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Influence of *KRAS* mutations, persistent organic pollutants, and trace elements on survival from pancreatic ductal adenocarcinoma

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Abstract

Introduction: Reasons why pancreatic ductal adenocarcinoma (PDAC) continues to have poor survival are only partly known. No previous studies have analyzed the combined influence of *KRAS* mutations, persistent organic pollutants (POPs), and trace elements upon survival in PDAC or in any other human cancer.

Objective: To analyze the individual and combined influence of *KRAS* mutations, POPs, and trace elements upon survival from PDAC.

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Methods: Incident cases of PDAC (n = 185) were prospectively identified in five hospitals in Eastern Spain in 1992–1995 and interviewed face-to-face during hospital admission. *KRAS* mutational status was determined from tumour tissue through polymerase chain reaction and artificial restriction fragment length polymorphism. Blood and toenail samples were obtained before treatment. Serum concentrations of POPs were analyzed by high-resolution gas chromatography with electron-capture detection. Concentrations of 12 trace elements were determined in toenail samples by inductively coupled plasma mass spectrometry. Multivariable Cox proportional hazards regression was used to assess prognostic associations.

Results: Patients with a *KRAS* mutated tumor had a 70% higher risk of early death than patients with a *KRAS* wild-type PDAC (hazard ratio [HR] = 1.7, p = 0.026), adjusting for age, sex, and tumor stage. *KRAS* mutational status was only modestly and not statistically significantly associated with survival when further adjusting by treatment or by treatment intention. The beneficial effects of treatment remained unaltered when *KRAS* mutational status was taken into account, and treatment did not appear to be less effective in the subgroup of patients with a *KRAS* mutated tumor. POPs did not materially influence survival: the adjusted HR of the highest POP tertiles was near unity for all POPs. When considering the joint effect on survival of POPs and *KRAS*, patients with *KRAS* mutated tumors had modest and nonsignificant HRs (most HRs around 1.3 to 1.4). Higher concentrations of lead, cadmium, arsenic, vanadium, and aluminium were associated with better survival. When *KRAS* status, POPs, and trace elements were simultaneously considered along with treatment, only the latter was statistically significantly related to survival.

Conclusions: In this study based on molecular, clinical, and environmental epidemiology, *KRAS* mutational status, POPs, and trace elements were not adversely related to PDAC survival when treatment was simultaneously considered; only treatment was independently related to survival. The lack of adverse prognostic effects of POPs and metals measured at the time of diagnosis provide scientific and clinical reassurance on the effects of such exposures upon survival of patients with PDAC. The weak association with *KRAS* mutations contributes to the scant knowledge on the clinical implications of a genetic alteration highly frequent in PDAC.

Keywords

persistent organic pollutants (POPs); trace elements; metals; pancreatic cancer; pancreatic ductal adenocarcinoma (PDAC); survival; *KRAS* oncogene

1. Introduction

Reasons why pancreatic ductal adenocarcinoma (PDAC) generally continues to have such dismal survival are only partly known (Huang et al., 2019; Tempero et al., 2017). Among numerous potential explanations, the influence of *KRAS* mutations remains unclear. Even less studied is the role of the body burden of environmental agents such as persistent organic pollutants (POPs) and trace elements (Benetou et al, 2018).

Mutations in the *KRAS* oncogene are an early event in several neoplasms (Deramaudt and Rustgi, 2005; Eser et al., 2014; Rachakonda et al., 2013; Waddell et al., 2015). In PDAC, codon *KRAS* 12 mutations were traditionally thought to be present in 70% to 90% of

tumours (Deramaudt and Rustgi, 2005; Parker et al., 2011a, 2011b; Porta et al., 1999; Waddell et al., 2015). At present, older techniques as polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis are known to yield a lower sensitivity to detect *KRAS* mutations than more recent techniques as droplet digital (dd) PCR, high resolution melting analysis or next-generation sequencing. Thus, tumours identified as *KRAS* wild-type and *KRAS* mutated by traditional techniques may have levels of *KRAS* mutations below and above 1 in 50 alleles, respectively (Bittoni et al., 2015; Buscail et al., 2019; Collisson et al., 2011, 2012; Deramaudt and Rustgi, 2005; Porta et al., 1999; Schlitter et al., 2017). Mutated and wild-type *KRAS* PDAC may have distinct gene expression patterns, perhaps in part as a result of different gene-environment interactions, some of which may involve POPs and trace elements (Gómez-Tomás et al., 2019; Porta et al., 1999, 2009b); such processes might influence tumor progression and clinical prognosis in PDAC differentially according to *KRAS* mutational status (Bittoni et al., 2015; Collisson et al., 2011, 2012; Crous-Bou, 2009; O'Brien et al., 2013; Porta et al., 1999, 2009b; Qian et al., 2018; Rachakonda et al., 2013; Schlitter et al., 2017).

POPs are highly lipophilic and degradation-resistant synthetic chemicals. They bioaccumulate in the environment, food webs and living organisms, and may contribute to cause clinically and socially significant health effects. Exposed to such agents throughout life, mostly from the ingestion of animal fats, virtually all humans store POP mixtures in adipose tissues. Although production of some POPs was banned decades ago −and their environmental, food, and human concentrations subsequently decreased− human exposure, contamination, and health effects remain relevant (Alonso-Magdalena et al., 2011; Department of Health and Human Services, 2019; Gore et al., 2015; Henkler and Luch, 2011; Porta, 2015; Quinn and Wania, 2012; Quinn et al., 2011; Stein, 2012; Vandenberg et al., 2012; Wu et al., 2013). While it is biologically and clinically plausible that the toxic effects of POPs could adversely affect prognosis in PDAC and other cancer types, very few studies assessed the influence of POPs on cancer survival (Hardell et al., 2007; Parada et al., 2016a, 2016b, 2019; Roswall et al., 2018).

