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Plasma IL8 Is a Biomarker for TAK1 Activation and Predicts Resistance to Nanoliposomal Irinotecan in Patients with Gemcitabine-Refractory Pancreatic Cancer

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ABSTRACT

Purpose: Pancreatic cancer is one of the most lethal solid tumors, mainly because of its intrinsic chemoresistance. We identified TAK1 as a central hub sustaining this resistance. Nanoliposomal irinotecan (nal-IRI) is a novel treatment for metastatic gemcitabine-refractory pancreatic cancer. We endeavored to identify circulating markers for TAK1 activation predicting chemoresistance in this setting.

Experimental design: *In vivo* activity of nal-IRI was validated in an orthotopic nude murine model expressing TAK1-specific shRNA. Plasma concentration of 20 different cytokines were measured by a multiplex xMAP/Luminex technology in patients prospectively enrolled to receive nal-IRI plus 5-fluorouracil/leucovorin (5-FU/LV). The optimal cutoff thresholds able to significantly predict patients' outcome were obtained on the basis of the maximization of the Youden's statistics.

Results: Differential expression profiling revealed the gene coding for IL8 as the most significantly downregulated in shTAK1 pancreatic cancer cell lines. Mice bearing shTAK1 tumors had significantly lower plasma levels of IL8 and experienced a significant reduction in tumor growth if treated with nal-IRI, whereas those bearing TAK1-proficient tumors were resistant to this agent. In a discovery cohort of 77 patients, IL8 was the circulating factor most significantly correlated with survival (plasma levels lower vs higher than cutoff: mPFS 3.4 months vs 2.8 months; hazard ratio [HR], 2.55; 95% CI, 1.39–4.67; $P = 0.0017$; median overall survival 8.9 months vs 5.3 months; HR, 3.51; 95% CI, 0.84–6.68; $P = 4.9e{-}05$). These results were confirmed in a validation cohort of 50 patients.

Conclusions: Our study identified IL8 as the most significant circulating factor for TAK1 pathway activation and candidates IL8 as a potential predictive biomarker of resistance to nal-IRI in gemcitabine-refractory patients with pancreatic cancer.

Introduction

Pancreatic cancer remains one of the most lethal and poorly understood human malignancies and will continue to be a major unsolved health problem in the 21st century (1). Pancreatic cancer has still the lowest 5-year relative survival rate among solid tumors at 7%, and is projected to become the second leading cause of cancer-related death by 2030 in Western countries (2, 3). The poor prognosis for patients with pancreatic cancer could be mainly attributed to the limited efficacy of currently approved classic chemotherapeutic treatments (4).

We recently identified the serine/threonine kinase TGFβ-activated kinase 1 (TAK1) as a central hub integrating the most relevant signals from various cytokines (5, 6) and sustaining, in turn, resistance to chemotherapeutic treatments through the activation of different transcription factors, including AP-1, NF-κB (7, 8), and YAP/TAZ (9). In particular, we demonstrated that targeting the kinase activity of TAK1 dramatically led to a proapoptotic phenotype and, in turn, to a significantly higher sensitivity to chemotherapy and radiotherapy in pancreatic (10) and esophageal carcinoma (11).

Nanoliposomal irinotecan (nal-IRI; Onivyde; MM-398) is a liposomal formulation of the topoisomerase I inhibitor irinotecan. Efficacy of nal-IRI was recently demonstrated in the NAPOLI-1 study, a phase III, randomized, open-label, multicenter study that tested nal-IRI monotherapy or nal-IRI plus 5-fluorouracil (5-FU) and leucovorin (LV) versus 5-FU/LV alone in patients with metastatic pancreatic cancer previously treated with gemcitabine-based therapy (12). nal-IRI plus 5-FU/LV led to a significant improvement in median overall survival (mOS) over 5-FU/LV alone (13, 14), becoming a novel standard of care for second-line treatment of patients with metastatic pancreatic cancer (15).

We aimed this study at identifying circulating markers of TAK1 pathway activation, which could potentially serve as resistance biomarkers in this clinical setting.

Materials and Methods

Cell cultures and reagents

AsPC1, Panc1, and MDA-Panc28 human pancreatic cancer cell lines were purchased from the ATCC. MDA-Panc28 cell line was a kind gift by Dr. Paul J. Chiao. Panc1, AsPC1, and MDA-Panc28

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

The poor prognosis of patients with pancreatic cancer could be mainly attributed to its intrinsic resistance to the currently approved classic chemotherapeutic treatments. In the last decade, we contributed to this field by demonstrating that TAK1 pathway has one of the most essential roles in pancreatic cancer treatment resistance. Nanoliposomal irinotecan (nal-IRI) is a novel standard treatment for metastatic gemcitabine-refractory pancreatic cancer. However, the exceeding cost-effectiveness ratio limited the recommendation for marketing authorization of this regimens and, thus, the identification of biomarkers for patients' selection remains a significant priority. Here, we demonstrated that the chemokine interleukin-8 is the circulating factor most significantly regulated by TAK1 activation. By demonstrating that interleukin-8 could be, indeed, a useful biomarker for identifying patients that could take the larger advantage by nal-IRI, our findings cover one of the most significant unmet need in the selection of second- and further lines of treatment for pancreatic cancer.

pancreatic cancer cell lines silenced for the expression of TAK1 were established as described in ref. 10. All cell lines were cultured as monolayers at 37°C, 5% CO_2 in high glucose DMEM (catalog No. 41966-029; Life Technologies), supplemented with 10% heat-inactivated FBS (catalog No. 10270-106; Life Technologies), 2 mmol/L L-glutamine (catalog No. BE17-605E; Lonza), 100 IU/mL penicillin and 100 μg/mL streptomycin (catalog No. 15140-122; Life Technologies), and cultured for maximum 1 month. Cell lines were authenticated by DNA fingerprinting at the genomic core facility at Wayne State University (2009) and routinely tested for mycoplasma presence using the MycoAlert Mycoplasma Detection Kit (catalog No. LT07-118; Lonza).

