysis is objective and not swayed by expectations or preconceived notions. By keeping the analysts blinded, the investigation aims to produce more reliable and unbiased results.

**Human studies**

**Institutional review board (IRB) statement**

The study was conducted in accordance with the U.S. Common Rule ethical principles and received approval and oversight from the Emory University Institutional Review Board (Phase II trial, IRB Protocol ID # Winship5079-20; Atlanta, GA). All participants provided written informed consent prior to any research-related procedures. Additionally, the study is registered at www.clinicaltrials.gov (NCT ID # NCT04524702).

**Patients and attrition**

Eligible participants, the heart of our study, were adults aged 18 years or older diagnosed with metastatic pancreatic adenocarcinoma with measurable disease as per RECIST version 1.1 criteria. They were required to have an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0-2 or a Karnofsky score of at least 60%. Before initiating study treatment, patients needed to demonstrate adequate organ and bone marrow function, assessed within four weeks, meeting specific criteria: hemoglobin levels ≥ 9.0 g/dL with no recent blood transfusions, absolute neutrophil count ≥ 1.5 x 109/L without recent growth factor support, platelet count ≥ 100 x 109/L, total bilirubin ≤ 1.5 x upper limit of normal (ULN), AST (SGOT) and ALT (SGPT) ≤ 5 x ULN, serum creatinine ≤ 1.5 x ULN or creatinine clearance ≥ 60 mL/min/1.73 m2 for patients with higher creatinine levels. Patients were excluded if they had known G6PD lack, porphyria, severe psoriasis, severe diabetic retinopathy, or macular degeneration due to likely heightened toxicity risks associated with HCQ. Additionally, individuals with pre-existing hypercalcemia or a recent history (within six months) of HCQ (aminoquinolines) or Paricalcitol use were not eligible. Upon enrollment and consent, patients were required to discontinue any vitamin D or calcium-containing supplements to mitigate the risk of hypercalcemia during the study period.

Patients in the study received paricalcitol at a dose of 25 mcg intravenously (i.v.) three times weekly, and hydroxychloroquine dosing began with 400 mg orally for one week, followed by 600 mg twice daily. Gemcitabine was administered I.V. at 1000 mg/m2 over 30 minutes and nab paclitaxel at 100 mg/m2 on Days 1, 8, and 15 of each 28-day cycle. Treatment continued until there was clinical or radiographic evidence of disease progression, withdrawal due to unacceptable side effects, patient preference, or recommendation from the physician.

Patients who met eligibility criteria but did not contribute to efficacy assessments were classified as non-evaluable. This classification was applied when data on the primary endpoint were unavailable due to premature study departure before undergoing post-treatment radiographic evaluation and clinical response assessment. These patients' data were included in safety analyses but excluded from efficacy assessments.

**Study endpoints**

The primary endpoint focused on the investigator-assessed objective response rate (ORR), which measured complete (CR) and partial responses (PR) based on RECIST version 1.1 criteria. Monitoring of cancer antigen (CA) 19-9 protein levels did not occur routinely. Secondary endpoints included progression-free survival (PFS), overall survival (OS), and safety. PFS spanned from treatment initiation to clinical or radiographic progression or death, with censoring at the last progression-free clinical evaluation. OS measured from treatment initiation to death or last follow-up.

Radiographic assessments and clinical evaluations occurred 8- and 16-weeks post-treatment initiation, followed by evaluations every eight weeks while patients remained on therapy. Independent imaging review did not take place. Serious adverse events (SAEs) and treatment-related adverse events (AEs) graded 3 to 5 were reported following CTCAE version 4.0.

For biopsies of metastasized pancreatic ductal adenocarcinoma (PDAC) in the liver, samples underwent dissociation using the GentleMACs dissociation kit (Miltenyi Biotec) as per manufacturer instructions, followed by viably freezing. Single-cell RNA sequencing utilized the NextGem 3’V3.1 kit (10x Genomics) for preliminary analysis, distinguishing PDAC clusters alongside T cells and endothelial cells from pre-treatment (1537 cells) and post-treatment (367 cells) samples of patient D.

**Statistical considerations**

The statistical methodology employed Simon's optimal two-stage Minimax design, hypothesizing an ORR of 23% under null versus 45% under alternative hypotheses, with power and alpha set at 80% and 10%, respectively. Stage I required enrollment of 15 patients, closing if fewer than four achieved objective responses or expanding to stage II if warranted. The null hypothesis was rejected with at least eight objective responses, with primary endpoint summarization adjusted per the study's two-stage design and 90% confidence intervals. Kaplan-Meier curves estimated PFS and OS distributions and safety analyses encompassed all treated patients.

**PDAC Biopsies Immunofluorescence Assay**

The spatial composition of PDAC metastases was assessed using an AKOYA bioscience CODEX immunofluorescence assay. Using the CodexMAV plugin for Image J, individual cells were segmented based on their DAPI expression. A Voronoi diagram was computed for each cell to establish cell area and the average fluorescent intensity of each marker in this region to generate per-cell expression profiles. These cells were clustered using X-shift clustering using the CodexMAV plugin, and clusters were annotated based on per-cell average marker expression. Clusters, such as T cells, were manually refined by gating for canonical marker expression, such as CD4 vs CD8 expression. Clustering was performed independently on the baseline and post-treated samples, which were imaged in different rounds. Post-treated sample A contained an artifact from a fiber overlapping part of the visible region, which displayed autofluorescence for a subset of the stains. Regions from this artifact were manually marked and omitted from any downstream analysis.