Some trace elements considered in this report are carcinogenic or probably carcinogenic to humans. For instance, cadmium and cadmium compounds, as well as arsenic and inorganic arsenic compounds, are carcinogenic to humans (IARC group 1); inorganic lead compounds are probably carcinogenic to humans (IARC group 2A). Elements such as arsenic, cadmium and lead, which have been associated with PDAC risk (Amaral et al. 2012), are weak mutagens, and non-genotoxic mechanisms are their predominant carcinogenic mode of action (Hartwig, 2010; Straif et al., 2009). In addition to their etiopathogenic roles, they may adversely affect the clinical course and prognosis because they induce oxidative stress, defective DNA repair, genomic instability, post-translational histone modifications, and altered methylation of tumour-suppressor genes and oncogenes (Camargo et al., 2019; Chervona et al., 2012; Gómez-Tomás et al., 2019; Henkler and Luch, 2011; Henkler et al., 2010; Hernández et al., 2009; Stein, 2012). Despite these pieces of evidence, studies that have analyzed the influence of biomarkers of trace elements on the survival of cancer patients are scarce (Du et al., 2019; He et al, 2017). Furthermore, no study has analyzed the combined influence of *KRAS* mutations, POPs, and trace elements upon survival in any human cancer.

Therefore, the present study aimed to investigate the individual and combined effects of these three factors on the survival of patients with PDAC.

2. Material and Methods

2.1. Study population

Methods of the PANKRAS II study have been described in detail (Camargo et al., 2019; Crous-Bou, 2009; Gasull et al., 2010; Gómez-Tomás et al., 2019; López et al., 2014; Porta et al., 1999, 2000, 2005, 2008, 2009b, 2009c, 2012; Soler et al., 1999). Briefly, subject recruitment took place between 1992 and 1995 at five general university hospitals in eastern Spain, where 602 patients with biliopancreatic diseases, including 185 incident cases of PDAC, were prospectively identified. Of the 185 patients with PDAC, 121 had results on *KRAS* status, 144 on serum levels of persistent organic pollutants (POPs) (103 on the two types of variables) (see below), 118 on toenail levels of trace elements (78 on elements and *KRAS*), and 72 on all three factors (Supplemental Figure). There were no significant differences between the 72 cases and the remaining cases in a broad range of sociodemographic and clinical variables (including sex, education, occupation, hospital, tumour stage, signs and symptoms of pancreatic cancer at presentation, diet, consumption of coffee, tobacco and alcohol, and duration of the interview), except that the 72 cases were slightly younger (Camargo et al., 2019; Crous-Bou, 2009; Gómez-Tomás et al., 2019; Porta et al., 2009b), had more often treatments with a radical intention, and had a longer survival than the other 113 PDAC patients (Supplemental Table). While our main strategy was to analyse and present results for all subjects with available data in each phase of the analyses (Supplemental Figure), we also analysed and will summarize results for the subgroup of 72 patients with data on *KRAS*, POPs and metals.

The clinico-pathological information of all cases, including diagnoses, was reviewed by a panel of experts and by the study reference pathologists, blinded to the original diagnoses and to molecular results (Porta et al., 2000, 2008; Soler et al., 1999). The Ethics Committees of participating hospitals approved the study protocol, and patients gave informed consent to participate.

2.2. Clinicopathological information and personal interviews

More than 89% of the 185 PDAC patients were interviewed face-to-face by trained monitors during hospital stay, close to the time of diagnosis (Crous-Bou, 2009; Porta et al., 2005). Interviews included questions about past medical conditions, symptoms, and coffee, tobacco and alcohol consumption (Crous-Bou, 2009; Porta et al., 2005, 2008, 2009b). A structured form was used to collect clinicopathological information from medical records, including details on past medical conditions, diagnostic procedures, signs and symptoms of pancreatic cancer, tumour stage, laboratory results, treatment, and follow-up (Crous-Bou, 2009; Porta et al., 2000, 2005, 2009b; Soler et al., 1999). All items concerning medical conditions were further reviewed by study physicians and checked for consistency (Crous-Bou, 2009; Porta et al., 2000). Hospital discharge diagnoses and tumour's clinical stage were also recorded in the form (Crous-Bou, 2009; Porta et al., 1999, 2000). The tumour's clinical stage at diagnosis was classified according to the tumor-node-metastasis (TNM) system. All items

concerning the presence of signs and symptoms were further reviewed by two experienced oncologists and checked for consistency (Porta et al., 2005). Cholestatic syndrome involved jaundice, hypocholia and choluria and the constitutional syndrome comprised fatigue, anorexia and weight loss (Porta et al., 2005, 2008).

2.3. Detection of KRAS mutations

Cytohistological samples from patient tumors were obtained during hospital stay. Details of tissue specimens and laboratory protocols have been described in detail elsewhere (Crous-Bou 2009; Gómez-Tomás et al., 2019; Malats et al. 1995, 1997; Porta et al., 1999, 2009c). Briefly, mutations in codon 12 of the *KRAS* oncogene were studied using DNA extracted from paraffin-embedded tumour tissue. Tumor DNA was extracted and analyzed immediately after the end of patient recruitment (i.e, from a few weeks to about three years after tumor procurement). Amplifications were done in two steps by nested polymerase chain reaction; in the second amplification reaction, an artificial BstNI restriction endonuclease site was created to discriminate between wild-type and mutated *KRAS* codon 12 sequences. The 103 bp product of this amplification reaction was digested overnight. Wild-type sequences were cleaved, resulting in two fragments of 82 and 21 bp, whereas codon 12 mutated sequences were not. Products were analyzed by acrylamide gel electrophoresis and ethidium bromide staining. This technique was able to detect 1 heterozygously mutated cell per 50 wild-type cells (i.e., 1 mutant allele per 50 wild-type alleles). Interpretation of digestion products' electrophoresis was performed independently by two investigators. When discordant results were obtained, the analysis was repeated and results evaluated again. This strategy has been shown to yield an agreement of >95% for all enzyme digestions (Malats et al., 1995, 1997).

2.4. Analysis of serum concentrations of POPs

Methods to analyze POPs have also been described (Gasull et al., 2010; López et al., 2014; Porta et al., 1999, 2009b, 2009c). Briefly, blood samples were obtained before treatment, gas chromatography analyses were performed, and selected samples were analysed by negative-ion chemical ionization gas chromatography–mass spectrometry (Porta et al., 1999, 2009b). Analyses were carried out in the Department of Environmental Chemistry (IIQAB-CSIC) in Barcelona, Spain. In this report, statistical analyses are limited to the 7 POPs that were detected above the detection limit in >85% of the 144 PDAC cases with POPs determined: *p,p'*-dichlorodiphenyltrichloroethane (DDT), *p,p'*-dichlorodiphenyldichloroethene (DDE), polychlorinated biphenyls (PCBs) 138, 153 and 180, hexachlorobenzene (HCB) and β-hexachlorocyclohexane (HCH) (Porta et al., 1999, 2009b, 2009c).