Gene expression and pathway analyses

Differences in gene expression between control and TAK1-silenced cells (GEO accession No. GSE137265) were examined by using Illumina Human 48k gene chips (D-103-0204; Illumina) as previously described in ref. 9.

Protein extraction and Western blotting

Western blot analyses were performed as described previously (16). Briefly, total protein extracts were prepared by lysing cells in radioimmunoprecipitation assay buffer [50 mmol/L Tris HCl (pH 8), 150 mmol/L NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS]. All protein extracts were quantified by BCA Protein Assay Kit (23225; Thermo Fisher Scientific) and equal amounts (20–50 μg of protein extract) were loaded onto SDS-PAGE (4–20%) and transferred to polyvinylidene fluoride membranes (Immobilon-P, catalog No. IPVH00010; Millipore). Immunoblots were performed using the indicated antibodies. Anti-TAK1 antibody (ab109526, 1:1,000) was purchased from Abcam. Secondary anti-mouse and anti-rabbit antibodies were purchased from Jackson Immunoresearch. All antibodies were diluted in 5% nonfat dry milk dissolved in Tris-buffered saline/0.1% Tween-20. Immunoreactive proteins were visualized with Immobilion Western Kit (catalog No. WBKLS0500; EMD MIllipore) according to the manufacturer's instructions. Images were acquired using ImageQuant LAS 4000 mini (GE Healthcare Life Sciences).

Nude mouse orthotopic xenograft models

Five-week-old female athymic nude mice (Crl:CD1-Foxn1nu, CDNSSFE05S) were purchased from Charles River. University of Verona Animal Ethic Committee approved this study. Subconfluent cultures of pancreatic cancer cells were collected using 0.05% trypsin-EDTA (GIBCO, ref. 25300-054), that was inhibited with 10% FBS in DMEM. Tumor cells were resuspended in a solution of 1:1 Matrigel: PBS at 1.0×10^4 cells/μL concentration (Matrigel Matrix Growth Factor, 356230; BD Biosciences). Orthotopic injection of pancreatic cancer cells was performed as described previously (17).

All mice were weighed weekly and observed for tumors becoming palpable. When a tumor mass reached the diameter of 10 mm became palpable and measurable with a standard caliper. Tumor diameter was assessed with a Vernier caliper, and tumor volume (mm^3) was calculated as $d2 \times D/2$, wherein d and D represent the shortest and longest diameters, respectively. Bulky disease was considered present when tumor burden was prominent in the mouse abdomen (tumor volume, >2,000 mm^3). When at least three of five mice in a treatment group presented with bulky disease, the median survival duration for that group was considered to be reached. At the median survival duration of the control groups, all mice of control and treatment groups were euthanized by deep terminal anesthesia and blood and tumor samples were collected. Differences in mean weight were considered for the effects of treatments between different groups.

Immunohistochemistry

Immunostaining was performed in formalin-fixed paraffin-embedded tissue sections using the polymeric HRP staining system (UltraTek HRP Anti-Polyvalent DAB Staining System, AMF080; Histoline) as described ref. 18. Citrate pH 6.0 was used as antigen retrieval buffer. Primary monoclonal antibodies to IL8 (ab106350, 1:500) or to CD68 (ab31630; Abcam), or polyclonal antibody to CES2 (orb214832; Biorbyt) were incubated in a humified chamber at 4°C overnight. Signal detection was performed incubating sections with UltraTek Anti-Polyvalent and UltraTek HRP for 10 minutes at room temperature (UltraTek HRP Anti-Polyvalent DAB Staining System, AMF080; Histoline). Section were counterstained with Mayer's hematoxylin and slides were mounted using Entellan Neo Rapid nonaqueous mounting medium (Merck Millipore).

Patients and samples analysis

Patients enrolled in this study had advanced histologically or cytologically confirmed pancreatic adenocarcinoma and were previously treated with gemcitabine-based therapy, received in localized or metastatic setting. Other eligibility criteria included adequate hepatic, renal, and hematologic function, an ECOG performance status ≤2, and no other clinically significant disorders. Eligible patients were prospectively included in an Expanded Access Program to receive nal-IRI 80 mg/m^2 intravenously over 90 minutes, followed by LV 400 mg/m^2 intravenously over 30 minutes and 5-FU 2,400 mg/m^2 intravenously over 46 hours, every 2 weeks. We analyzed patients of two different cohorts on the basis of site of enrollment. Patients from the University Hospital Trust in Verona comprised the discovery cohort ($n = 77$), and those from Veneto Institute of Oncology (IOV-IRCCS) in Padua comprised the validation cohort ($n = 50$). At baseline, before first dose of nal-IRI, peripheral blood samples were collected using EDTA-containing tubes. All data were anonymized assigning a progressive number to every patient and the corresponding blood sample. Plasma aliquots were isolated from the blood samples by centrifugation with 1,600 rcf for 10 minutes and subsequently with 3,000 g for 10 minutes. Plasma samples were stored at −80°C. Written informed consent was

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obtained from all subjects. The study was conducted in accordance with the Declaration of Helsinki and approved by local ethics committee. Tumor response was assessed according to RECIST 1.1. Disease was assessed by CT (or MRI) every 12 weeks until disease progression.

Multiplex cytokines profiling

Plasma concentration of IL1β, IL2, IL4, IL5, IL6, IL8, IL12p70, IL13, IL18, eotaxin, GM-CSF, IFNγ, IP-10 (CXCL10), monocyte chemoattractant protein (MCP1; CCL2), macrophage inflammatory protein 1 α (MIP-1a; CCL3), MIP1-β (CCL4), TNFα, Rantes, SDF1α, GROα were measured by using a 20-plex Luminex Kit (Life Technologies, catalog No. EPX200-12173-901). All Luminex assays were performed according to the instructions provided by the manufacturer (Bio-Rad Laboratories). Median fluorescence intensities were collected on a Luminex-200 instrument, using Bio-Plex Manager software version 6.2. Standard curves for each cytokine were generated using the premixed lyophilized standards provided in the kits. Cytokine concentrations in samples were determined from the standard curve using a 5-point regression to transform mean fluorescence intensities into concentrations.

