

RESEARCH ARTICLE

Cancer Genetics and Epigenetics

Polymorphisms within autophagy-related genes as susceptibility biomarkers for pancreatic cancer: A meta-analysis of three large European cohorts and functional characterization

Fernando Gálvez-Montosa¹ | Giulia Peduzzi² | José Manuel Sanchez-Maldonado^{3,4,5,6} | Rob ter Horst^{7,8} | Antonio J. Cabrera-Serrano^{4,5} | Manuel Gentiluomo²  | Angelica Macaudo⁶ | Natalia Luque¹ | Pelin Ünal⁶ | Francisco José García-Verdejo¹ | Yang Li^{7,57} | José Antonio López López¹ | Angelika Stein⁶ | H. Bas Bueno-de-Mesquita^{9†} | Paolo Giorgio Arcidiacono¹⁰ | Dalila Luciola Zanette¹¹ | Christoph Kahlert¹² | Francesco Perri¹³ | Pavel Soucek¹⁴  | Renata Talar-Wojnarowska¹⁵ | George E. Theodoropoulos¹⁶ | Jakob R. Izicki¹⁷ | Hussein Tamás^{18,19} | Hanneke Van Laarhoven^{20,21} | Gennaro Nappo^{22,23} | Maria Chiara Petrone¹⁰ | Martin Lovecek²⁴ | Roel C. H. Vermeulen²⁵ | Kestutis Adamonis²⁶ | Fernando Jesus Reyes-Zurita³ | Bernd Hollecsek^{27,28} | Jolanta Sumskiene²⁶ | Beatrice Mohelníková-Duchoňová²⁹ | Rita T. Lawlor^{30,31} | Raffaele Pezzilli³² | Mateus Nobrega Aoki¹¹ | Claudio Pasquali³³ | Vitalija Petrenkiene²⁶ | Daniela Basso³⁴ | Stefania Bunduc^{18,19,35,36} | Annalisa Comandatore³⁷ | Hermann Brenner^{28,38}  | Stefano Ermini² | Giuseppe Vanella^{39,40} | Mara R. Goetz¹⁷ | Livia Archibugi^{39,40} | Maurizio Lucchesi⁴¹ | Faik Guntac Uzunoglu¹⁷ | Olivier Busch^{21,42} | Anna Caterina Milanetto³³ | Marta Puzzono⁴³ | Juozas Kupcinskas²⁶ | Luca Morelli³⁷ | Cosimo Sperti³³ | Silvia Carrara⁴⁴ | Gabriele Capurso^{39,40} | Casper H. J. van Eijck⁴⁵ | Martin Oliverius⁴⁶ | Susanne Roth¹² | Francesca Tavano¹³  | Rudolf Kaaks⁴⁷  | Andrea Szentesi⁴⁸ | Ludmila Vodickova^{49,50,51} | Claudio Luchini^{30,52} | Ben Schöttker²⁸  | Stefano Landi²  | Orsolya Dohan¹⁹ | Matteo Tacelli¹⁰ |

Fernando Gálvez-Montosa, Giulia Peduzzi, and José Manuel Sanchez-Maldonado share first authorship.

Daniele Campa and Juan Sainz share last authorship.

† Deceased researcher.

For affiliations refer to page 348. [Correction added on 2 June 2025, after first online publication: The affiliation of Yang Li has been updated.]

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William Greenhalf⁵³ | Maria Gazouli⁵⁴  | John P. Neoptolemos¹² |
 Giulia Martina Cavestro⁴³ | Ugo Boggi⁵⁵ | Anna Latiano¹³ |
 Péter Hegyi^{18,19,48,56} | Laura Ginocchi⁴¹ | Mihai G. Netea^{57,58} |
 Pedro Sánchez-Rovira¹ | Federico Canzian⁶  | Daniele Campa²  |
 Juan Sainz^{3,4,5,59}  

Correspondence

Juan Sainz, Department of Biochemistry and Molecular Biology I, University of Granada, Avenida Fuente Nueva, s/n, 18071, Granada, Spain.

Email: jsainz@ugr.es

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal cancers with patients having unresectable or metastatic disease at diagnosis, with poor prognosis and very short survival. Given that genetic variation within autophagy-related genes influences autophagic flux and susceptibility to solid cancers, we decided to investigate whether 55,583 single nucleotide polymorphisms (SNPs) within 234 autophagy-related genes could influence the risk of developing PDAC in three large independent cohorts of European ancestry including 12,754 PDAC cases and 324,926 controls. The meta-analysis of these populations identified, for the first time, the association of the *BID*_{rs9604789} variant with an increased risk of developing the disease ($OR_{Meta} = 1.31$, $p = 9.67 \times 10^{-6}$). We also confirmed the association of *TP63*_{rs1515496} and *TP63*_{rs35389543} variants with PDAC risk ($OR = 0.89$, $p = 6.27 \times 10^{-8}$ and $OR = 1.16$, $p = 2.74 \times 10^{-5}$). Although it is known that *BID* induces autophagy and *TP63* promotes cell growth, cell motility and invasion, we also found that carriers of the *TP63*_{rs1515496G} allele had increased numbers of FOXP3+ Helios+ T regulatory cells and CD45RA+ T regulatory cells ($p = 7.67 \times 10^{-4}$ and $p = 1.56 \times 10^{-3}$), but also decreased levels of CD4+ T regulatory cells ($p = 7.86 \times 10^{-4}$). These results were in agreement with research suggesting that the *TP63*_{rs1515496} variant alters binding sites for FOXA1 and CTCF, which are transcription factors involved in modulating specific subsets of regulatory T cells. In conclusion, this study identifies *BID* as new susceptibility locus for PDAC and confirms previous studies suggesting that the *TP63* gene is involved in the development of PDAC. This study also suggests new pathogenic mechanisms of the *TP63* locus in PDAC.

KEYWORDS

autophagy, functional characterization, genetic variants, pancreatic cancer, polymorphisms, susceptibility

What's new?

The etiology of pancreatic ductal adenocarcinoma (PDAC), among the most aggressive and deadliest cancers worldwide, remains largely unknown. Here, using data from cohorts of European ancestry, the authors investigated the influence on PDAC risk of single nucleotide polymorphisms (SNPs) in genes associated with autophagy. Analyses identified multiple SNPs associated with PDAC risk, including variants within *BID* and *TP63*. Variants in *BID* potentially dysregulate *BID*-dependent autophagy, while those in *TP63* may influence PDAC risk by modulating levels of T regulatory cells involved in host immune responses against tumor cells. The variants warrant further study to better elucidate their involvement in PDAC.