Concentrations of total cholesterol and triglycerides were determined enzymatically, using serum obtained at the same time as the serum used for the organochlorine analyses (Gasull et al., 2010; López et al., 2014; Porta et al., 1999, 2009b, 2009c). Total serum lipids (TL) were calculated by the standard formula 2 of Phillips et al. (1989), based on total cholesterol and triglycerides (Bernert et al., 2007; Phillips et al., 1989). POP concentrations were individually corrected for TL and are expressed in nanograms per gram lipid (ng/g of lipid) (Bernert et al., 2007; López et al., 2014; Phillips et al., 1989; Porta et al., 1999, 2009b).

2.5 Analysis of trace elements

Toenail samples were also obtained during hospital stay before treatment (Amaral et al., 2012; Camargo et al., 2019; Gómez-Tomás et al., 2019). Toenails are not altered with long-term storage, and they provide a valid measure of cumulative exposure to trace elements 6–18 months prior to their clipping (He, 2011; Laohaudomchok et al., 2011; Slotnick and Nriagu, 2006; Slotnick et al., 2007; Wickre et al., 2004). The rationale for their use in the present study is that the ranking of toenail concentrations in a group of individuals (e.g., in *KRAS* mutated and non-mutated cases) at one point in time will be highly similar to the ranking in the more distant past.

Toenails were stored at room temperature until analysis. In 2009, after careful cleaning and washing to remove external contaminants, twelve trace elements (lead, cadmium, arsenic, selenium, zinc, vanadium, manganese, aluminium, chromium, iron, nickel, and copper) were quantified at the Trace Element Analysis Core (Dartmouth College, NH, USA) using inductively coupled plasma-mass spectrometry. Toenails were acid digested with Optima nitric acid (Fisher Scientific, St. Louis, MO) at 105°C followed by addition of hydrogen peroxide and further heating the dilution with deionized water. All sample preparation steps were recorded gravimetrically. As a quality control, each batch of analyses included six standard reference material (SRM) samples with known trace element concentrations and six analytic blanks, along with the study samples. The amount of SRM used ranged from less than 10 to 50 mg to mimic the mass of toenails (Amaral et al., 2012; Camargo et al., 2019; Gómez-Tomás et al., 2019). Concentrations of trace elements are expressed as μg/g.

2.6. Follow-up and survival analysis

The date of death was obtained from hospital records, interviews with relatives, and the mortality registry of Catalonia (Macià et al., 2013; Puigdefàbregas et al., 2013). 90.0% of patients died of causes related to the pancreatic cancer. Follow-up for all participants in the PANKRAS II study was 17.5 years. We lost to follow-up only three of the 185 PDAC patients (one patient 5 years after diagnosis, one after 2.5 years, and one after 8 days). 49.2% of PDAC patients died during the first 3 months after the diagnosis of PDAC, whereas survival at 6 and 12 months was 29.7% and 15.1%, respectively; at 2 years it was 6.5%, and at 5 years, 2.2%.

Univariate statistics were computed as customary (Armitage et al., 2002). Survival curves were estimated by the Kaplan-Meier method and compared by the log-rank test and Tarone's trend test (Armitage et al., 2002). Cox proportional hazards regression was applied to estimate the hazard ratio (HR). HRs were used to describe the relationship between the explanatory variable and survival time adjusted for potential confounding and effect-modifying variables (e.g., age, sex, tumour stage). The assumption of proportional hazards was checked for each variable. The level of statistical significance was set at 0.05 and all tests were two tailed. Statistical analyses were performed using SPSS version 22.0.0.0 (IBM SPSS Statistics, Armonk, NY, USA, 2013) and R version 3.5.2 (2018).

3. Results

Median survival after diagnosis was 3.1 months, with significant differences by age (but not sex), tumour stage, treatment, and history of pancreatitis in models adjusted for age, sex and tumour stage (Table 1). In such models, patients who underwent radical surgery had a 66% lower risk of death (HR = 0.34, 95% CI: 0.19, 0.60, *p*-value <0.001) than patients with no specific treatment; for patients who received chemotherapy or other specific treatment the HR was 0.19 (95% CI: 0.09, 0.41, *p*-value <0.001) (Table 1). As expected, treatment intention was also significantly associated with survival. Cox's multivariate regression models did not reveal significant differences by sex, alcohol drinking, coffee intake, or tobacco smoking. No differences were observed either by individual signs and symptoms (nor by syndromes) preceding diagnosis. History of pancreatitis was not statistically significantly associated with survival when unadjusted by stage.

Patients with a *KRAS* mutated tumor had a 70% higher risk of death than patients with a *KRAS* wild-type PDAC (HR = 1.70, 95% CI: 1.07, 2.70, *p*-value = 0.026), adjusting for age, sex, and stage (Table 1 and Figure 1). However, *KRAS* mutational status was only modestly and not significantly associated with survival when further adjusted by treatment or by treatment intention (in two separate models, Table 2): the HRs for patients with a *KRAS* mutated tumor (vs. *KRAS* wild-type PDAC) were then increased by 24% and 52%, respectively. In the subgroup of 72 patients with data on *KRAS*, POPs and metals (Supplemental Figure), the respective HRs for *KRAS* were 1.88 (95% CI: 0.89, 3.95, *p*-value = 0.097), and 2.33 (95% CI: 1.18, 4.57, *p*-value = 0.014).

Adjusting by treatment or by treatment intention, PDAC patients with a *KRAS* mutated, stage IV tumor had a 49% and a 92% higher chance of death, respectively, than PDAC patients with a *KRAS* wild-type, stage I tumor (HR = 1.49, 95% CI: 0.55, 4.02, *p*-value = 0.429 when adjusted by treatment and stage, and HR = 1.92, 95% CI: 0.72, 5.10, *p*-value = 0.193 when adjusted by treatment intention and stage).

The beneficial effects of treatment remained unaltered (and they even increased slightly) when *KRAS* mutational status was taken into account: compare HRs for treatment and treatment intention in Tables 1 and 2.