Statistical analysis

Provided the exploratory design of our study, the sample size was not predetermined. Results are shown as means and SDs. Survival curves were estimated using the Kaplan–Meier method and compared by log-rank test. Univariate and multivariate analyses of median progression-free survival (mPFS) and mOS, with stepwise variable selection, were conducted by Cox's proportional hazard regression models. Multivariate analysis was conducted using the clinical–pathologic variables with a P value <0.05 and the strongest significant molecular variables in univariate analysis (P value <0.01). Fisher exact test was used for pairwise comparisons of objective response.

The optimal cutoff thresholds for soluble biomarkers were obtained on the basis of the maximization of the Youden's statistics (J = sensitivity + specificity + 1; ref. 19) using an R-based software as described in ref. 20. Statistical analyses were performed using SPSS 24.0 statistical software (SPSS, Inc.), GraphPad Prism software program (version 6.0; GraphPad Software), and the statistical language R.

Results

Identification of IL8 as the most significant TAK1-regulated secreted protein in nal-IRI–resistant pancreatic cancer *in vivo* models

To identify novel circulating markers of TAK1-pathway activation, which could serve as biomarkers for chemoresistance, we compared gene expression profiles in AsPC1, Panc1, and MDA-Panc28 pancreatic cancer cell lines transduced with lentivirus expressing TAK1-specific shRNA (shTAK1) or scramble sequence (shctrl) as control by microarray analysis (**Fig. 1A**). We analyzed data of the genes with significantly different levels of expression by using the Ingenuity Pathway Analysis software program, which identifies groups of genes associated with specific molecular pathways. We found a significant association of the genes in the TAK1-proficient pancreatic cancer cells lines with the IL8 signaling pathway (**Fig. 1B**), and we identified *CXCL8*, the gene coding for IL8, as the most significant downregulated gene coding for secreted protein in all shTAK1 pancreatic cancer cell lines as compared with their respective scramble controls (**Fig. 1C**). We confirmed a significant downregulation of *CXCL8* mRNA in all shTAK1 pancreatic cancer cell lines (**Fig. 1D**) and a significant reduction in the IL8 expression in conditional medium from shTAK1 cell lines (**Fig. 1E**).

To measure whether the expression of tumor TAK1 could correlate *in vivo* with circulating levels of IL8, and to demonstrate that TAK1 kinase is relevant to sustain resistance to nal-IRI, we used an orthotopic xenograft nude mouse model. AsPC1^{shctrl} and AsPC1^{shTAK1} orthotopic tumor-bearing mice were randomly assigned ($n = 5$) to receive nal-IRI 10 mg/kg i.p. once a week or its intraperitoneal vehicle as control. At median survival duration of mice in the respective control groups, all of the mice were sacrificed and tumors and blood samples collected for analyses. We measured a significant downregulation for IL8 expression in tumor specimens excised from mice bearing shTAK1 AsPc1 tumors if compared with that in control tumors (**Fig. 2A**). Consistently, we demonstrated significantly lower plasma levels of IL8 in mice bearing TAK1 knockdown AsPc1 tumors than in those bearing TAK1-proficient tumors (**Fig. 2B**).

Most importantly, mice bearing TAK1-proficient AsPc1 tumors treated with nal-IRI had tumor growth rates comparable with those in vehicle-treated control mice [tumor weight mean ± SEM (g) = 0.94 ± 0.15 vs 1.615 ± 0.305, P value = 0.0973, unpaired t test; **Fig. 2C**]. Conversely, mice bearing TAK1 knockdown AsPc1 tumors treated with nal-IRI experienced a statistically significant reduction in tumor growth if compared with their respective vehicle-treated controls [mean ± SEM (g) = 0.5288 ± 0.1974 vs 3.036 ± 0.7067, P value = 0.0091, unpaired t test; **Fig. 2D**]. We ruled out the possibility that different levels of IL8 could modulate the infiltration of tumor-associated macrophages (TAMs), and the local conversion of nal-IRI in SN-38 by measuring similarly minimal levels (less than 1 cell/field) of $CD68^+$ cells and carboxylesterase-2 in tumor specimens excised from mice bearing shTAK1 AsPc1 tumors if compared with that in control tumors (Supplementary Fig. S1).

Altogether, these data demonstrate that IL8 is the most relevant secreted marker *in vitro* and *in vivo* for the activation of the TAK1 pathway that is responsible for a tumor cells autonomous resistance of pancreatic cancer to nal-IRI.

IL8 is the most significant predictive marker of survival in patients with metastatic pancreatic cancer treated with nal-IRI plus 5-FU/LV

To demonstrate the clinical relevance of IL8 in predicting resistance to nal-IRI, we initially collected plasma at baseline in a discovery cohort of 77 patients with gemcitabine-refractory metastatic pancreatic cancer that were prospectively enrolled between November 2016 and June 2018 within an Expanded Access Program to receive nal-IRI plus 5-FU/LV as second or further line therapy. Compliance with REMARK guidelines is reported in Supplementary Table S1. Patients' characteristics are shown in Supplementary Table S2. In this discovery cohort, median age was 64 years and 52% were male. Most of them had a normal ECOG performance status (58.4%). Half of the patients had tumors in the head of pancreas (52%). The majority of patients had increased levels of Ca19.9 (83.1%). More than one third of patients had two or more site of metastasis, and liver was the most common secondary site (70.1%). 76.7% of patients received nal-IRI + 5-FU/LV as second-line, 22.0% as third-line, and one patient (1.2%) as fourth-line treatment for metastatic disease (Supplementary Table S2). After a median follow-up of 26 months, the mPFS was 3.3 months (95% CI, 3.040–3.560) and the mOS was 7.4 months (95% CI, 8.309–12.251) for the overall population (Supplementary Figs. S2A and S2B).