1 | INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is the most common form of pancreatic cancer^{1–3} with a worldwide incidence that has been increasing over decades and it is somewhat higher in males than females (6.7 vs. 6.0 cases per 100,000 people per year, respectively; <https://gco.iarc.fr/today/home>). Early diagnosis of PDAC is hampered by the lack of specific screening tests and by the fact that most of the patients do not have symptoms or if they do, they are very unspecific.^{3,4} Consequently, PDAC is frequently diagnosed at late stages leading to an extremely poor prognosis and a 5-year survival rate that ranges from 2% to 10%.⁵ Several environmental factors (including exposure to pesticides, asbestos, benzene, and chlorinated hydrocarbons) and lifestyle risk factors (cigarette smoking, obesity, family history of cancer, pancreatitis, type 2 diabetes, heavy alcohol consumption, low physical activity, stress, mobile phone use, allergies, and poor oral health) have been identified for PDAC.^{2,6–8} Furthermore, around 30 genetic susceptibility loci have been identified through genome wide association studies (GWAS) and large candidate gene or candidate region studies.^{9–16} However, PDAC continues to be one of the most aggressive and lethal diseases with still a relatively unknown etiology.^{17,18}

Recent evidence suggests that autophagy, a lysosome-dependent catabolic degradation process involved in removing toxic air pollutants, particulate particles and heavy metals, but also toxic aggregated cytosolic proteins and malfunctioning organelles from normal cells, might influence the onset of solid tumors and blood malignancies.^{19–21} In PDAC, several studies have demonstrated that pancreatic tumoral tissues have an increased autophagy flux and a greater number of autophagosomes²² in comparison to normal pancreatic cells.^{23,24} Furthermore, it has been shown that autophagy participates in controlling multiple processes including tumor cell growth, metabolism, MHC-I and MHC-II presentation,^{25,26} cell migration and metastasis.²⁷ These findings, along with the fact that treatment with chloroquine (an autophagy inhibitor) or the genetic ablation of autophagy initiation genes (ATG5 or ATG7) reduced the growth of human PDAC cell lines,²³ suggest that autophagy might represent a potential target for PDAC treatment. In support of this hypothesis, studies using xenografts or animal models have shown the benefit of inhibiting autophagy to induce tumor regression and prolong survival.^{28–30} In addition, autophagy is implicated in controlling the resistance to apoptosis³¹ and, therefore, it might represent a poor prognostic factor for PDAC.³² Nonetheless, several studies have also suggested an anti-tumorigenic effect of autophagy in PDAC^{33,34} and some clinical trials using autophagy inhibitors have shown limited success.^{35,36}

Considering the above-reported results, but also previous findings suggesting that genetic variation within autophagy-related genes influences autophagic flux and the susceptibility to solid and hematological cancers,^{37,38} we decided to comprehensively evaluate the impact of 55,583 common genetic variants within 234 autophagy-related genes in determining the risk of developing PDAC. Understanding the impacts of environmental exposure through autophagy and how this catabolic process is genetically regulated might offer

new approaches for risk assessment, protection and preventive actions against cancer. Given the known role of autophagy in shaping immune responses, inflammation and immune tolerance³⁹ and its potential role in regulating immunotherapy efficacy,⁴⁰ we also assessed the correlation of the most promising autophagy variants with cytokine production after *in vitro* stimulation of whole blood (WB), peripheral blood mononuclear cells (PBMCs), or monocyte-derived macrophages (MDM) with lipopolysaccharide (LPS), phytohemagglutinin (PHA), Pam3Cys or CpG. Additionally, we assessed the impact of genetic variants in autophagy genes on the absolute numbers of 91 blood-derived cell populations, 103 serum immunological proteins, and 7 steroid hormones in a large cohort of 408 healthy donors (500FG) from the Human Functional Genomic Project (HFGP).⁴¹

2 | MATERIALS AND METHODS

A workflow diagram of the study is included as Figure 1.

2.1 | Study populations

The discovery population consisted of 15,776 subjects from previous GWAS on PDAC conducted by the Pancreatic Cancer Cohort (PanScan I–III),^{42–44} and the Pancreatic Cancer Case–Control (PanC4) consortia⁴⁵ (Figure 1). In these studies, the PDAC cases presented an established diagnosis and controls were blood donors or healthy subjects randomly selected from the general population and with no history of previous cancers. As part of the discovery cohorts, we also included the FinnGen research genomic project, which arose from Finnish biobanks and digital health record data from Finnish health registries (<https://www.finnngen.fi/en>). At the time this study was conducted, FinnGen (Data Freeze 10 or Ristey10) included genetic data of 314,924 individuals (731 PDAC cases and 314,193 controls).

2.2 | SNP selection and meta-analysis of discovery cohorts

A total of 234 autophagy-related genes were selected based on their presence in the autophagy database (<http://autophagy.lu/clustering/index.html>; Table S1) and association of 55,583 genotyped or imputed SNPs within or near these genes (5 kb upstream and 3 kb downstream)^{46–48} with PDAC risk was computed from a GWAS conducted in the PanScan I–III and the PanC4 population. GWAS datasets were downloaded from the NCBI database of genotypes and phenotypes (dbGaP; study accession numbers phs000206.v5.p3 and phs000648.v1.p1; project reference no. 12644), namely PanScan I, PanScan II and PanC4. These PanScan I–III + PanC4 GWAS were subjected to rigorous standard quality control protocols before imputation using the Michigan imputation server (based on the Haplotype Reference Consortium).⁴⁹ After imputation, GWAS data were filtered to include only