**Statistical analysis**

One way ANOVA, followed by Tukey’s test for pairwise tests (GraphPad Prism 10.0.2; RRID # SCR\_002798), was run for various group comparisons of means. If the parametric test assumptions were not met, non-parametric test like Kruskal-Wallis (for three or more groups). Two-way ANOVA was considered for mean comparisons across two factors: treatment and phenotype. Results were shown as mean ± SD. Or SE. Survival was estimated using the Kaplan-Meier method, and survival distributions were compared using the log-rank test (R v. 4.3.2). A statistically significant deviation was deemed at p<0.05. All tests were two-sided unless otherwise noted. The sample number for the power of 0.8 at α value of 0.05 was determined using G-power 3.1.9 (RRID:SCR\_013726).

**Data Accessibility**. The data (scRNA-seq raw) produced in this manuscript are presented in Zenodo (<https://doi.org/10.5281/zenodo.13145221>; <https://doi.org/10.5281/zenodo.13145487>; <https://doi.org/10.5281/zenodo.13145761> and Jupyter notebooks documenting the computational pipeline are available on GitHub (https://github.com/dbetel/PDAC\_Singhal). The proteomics raw data produced in this manuscript are presented in SynID: syn61942767   DOI: <https://doi.org/10.7303/syn61942767>. All other raw data are available upon request from the corresponding author.

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**Author contributions**

G.P.N., G.B.L., V.P.S., and B.E., conceived the project and supervised the project. G.P.N., M.S.S., M.K.B., J.B.F., A.M.K., Y.G., D.S.R.B., S.S., S.P.R., J.S., C.H., and O.B.A., performed the experiments and analysis. G.P.N., M.S.S., J.B.F., O.B.A., M.K.B., and B.E., wrote the manuscript with input from A.M., J.L.C., M.A., K.J.Y., U.M., G.B.L., V.P.S., and B.E.

**REFERENCES**

1. Rahib L, Wehner MR, Matrisian LM, Nead KT. Estimated projection of US cancer incidence and death to 2040. JAMA network open **2021**;4(4):e214708-e.

2. Siegel RL, Miller KD, Wagle NS, Jemal A. Cancer statistics, 2023. CA: a cancer journal for clinicians **2023**;73(1):17-48.

3. Jiang Y, Sohal DP. Pancreatic adenocarcinoma management. JCO Oncology Practice **2023**;19(1):19-32.

4. Di Costanzo F, Di Costanzo F, Antonuzzo L, Mazza E, Giommoni E. Optimizing First-Line Chemotherapy in Metastatic Pancreatic Cancer: Efficacy of FOLFIRINOX versus Nab-Paclitaxel plus Gemcitabine. Cancers **2023**;15(2):416.

5. Sherman MH, Beatty GL. Tumor microenvironment in pancreatic cancer pathogenesis and therapeutic resistance. Annual Review of Pathology: Mechanisms of Disease **2023**;18:123-48.

6. Luong T, Golivi Y, Nagaraju GP, El-Rayes B. Fibroblast heterogeneity in pancreatic ductal adenocarcinoma: Perspectives in immunotherapy. Cytokine & Growth Factor Reviews **2022**.

7. Singh SS, Vats S, Chia AY-Q, Tan TZ, Deng S, Ong MS*, et al.* Dual role of autophagy in hallmarks of cancer. Oncogene **2018**;37(9):1142-58.

8. Singh BN, Kumar D, Shankar S, Srivastava RK. Rottlerin induces autophagy which leads to apoptotic cell death through inhibition of PI3K/Akt/mTOR pathway in human pancreatic cancer stem cells. Biochemical pharmacology **2012**;84(9):1154-63.

9. Zachari M, Ganley IG. The mammalian ULK1 complex and autophagy initiation. Essays in biochemistry **2017**;61(6):585-96.

10. Cui B, Lin H, Yu J, Yu J, Hu Z. Autophagy and the immune response. Autophagy: Biology and Diseases: Basic Science **2019**:595-634.

11. Münz C. Autophagy beyond intracellular MHC class II antigen presentation. Trends in immunology **2016**;37(11):755-63.

12. Zhao Z-x, Zhang Y-Q, Sun H, Chen Z-Q, Chang J-j, Wang X*, et al.* Calcipotriol abrogates cancer-associated fibroblast-derived IL-8-mediated oxaliplatin resistance in gastric cancer cells via blocking PI3K/Akt signaling. Acta Pharmacologica Sinica **2023**;44(1):178-88.

13. Liu X, Iovanna J, Santofimia-Castaño P. Stroma-targeting strategies in pancreatic cancer: a double-edged sword. Journal of Physiology and Biochemistry **2022**:1-10.

14. Yu Y, Schuck K, Friess H, Kong B. Targeting aggressive fibroblasts to enhance the treatment of pancreatic cancer. Expert Opinion on Therapeutic Targets **2021**;25(1):5-13.

15. Gorchs L, Ahmed S, Mayer C, Knauf A, Fernández Moro C, Svensson M*, et al.* The vitamin D analogue calcipotriol promotes an anti-tumorigenic phenotype of human pancreatic CAFs but reduces T cell mediated immunity. Scientific reports **2020**;10(1):1-15.

16. Kong W, Liu Z, Sun M, Liu H, Kong C, Ma J*, et al.* Synergistic autophagy blockade and VDR signaling activation enhance stellate cell reprogramming in pancreatic ductal adenocarcinoma. Cancer Letters **2022**;539:215718.

17. Tavera-Mendoza LE, Westerling T, Libby E, Marusyk A, Cato L, Cassani R*, et al.* Vitamin D receptor regulates autophagy in the normal mammary gland and in luminal breast cancer cells. Proceedings of the National Academy of Sciences **2017**;114(11):E2186-E94.