Thus, we measured the plasma concentration of a panel of 20 different cytokines, chemokines, and growth factors. The optimal cutoff thresholds able to significantly predict patients' outcomes were

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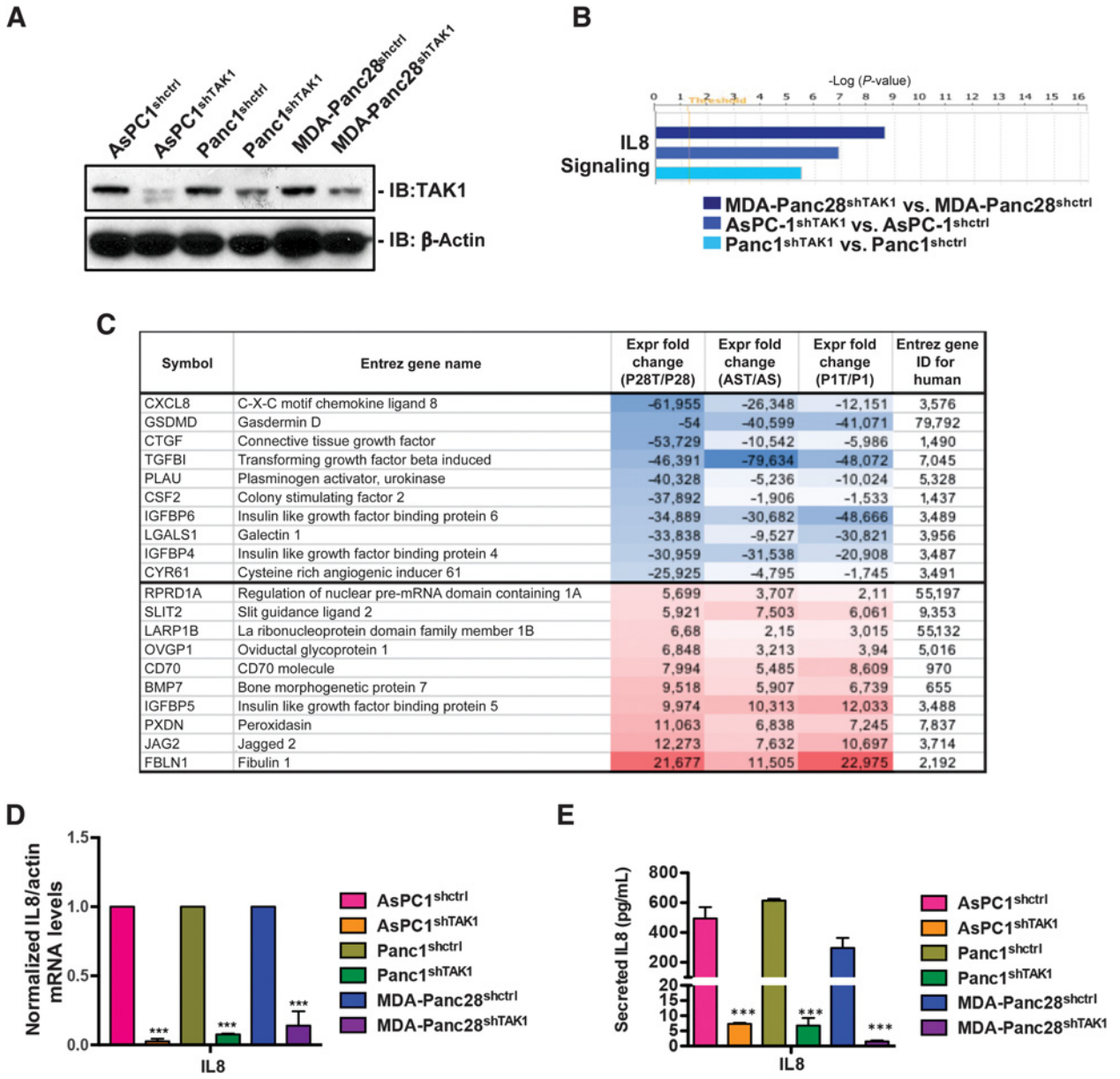

Symbol	Entrez gene name	Expr fold change (P28T/P28)	Expr fold change (AST/AS)	Expr fold change (P1T/P1)	Entrez gene ID for human
CXCL8	C-X-C motif chemokine ligand 8	-61,955	-26,348	-12,151	3,576
GSDMD	Gasdermin D	-54	-40,599	-41,071	79,792
CTGF	Connective tissue growth factor	-53,729	-10,542	-5,986	1,490
TGFBI	Transforming growth factor beta induced	-46,391	-79,634	-48,072	7,045
PLAU	Plasminogen activator, urokinase	-40,328	-5,236	-10,024	5,328
CSF2	Colony stimulating factor 2	-37,892	-1,906	-1,533	1,437
IGFBP6	Insulin like growth factor binding protein 6	-34,889	-30,682	-48,666	3,489
LGALS1	Galectin 1	-33,838	-9,527	-30,821	3,956
IGFBP4	Insulin like growth factor binding protein 4	-30,959	-31,538	-20,908	3,487
CYR61	Cysteine rich angiogenic inducer 61	-25,925	-4,795	-1,745	3,491
RPRD1A	Regulation of nuclear pre-mRNA domain containing 1A	5,699	3,707	2,11	55,197
SLIT2	Slit guidance ligand 2	5,921	7,503	6,061	9,353
LARP1B	La ribonucleoprotein domain family member 1B	6,68	2,15	3,015	55,132
OVGP1	Oviductal glycoprotein 1	6,848	3,213	3,94	5,016
CD70	CD70 molecule	7,994	5,485	8,609	970
BMP7	Bone morphogenetic protein 7	9,518	5,907	6,739	655
IGFBP5	Insulin like growth factor binding protein 5	9,974	10,313	12,033	3,488
PXDN	Peroxidasin	11,063	6,838	7,245	7,837
JAG2	Jagged 2	12,273	7,632	10,697	3,714
FBLN1	Fibulin 1	21,677	11,505	22,975	2,192

Figure 1.