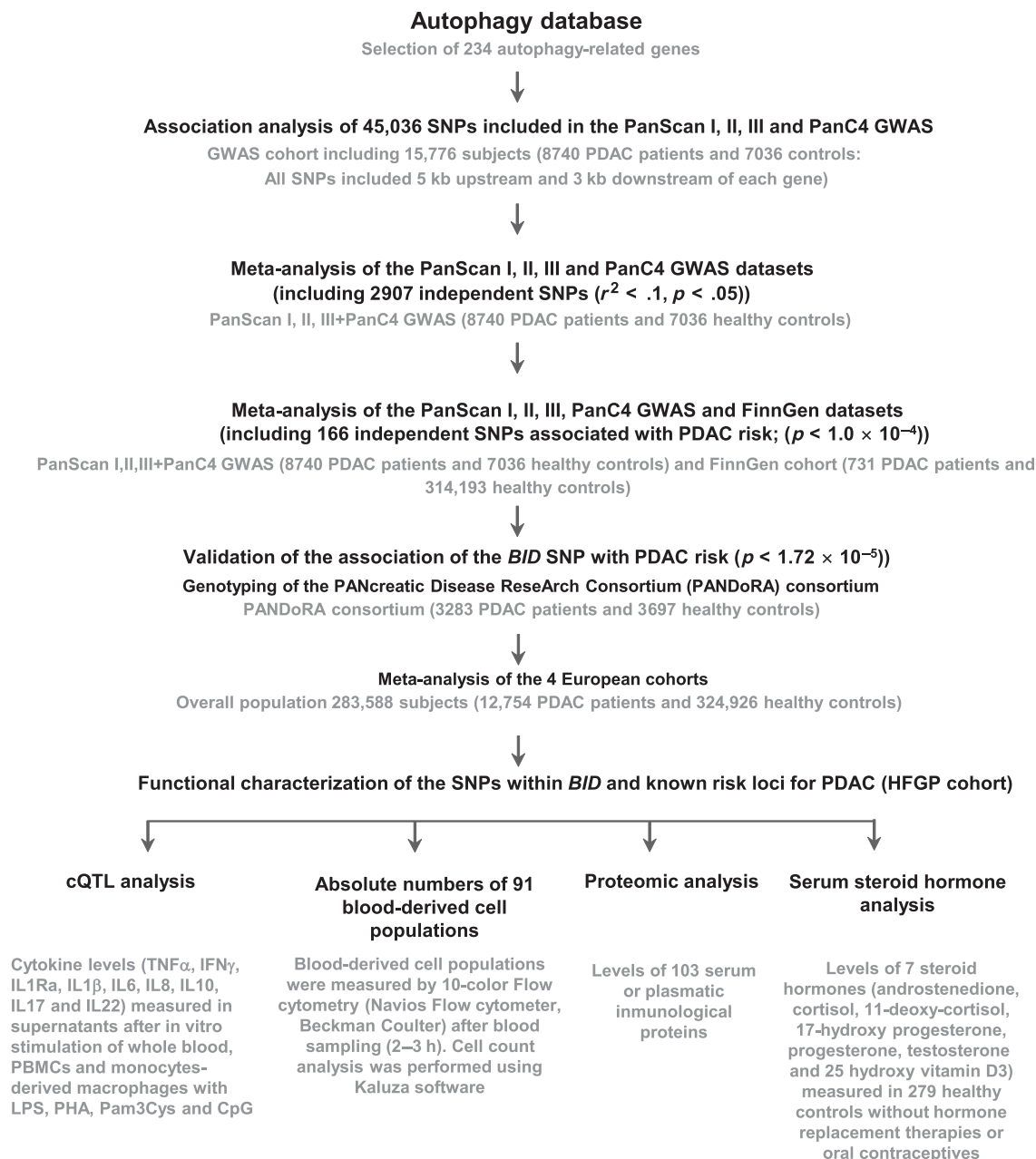


FIGURE 1 Flow diagram of the study.

high-quality imputed variants (info score >0.8). Further quality-control checks were implemented including checks for missingness, duplicates, abnormal heterozygosity, cryptic relatedness, population outliers (evaluated by principal components analyses using Eigenstrat software), and genomic inflation ($\lambda = 1.00$).⁵⁰ Detailed information about the genotyping technologies used, quality control, imputation, and ethnic composition of the discovery cohorts is provided as Data S1. A fixed-effect meta-analysis of the PanScan I–III + PanC4 and publicly available FinnGen GWAS data was conducted using METAL.⁵¹ The I^2 statistic was used to assess statistical heterogeneity between the study cohorts and pooled odds ratios (ORs) were computed using the fixed-effect model. Among the 55,583 selected variants, a total of 45,036 SNPs were shared by the GWAS

platforms and were, therefore, available for association analysis. Of those 45,036 variants, a total of 2907 SNPs were considered independent ($r^2 < .1$) according to LDLink data for European cohorts (<https://ldlink.nci.nih.gov/?tab=snpclip>), and therefore, the multiple testing significance threshold for the study was set to $p = 1.72 \times 10^{-5}$ ($0.05/2907$ SNPs; Table S2). To select the most interesting markers for further validation, we excluded those SNPs that were previously reported as susceptibility markers for PDAC and we advanced for replication in the PANcreatic Disease ReseArch (PANDoRA) consortium only those markers that showed a significant association with PDAC risk after multiple testing correction ($p < 1.72 \times 10^{-5}$). Genotyping of the genetic markers included 3283 PDAC cases and 3697 controls.

2.3 | Genotyping and meta-analysis

Genotyping of genetic variants advanced for replication in PANDoRA was carried out at University of Pisa (Department of Biology, University of Pisa, Pisa, Italy) using KASPar (LGC Genomics, Hoddesdon, UK) according to previously reported protocols.^{52,53} For internal quality control, ~5% of samples were randomly selected and included as duplicates. Concordance between the original and the duplicate samples for the SNPs tested was ≥99.0%. Selected SNPs showed genotype frequencies in the control population similar to those found in the 1000 Genomes database (data not shown) and were in Hardy-Weinberg equilibrium (HWE). After the genotyping of selected markers in PANDoRA, an overall meta-analysis of the association estimates of PANDoRA with those from the PanScan I-III, PANC4, and FinnGen studies was conducted in R using the Meta package.⁵⁴

2.4 | Functional effect of the autophagy-related variants

To provide insight into the functional role of those SNPs that remained statistically significant after multiple correction ($p = 1.72 \times 10^{-5}$), but also those SNPs that are well-established susceptibility markers for PDAC, we tested if any of them were associated with cytokine expression quantitative trait loci (cQTL) data from in vitro stimulation experiments. We also tested the association with the absolute numbers of 91 blood-derived cell populations, 103 serum or plasmatic inflammatory proteins and 7 steroid hormones quantified in 408 volunteers from the 500 Functional Genomics (500FG) cohort from the HFPG.

2.4.1 | Correlation of autophagy-related SNPs with cQTL data

The cQTL data included cytokine levels (IFN γ , IL1 β , IL6, TNF α , IL17 and IL22) measured when peripheral blood mononuclear cells (PBMCs), monocyte-derived macrophages (MDM), or whole blood from 408 healthy subjects were left untreated or stimulated for 24 h with LPS (1 or 100 ng/mL; Sigma Aldrich, St. Louis, MO), PHA (10 μ g/mL, Sigma, St. Louis, MO), Pam3Cys (10 μ g/mL, EMC microcollections, Tübingen, Germany) or CpG (100 ng/mL, InvivoGen, San Diego, CA). Detailed protocols for PBMCs isolation, macrophage differentiation and stimulation assays have been reported elsewhere.^{38,55} Briefly, PBMCs were washed twice in saline and suspended in medium (RPMI 1640) supplemented with gentamicin (10 mg/mL), L-glutamine (10 mM) and pyruvate (10 mM). PBMC stimulations were performed with 5×10^5 cells/well in round-bottom 96-well plates (Greiner Bio-one, Frickenhausen, Germany) for 24 h in the presence of 10% human pool serum at 37°C and 5% CO $_2$. Supernatants were collected and stored at -20°C until used for ELISA. Concentrations of human IFN γ , IL1 β , IL6, TNF α , IL17 and IL22 were determined using specific commercial ELISA kits (PeliKine Compact, Amsterdam or R&D

Systems), in accordance with the manufacturers' instructions. When values were below or above the detection limit of the ELISA, the corresponding limit was used. After log transformation, linear regression analyses adjusted for age and sex were used to determine the correlation of the selected SNPs with cQTL data.

2.4.2 | Correlation of autophagy SNPs and blood cell counts and serum/plasmatic proteomic profile

Next, we evaluated the impact of selected SNPs on cell-level variation. A total of 91 blood-derived cell populations were measured by 10-color flow cytometry (Navios flow cytometer, Beckman Coulter, Miami, FL) after blood sampling (2–3 h), and cell count analysis was performed using Kaluza software (Beckman Coulter, v.1.3). To reduce inter-experimental noise and increase statistical power, cell count analysis was performed by calculating parental and grandparental percentages, which were defined as the percentage of a certain cell type within the subpopulation of the cells from which it was isolated (Table S3).⁵⁵ Detailed laboratory protocols for cell isolation, reagents, gating, and flow cytometry analysis have been reported elsewhere⁵⁶ and raw flow cytometry data and analyzed data files are available upon reasonable request to the authors (<http://hfgp.bbmri.nl>, accessed on 13 February 2024). A proteomic analysis was also performed in serum and plasma samples from the HFPG study. Circulating proteins were measured using the proximity extension assay (Olink Inflammation panel, Olink, Sweden) that resulted in the measurement of 103 different biomarkers (Table S4). Protein levels were expressed on a log $_2$ -scale as normalized protein expression values and normalized using bridging samples to correct for batch variation. Considering the number of proteins ($n = 103$), blood-derived cell populations ($n = 91$), and SNPs ($n = 4$) tested, significance p -values were set to be 1.21×10^{-4} and 1.37×10^{-4} for the proteomic and blood cell count analyses, respectively.