Treatment did not appear to be less effective in patients with a *KRAS* mutated tumor than in patients with a *KRAS* wild-type tumor. Among patients with a *KRAS* mutated tumour, those who underwent radical surgery had a 77% lower chance of death (HR = 0.23, 95% CI: 0.10, 0.50, *p*-value <0.001) than patients with no specific treatment; the corresponding figures among patients with a *KRAS* wild-type tumour were 35% (HR = 0.65; 95% CI: 0.04, 11.24, *p*-value = 0.765) (adjusting for age, sex, and stage). Among patients with a *KRAS* mutated tumour, those who received chemotherapy or another specific treatment had a 91% lower chance of death (HR = 0.09, 95% CI: 0.01, 0.71, *p*-value = 0.023) than patients with no specific treatment; the corresponding figures among patients with a *KRAS* wild-type tumour were 88% (HR = 0.12; 95% CI: 0.02, 0.81, *p*-value = 0.029) (again adjusting for age, sex, and stage).

Some POPs appeared to weakly and negatively influence survival in crude (unadjusted) analyses. Thus, PDAC patients who had serum concentrations of *p,p'*-DDT, HCB and *β*-HCH in the highest tertile had a modestly lower median survival than patients with concentrations in the other tertiles (Table 3). The unadjusted HR of the highest tertile was slightly increased for all 7 POPs (from 1.12 to 1.39, all p-values >0.12). But when adjusted for age, sex, tumour stage, and treatment, such HR of the highest tertile was slightly below or near unity for all 7 POPs (Table 3). With the example of PCB 153, Figure 2 illustrates that survival curves can give the impression of a weak association between higher POP concentrations and lower survival, similar to the relationship suggested by the unadjusted HRs, which is not corroborated by the adjusted HRs. In the subgroup of 72 patients with data on *KRAS*, POPs and metals, the HRs for all POPs were slightly closer to unity than figures shown in Table 3.

In models including both POP concentrations and *KRAS* mutational status, as well as age, gender, stage, and treatment, neither POPs nor *KRAS* were statistically significant, although patients with *KRAS* mutated tumors had consistently and modestly increased HRs (Table 4). All these HRs for patients with *KRAS* mutated tumours increased in the subgroup of 72 patients, ranging from 1.57 to 2.51 in the case of *KRAS* adjusted for DDT and the other mentioned factors (95% CI: 1.05, 5.97, *p*-value = 0.038). By contrast, the HRs for POPs shown in Table 4 did not materially change in the 72 patients.

There were few significant correlations between concentrations of POPs and trace elements, all inverse. The strongest correlations were between arsenic and DDT, DDE, HCB and β-HCH (all Spearman's ρ less than –0.3 and $p<0.05$).

Higher concentrations of several trace elements (lead, cadmium, arsenic, zinc) were significantly associated with better survival (Table 5 and Figure 3). The HRs for the upper tertile were from 0.55 to 0.60 (i.e., 45% to 40% better survival than patients with concentrations in the lowest tertile) (all $p<0.04$). The HR of the upper tertile was also ≥20% lower than the HR of the lower tertile for vanadium, manganese, aluminium, chromium, iron, and copper (all statistically nonsignificant). In adjusted models (again including treatment), patients with concentrations of lead, cadmium, arsenic, and vanadium in the upper tertile had HRs around 0.5 – 0.6 (p-values from 0.057 to 0.011) (Table 5 and Figure 3).

When *KRAS* status was also included in the models, concentrations of lead (HR = 0.45, 95% CI: 0.20, 1.01) and arsenic (HR = 0.51, 95% CI: 0.26, 0.99) in the upper tertile remained associated with better survival. In these models the HR for mutated *KRAS* was 1.56 (0.78, 3.13), and 1.52 (0.75, 3.07), respectively.

POPs and trace elements did not substantively change their respective associations with survival when examined simultaneously; e.g., the HRs for POPs adjusted by selenium were similar to those unadjusted by this trace element (Table 3), and the HRs for trace elements adjusted by POPs were similar to those shown in Table 5. We did not find consistent interactions upon survival between POPs and KRAS, between metals and KRAS, or between POPs and metals.

When *KRAS* mutational status, concentrations of POPs and of trace elements were simultaneously considered in the same adjusted model (which continued to include age, sex, stage and treatment), only treatment was statistically significantly related to survival, with the HRs for treatment being very similar to those shown in Table 2, even though this model included only 70 PDAC patients.

4. Discussion

We did not find strong influences of *KRAS* mutational status, POPs and metals on the survival of the study participants. By contrast, two well-established clinical factors –stage, and therapeutic interventions– remained the strongest predictors of survival. The lack of adverse prognostic effects of POPs and metals measured at the time of diagnosis provide scientific and clinical reassurance on the effects of such exposures upon survival of patients with PDAC. The weak or null association with *KRAS* mutations contributes to the scant knowledge on the clinical implications of a genetic alteration frequent in PDAC and other cancers (Porta et al., 2009a).

However, the possible relevance of the findings is constrained by the study limitations: diagnostic and therapeutic procedures common more than 20 years ago; low survival; low sensitivity of the laboratory method to detect *KRAS* mutations; lack of detailed information on surgical and nonsurgical treatments, on BMI and weight loss at diagnosis, treatment onset, and during follow-up; relatively low number of POPs analyzed; modest numbers of patients and, therefore, limited precision of estimates, even when their magnitude was potentially important; blood samples obtained at the time of diagnosis and, hence, possibility of disease progression bias (Camargo et al., 2019; Crous-Bou, 2009; Gasull et al., 2010; Gómez-Tomás et al., 2019; Porta, 2001).

During recent decades, diagnostic and therapeutic procedures for PDAC have improved considerably (Gobbi et al., 2013; Huang et al., 2019; Tempero et al., 2017), and have benefitted many patients with access to quality care; nonetheless, in unselected series of patients survival remains quite stable and low, unfortunately. As just mentioned, our study did not collect detailed information on treatments or on other variables that might influence survival; e.g., on ABO blood groups or circulating nucleic acids (Rizzato et al., 2013; Bernard et al., 2019). Still, there is little evidence that such unmeasured variables could be confounders in the processes we focused on: the combined influence upon PDAC survival of *KRAS* mutations, POPs and trace elements. Today, few studies assess the role of such genetic and environmental factors on clinical outcomes (Porta and Vandenberg, 2019), and this is the most original, central component of the present report.