18. Suares A, Tapia C, González-Pardo V. VDR agonists down regulate PI3K/Akt/mTOR axis and trigger autophagy in Kaposi's sarcoma cells. Heliyon **2019**;5(8):e02367.

19. Kanat Ö, Ertaş H. Shattering the castle walls: Anti-stromal therapy for pancreatic cancer. **2018**.

20. Schwartz GG, Eads D, Naczki C, Northrup S, Chen T, Koumenis C. 19-nor-1α, 25-Dihydroxyvitamin D2 (Paricalcitol) inhibits the proliferation of human pancreatic cancer cells in vitro and in vivo. Cancer biology & therapy **2008**;7(3):430-6.

21. Mukai Y, Yamada D, Eguchi H, Iwagami Y, Asaoka T, Noda T*, et al.* Vitamin D supplementation is a promising therapy for pancreatic ductal adenocarcinoma in conjunction with current chemoradiation therapy. Annals of surgical oncology **2018**;25:1868-79.

22. Abdel-Aziz AK, Saadeldin MK, Salem AH, Ibrahim SA, Shouman S, Abdel-Naim AB*, et al.* A Critical Review of Chloroquine and Hydroxychloroquine as Potential Adjuvant Agents for Treating People with Cancer. Future Pharmacology **2022**;2(4):431-43.

23. Chen D, Xie J, Fiskesund R, Dong W, Liang X, Lv J*, et al.* Chloroquine modulates antitumor immune response by resetting tumor-associated macrophages toward M1 phenotype. Nature communications **2018**;9(1):873.

24. Principe DR, Timbers KE, Atia LG, Koch RM, Rana A. TGFβ signaling in the pancreatic tumor microenvironment. Cancers **2021**;13(20):5086.

25. AlMasri SS, Zenati MS, Desilva A, Nassour I, Boone BA, Singhi AD*, et al.* Encouraging long‐term survival following autophagy inhibition using neoadjuvant hydroxychloroquine and gemcitabine for high‐risk patients with resectable pancreatic carcinoma. Cancer Medicine **2021**;10(20):7233-41.

26. Fei N, Wen S, Ramanathan R, Hogg ME, Zureikat AH, Lotze MT*, et al.* SMAD4 loss is associated with response to neoadjuvant chemotherapy plus hydroxychloroquine in patients with pancreatic adenocarcinoma. Clinical and translational science **2021**;14(5):1822-9.

27. Karasic TB, O’Hara MH, Loaiza-Bonilla A, Reiss KA, Teitelbaum UR, Borazanci E*, et al.* Effect of gemcitabine and nab-paclitaxel with or without hydroxychloroquine on patients with advanced pancreatic cancer: a phase 2 randomized clinical trial. JAMA oncology **2019**;5(7):993-8.

28. Deng J, Guo Y, Du J, Gu J, Kong L, Tao B*, et al.* The intricate crosstalk between insulin and pancreatic ductal adenocarcinoma: a review from clinical to molecular. Frontiers in Cell and Developmental Biology **2022**;10:844028.

29. Elyada E, Bolisetty M, Laise P, Flynn WF, Courtois ET, Burkhart RA*, et al.* Cross-species single-cell analysis of pancreatic ductal adenocarcinoma reveals antigen-presenting cancer-associated fibroblasts. Cancer discovery **2019**;9(8):1102-23.

30. Ma C, Yang C, Peng A, Sun T, Ji X, Mi J*, et al.* Pan-cancer spatially resolved single-cell analysis reveals the crosstalk between cancer-associated fibroblasts and tumor microenvironment. Molecular Cancer **2023**;22(1):170.

31. Nakajima Y, Chamoto K, Oura T, Honjo T. Critical role of the CD44lowCD62Llow CD8+ T cell subset in restoring antitumor immunity in aged mice. Proceedings of the National Academy of Sciences **2021**;118(23):e2103730118.

32. Xu C, Wang Y, Tu Q, Zhang Z, Chen M, Mwangi J*, et al.* Targeting surface nucleolin induces autophagy-dependent cell death in pancreatic cancer via AMPK activation. Oncogene **2019**;38(11):1832-44.

33. Xavier CB, Marchetti KR, Castria TB, Jardim DL, Fernandes GS. Trametinib and Hydroxychloroquine (HCQ) combination treatment in KRAS-mutated advanced pancreatic adenocarcinoma: detailed description of two cases. Journal of Gastrointestinal Cancer **2021**;52:374-80.

34. Cao W, Li J, Yang K, Cao D. An overview of autophagy: Mechanism, regulation and research progress. Bulletin du cancer **2021**;108(3):304-22.

35. Li X, He S, Ma B. Autophagy and autophagy-related proteins in cancer. Molecular cancer **2020**;19(1):1-16.

36. Modi S, Kir D, Banerjee S, Saluja A. Control of apoptosis in treatment and biology of pancreatic cancer. Journal of cellular biochemistry **2016**;117(2):279-88.

37. Vivot K, Pasquier A, Goginashvili A, Ricci R. Breaking bad and breaking good: β-cell autophagy pathways in diabetes. Journal of molecular biology **2020**;432(5):1494-513.

38. Luong T, Golivi Y, Nagaraju GP, El-Rayes BF. Fibroblast heterogeneity in pancreatic ductal adenocarcinoma: Perspectives in immunotherapy. Cytokine & Growth Factor Reviews **2022**;68:107-15.

39. Jacobetz MA, Chan DS, Neesse A, Bapiro TE, Cook N, Frese KK*, et al.* Hyaluronan impairs vascular function and drug delivery in a mouse model of pancreatic cancer. Gut **2013**;62(1):112-20.