2.4.3 | Correlation between autophagy-related SNPs and serum steroid hormone levels

Besides the immunological experiments, we also evaluated the correlation of autophagy SNPs with serum steroid hormone levels (androstenedione, cortisol, 11-deoxy-cortisol, 17-hydroxyprogesterone, progesterone, testosterone and 25 hydroxy vitamin D3) from 279 healthy controls of the 500FG cohort without hormone replacement or oral contraceptive therapies. Serum steroid hormone levels were determined by ELISA following the manufacturer's instructions. Correlation between levels of 7 serum steroid hormones and autophagy-related SNPs was evaluated by linear regression analysis adjusted for age and sex. The significance threshold was set to $p = .00178$ considering the number of independent SNPs tested ($n = 4$) and the number of hormones determined ($n = 7$).

All analyses of functional data were performed using R software (<http://www.r-project.org>), using custom scripts in the R programming

language based on existing functions such as `lm (stats)`. Functional plots were displayed using the Prism software.

2.5 | In silico functional analysis

Haploreg (<http://www.broadinstitute.org/mammals/haploreg/haploreg.php>)⁵⁷ was also used to predict the functional role of the autophagy SNPs. We also tested whether all these autophagy SNPs could represent expression quantitative trait loci (eQTL) for different cell types and tissues using publicly available GTex portal data (<https://gtexportal.org/home/>) and information available at the Blood eQTL browser (<https://genenetwork.nl/bloodeqtlbrowser/>).

3 | RESULTS

This study included 337,680 individuals consisting of 12,754 cases of PDAC and 324,926 controls, 8740 cases and 7036 controls from the PanScan I–III and PanC4 cohort, 731 cases and 314,193 controls from the FinnGen study and 3283 patients and 3697 healthy controls from the PANDoRA cohort. A total of 45,036 SNPs showing no deviation from HWE ($p < .001$) neither in the PanScan I–III + PanC4 and FinnGen cohorts were selected for association analysis. After testing HWE in the control group by a standard observed-expected chi-square (χ^2) test ($p < 10^{-5}$) and filtering by linkage disequilibrium (LD) values ($r^2 < .1$) and a minor allele frequency (MAF) of 0.01, the meta-analysis of the association estimates for these SNPs in the PanScan I–III + PanC4 and FinnGen cohorts showed that nine polymorphisms were associated with the risk of developing PDAC at $p \leq 10^{-4}$ level (Table 1).

Importantly, among these 9 SNPs, we found a statistically significant association for a SNP within the *BID* locus with PDAC risk ($p < 1.72 \times 10^{-5}$). Therefore, it was advanced for replication and genotyped in PANDoRA. Association results for this SNP are reported in Table 2. After the meta-analysis of the three European cohorts, we found that carriers of the *BID*_{rs9604789G} allele had an increased risk of developing PDAC when compared with those carrying the most frequent allele (OR = 1.31, $p = 9.67 \times 10^{-6}$; Table 2). The association of the *BID*_{rs9604789} SNP with PDAC risk remained significant after correction for multiple testing, which suggested that this variant might have a functional role in modulating PDAC risk. In support of this hypothesis, it has been reported that the *BID*_{rs9604789} SNP affects chromatin states in multiple primary cell types including primary monocytes from peripheral blood, T regulatory cells, T helper cells, CD8+ T cells, B cells, NK cells, and neutrophils.

Besides these results, we found three autophagy-related SNPs within the *TP63* locus that have been previously identified as susceptibility markers for PDAC. Our results confirmed that carriers of the *TP63*_{rs1515496G} *TP63*_{rs35389543C} alleles had decreased risk of developing PDAC (OR = 0.89, $p = 6.27 \times 10^{-8}$ and OR = 1.16, $p = 2.74 \times 10^{-5}$; Table 1). As expected, the association of the *TP63*_{rs1515496} SNP remained significant after correction for multiple

TABLE 1 Association analysis of autophagy-related SNPs in the discovery cohorts.

SNP	Gene	Chr	A1	MAF	PanScan + PanC4 (n = 15,776)			FinnGen R10 (n = 314,924)			Meta-analysis (n = 330,700) ^a			Direction	P _{Het}
					OR (95% CI)	p		OR (95% CI)	p		OR (95% CI)	p			
rs9604789	<i>BID</i>	22	A	0.017	1.33 (1.13–1.56)	4.76E-04		1.34 (1.07–1.68)	.0107		1.33 (1.17–1.52)	1.52E-05	++	++	.9375
rs11533998	<i>ERBB2</i>	17	C	0.335	1.09 (1.04–1.14)	7.34E-04		1.08 (0.98–1.18)	.1196		1.09 (1.04–1.13)	2.02E-04	++	++	.9283
rs11540923	<i>MAP1LC3B</i>	16	C	0.029	0.73 (0.62–0.86)	1.30E-04		0.52 (0.23–1.16)	.1087		0.72 (0.61–0.84)	4.72E-05	–	–	.4182
rs72552382	<i>MTOR</i>	1	C	0.015	1.52 (1.23–1.88)	1.33E-04		0.67 (0.22–2.09)	.4911		1.48 (1.2–1.82)	2.89E-04	+-	+-	.1659
rs75447274	<i>NBR1</i>	17	T	0.031	0.77 (0.67–0.90)	6.50E-04		0.91 (0.67–1.24)	.5697		0.80 (0.70–0.91)	8.94E-04	–	–	.3368
rs78344309	<i>PEX14</i>	1	T	0.070	0.81 (0.72–0.92)	9.99E-04		0.87 (0.69–1.10)	.2530		0.83 (0.74–0.92)	5.67E-04	–	–	.6135
rs1515496	<i>TP63</i>	3	G	0.389	0.88 (0.84–0.92)	3.50E-08		1.05 (0.94–1.17)	.3907		0.89 (0.85–0.93)	6.27E-08	++	++	.1725
rs35389543	<i>TP63</i>	3	C	0.099	1.17 (1.08–1.26)	1.48E-04		1.15 (0.99–1.34)	.0727		1.16 (1.08–1.25)	2.74E-05	–	–	.8619
rs9882798	<i>TP63</i>	3	A	0.096	1.17 (1.08–1.26)	1.39E-04		1.05 (0.90–1.22)	.5464		1.14 (1.06–1.22)	2.61E-04	++	++	.2141

Note: The *TP63*_{rs1515496} SNPs (or other SNPs in strong LD) is a well-established susceptibility markers for PDAC, $p < .05$ in bold. Abbreviations: A1, effect-allele; SNP, single nucleotide polymorphism.

^aMeta-analysis: PanScan + PanC4 + FinnGen; FinnGen releases DF10 according to a random effect model.

TABLE 2 Meta-analysis of the association between the BID SNP and PDAC risk.

SNP	Gene	Chr.	EA	MAF	PanScan + PanC4 (n = 15,776)		FinnGen [#] (n = 314,924)		PANDoRA (n = 6,980)		Meta-analysis (n = 337,680)		P _{Het}
					OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)	p _{Meta}	
rs9604789	BID	22	A	0.02	1.33 (1.13–1.56)	4.70E-04	1.34 (1.07–1.68)	.0107	1.19 (0.89–1.60)	.251	1.31 (1.16–1.47)	9.674E-06	.7868

Note: $p < .05$ in bold.
Abbreviations: SNP, single nucleotide polymorphism; EA, effect allele; MAF, minor allele frequency according to the 1000genome project.

testing (with a threshold of $p_{\text{Bonferroni}} = 1.72 \times 10^{-5}$) whereas the $TP63_{\text{rs35389543}}$ SNP remained borderline significant.