Identification of IL8 as the most significant TAK1-regulated secreted protein. **A,** Western blot analysis for the expression of TAK1 in AsPc-1, PANC-1, and MDAPanc-28 pancreatic cancer cells transduced with lentiviruses expressing TAK1-specific small hairpin RNA (shTAK1) or a scramble sequence as control (shctrl). β-Actin was used as loading control. **B,** Selection of relevant biological processes and secreted protein genes by using global transcript profiling. IL8 signaling was enriched among genes differentially expressed in pancreatic cancer cells transduced with lentiviruses expressing shTAK1 or a shctrl by ingenuity pathway analyses (IPA) software. The X-axis represents the log (10) *P* value for enrichment, with the threshold drawn at $P = 0.05$. **C,** Levels of the most significantly downregulated (blue) and upregulated (red) genes coding for secreted proteins in pancreatic cancer cells transduced with lentiviruses expressing shTAK1 (P28T, AST, P1T) versus those expressing a shctrl (P28, AS, P1). **D,** Histogram shows normalized mRNA levels of IL8/β-actin in the indicated pancreatic cancer cell lines. Means and SDs are shown, $^{***}P < 0.001$, as determined by unpaired Student *t* test. **E,** Histogram shows IL8 protein levels in the conditioned media of the indicated pancreatic cancer cell lines. Means and SDs are shown, ***, $P < 0.001$, as determined by unpaired Student *t* test.

evaluated for each cytokine (**Table 1**). This analysis confirmed IL8 as the circulating factor most significantly able to predict mPFS and mOS in this discovery cohort of patients. The mPFS was 3.4 months compared with 2.8 months (HR, 2.55; 95% CI, 1.39–4.67; $P = 0.0017$) (**Fig. 3A**), and the mOS was 8.9 months compared with 5.3 months (HR, 3.51; 95% CI, 1.84–6.68; $P = 4.9e{-}05$; **Fig. 3B**) for patients with plasma levels of IL8 at baseline lower versus higher than cutoff of 16.68 pg/mL, respectively (Supplementary Figs. S3A–S3F). Furthermore, in those 15 patients with plasma levels of IL8 at baseline higher than cutoff, we measured only one (6.7%) stable disease (SD), and no partial (PR) or complete responses. On the contrary, among those 62 patients with plasma levels of IL8 at baseline lower than cutoff,

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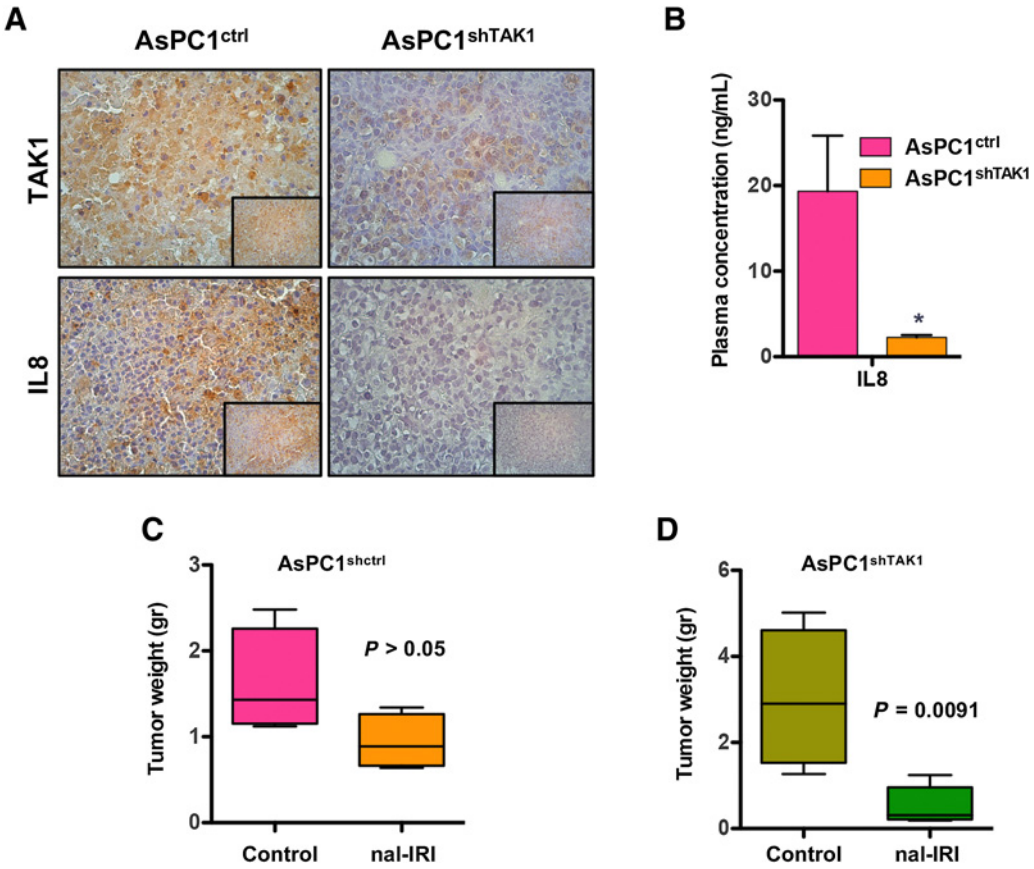

Figure 2.

TAK1 sustains high circulating levels of IL8 and resistance to nal-IRI- in pancreatic cancer *in vivo* models. **A,** IHC analysis. Serial paraffin sections from tumors excised from mice orthotopically inoculated with pancreatic cancer cells transduced with lentiviruses expressing shTAK1 or a shctrl were stained with antibodies to TAK1 and IL8. **B,** Histogram shows IL8 plasmatic concentration in mice inoculated with pancreatic cancer cells transduced with lentiviruses expressing shTAK1 or a shctrl. Means and SDs ($n = 3$) are shown, *, $P < 0.05$, as determined by unpaired Student *t* test. **C,** Antitumor activity of nal-IRI *in vivo* in AsPc-1 expressing shctrl pancreatic tumor orthotopic xenografts ($n = 10$, five mice per group). When at least three of five mice in a treatment group presented with bulky disease (tumor volume, >2,000 mm^3), the median survival duration for that group was considered to be reached. At the median survival duration of the control group, all mice of control and treatment groups were euthanized by deep terminal anesthesia and blood and tumor samples were collected. Differences in weight were considered for the effects of treatments between different groups. Box-and-whiskers plot shows median and 25th to 75th percentiles of tumors weight in each group. *P* value not significant (>0.05), as determined by unpaired Student *t* test. **D,** Antitumor activity of nal-IRI *in vivo* in AsPc-1 expressing shTAK1 pancreatic tumor orthotopic xenografts ($n = 10$, five mice per group). Analysis conducted as described above. Box-and-whiskers plot shows median and 25th to 75th percentiles of tumors weight in each group. *P* value = 0.0091 as determined by unpaired Student *t* test.

we measured 14 (23%) SD, 5 (8.1%) PR, and 1 (1.7%) CR, counting for a disease control rate of 32.7% (Supplementary Table S3).