40. Thuwajit C, Ferraresi A, Titone R, Thuwajit P, Isidoro C. The metabolic cross‐talk between epithelial cancer cells and stromal fibroblasts in ovarian cancer progression: Autophagy plays a role. Medicinal Research Reviews **2018**;38(4):1235-54.

41. Chi M-S, Lee C-Y, Huang S-C, Yang K-L, Ko H-L, Chen Y-K*, et al.* Double autophagy modulators reduce 2-deoxyglucose uptake in sarcoma patients. Oncotarget **2015**;6(30):29808.

42. Sherman MH, Ruth TY, Engle DD, Ding N, Atkins AR, Tiriac H*, et al.* Vitamin D receptor-mediated stromal reprogramming suppresses pancreatitis and enhances pancreatic cancer therapy. Cell **2014**;159(1):80-93.

43. Zhu Y-H, Zheng J-H, Jia Q-Y, Duan Z-H, Yao H-F, Yang J*, et al.* Immunosuppression, immune escape, and immunotherapy in pancreatic cancer: focused on the tumor microenvironment. Cellular Oncology **2023**;46(1):17-48.

44. Karamitopoulou E. Tumour microenvironment of pancreatic cancer: immune landscape is dictated by molecular and histopathological features. British journal of cancer **2019**;121(1):5-14.

45. Bayne LJ, Beatty GL, Jhala N, Clark CE, Rhim AD, Stanger BZ*, et al.* Tumor-derived granulocyte-macrophage colony-stimulating factor regulates myeloid inflammation and T cell immunity in pancreatic cancer. Cancer cell **2012**;21(6):822-35.

46. Peng H, James CA, Cullinan DR, Hogg GD, Mudd JL, Zuo C*, et al.* Neoadjuvant FOLFIRINOX therapy is associated with increased effector T cells and reduced suppressor cells in patients with pancreatic cancer. Clinical Cancer Research **2021**;27(24):6761-71.

47. Sams L, Kruger S, Heinemann V, Bararia D, Haebe S, Alig S*, et al.* Alterations in regulatory T cells and immune checkpoint molecules in pancreatic cancer patients receiving FOLFIRINOX or gemcitabine plus nab-paclitaxel. Clinical and Translational Oncology **2021**;23:2394-401.

48. Pawlik A, Anisiewicz A, Filip-Psurska B, Nowak M, Turlej E, Trynda J*, et al.* Calcitriol and its analogs establish the immunosuppressive microenvironment that drives metastasis in 4T1 mouse mammary gland cancer. International Journal of Molecular Sciences **2018**;19(7):2116.

49. Hirsch D, Archer FE, Joshi-Kale M, Vetrano AM, Weinberger B. Decreased anti-inflammatory responses to vitamin D in neonatal neutrophils. Mediators of inflammation **2011**;2011.

50. Agraz-Cibrian JM, Giraldo DM, Urcuqui-Inchima S. 1, 25-Dihydroxyvitamin D3 induces formation of neutrophil extracellular trap-like structures and modulates the transcription of genes whose products are neutrophil extracellular trap-associated proteins: A pilot study. Steroids **2019**;141:14-22.

51. Liu X, Kwon H, Li Z, Fu Y-x. Is CD47 an innate immune checkpoint for tumor evasion? Journal of hematology & oncology **2017**;10:1-7.

52. Alausa A, Lawal KA, Babatunde OA, Obiwulu E, Oladokun OC, Fadahunsi OS*, et al.* Overcoming immunotherapeutic resistance in PDAC: SIRPα-CD47 blockade. Pharmacological Research **2022**;181:106264.

53. Prakash H, Nadella V, Singh S, Schmitz-Winnenthal H. CD14/TLR4 priming potentially recalibrates and exerts anti-tumor efficacy in tumor associated macrophages in a mouse model of pancreatic carcinoma. Scientific reports **2016**;6(1):31490.

54. Jin L, Kim HS, Shi J. Neutrophil in the pancreatic tumor microenvironment. Biomolecules **2021**;11(8):1170.

55. Burugu S, Dancsok AR, Nielsen TO. Emerging targets in cancer immunotherapy. 2018. Elsevier. p 39-52.

56. Cinier J, Hubert M, Besson L, Di Roio A, Rodriguez C, Lombardi V*, et al.* Recruitment and Expansion of Tregs Cells in the Tumor Environment—How to Target Them? Cancers **2021**;13(8):1850.

57. Zeh HJ, Bahary N, Boone BA, Singhi AD, Miller-Ocuin JL, Normolle DP*, et al.* A randomized phase II preoperative study of autophagy inhibition with high-dose hydroxychloroquine and gemcitabine/nab-paclitaxel in pancreatic cancer patients. Clinical Cancer Research **2020**;26(13):3126-34.

58. Bigelsen S. Evidence-based complementary treatment of pancreatic cancer: A review of adjunct therapies including paricalcitol, hydroxychloroquine, intravenous vitamin C, statins, metformin, curcumin, and aspirin. Cancer Management and Research **2018**:2003-18.

59. Horvat NK, Karpovsky I, Phillips M, Wyatt MM, Hall MA, Herting CJ*, et al.* Clinically relevant orthotopic pancreatic cancer models for adoptive T cell transfer therapy. Journal for Immunotherapy of Cancer **2024**;12(1).

60. Foote JB, Mattox TE, Keeton AB, Chen X, Smith FT, Berry KL*, et al.* A Novel Pan-RAS Inhibitor with a Unique Mechanism of Action Blocks Tumor Growth in Mouse Models of GI Cancer. bioRxiv **2024**:2023.05.17.541233 doi 10.1101/2023.05.17.541233.