Given that the $TP63_{\text{rs1515496}}$ SNP is a well-established risk marker for PDAC, we investigated, for the first time, its correlation with host immune responses. Of note, we found a potentially interesting correlation of the $TP63_{\text{rs1515496}}$ SNP with increased numbers of FOXP3+ Helios+ T regulatory cells ($p = 7.67 \times 10^{-4}$; Figure 2A) and CD45RA + T regulatory cells ($p = 1.56 \times 10^{-3}$; Figure 2B) but decreased levels of CD4+ T regulatory cells ($p = 7.86 \times 10^{-4}$; Figure 2C). Although these associations did not remain significant after correction for multiple testing ($p_{\text{Bonferroni}} = p = 6.1 \times 10^{-5}$, 0.05/9SNPs/91 blood-derived cell populations), these results, together with our genetic findings, might suggest a modest but still functional effect of the $TP63$ locus on determining PDAC risk by regulating absolute numbers of specific subpopulations of regulatory T cells.

Interestingly, we could not find significant correlations between the BID and $TP63$ variants and levels of circulating inflammatory proteins or steroid hormones, which also suggests that these loci do not impact PDAC risk through the modulation of circulating hormone and inflammatory protein levels. However, further investigation into these aspects is warranted to fully understand their roles in PDAC development.

4 | DISCUSSION

This study comprehensively evaluated the role of autophagy-related variants in modulating PDAC risk in three large and independent cohorts including a total of 13,215 PDAC cases and 270,274 healthy controls. The meta-analysis of these European populations identified for the first time that the $BID_{\text{rs9604789}}$ SNP was significantly associated with an increased risk of PDAC. This association remained significant after Bonferroni correction (multiple testing). The BID gene (BH3 Interacting Domain Death Agonist) is located on chromosome 22 and it encodes for a Bcl-2 family member that promotes autophagy-mediated cell death and apoptosis through different mechanisms involving caspase-8, but also Bak and Bax proteins.^{58–60} Interestingly, it has been proposed that BID also acts as a mediator in modulating the autophagic flux and host immune responses,^{61,62} which underlines the central role of this gene in tumorigenesis. In this regard, it has been described that the $BID_{\text{rs9604789}}$ SNP alters binding motifs for multiple transcription factors including NFκB, POL2, TAF1, TCF12, EGR1, GABP, TBP, POL24H8 and PAX5N19 that are very well-known regulators of key immune processes involving multiple immune cell types.⁶³ Furthermore, it has been shown that the $BID_{\text{rs9604789}}$ SNP affect chromatin states and modulates histone marks in multiple primary immune cell types,⁵⁷ which might suggest a role of this SNP in modulating BID expression in specific immune cells. Likewise, it has been reported that the BID protein interacts with NOD1, NOD2 and IKK complex to promote the activation of NFκB and the induction of the extracellular signal-regulated kinase (ERK) signaling pathway. Previous studies have reported that the constitutive activation of ERK/MAPK signaling pathway (resulting from

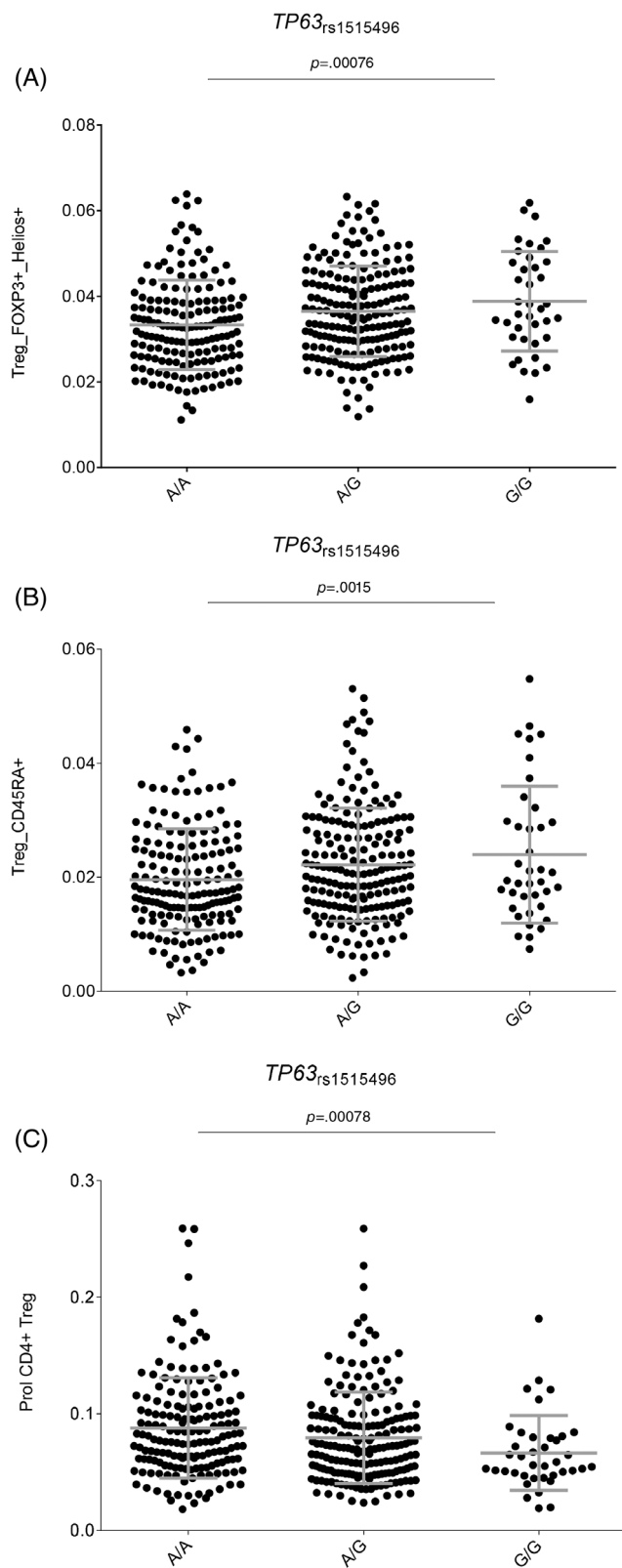


FIGURE 2 (A–C) Correlation of the *TP63*_{rs1515496} SNP with numbers of specific T regulatory T cell subsets.

KRAS mutation) has a relevant role in modulating autophagy, which confirms that *BID* is an autophagy regulator gene that has a relevant role in PDAC.

This activation is linked to macrophage cell survival⁶⁴ and it is independent of the *BID*-induced apoptosis.⁶¹ Furthermore, it has been demonstrated that macrophages from *BID*^{−/−} mice are markedly defective in the production of IL6 and TNFα after stimulation with a NOD2 agonist,⁶¹ which suggests that any disruption of *BID* function might modulate cytokine production. In agreement with these findings, it has been reported that different mutant forms of the *BID* gene affecting protein phosphorylation regulated IL-6 production after stimulation of macrophages with muramyl-dipeptide, a NOD2 agonist.⁶⁵ Importantly, it has been also shown that *BID* modulates myeloid homeostasis and tumor suppression⁶⁶ and acts along with BIM to regulate T cell expansion following acute and persistent infection.⁶² Therefore, considering the above information, it seems plausible to suggest that the *BID*_{rs9604789} SNP might dysregulate *BID*-dependent autophagy, host innate immune responses and survival of macrophages and T cells and, thereby, influence cancer development. However, although tempting, we could not demonstrate the immunological role of this marker to modulate PDAC risk as our functional experiments did not show any significant effect on cytokine production, absolute numbers of blood-derived cell populations and serum inflammatory mediators.