Different circulating factors and cytokines with levels higher than cutoff were also significantly associated with either a shorter patients' mPFS and mOS, including the chemokines MCP-1, IP-10, MIP-1β, and SDF-1α, and the T_H1 cytokines IL18 and INFγ. The same associations were not proven for the other cytokines (**Table 1**; Supplementary Figs. S4–S11).

Univariate analysis of correlation between IL8 plasma levels, clinical features, and survival outcomes is shown in **Table 2**. Among the clinical parameters analyzed, patients who previously underwent surgery had a significantly longer mOS (HR, 0.387; $P = 0.012$) and mPFS (HR, 0.437; $P = 0.017$) compared with unresectable patients. Furthermore, patients with a normal ECOG PS had a longer mPFS compared with patients with ECOG PS 1-2 (HR, 1.884; $P = 0.010$). To confirm the independence of the predictive value for IL8 plasma levels, we performed a multivariate analysis including IL8 and those clinical features significant at univariate analysis ($P < 0.05$). This analysis revealed IL8 as an independent predictor of PFS and OS in the discovery cohort of patients (**Table 2**).

To validate the negative predictive value of IL8 in patients treated with nal-IRI plus 5-FU/LV, we analyzed the association of IL8 levels and clinical outcomes in an external validation cohort of patients treated in a different center. Patients' characteristics are shown in Supplementary Table S4. The mPFS was 3.3 months (95% CI, 4.504-7.804) and the mOS was 7.4 months (95% CI, 8.309–12.251) for the overall population (Supplementary Figs. S12A and S12B). In this validation cohort, we confirmed IL8 as significantly associated with mPFS and mOS. The mPFS was 4.6 months compared with 1.8 months (HR, 2.84; 95% CI, 1.41–5.72; $P = 0.0023$; **Fig. 4A**), and the mOS was 9.7 months compared with 5.4 months (HR, 2.32; 95% CI, 1.18–4.56; $P = 0.012$; **Fig. 4B**) for patients with serum levels of IL8 at baseline lower versus higher than cutoff, respectively (Supplementary Fig. S13).

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Table 1. Plasmatic cytokines able to significantly predict PFS and OS in the discovery cohort population.

				PFS			OS		
	Median concentration (pg/mL)	Range		Cutoff (pg/mL)	HR (95% CI)	*P*	Cutoff (pg/mL)	HR (95% CI)	*P*
Chemokines									
IL8	5.96	0	54.67	16.68	2.55 (1.39–4.66)	0.0017	16.68	3.51 (1.84–6.68)	4.9e-05
MCP-1	55.27	20.41	237.7	60.02	2.21 (1.35–3.62)	0.0013	58.07	2.38 (1.46–3.89)	0.00038
IP-10	19.56	9.73	46.85	25.49	2.11 (1.24–3.6)	0.005	25.35	2.52 (1.47–4.34)	0.00055
MIP-1β	36.24	20.29	121.8	56.21	2.51 (1.3–4.85)	0.0046	56.21	2.39 (1.23–4–65)	0.0079
SDF-1α	258	94.29	647.6	334	2.38 (1.24–4.55)	0.0069	334	2.06 (1.09–3.88)	0.023
RANTES	139.31	36.67	255.1	—	—	—	—	—	—
Eotaxin	23.07	12.25	56.84	—	—	—	—	—	—
GROAα	0	0	102.91	—	—	—	—	—	—
MIP-1α	1.79	0	54.33	—	—	—	—	—	—
T_H1									
IL2	12.93	0	31.27	—	—	—	6.775	0.5 (0.27–0.93)	0.026
IL18	31.55	0	136.71	54	2.14 (1.21–3.8)	0.0076	25.21	2 (1.2–3.33)	0.0065
IL12p70	4.28	0	72.33	—	—	—	1.755	0.53 (0.32–0.86)	0.0098
IFNγ	24.53	0	137.69	44.27	2.62 (1.4–4.88)	0.0017	20.32	1.88 (1.11–3.2)	0.018
TNFα	11.75	2.46	47.04	—	—	—	—	—	—
T_H2									
IL4	0	0	218.39	6.195	2.43 (1.2–4.88)	0.01	—	—	—
IL5	7.23	0	44.52	—	—	—	—	—	—
IL6	0.5	0	193.81	—	—	—	6.175	2 1.21–3.3	0.0061
IL13	0	0	123.7	7.93	2.31 (1.17–4.59)	0.014	—	—	—
Other cytokines and growth factors									
IL1β	0	0	13.13	—	—	—	—	—	—
GM-CSF	0	0	102.98	—	—	—	—	—	—

Note: Median concentration, range, cutoff identified, hazard ratio (HR) and 95% CI, and *P* value are reported.

Discussion

In this study, we sought to identify circulating biomarkers useful to estimate the activation of TAK1 pathway, one of the most relevant molecular mechanisms responsible for the resistance of pancreatic cancer to the proapoptotic activity of chemotherapeutic treatments (5). We demonstrated that the chemokine IL8 is the secreted factor most significantly regulated by TAK1 activation. Most importantly, we demonstrated for the first time that IL8 could be, indeed, a useful biomarker to predict resistance to the novel nanotechnologic agent nal-IRI in two large discovery and external validation cohorts of patient affected by gemcitabine-refractory advanced pancreatic cancer.