61. Miller AL, Garcia PL, Gamblin TL, Vance RB, Yoon KJ. Development of gemcitabine-resistant patient-derived xenograft models of pancreatic ductal adenocarcinoma. Cancer Drug Resistance **2020**;3(3):572.

**Figure Legends**

**Figure 1.** **The combination of gemcitabine (G) with paricalcitol (P) and hydroxychloroquine (H) enhances growth arrest of human and mouse PDAC cell lines *in vivo*.**

**(A-B)** KPC-Luc cells (1×105) were injected into the central section of the pancreas (orthotropic) of C57BL/6JJJ male mice. The first group received PBS and served as a sham; the second group received G (60mg/kg; weekly twice); the third group received P (0.3μg/kg; weekly twice) plus H (60 mg/kg; oral daily for fourteen days), and the fourth group received GPH. The development of tumors (KPC-Luc cells) was evaluated by the IVIS system (bioluminescent imaging). One-way ANOVA run, followed by Tukey's multiple comparisons test, determined p values. Asterisks represent the level of significance between the sham and treatment groups (\*\*p<.001; \*\*\*p<.0001 and \*\*\*\*p<0.00001), Error bars indicate SD.

**(C-D)** KPC-Luc cells (0.5×106) were injected subcutaneously into C57BL/6JJJ male mice. The first group received PBS and served as a sham; the second group received GnPH (G, 60mg/kg (i.P); nP, 10mg/kg (i.v) weekly once; H (60 mg/kg; oral daily for fourteen days); the third group received GnPP (P, 0.3μg/kg; weekly twice), and the fourth group received GnPPH for four weeks. Tumor sizes were measured once weekly with digital calipers. Terminal tumor weight of treated and untreated mice was noted. One-way ANOVA run, followed by Tukey's multiple comparisons test, determined p values. Asterisks represent the significance level between the sham and treatment groups (\*\*\*\*p<0.00001). Error bars indicate SD.

**(E-F)** PDX mouse model. KRASG12V mutated pancreaticobiliary-PDX cell suspension was injected into the pancreas of NSG mice. Mice were randomized into four groups and treated as indicated (A-B) above. Terminal tumor weight and pictures of treated and untreated mice were noted. One-way ANOVA run, followed by Tukey's multiple comparisons test, determined p values. Asterisks represent the level of significance between the sham and treatment groups (\*\*\*p<0.0001, \*\*\*\*p<0.00001; Error bars indicate SD).

**(G-H)** PDX mouse model. KRASG12D mutated pancreatic cancer-PDX (~10 mg) was implanted into the subcutaneous NSG mice. Mice were randomized into four groups and treated as indicated (A-B) above. Terminal tumor volume and weight of treated and untreated mice were noted. One-way ANOVA run, followed by Tukey's multiple comparisons test, determined p values. Asterisks represent the level of significance between the sham and treatment groups (\*\*\*\*p<0.00001). Error bars indicate SD.

**(I)** Kaplan-Meier survival curves for KPC-Luc cells (1×105) were injected into the central section of the pancreas (orthotropic) of C57BL/6JJJ male mice. GPH treatment significantly (p<0.001) increased the survival of mice as compared to mice receiving the other individual (G) and combination (PH) treatments. No significant change observed between treated and sham received mouse body weight. Error bars indicate SD.

**Figure 2. The combination of gemcitabine (G) with paricalcitol (P) and hydroxychloroquine (H) enhances autophagy in human and mouse PDAC models.**

**(A)** Autophagy markers expression in KPC-Luc and MIA PaCa-2 cells was evaluated using Immunoblot. MIA PaCa-2 and KPC-Luc cell lines were exposed to P (300 nM) plus H (25 μM) and G (7.5 nM) for 36 hrs. Lysates were freshly prepared from treated and sham-received cell lines. The expression of autophagy markers LC3A/B, Atg7, BECN-1, and VDR were examined by Western blotting in KPC-Luc and MIA PaCa-2 cells. Results are repeated in three independent experiments.

**(B)** Immunoblot to evaluate the expression of autophagy markers in tumor tissues. Autophagy proteins in lysates from freshly isolated KPC-Luc tumors ex vivo (from 3 demonstrative mice per group). β-Actin was used as a loading control. Atg5 indicates autophagy-related 5; Atg7 indicates autophagy-related 7; LC3, light chain 3; and VDR, vitamin D receptor.

**(C-H)** Transmission electron microscopy (TEM). The three PDAC (MIA PaCa-2, PANC-1, and KPC-Luc) cell line specimens were examined using a Hitachi H-7500 electron microscope operated at 75 kV. Autophagosomes (indicated “arrow”) are indicated in PH, G, and GPH-treated PDAC cells. Bars = 50 nm (PANC-1 cells) and 1.0 μm (MIA PaCa-2 and KPC-Luc), respectively. \*D, F, and H Quantification of autophagosome puncta.

**Figure 3. Single-cell RNA sequence analysis reveals a marked reduction in** orthotopic KPC-Luc **intratumoral cell populations in mice treated with gemcitabine (G), paricalcitol (P), and hydroxychloroquine (H). (A)**. The t-distributed stochastic neighbor embedding (t‑SNE) plot showed 0 to 26 clusters (7 identified main cell types in orthotopic KPC-Luc tumors). **(B)**. Violin plots display the well-established 21 gene markers representing seven main cell types. **(C).** Number and percentage of orthotopic KPC-Luc intratumoral cell populations in sham and GPH or PH or G treatments.