Considering these results, it seems more plausible to suggest that the role of *BID*_{rs9604789} SNP in modulating the risk of PDAC is mediated by its effect on the modulating of autophagy-mediated cell death and/or apoptosis.⁶⁷ In support of this hypothesis, Li et al. (1998) reported that *BID* is a specific proximal substrate of CASP8 in the Fas apoptotic signaling pathway that mediates mitochondrial damage induced by CASP8.⁶⁸ Conversely, although *BID* is mainly known as a pro-apoptotic protein,⁶⁵ it has been reported that it may also lead to the inhibition of apoptosis and induce a shift of toward autophagy-mediated cell death especially in cells resistant to apoptosis.⁶⁹ In addition, it has been reported that *BID* is a target gene of p53,⁷⁰ which suggests its implication in the regulation of autophagy-dependent cell death. However, despite the above-reported information, it is difficult to draw definitive conclusions on how the *BID*_{rs9604789} SNP within the *BID* gene might determine *BID* function and, thereby, cancer risk. Therefore, additional functional studies are now warranted to decipher the specific role of this SNP in modulating the risk of PDAC.

Besides the identification of the *BID* SNP in determining PDAC risk, the meta-analysis of the three cohorts validated results from previous studies demonstrating that autophagy-related variants within the *TP63* locus (or specific SNPs in strong LD with them) are susceptibility biomarkers for PDAC risk.^{16,45,71} The strongest effect was found for the *TP63*_{rs1515496} SNP that survived correction for multiple testing ($p = 5.0 \times 10^{-8}$). Although the association of the *TP63*_{rs1515496} SNP with PDAC risk has been previously established using among others PanScan, PanC4 and PANDoRA populations,^{45,72} its specific biological function has not been completely elucidated. In this regard, we could demonstrate that the *TP63*_{rs1515496} SNP was associated with increased absolute numbers of FOXP3+ Helios+ T regulatory cells, and CD45RA+ T regulatory cells, but also with decreased numbers of CD4+ T regulatory cells, the latter being a subset of regulatory T cells with potentially suppressing immunological activities. These findings

are in agreement with previous studies demonstrating that higher counts of T regulatory cells were significantly associated with an increased risk of PDAC in participants diagnosed within the first 5 years of follow-up⁷³ and even with poor prognosis.^{74,75} Furthermore, functional data from Haploreg showed that the *TP63*_{rs1515496} SNP alters binding sites for FOXA1 and CTCF transcription factors, which are involved in modulating T regulatory cell numbers and, therefore, host immune responses.⁶³ In addition to this, it has also been shown that *TP63* is frequently overexpressed in PDAC tissues⁷⁶ and that its expression correlates with disease aggressiveness.⁷⁶ Likewise, besides the role of the *TP63*_{rs1515496} variant in determining absolute numbers of regulatory T cell subsets and host immune responses, it is conceivable that this intronic variant (or any other polymorphism in strong LD with it) could influence *TP63* function through alteration of splicing sites and thus give rise to functionally different *TP63* isoforms. In this regard, a recent study has demonstrated that whereas the TAp63 isoform induces cell death and cell cycle arrest with tumor suppressor features,⁶⁵ the DNp63 isoform, which is the most common isoform in PDAC cell lines, has an opposite effect inducing pancreatic cancer growth, motility and invasion.^{77,78}

Finally, even though the association of genetic markers within the *MAP1LC3B*, *ERBB2*, *MTOR*, *PEX14* and *NRB1* loci did not reach the multiple testing significance threshold and, therefore, were not advanced for replication in the PANDORA cohort, we think that they are potentially interesting markers that need to be further analyzed in future studies. In particular, it has been reported that the *MAP1LC3B* and *ERBB2* genes, which showed SNPs associated with PDAC risk close to the Bonferroni significance threshold, may play a role in modulating tumor cell survival and resistance to treatments. Rouschop et al. (2010) demonstrated that the unfolded protein response protects human tumor cells during hypoxia by regulating *MAP1LC3B*, which is involved in phagophore expansion and autophagosome formation.⁷⁹ In addition, expression of the *MAP1LC3B* protein in carcinoma-associated fibroblasts (CAFs) has been linked to poor survival in PDAC patients,⁸⁰ suggesting its involvement in modulating adaptive immune resistance of tumor cells.

Similarly, *ERBB2* has also been consistently implicated in PDAC onset and patient survival. Previous studies have demonstrated that this gene is frequently amplified in PDAC patients,⁸¹ and its oncogenic effect is mediated not only by gene amplification but also by overexpression,⁸² highlighting the value of studying this component from a genetic perspective. Additionally, Ortega et al. (2015) recently demonstrated that *ERBB2* is upregulated in a high proportion of PDAC patients,⁸³ suggesting its involvement not only in disease onset but also in disease progression^{84–86} and patient survival.⁸³ However, further studies are needed to delineate the precise roles of *MAP1LC3B* and *ERBB2* in PDAC pathogenesis, as some previous studies have indicated a lack of influence of these genes on disease progression.^{87,88}

This study has both strengths and limitations. The most important strengths of our study are the comprehensive analysis of autophagy-related SNPs and the inclusion of three large independent populations of European ancestry for a total of 276,608 study participants. Furthermore, we comprehensively analyzed the functional impact of

autophagy-related SNPs in modulating host immune responses, absolute numbers of blood-derived cell populations, serum and plasma metabolites, and steroid hormones in a large study of healthy subjects ascertained through the HFGP. However, this study also has limitations. Even though the 5 kb upstream and 3 kb downstream range used to select SNPs is generally sufficient to capture most relevant variants, including those in promoter regions and untranslated regions that can impact gene function, we acknowledge that regulatory elements, such as enhancers and repressors, can exist outside these regions. Therefore, our study might miss some potentially interesting and functional markers that affect the regulation of autophagy genes. Another limitation of our study is that it included only populations of European ancestry, which restricted the translation of the above-reported results to other ethnicity groups. Although we attempted to validate the association of the *BID* SNP with PDAC risk in the JaPAN consortium, this cohort did not have genotyping information of the *BID* marker (or its proxies).⁸⁹ Additionally, the lack of environmental variables in the PANDORA database prevented us from conducting gene-environment interaction analyses to explore the relationship between genetic factors and environmental influences in PDAC development.

5 | CONCLUSION

In summary, our study has identified, for the first time, the association of the *BID*_{rs9604789} SNP with an increased risk of developing the disease. In addition, it has confirmed the association of *TP63* SNPs with the risk of developing PDAC. This study points to a functional role of the *BID* and *TP63* loci in modulating PDAC onset likely through the regulation autophagy and host immune responses mediated by different subsets of T regulatory cells. Finally, this work has underlined the need of additional studies to elucidate the functional role of the *MAP1LC3B*, *ERBB2*, *MTOR*, *PEX14* and *NRB1* loci to determine PDAC risk.