The most appropriate systemic regimen for patients with advanced pancreatic cancer progressing under a first-line gemcitabine-containing regimen largely remains a matter of debate. Two randomized phase III trials that explored the role of oxaliplatin-based chemotherapy in patients progressing to a first-line single-agent gemcitabine therapy reached conflicting results. No randomized phase III trials explored standard irinotecan-containing regimens in this setting (21, 22) To date, NAPOLI-1 remains the largest phase III study in second-line metastatic pancreatic cancer (12). This trial led to the regulatory approval of nal-IRI in the United States in 2015 for the treatment of metastatic pancreatic cancer in combination with 5-FU/LV after progression on gemcitabine-based therapy. Nonetheless, the lack of randomized trials comparing nal-IRI plus 5-FU/LV to standard irinotecan-containing regimens such as FOLFIRI, and the exceeding cost-effectiveness ratio per quality-adjusted life year gained for nal-IRI plus 5-FU/LV limited the recommendation for marketing authorization of this regimen in several European countries and in United Kingdom (23). In this regard, the identification of biomarkers for the selection of those patients that could gain the largest advantage by this regimen remains a significant priority. Here, we demonstrated that IL8 is the most significant independent factor for PFS and OS in two different cohorts of patients among a series of cytokines in patients with advanced pancreatic cancer receiving nal-IRI plus 5-FU/LV treatment, representing a potential biomarker for patients' selection and, in turn, for potentially improving clinical-effectiveness evidence and cost-effectiveness results for this regimen.

IL8 is a chemokine mainly produced by tumor cells, and its main functions include angiogenesis, survival signaling for cancer stem cells, and attraction of myeloid-derived suppressor cells (24). We recently discovered IL8 as part of a proinflammatory cytokine signature inducing resistance to antiangiogenic drugs in patients with pancreatic and colorectal cancer through the recruitment of myeloid $CD11b^+$ cells (25–27). Previous studies demonstrated a central role for TAK1 in sustaining IL1-induced transcription and mRNA stabilization, as well as expression of endogenous IL8 (28). TAK1 is also part of an intracellular signaling axis responsible for Death receptor 3-mediated expression of IL8 (29). More recently, an autocrine loop between IL8 and TAK1 has been demonstrated given that IL8 can activate TAK1/NF-κB signaling via the CXCR2 receptor in models of ovarian cancer (30). In this study, we demonstrated that IL8 is the secreted factor most significantly regulated by TAK1 activation, one of the most

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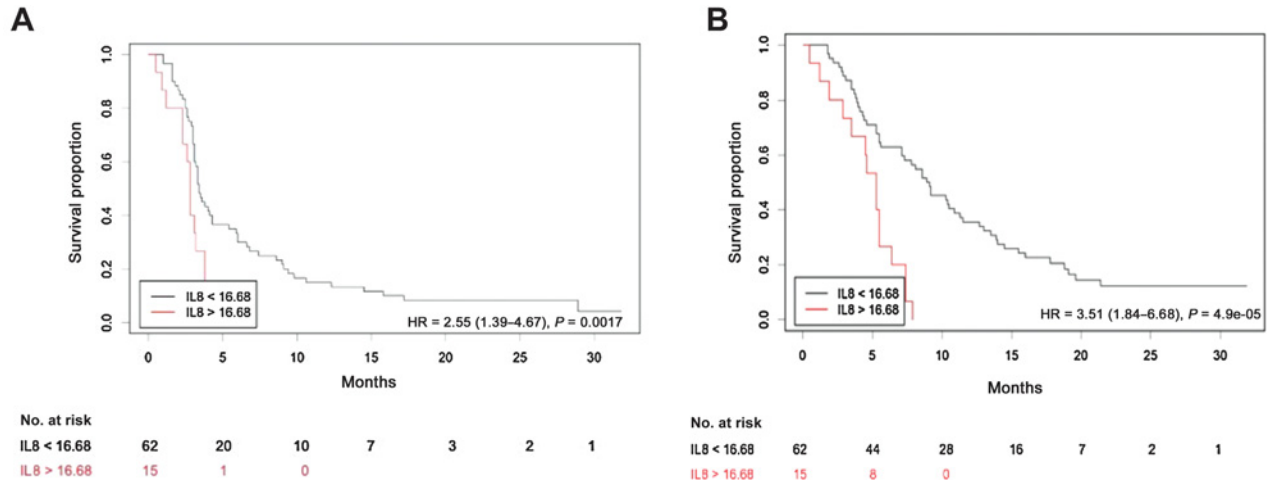

Figure 3.

Kaplan–Meier analysis of PFS (**A**) and OS (**B**), according to cut-off value of IL8 in the discovery cohort. **A,** In the discovery cohort, the median PFS was 3.4 months in low IL8 group, as compared with 2.8 months in high IL8 group. **B,** The median OS was 8.9 months in low IL8 group, as compared with 5.3 months in high IL8 group.

potent mediators of chemoresistance in pancreatic cancer through the activation of AP-1 and NF-κB transcription factors. AP-1 and NF-κB are, indeed, recognized as potent transcription factors cooperating for the induction of IL8 in human pancreatic cancer cells by hypoxia (31).

The stable nanoliposome formulation of nal-IRI has been developed to improve the therapeutic index of irinotecan throughout an active accumulation and conversion in TAMs, allowing for a more efficient delivery of its active metabolite SN-38 within the tumor microenvironment. However, the effect of tumor derived IL8 on TAMs in murine models has been only limitedly explored (32) as an homologue of human CXCL8 gene is completely absent from the genome of rodents (33). Previous studies in mice carrying a bacterial artificial chromosome encompassing the entire human CXCL8 gene showed an increased mobilization of immature $CD11b^{+}Gr\text{-}1^{+}$ myeloid cells (34). In our study, we ruled out a putative mechanistic association between the infiltration and the carboxylesterase activity of TAMs and IL8 levels as cause for the lack of activity of nal-IRI in pancreatic cancer models. The activation of transcription factors, including AP-1, NF-κB, and YAP/TAZ (7–9), remains the most relevant mechanism for the treatment resistance sustained by TAK1, and IL8 is the most reliable circulating factor to measure the activation of this pathway.