Without any doubt, it is necessary to refute or to replicate the present findings in a more contemporary and large series of patients (Carrato et al., 2015; Huang et al., 2019). If possible, incident cases arising from a healthy cohort followed prospectively for 15 or more years before PDAC diagnosis; with detailed information on PDAC histopathology, stage, treatments, comorbidity, and lifestyle; with information on changes in BMI and health status during follow-up (i.e, long before diagnosis); and with biological samples collected at entry into the cohort and at some other intervals (again, long before diagnosis), to avoid possible

reverse causation: the validity of prognostic estimates based on measurements of POPs and metals close to the time of diagnosis is uncertain. Thus, two strengths of our study that future research should incorporate are the study of incident PDAC cases, and the biological measurement of POPs in serum (or fat tissue) and of trace elements in toenails. The latter feature is relevant because most studies have determined trace elements in serum or in pancreatic juice at the time of diagnosis (Carrigan et al., 2007; Farzin et al., 2013; Kriegel et al., 2006; Laohaudomchok et al., 2011; Lener et al., 2016). No such studies have also assessed *KRAS* mutations.

A related, third strength is the assessment of *KRAS* mutations in tumour tissue, which is known to yield better sensitivity than detection in serum (Brychta et al., 2016; Parker et al., 2011a, 2011b; Takai et al., 2015). However, as mentioned earlier, our PCR and RFLP analysis yields a lower sensitivity to detect *KRAS* mutations than more recent techniques such as droplet digital (dd) PCR, high resolution melting analysis or next-generation sequencing (Buscail et al., 2019; Schlitter et al., 2017). Thus, many or perhaps most samples we characterized as wild-type probably had some *KRAS* mutations. Therefore, *KRAS* wild-type and *KRAS* mutated tumors likely had non-zero levels of *KRAS* mutations less than and greater than 1 in 50 alleles, respectively. Today, studies could overcome this possible misclassification, although, to avoid selection bias, they will likely need to collect tumor tissue from >80–90% of cohort members diagnosed with incident PDAC –which is feasible, though not easy (Hoppin et al., 2002; Porta et al., 2002).

Lipid mobilization and the other metabolic changes characteristic of PDAC progression may have altered serum concentrations of POPs (Gasull et al., 2019), but are unlikely to have influenced toenail levels of the trace elements. Hair and nail samples reflect the concentration of elements in the organism over several months, and are thus useful for the evaluation of chronic exposure (Golasik et al., 2015; Gómez-Tomás et al., 2019; Goyer and Clarkson, 2001; He, 2011; Hopps, 1977; Slotnick and Nriagu, 2006). Trace elements in nails are incorporated during their formation (12–18 months) from blood, lymph vessels, body tissues and epidermis; thus, they reflect exposures or body burdens that have occurred in such months (Goyer and Clarkson, 2001; Hopps, 1977; Slotnick and Nriagu 2006). The rationale for their use in studies on disease survival is that the ranking of toenail concentrations in a group of individuals remains relatively stable over the etiologically relevant subclinical or clinical time period (Camargo et al., 2019; Gómez-Tomás et al., 2019). In contrast with etiologic studies, for prognostic studies both the ranking of patients based on their concentrations of trace elements and POPs at diagnosis, and the absolute concentrations at diagnosis themselves, may be a valid option.

Patients with a *KRAS* mutated tumor had a HR of 1.70 when adjusting only for age, sex, and stage, which is similar to findings from other studies (Rachakonda et al., 2013; Qian et al., 2018). However, *KRAS* mutational status was not significantly associated with survival when further adjusted by treatment; such adjustment answers a different question, which we suggest is more clinically significant than the question addressed when treatment is not taken into account.

Treatment did not appear to be less effective in patients with a *KRAS* mutated tumor than in patients with a *KRAS* wild-type tumor. The positive effect of radical surgery on survival was clear among patients with a *KRAS* mutated tumour and statistically nonsignificant among patients with a *KRAS* wild-type tumour (HR = 0.23 and 0.65, respectively). The positive effect of chemotherapy or other specific treatments was similar among patients with a *KRAS* mutated and a *KRAS* wild-type tumour (HR = 0.09 and 0.12, respectively). When assessing these results it is important to keep in mind the wide confidence intervals, especially among wild-type cases. In principle, results rule out confounding by indication. Furthermore, during the study years, as at present, *KRAS* mutational status did not influence treatment decisions. Also, we are not aware that the environmental exposures may affect PDAC in a way that could affect choice of treatment (if that were the case, when adjusting by treatment, as we did, we would be adjusting away some of the effect of the exposures on survival).

Low numbers of cases did not allow to analyze the different *KRAS* codon 12 mutations (i.e., their spectrum); nor did we assess *KRAS* codon 13 mutations (which are uncommon in PDAC) (Rachakonda et al., 2013). Different *KRAS* mutations have different downstream signaling effects (Céspedes et al., 2006; Porta et al. 2003, 2009b; Shields et al., 2000), and might differently influence prognosis.

Evidence on associations between *KRAS* mutations, downstream cell signaling, and oxidative stress should be considered when assessing the present findings (Parsons et al., 2013; Boldogh et al., 2012). Certainly, it is beyond the scope of an observational study in humans to address in detail such mechanistic scenarios. However, it is interesting to consider how *KRAS* mutations participate in and are themselves impacted during tumor progression. *KRAS* mutation is an early driver of a large percentage of PDAC (Rachakonda et al., 2013; Waters and Der, 2018), but *KRAS* mutant cells may also be selected against during tumor progression (Parsons and Myers, 2013). Our publications have long underlined that we assess the prevalence of *KRAS* mutations at diagnosis (i.e., their occurrence and persistence), not their incidence during preclinical or clinical phases.

When *KRAS* status and trace elements were jointly included in the models, higher concentrations of lead and arsenic were significantly associated with better survival. Although statistically the finding is clear, mechanistic interpretations remain open. We previously reported that concentrations of several trace elements were higher in *KRAS* wild-type PDAC cases than in *KRAS* mutated cases (Gómez-Tomás et al., 2019).

Indeed, our final sample size was small, and estimates were often statistically imprecise; this limitation also hampered a wider quantitative assessment of interactions and combined effects. Furthermore, for studies integrating environmental, clinical, anatomopathological and genetic information it remains a challenge to overcome incomplete overlapping of the data; i.e., to have data available on all types of factors for a high proportion of subjects (Hoppin et al., 2002; Porta et al., 2002). It is important to report transparently these issues (Gallo et al., 2011), as we do in the section on Methods, in the Supplemental Table and in the Supplemental Figure. Results in the subgroup of 72 patients with data on *KRAS*, POPs and metals are not necessarily more valid than results on the larger subsets of patients, but

presentation of results in the 72 patients is warranted to interpret changes in estimates when adjusting for multiple factors.