AUTHOR CONTRIBUTIONS

Fernando Gálvez-Montosa: Formal analysis; investigation; resources; writing – original draft. **Giulia Peduzzi:** Data curation; formal analysis; investigation; resources; writing – original draft. **José Manuel Sanchez-Maldonado:** Formal analysis; investigation; writing – original draft. **Rob ter Horst:** Data curation; formal analysis; investigation; resources; writing – review and editing. **Antonio J. Cabrera-Serrano:** Formal analysis; investigation; writing – review and editing. **Manuel Gentiluomo:** Resources; writing – review and editing. **Angelica Macaudo:** Resources; writing – review and editing. **Natalia Luque:** Resources; writing – review and editing. **Pelin Ünal:** Resources; writing – review and editing. **Francisco José García-Verdejo:** Resources; writing – review and editing. **Yang Li:** Resources; writing – review and editing. **José Antonio López López:** Resources; writing – review and editing. **Angelika Stein:** Resources; writing – review and editing. **H. Bas Bueno-de-Mesquita:** Resources; writing – review and editing. **Paolo Giorgio Arcidiacono:** Resources; writing – review and editing.

Dalila Luciola Zanette: Resources; writing – review and editing. **Christoph Kahlert:** Resources; writing – review and editing. **Francesco Perri:** Resources; writing – review and editing. **Pavel Soucek:** Resources; writing – review and editing. **Renata Talar-Wojnarowska:** Resources; writing – review and editing. **George E. Theodoropoulos:** Resources; writing – review and editing. **Jakob R. Izbecki:** Resources; writing – review and editing. **Hussein Tamás:** Resources; writing – review and editing. **Hanneke Van Laarhoven:** Resources; writing – review and editing. **Gennaro Nappo:** Resources; writing – review and editing. **Maria Chiara Petrone:** Resources; writing – review and editing. **Martin Lovecek:** Resources; writing – review and editing. **Roel C. H. Vermeulen:** Resources; writing – review and editing. **Kestutis Adamonis:** Resources; writing – review and editing. **Fernando Jesus Reyes-Zurita:** Resources; writing – review and editing. **Bernd Holleczek:** Resources; writing – review and editing. **Jolanta Sumskiene:** Resources; writing – review and editing. **Beatrice Mohelníková-Duchonová:** Resources; writing – review and editing. **Rita T. Lawlor:** Resources; writing – review and editing. **Raffaele Pezzilli:** Resources; writing – review and editing. **Mateus Nobrega Aoki:** Resources; writing – review and editing. **Claudio Pasquali:** Resources; writing – review and editing. **Vitalija Petrenkiene:** Resources; writing – review and editing. **Daniela Basso:** Resources; writing – review and editing. **Stefania Bunduc:** Resources; writing – review and editing. **Annalisa Comandatore:** Resources; writing – review and editing. **Hermann Brenner:** Resources; writing – review and editing. **Stefano Ermini:** Resources; writing – review and editing. **Giuseppe Vanella:** Resources; writing – review and editing. **Mara R. Goetz:** Resources; writing – review and editing. **Livia Archibugi:** Resources; writing – review and editing. **Maurizio Lucchesi:** Resources; writing – review and editing. **Faik Guntac Uzunoglu:** Resources; writing – review and editing. **Olivier Busch:** Resources; writing – review and editing. **Anna Caterina Milanetto:** Resources; writing – review and editing. **Marta Puzzono:** Resources; writing – review and editing. **Juozas Kupcinskas:** Resources; writing – review and editing. **Luca Morelli:** Resources; writing – review and editing. **Cosimo Sperti:** Resources; writing – review and editing. **Silvia Carrara:** Resources; writing – review and editing. **Gabriele Capurso:** Resources; writing – review and editing. **Casper H. J. van Eijck:** Resources; writing – review and editing. **Martin Oliverius:** Resources; writing – review and editing. **Susanne Roth:** Resources; writing – review and editing. **Francesca Tavano:** Resources; writing – review and editing. **Rudolf Kaaks:** Resources; writing – review and editing. **Andrea Szentesi:** Resources; writing – review and editing. **Ludmila Vodickova:** Resources; writing – review and editing. **Claudio Luchini:** Resources; writing – review and editing. **Ben Schöttker:** Resources; writing – review and editing. **Stefano Landi:** Resources; writing – review and editing. **Orsolya Dohan:** Resources; writing – review and editing. **Matteo Tacelli:** Resources; writing – review and editing. **William Greenhalf:** Resources; writing – review and editing. **Maria Gazouli:** Resources; writing – review and editing. **John P. Neoptolemos:** Resources; writing – review and editing. **Giulia Martina Cavestro:** Resources; writing – review and editing. **Ugo Boggi:** Resources; writing – review and editing. **Anna Latiano:** Resources; writing – review and editing. **Péter Hegyi:** Resources;

writing – review and editing. **Laura Ginocchi:** Resources; writing – review and editing. **Mihai G. Netea:** Resources; writing – review and editing. **Pedro Sánchez-Rovira:** Resources; writing – review and editing. **Federico Canzian:** Conceptualization; investigation; resources; writing – review and editing. **Daniele Campa:** Conceptualization; funding acquisition; resources; writing – original draft; writing – review and editing. **Juan Sainz:** Conceptualization; formal analysis; funding acquisition; investigation; resources; writing – original draft; writing – review and editing.

AFFILIATIONS

¹Department of Medical Oncology, Complejo Hospitalario de Jaén, Jaén, Spain

²Department of Biology, University of Pisa, Pisa, Italy

³Department of Biochemistry and Molecular Biology I, University of Granada, Granada, Spain

⁴Genomic Oncology Area, GENYO, Centre for Genomics and Oncological Research: Pfizer/University of Granada/Andalusian Regional Government, PTS, Granada, Spain

⁵Instituto de Investigación Biosanitaria Ibs.Granada, Granada, Spain

⁶Genomic Epidemiology Group, German Cancer Research Center (DKFZ), Heidelberg, Germany

⁷Department of Internal Medicine and Radboud Center for Infectious Diseases, Radboud University Medical Center, Nijmegen, The Netherlands

⁸CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, Austria

⁹National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands

¹⁰Pancreatico/Biliary Endoscopy & Endosonography Division, Pancreas Translational & Clinical Research Center, San Raffaele Scientific Institute, Milan, Italy

¹¹Laboratory for Applied Science and Technology in Health, Carlos Chagas Institute, Oswaldo Cruz Foundation (Fiocruz), Curitiba, Brazil

¹²Department of General Surgery, University of Heidelberg, Heidelberg, Baden-Württemberg, Germany

¹³Division of Gastroenterology and Research Laboratory, Fondazione IRCCS “Casa Sollievo della Sofferenza” Hospital, Foggia, Italy

¹⁴Biomedical Center, Faculty of Medicine in Pilsen, Charles University, Pilsen, Czech Republic

¹⁵Department of Digestive Tract Diseases, Medical University of Lodz, Lodz, Poland

¹⁶Colorectal Unit, First Department of Propaedeutic Surgery, Medical School of National and Kapodistrian University of Athens, Hippocraton General Hospital, Athens, Greece

¹⁷Department of General, Visceral and Thoracic Surgery, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