**(D).** The t-SNE plot analysis showed that GPH treatment in orthotopic KPC-Luc tumors decreased all clusters and increased 8 and 9 PDAC clusters.

**Figure 4. The combination of gemcitabine (G) with paricalcitol (P) and hydroxychloroquine (H) on autophagy and endoplasmic reticulum (ER) stress pathways in orthotopic KPC-Luc tumors.**

**(A-C)** Single-cell RNA sequence analysis reveals an increase in autophagy markers in GPH-treated orthotopic KPC-Luc tumors compared to PH and Sham. Cluster 8 or 9 or all other PDAC sub-clusters also showed higher expression of autophagy markers in GPH treatment.

**(D)** Proteomics data supports a single-cell RNA sequence. GPH treatment enhances the autophagy-associated markers inorthotopic KPC-Luc tumors.

**(E)** Single-cell RNA sequence analysis reveals an increase in ER stress pathway markers in GPH-treated orthotopic KPC-Luc tumors compared to PH and Sham.

**(F)** Gene-gene interaction analysis revealed that VDR is the master regulator for all autophagy-associated markers. VDR is connected to autophagy markers through Ins2, Caspase 3, Pdia3 and Sqstm1.

**(G-I)** To confirm the VDR function on autophagy, VDR knockdown in 3 PDAC cell lines and treated with Sham, PH, G, and GPH as indicated in Fig. 2A. Knockdown of PDAC cells did not show significant response on LC3A/B and VDR expression when treated with PH, G, and GPH. Results are repeated in three independent experiments.

**Figure 5. Gemcitabine (G) with paricalcitol (P) and hydroxychloroquine (H) modulate cancer-associated fibroblast (CAF) phenotype and abundance in the orthotopic KPC-Luc-tumors.**

**(A)** t-SNE plot analysis showed five subsets of CAFs: quiescent (qCAF), myofibroblastic (myCAF), inflammatory (iCAF), proliferative (pCAF), and antigen-present (apCAF) **(B).** Dot plot represents established gene markers for identifying qCAFs, myCAFs, iCAFs, pCAFs, and apCAFs

**(C-D).** t-SNE plot showed that GPH treatmentdecreases the percentage of active CAFs (iCAF, myCAF, pCAF, and apCAF) population and increases the percentage of qCAFs population.

**(E).** Dot plot showed that GPH treatmentdecreases the active CAFs (iCAF, myCAF, pCAF and apCAF) associated gene markers and α-smooth muscle actin expression

**(F).** Immunohistology showed that GPH treatment decreases theα-smooth muscle actin expression in orthotopic KPC-Luc tumors (\*\*\*p<0.0001, \*\*\*\*p<0.00001; Error bars indicate SD).

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**(G).** Dot plot showed that GPH treatmentdecreases the autophagy gene markers.

**(H).** Dot plot showed that ER stress molecules in various types of CAFs. GPH treatment decreases ER stress associated markers.

**Figure 6. The combination of gemcitabine (G) with paricalcitol (P) and hydroxychloroquine (H) promotes M1 polarization and T cell activation in the KPC-Luc-TME. (A)** At 14 days post-treatment, KPC-Luc tumors were digested, and single-cell suspensions were prepared for single-cell RNA sequence analysis. t-SNE plot analysis showed ten subset clusters representing five major cell types (M1 and M2 macrophages, monocytes, granulocytes, and dendritic cells.  **(B)** Well-established gene markers recognized sub-clusters for macrophages. **(C-D)** GPH treatmentincreases the percentage of M1 macrophages polarization, monocytes, and granulocytes.

**(E)** The percentages of CD45+ CD11b+ Ly6C- Ly6G- F4/80+ macrophage, CD11b+ Ly6G+ Ly6C+ gMDSC, and CD11b+ Ly6G- Ly6Chi mMDSC, CD11b- CD11chi MHCIIhi XCR1+ cDC1, CD11b+ CD11chi MHCIIhi CD172α+ cDC2, CD11b- CD11cint pDC subsets were evaluated in sham, PH, G, and GPH (N = 4 mice/group). Data is representative of a single experiment. One-way ANOVA run, followed by Tukey's multiple comparisons test, determined p values. Error bars indicate SD. **(F)** Dot plot showed that GPH treatmentmodulate the myeloid population representing gene markers. GPH treatment decreases the expression of M2 markers and increases dendritic cell (DC) markers. **(G)** The percentages of macrophages CD45+ CD11b+ Ly6G- Ly6C- F4/80+ macrophage and median fluorescent intensity of MHCII and CD206 were evaluated in sham, PH, G, and GPH (N = 4 mice/group). Data is representative of a single experiment. One-way ANOVA run, followed by Tukey's multiple comparisons test, determined p values. Error bars indicate SD.  **(H-I)** Well-established gene markers recognized sub-clusters for T cells.

**(J).** t-SNE plot showed that GPH treatmentdecreases the percentage of Cd4 and Cd8 Tregs and increases the percentage of Cd4+, and Cd8+ T-cells as well as NK cells.

**(K)** Proportions of CD4+ Foxp3-, CD4+ Foxp3+, CD8+ T cells, γδ T, and NK cells in KPC-Luc-TME were evaluated in sham, PH, G, and GPH (N = 4 mice/group). Data is representative of a single experiment. One-way ANOVA run, followed by Tukey's multiple comparisons test, determined p values. **(L)** Dot plot showed that GPH treatment increases the expression of the CD4, CD3e, NKG7, CTLA-4, PD-1 as well as IFNɣ, Prf1 and Tbx21 and decreases Foxp3 **(M)** CD4+ FOXP3+, **(N)** CD4+ Foxp3-, and **(O)** CD8+ T cell subsets from KPC-Luc-TME was graphed as mean fluorescent intensity per mg tumor weight. Data is representative of a single experiment. Welch’s t-test determined p values, ns, which indicate no significance. Error bars indicate SD.