This study based on molecular, clinical, and environmental epidemiology is the first one to analyze in any human cancer the combined influence upon survival of *KRAS* mutations and biomarkers of POPs and trace elements –three frequent cancer-related factors (Porta et al., 2009a). Only one previous study (Hardell et al., 2007) analysed the association between PDAC survival and POP concentrations; it did so in adipose tissue (a strength) of only 21 patients, and it observed a longer survival in patients with lower levels of some POPs. With six times more patients, our study did not replicate such findings.

Knowledge is available on possible adverse pancreatic effects of POPs and other contaminants (Amaral et al., 2012; Antwi et al., 2015; Barone et al., 2016; Benetou et al., 2018; Eriksen et al., 2009; Gasull et al., 2018, 2019; Gore et al., 2015; Hardell et al., 2007; Porta, 2006). There is also extensive evidence on the high number of toxic mixtures contaminating humans (Lee et al., 2014; Nøst et al., 2017; Porta et al., 2008, 2012; Pumarega et al., 2016; Tamayo-Uria et al., 2019). Therefore, another study limitation is the small number of POPs analysed, just the 7 POPs that were detected above the detection limit in more than 85% of the 144 PDAC cases with POPs determined. Some of the observed hazard ratios might be confounded –positively or negatively– by other, unmeasured environmental contaminants, such as dioxins and furans, phthalates, polybrominated diphenyl ethers (PBDEs), phenols, per- and polyfluorinated alkyl substances (PFAS) and others (Agier et al., 2019; Chen et al., 2019; Patel and Manrai, 2015; Porta et al., 2008; Robertson et al., 2001; Rosofsky et al., 2017).

Given the study limitations, it may be premature to definitely rule out significant roles for *KRAS* mutations, POPs and metals in the prognosis of PDAC. While inherited and acquired genetic alterations have been extensively studied, environmental factors also deserve so. Nevertheless, findings suggest that the well-established clinical factors –pathology, stage, and therapeutic interventions (Tempero et al., 2017)– remain the strongest predictors of PDAC survival. Results from models adjusting for treatment imply that therapeutic decisions influence PDAC survival more than *KRAS* mutations, and than environmental contaminants such as POPs and metals.

5. Conclusions

KRAS mutational status, POPs, and trace elements were not related to PDAC survival when simultaneously considered along with treatment; only the latter was independently related to survival. The lack of adverse prognostic effects of POPs and metals measured at the time of diagnosis provide scientific and clinical reassurance on the effects of such exposures upon survival of patients with PDAC. The weak association with *KRAS* mutations contributes to the scant knowledge on the prognostic implications of a genetic alteration highly frequent in PDAC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

CI	Confidence Interval
DDE	dichlorodiphenyldichloroethene
DDT	dichlorodiphenyltrichloroethane
HCB	hexachlorobenzene
HCH	hexachlorocyclohexane
HR	hazard ratio
PCBs	polychlorinated biphenyls
PCR	polymerase chain reaction
PDAC	pancreatic ductal adenocarcinoma
POPs	persistent organic pollutants
RFLP	restriction fragment length polymorphism
TL	total serum lipids

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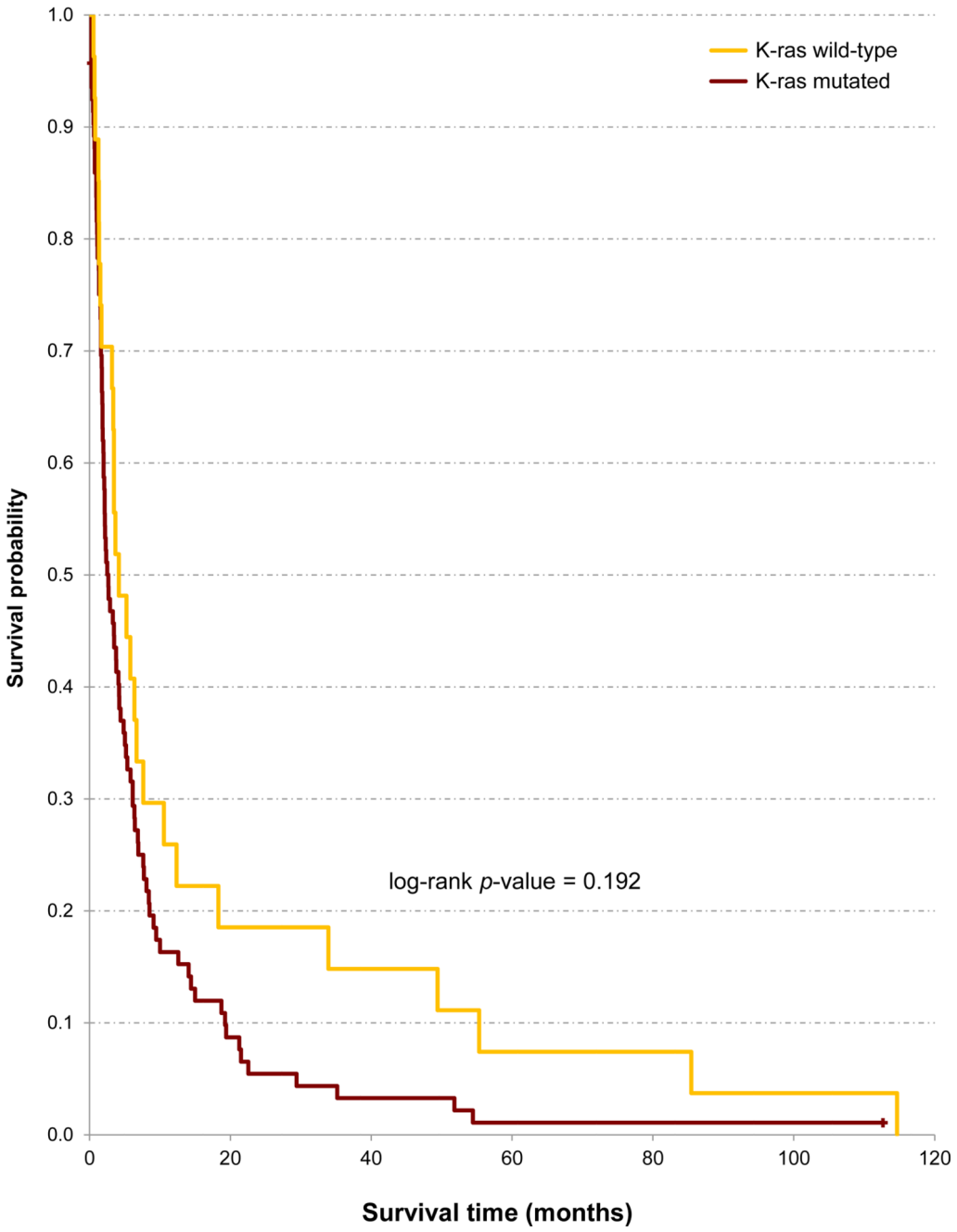

Figure 1.
Unadjusted Kaplan-Meier analysis of overall survival of 120 patients with pancreatic ductal adenocarcinoma according to *KRAS* mutational status.