¹⁸Center for Translational Medicine, Semmelweis University, Budapest, Hungary

¹⁹Division of Pancreatic Diseases, Heart and Vascular Center, Semmelweis University, Budapest, Hungary

²⁰Department of Medical Oncology, Amsterdam UMC location University of Amsterdam, Amsterdam, The Netherlands

²¹Cancer Center Amsterdam, Imaging and Biomarkers, Amsterdam, The Netherlands

²²Pancreatic Unit, IRCCS Humanitas Research Hospital, Milan, Italy

²³Department of Biomedical Sciences, Humanitas University, Milan, Italy

²⁴Department of Surgery I, University Hospital Olomouc, Olomouc, Czech Republic

²⁵University of Utrecht, Utrecht, The Netherlands

²⁶Gastroenterology Department and Institute for Digestive Research, Lithuanian University of Health Sciences, Kaunas, Lithuania

²⁷Saarland Cancer Registry, Saarbrücken, Germany

²⁸Division of Clinical Epidemiology and Aging Research, German Cancer Research Center (DKFZ), Heidelberg, Germany

²⁹Toxicogenomics Unit, National Institute of Public Health, Prague, Czech Republic

³⁰ARC-Net Centre for Applied Research on Cancer University of Verona, Verona, Italy

³¹Department of Diagnostics and Public Health, Section of Pathology, University of Verona, Verona, Italy

³²Potenza County Medical Association, Potenza, Italy

³³Department of DISCOG, University of Padova, Padova, Italy

³⁴Department of DIMED, Laboratory Medicine, University of Padova, Padova, Italy

³⁵Carol Davila University of Medicine and Pharmacy, Bucharest, Romania

³⁶Digestive Diseases and Liver Transplantation Center, Fundeni Clinical Institute, Bucharest, Romania

³⁷General Surgery Unit, Department of Translational Research and New Technologies in Medicine, University of Pisa, Pisa, Italy

³⁸German Cancer Consortium (DKTK), German Cancer Research Center (DKFZ), Heidelberg, Germany

³⁹Digestive and Liver Disease Unit, S Andrea Hospital, Rome, Italy

⁴⁰Pancreas Translational and Clinical Research Center, Pancreato-Biliary Endoscopy and Endoscopic Ultrasound, San Raffaele Scientific Institute IRCCS, Milan, Italy

⁴¹Oncologia Massa Carrara, Azienda USL Toscana Nord Ovest, Carrara, Italy

⁴²Department of Medical Oncology, Amsterdam UMC Location University of Amsterdam, Amsterdam, The Netherlands

⁴³Gastroenterology and Gastrointestinal Endoscopy Unit, Vita-Salute San Raffaele University, IRCCS San Raffaele Scientific Institute, Milan, Italy

⁴⁴Department of Gastroenterology, IRCCS Humanitas Research Hospital – Endoscopic Unit, Milan, Italy

⁴⁵Department of Surgery, Erasmus MC University Medical Center, Rotterdam, The Netherlands

⁴⁶Department of Surgery, University Hospital Kralovske Vinohrady, Third Faculty of Medicine, Charles University, Prague, Czech Republic

⁴⁷Division of Cancer Epidemiology, German Cancer Research Center (DKFZ), Heidelberg, Germany

⁴⁸Institute for Translational Medicine, Medical School, University of Pécs, Pécs, Hungary

⁴⁹Department of Molecular Biology of Cancer, Institute of Experimental Medicine, Czech Academy of Sciences, Prague, Czech Republic

⁵⁰Institute of Biology and Medical Genetics, First Faculty of Medicine, Charles University, Prague, Czech Republic

⁵¹Faculty of Medicine and Biomedical Center in Pilsen, Charles University, Pilsen, Czech Republic

⁵²Department of Engineering for Innovation in Medicine, University of Verona, Verona, Italy

⁵³Institute for Health Research Liverpool Pancreas Biomedical Research Unit, University of Liverpool, Liverpool, UK

⁵⁴Department of Basic Medical Science, Laboratory of Biology, Medical School, National and Kapodistrian University of Athens, Athens, Greece

⁵⁵Division of General and Transplant Surgery, Pisa University Hospital, Pisa, Italy

⁵⁶János Szentágothai Research Center, University of Pécs, Pécs, Hungary

⁵⁷Centre for Individualised Infection Medicine (CiIM) & TWINCORE, joint Ventures Between the Helmholtz-Centre for Infection Research (HZI) and the Hannover Medical School (MHH), Hannover, Germany

⁵⁸Department for Immunology & Metabolism, Life and Medical Sciences Institute (LIMES), University of Bonn, Bonn, Germany

⁵⁹Consortium for Biomedical Research in Epidemiology and Public Health (CIBERESP), Barcelona, Spain

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CONFLICT OF INTEREST STATEMENT

All authors have no competing interests to disclose.

DATA AVAILABILITY STATEMENT

The genotype data used in the present study come from three pancreatic cancer GWAS datasets downloaded from the NCBI database of genotypes and phenotypes (dbGaP; study accession numbers phs000206.v5.p3 and phs000648. v1.p1; project reference #12644), namely PanScan I, PanScan II and PanC4. Genotyping data of the genetic markers analyzed in the PANDORA cohort are available on request from the corresponding author. Functional data used in this project have been meticulously catalogued and archived in the BBMRI-NL data infrastructure (<https://hfgp.bbMRI.nl/>) using the

MOLGENIS open-source platform for scientific data. This allows flexible data querying and download, including sufficiently rich metadata and interfaces for machine processing (R statistics, REST API) and using FAIR principles to optimize Findability, Accessibility, Interoperability and Reusability. Further information is available from the corresponding author upon request.

ETHICS STATEMENT

Each participant in the PanScan and PanC4 studies obtained approval from the responsible institutional review board (IRB) and IRB certification permitting data sharing in accordance with the NIH Policy for sharing of Data Obtained in NIH-Supported or NIH-Conducted Genome Wide Association Studies. The PANDoRA study protocol was approved by the Ethics Commission of the Medical Faculty of the University of Heidelberg (S-565/2015, last update on April 3, 2017). In accordance with the Declaration of Helsinki, written informed consent was obtained from each participant. The PANDoRA cohort was set up in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans. Ethical approval for this study was obtained from each participating institution in PANDoRA and all participating subjects provided written informed consent (Data S1).^{90,91} The HFGP study was also approved by the Arnhem-Nijmegen Ethical Committee (no. 42561.091.12) and biological specimens were collected after informed consent was obtained.

ORCID

Manuel Gentiluomo  <https://orcid.org/0000-0002-0366-9653>

Pavel Soucek  <https://orcid.org/0000-0002-4294-6799>

Hermann Brenner  <https://orcid.org/0000-0002-6129-1572>

Francesca Tavano  <https://orcid.org/0000-0002-8831-7349>

Rudolf Kaaks  <https://orcid.org/0000-0003-3751-3929>

Ben Schöttker  <https://orcid.org/0000-0002-1217-4521>

Stefano Landi  <https://orcid.org/0000-0001-8364-6357>

Maria Gazouli  <https://orcid.org/0000-0002-3295-6811>

Federico Canzian  <https://orcid.org/0000-0002-4261-4583>

Daniele Campa  <https://orcid.org/0000-0003-3220-9944>

Juan Sainz  <https://orcid.org/0000-0002-9355-2423>

TWITTER

Juan Sainz  [JuanSainzUGR](https://twitter.com/JuanSainzUGR)

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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