**Figure 7. Human PDAC studies (scRNA and spatial studies). (A).** The waterfall plot denotes the maximum percentage change in target lesion size from the baseline. **(B).** Kaplan-Meier survival curves for enrolled patients. **(C).** Kaplan-Meier survival curves for progression free survival.(**D)** Percentage of cells observed in pre- and post-treatment samples from patient A and D. (**E)** The two T cell clusters identified in the human biopsy sample showed different expressions of **Figure S11A*.*** T cell function associated genes, **Figure S11B*.*** T cell exhaustion genes, in the baseline. **(F).** Comparison between CD8+ T cells and NK cells from TME (biopsy) and baseline level. **(G).** Spatial Analysis of liver metastases before and after therapy. Immunofluorescent stains of six markers covering various structural or immune characteristics. One region of each pre-treated sample is displayed. Markers displayed include DAPI (Nuclear), β-Actin (Structural), E-Cadherin (Normal, PDAC Tissue), Collagen-IV (Fibrotic), Pan-Cytokeratin (PCK, PDAC), CD3e (T Cell). A composite image including E-Cadherin (Green), Collagen-IV (Yellow), PCK (Cyan), and CD3e (Red). **(H)**. Cell annotations for select regions from pretreated samples A (left) and D (right). The left column shows the composite image with the same color scheme from (G). The right column displays the cell classification for each region. **(I).** Adjacency analysis for the cell types annotated in (H) for pretreated samples A and D. Each square displays the log-odds ratio for finding a cell of one type adjacent to another. Red indicates a higher likelihood of being found near each other than random, and blue indicates a lower probability. **(J).** Displaying individual fluorescent markers for select regions from post-treated samples A and D. The composite image uses the same color mappings as **(G)**. **(K).** A stacked barplot displays the percent area coverage of normal, fibrotic, and PCK+ malignant tissue for pre-treatment and post-treatment samples. **(L)**. Cell annotations for select regions from post-treated samples A and D, using the color scheme defined in **(H).** **(M)**. Adjacency analysis for the cell types annotated in F for the post-treated sample A (left) and D-Lower (right).

**Supplemental figure legends**

**Figure S1. The combination of gemcitabine (G) with paricalcitol (P) and hydroxychloroquine (H) enhances growth arrest of human and mouse PDAC cell lines *in vitro*.** MIA PaCa-2, PANC-1, KPC-Luc, and 5363cell lines were exposed to P (300 nM) plus H (25 μM) and G (7.5 nM) for 36 hrs.

**(A-D).** Proliferation was assessed with the 2,3-Bis-(2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide salt) (XTT) reagent. Results are stated as means and standard deviations from a minimum of 3 to 5 repeats per experiment. One-way ANOVA run, followed by Tukey's multiple comparisons test, determined p values. \*\*p<.001; and \*\*\*\*p<0.00001 for comparisons of sham with a G or with the combined treatment (PH or GPH).

**(E-H).** The clonogenic test assessed the colony-developing ability of the PDAC cells. The experiment was performed in triplicates, and the cell proliferation or colony formation percentage is shown relative to untreated cells. Results are stated as means and standard deviations from a minimum of 3 to 5 repeats per experiment. One-way ANOVA run, followed by Tukey's multiple comparisons test, determined p values. \*\*\*\*p<0.00001 for comparisons of sham with a G or with the combined treatment (PH or GPH).

**Figure S2. (A-C)** KPC-Luc cells (1×105) or 5363 were injected into the central section of the pancreas (orthotropic) of C57BL/6J male mice. The first group received PBS and served as a sham; the second group received G (60mg/kg; weekly twice); the third group received P (0.3μg/kg; weekly twice) plus H (60 mg/kg; oral daily for fourteen days), and the fourth group received GPH. The body weights were measured once every five days. The tumor weights (KPC-Luc and 5363) were measured at the end of the experiment. One-way ANOVA run, followed by Tukey's multiple comparisons test, determined p values. \*\*\*p<.0001 and \*\*\*\*p<0.00001 specify a significant tumor growth rate difference.

**(D-E)** KPC-Luc cells (0.5×106) were injected subcutaneously into the C57BL/6JJJ male mice. The first group received PBS and served as a sham; the second group received GnPH (G, 60mg/kg (i.P); nP,10mg/kg (i.v) weekly once; H (60 mg/kg; oral daily); the third group received GnPP (P, 0.3μg/kg; weekly twice), and the fourth group received GnPPH for four weeks. Terminal tumor pictures of treated and untreated mice were shown, and no significant change was observed between the treated and untreated body weight of mice.

**(F)** PDX mouse model. KRASG12V mutated pancreaticobiliary-PDX cell suspension was injected into the pancreas of NSG mice. Mice were randomized into four groups and treated as indicated (A-B) above. No significant change was observed between treated and untreated mouse body weight.

**(G-H)** PDX mouse model. KRASG12D mutated pancreatic cancer-PDX (~10 mg) was implanted into the subcutaneous NSG mice. Mice were randomized into four groups and treated as indicated (A-B) above. GPH significantly decreased tumor growth compared to other treatments (G and PH). Terminal tumor pictures of treated and untreated mice were shown, and no significant change was observed between treated and untreated mouse body weight.