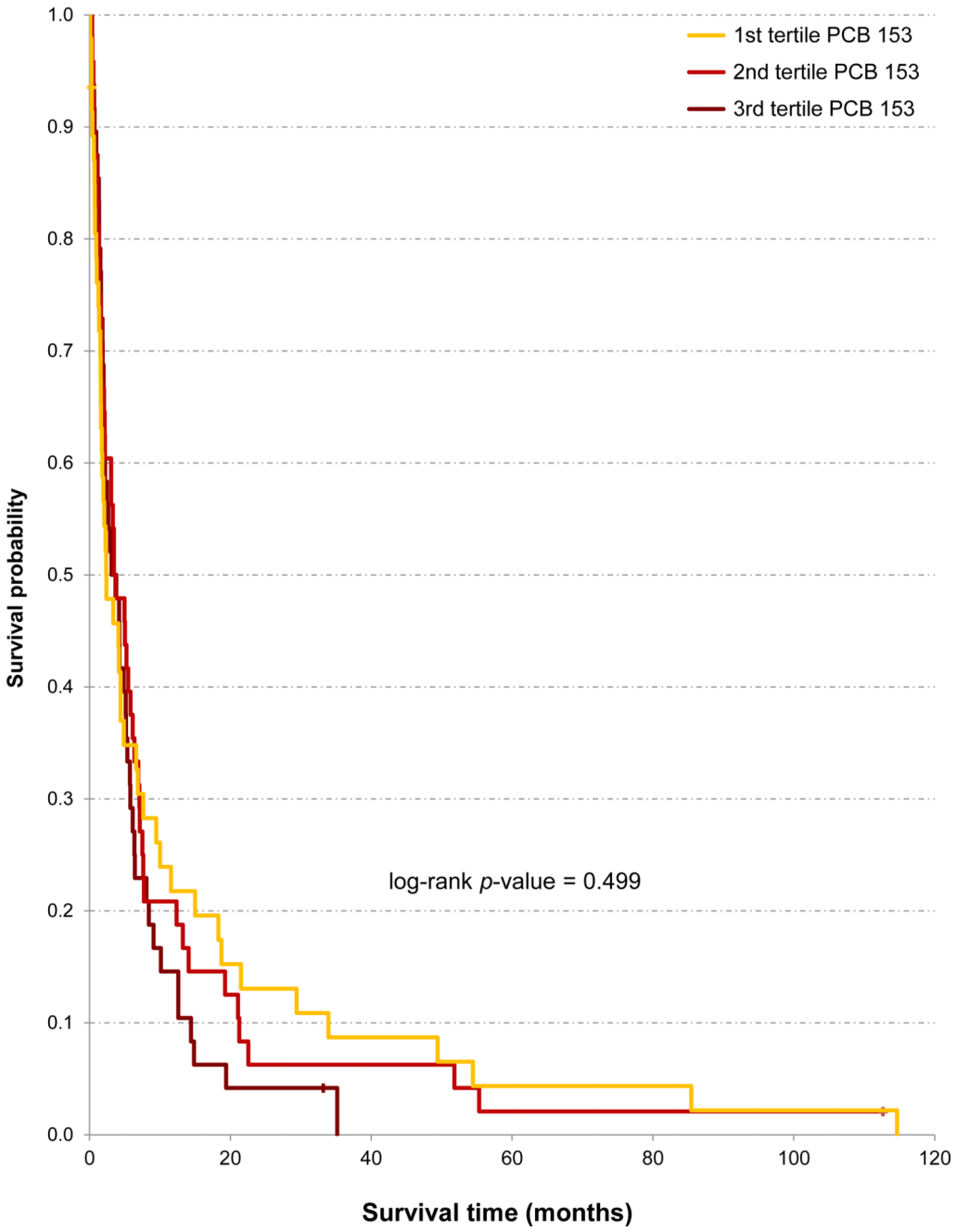

Figure 2.

Unadjusted Kaplan-Meier analysis of overall survival of 143 patients with pancreatic ductal adenocarcinoma according to serum concentrations of polychlorinated biphenyl (PCB) 153 (ng/g of lipid).