This study, however, had one limitation in lacking a control cohort including untreated patients with pancreatic cancer to distinguish between a merely prognostic or a predictive value for TAK1-regulated IL8. Although its relevant mechanistic role in cancer pathogenesis, a prognostic value for IL8 in pancreatic cancer has been, however, excluded by several previous studies. In one of the initial series

Table 2. Univariate and multivariate analyses of clinical characteristics and IL8 levels influencing PFS and OS of patients in the discovery cohort.

		OS							
		Univariate 95% CI				Multivariate 95% CI			
Variables		HR	Lower	Upper	*P*	HR	Lower	Upper	*P*
PS ECOG	0 vs 1–2	1.170	0.721	1.900	0.526				
Primary tumor location	Head vs body/tail	0.924	0.567	1.508	0.753				
Liver metastases	No vs yes	1.517	0.871	2.640	0.141				
CA19.9 (U/mL)	<40 vs ≥40	1.575	0.716	3.466	0.259				
N/L ratio	≤5 vs >5	1.368	0.696	2.689	0.363				
IL8	Low vs high	**3.406**	**1.790**	**6.481**	**0.000**				
		PFS							
		Univariate 95% CI				Multivariate 95% CI			
Variables		HR	Lower	Upper	*P*	HR	Lower	Upper	*P*
PS ECOG	0 vs 1–2	**1.884**	**1.164**	**3.049**	**0.010**	**2.216**	**1.346**	**3.649**	**0.002**
Primary tumor location	Head vs body/tail	0.850	0.529	1.366	0.502				
Liver metastases	No vs yes	0.925	0.538	1.591	0.778				
CA19.9 (U/mL)	<40 vs ≥40	1.832	0.828	4.057	0.135				
N/L ratio	≤5 vs >5	1.233	0.672	2.264	0.499				
IL8	Low vs high	**2.505**	**1.367**	**4.590**	**0.003**	**3.090**	**1.649**	**5.791**	**0.000**

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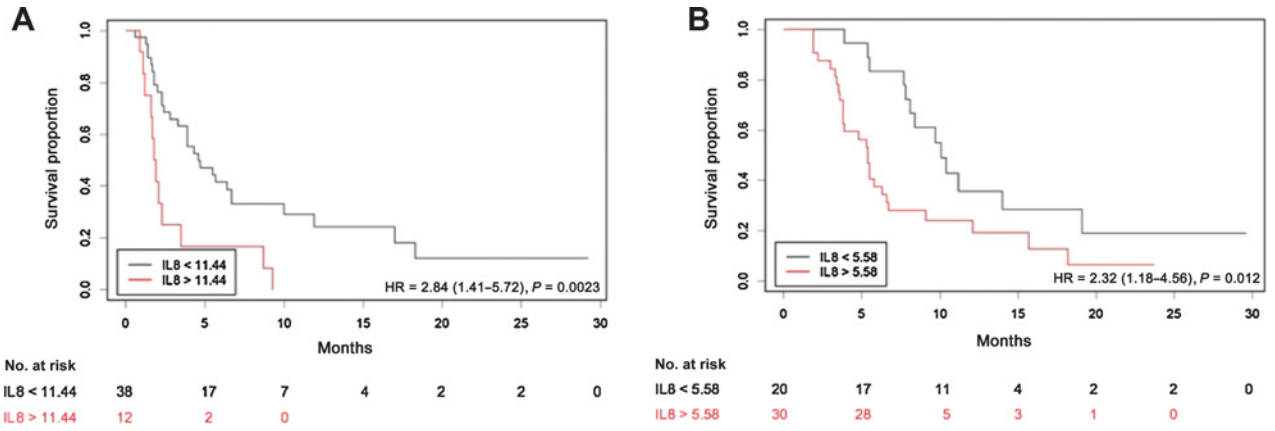

Figure 4.

Kaplan–Meier analysis of PFS (**A**) and OS (**B**), according to cut-off value of IL8 in the validation cohort. **A,** In the validation cohort, the median PFS was 4.6 months in low IL8 group, as compared with 1.8 months in high IL8 group. **B,** The median OS was 9.7 months in low IL8 group, as compared with 5.4 months in high IL8 group. IL8 cut off levels (pg/mL), HR and *P* value are indicated.

including healthy volunteers and patients with pancreatic cancer with resectable, locally advanced or metastatic disease, IL8 levels were higher in patients with cancer than in healthy controls but were not associated with survival differences (35). More recently, we conducted the most comprehensive profiling of cytokines in the largest prospective cohort of patients with resectable pancreatic cancer to date and IL8 had no prognostic value in this cohort of patients (36). Excluded a prognostic role for IL8, we acknowledge that its negative predictive value could be although extended also to other cytotoxic agents different from nal-IRI that induce in pancreatic cancer cells a proapoptotic stimuli by which a TAK1 activation mirrored by IL8 could protect.

In conclusion, we identified for the first time IL8 as the most significant circulating biomarker for predicting chemoresistance sustained by TAK1 pathway in pancreatic cancer. The expression of this chemokine could be useful to select those patients with pancreatic cancer that could benefit from the novel nanotechnologic agent nal-IRI in the larger extent.

Disclosure of Potential Conflicts of Interest

S. Lonardi reports grants and personal fees from Amgen, personal fees from Bayer and Merck Serono and personal fees from Lilly, Servier, Roche, and BMS outside the submitted work. D. Melisi also receives research funding by Servier and had a consulting role with Shire. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

V. Merz: Conceptualization, data curation, formal analysis, validation, investigation, methodology, writing-original draft, writing-review and editing. **C. Zecchetto:** Investigation. **R. Santoro:** Conceptualization, data curation, formal analysis, investigation, methodology, writing-original draft, writing-review and editing. **F. Simionato:** Investigation. **F. Sabbadini:** Investigation. **D. Mangiameli:** Investigation. **G. Piro:** Investigation. **A. Cavaliere:** Investigation. **M. Deiana:** Investigation. **M.T. Valenti:** Resources. **D. Bazan:** Resources and investigation. **V. Fedele:** Investigation. **S. Lonardi:** Resources and investigation. **D. Melisi:** Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, validation, methodology, writing-original draft, writing-review and editing.

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