**Figure S3. Combining gemcitabine with paricalcitol and hydroxychloroquine increases overall survival in KPC-Luc mouse models of PDAC.**

Kaplan-Meier survival curves for KPC-Luc cells (1×105) were injected into the central section of the pancreas (orthotropic) of C57BL/6JJJ male mice. GPH-treated mice group significantly (p<0.001) increased the survival of mice from the other individual (G) and combination (PH) treatments. **(A)** Sham vs. GPH, **(B)** PH vs. GPH, **(C)** G vs. GPH, **(D)** GP vs. GPH, and **(E)** GH vs. GPH. The survival assessment was done by Log Rank assessment (R v.4.3.2). A statistically significant deviation was deemed at p<0.05.

**Figure S4.** **(A-B)** PANC-1, KPC-Luc, and 5363 cell lines were exposed to P (300 nM) plus H (25 μM) and G (7.5 nM) for 36 hrs. Results are stated as means and standard deviations from a minimum of 3 repeats per experiment. LC3 A/B expression in PDAC cell lines was evaluated using Immunofluorescence. LC3A/B expression and localization are displayed in red, and nuclear staining (DAPI) is presented in blue. The Image J program quantified the intensity of LC3A/B. \*p<.05; \*\*p<.001; and \*\*\*\*p<0.00001; ns, non-significant for comparisons of sham with a G or for comparisons of a G with the combined treatment (PH or GPH).

**Figure S5. (A)** A Dot plot shows autophagy gene expressions in various CAFs. **(B-F)** A Dot plot shows that GPH treatmentdecreases the autophagy gene markers in various subclusters of CAFs.

**Figure S6.** Multiparameter FACS gating strategy for myeloid cells. Multiparameter gating **(A-H).** to identify **(A-C).** Live, CD45+ lymphoid cells, **(D).** myeloid cells (CD11b+ and CD11c+). Myeloid cells were further subsetted into **(E).** monocytes, granulocytes, and macrophages based on Ly6C and F4/80 expression. From the Ly6C- F4/80- cells, **(F-H)**. dendritic cell subsets were identified (MHCII, CD11c) based on CD103, XCR1, and CD172a into CDD103+ (CD103+ CD11c+), cDC1 (XCR1+ CD11b-), and cDC2 (CD172a+ CD11b+) dendritic cell subsets.

**Figure S7. Multiparameter FACS gating strategy for T and NK cells.** Multiparameter gating **(A-F).** to identify (**A-B**). Live, CD45+ lymphoid cells, (**C**). T and B cells (CD3 and CD19). T and NK cells were further subsetted into (**D-E**). CD4+, CD8+, and CD4- CD8- populations based on CD4 (Foxp3+ and Foxp3-) and CD8 expression. From the CD4- CD8- cells, (**F**). NK and Gamma Delta T cells were identified by NK1.1 and TCRgd expression.

**Figure S8. Multiparameter FACS gating strategy for Intracellular Cytoine stimulation study.** Multiparameter gating **(A-D)** to identify (**A-B**) Live, CD45+ lymphoid cells, (**C**) T cell population (CD4+ and CD8+). (D) Gating in CD8+ T cells secreting IFNγ, similar gating scheme was adopted to determine the percent CD8+ T cells secreting IL17, TNFα, IL2, Granzyme B.

**Figure S9. Combining gemcitabine (G) with Paricalcitol (P) and hydroxychloroquine (H) increases M1 phenotypes in myeloid subsets and reduces cytokine production from CD4+ and CD8+ T cells.** Expression of MHCII and CD206 (median fluorescence intensity per mg tumor weight) on **(A).** Monocytes, **(B).** Granulocytes, **(C).** cDC1, **(D).** cDC2, and **(E).** pDC were evaluated in sham, PH, G, and GPH (n = 4 mice/group). **(F).** Expression of CD44, CD62L, and CD69 were evaluated on CD4+ and CD8+ T cells to determine proportions of naïve (CD44- CD62L+), pre-effector like (CD44-, CD62L-), effector memory (CD44+, CD62L-), and central memory (CD44+ CD62L+). CD69 expression can be an indication of recent activation or tissue-resident memory status. Expression of intracellular cytokines, granzyme B, and cell surface of expression of the degranulation marker CD107β in **(G).** CD4+ and **(H).** CD8+ T cells. Data is representative of a single experiment—a one-way ANOVA run, followed by Tukey's multiple comparisons test, determined p values. Error bars indicate SD.

**Figure S10.** *Ex vivo* intracellular cytokine stimulation of immune cells demonstrated an increasing trend of cytokine production in CD4+ T cells in GPH treated in orthotopically implanted KPC-Luc mice (n=4). **Figures** **A–E** proportions of CD4+ cells secreting 107a (Degranulation marker), Granzyme B, IFNγ, TNFα, and IL17. **Figures** **F–**J proportions of CD8+ cells secreting 107a (Degarnualtion marker), Granzyme B, IFNγ, TNFα, IL17, respectively, in each group. Data represented as mean ± SD, one-way ANOVA was performed with Tukey’s post hoc test for multiple comparisons. When parametric assumptions were not satisfied, the Kruskal-Wallis test with Dunnett’s post hoc test was utilized, ns indicates not significant.

**Figure S11. (A).** Needle biopsies of metastasized PDAC in the liver were used for single-cell analysis. UMAP plot of single cell profiles of baseline (pre-treatment) and treated (post-treatment; GnPPH) samples. The orange color arrow denotes increased cell numbers. (**B)** Top genes in the human biopsy.

**Graphical abstract.** The combination of gemcitabine (G) with paricalcitol (P) and hydroxychloroquine (H) enhances growth arrest and autophagy of human and mouse PDAC models through activation of the VDR-AMPK-ER stress axis.