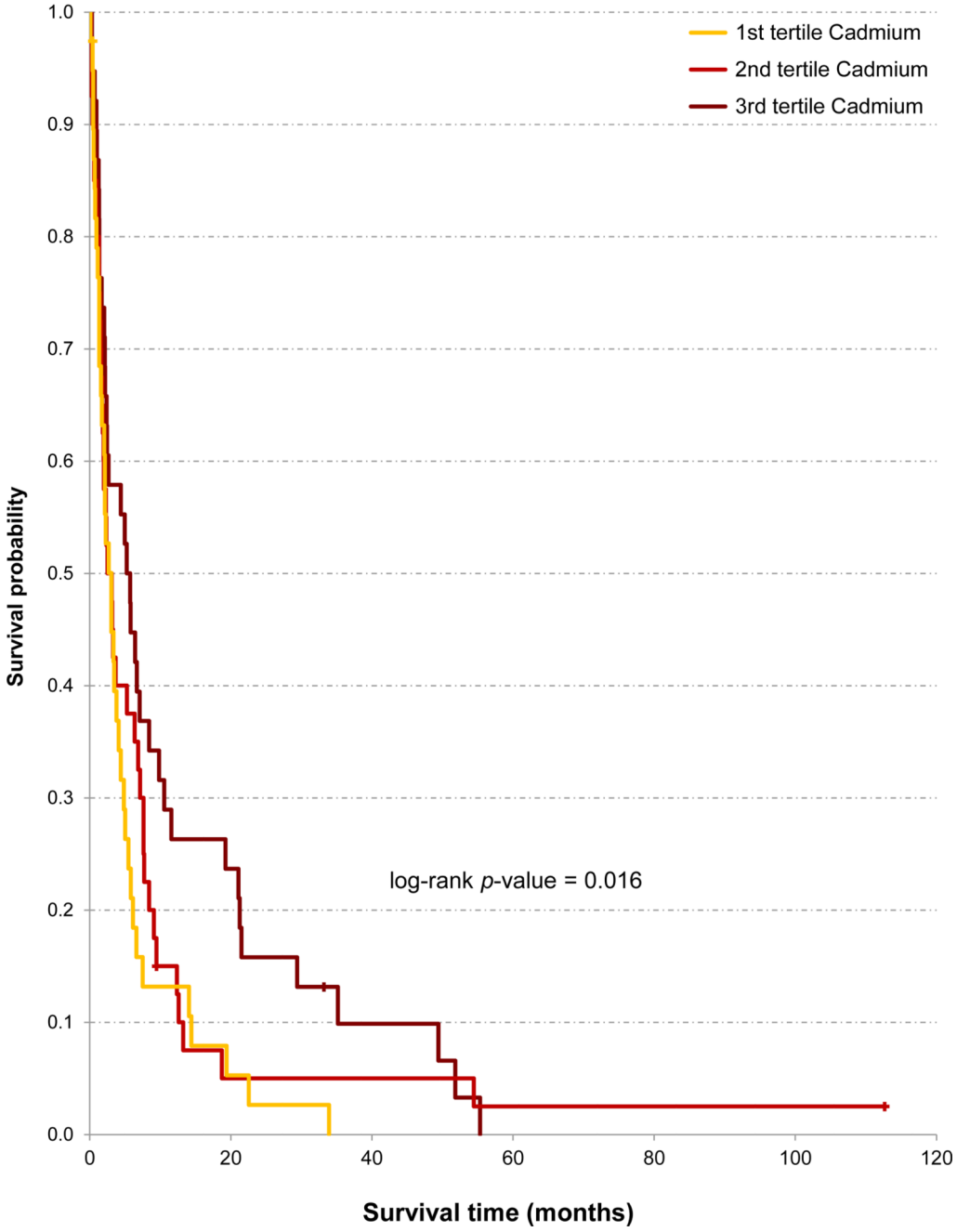

Figure 3.
Unadjusted Kaplan-Meier analysis of overall survival of 117 patients with pancreatic ductal adenocarcinoma according to toenail concentrations of cadmium (μg/g).

Table 1.

Survival of patients with pancreatic ductal adenocarcinoma according to clinical and sociodemographic characteristics.

Characteristics	N	(%)	Median survival (months)	Hazard ratio[a]	(95% CI)	*p*-value
Total	185		3.1			
Age						
<66 years	85	(45.9)	4.1[b]	1.00	–	0.005
≥66 years	100	(54.1)	2.2	1.53	(1.13, 2.07)	
Sex						
Men	110	(59.5)	2.9	1.00	–	0.698
Women	75	(40.5)	3.1	0.94	(0.68, 1.29)	
Tumour stage at diagnosis						
Stage I	45	(24.6)	5.8[b]	1.00	–	<0.001
Stage II	23	(12.6)	3.9	1.04	(0.62, 1.75)	
Stage III	23	(12.6)	4.2	1.39	(0.83, 2.33)	
Stage IV	92	(50.3)	1.7	2.34	(1.62, 3.39)	
Treatment						
No specific treatment	69	(38.3)	1.6[b]	1.00	–	<0.001
Radical surgery	24	(13.3)	8.4	0.34	(0.19, 0.60)	
Palliative surgery	45	(25.0)	3.7	0.49	(0.33, 0.75)	
Symptomatic surgery	31	(17.2)	3.4	0.63	(0.40, 0.98)	
Chemotherapy or other specific	11	(6.1)	6.4	0.19	(0.09, 0.41)	
Treatment intention						
Radical	25	(14.0)	12.6[b]	1.00	–	<0.001
Palliative	53	(29.8)	4.4	1.07	(0.63, 1.83)	
Symptomatic	100	(56.2)	2.0	2.23	(1.32, 3.78)	
History of pancreatitis						
No	176	(96.2)	2.7	1.00	–	0.038
Yes	7	(3.8)	6.4	0.44	(0.20, 0.95)	
History of diabetes mellitus						
No	138	(74.6)	2.7	1.00	–	0.846
Yes	46	(24.9)	3.4	0.94	(0.68, 1.29)	
Cholestatic syndrome at presentation						
Absent	64	(34.6)	3.1	1.00	–	0.148
Partial	39	(21.1)	2.0	1.48	(0.98, 2.25)	
Complete	82	(44.3)	3.4	1.33	(0.90, 1.94)	
Constitutional syndrome at presentation						
Absent	12	(6.5)	2.1	1.00	–	0.594
Partial	39	(21.1)	3.4	0.86	(0.44, 1.69)	
Complete	134	(72.4)	2.9	1.05	(0.57, 1.92)	

Characteristics	N	(%)	Median survival (months)	Hazard ratio[a]	(95% CI)	*p*-value
Alcohol consumption						
No and occasional	43	(26.2)	2.4	1.00	–	0.807
Moderate and heavy	121	(73.8)	3.7	0.95	(0.64, 1.42)	
Tobacco smoking						
Never	73	(44.2)	3.2	1.00	–	0.275
Former	37	(22.4)	4.2	0.64	(0.34, 1.20)	
Current	55	(33.3)	3.8	0.89	(0.52, 1.53)	
Coffee intake						
Non-regular drinkers	24	(14.6)	4.3	1.00	–	0.581
Regular drinkers	140	(85.4)	3.1	1.13	(0.73, 1.76)	
KRAS status						
Wild-type	27	(22.3)	4.2	1.00	–	0.026
Mutated	94	(77.7)	2.7	1.70	(1.07, 2.70)	

[a] All factors adjusted for age, sex and tumour stage.

[b] *p*-value <0.001 (log-rank test).

Table 2.

Influence on survival of *KRAS* mutational status, tumour stage, and treatment.

Model	Variables	Hazard ratio[a]	(95% CI)	*p*-value[b]
1	*KRAS*			
	Wild-type	1.00	-	0.416
	Mutated	1.24	(0.74, 2.07)	
	Tumour stage			
	Stage I	1.00	-	0.046
	Stage II	0.66	(0.33, 1.34)	
	Stage III	1.07	(0.54, 2.11)	
	Stage IV	1.47	(0.79, 2.72)	
	Treatment			
	No specific treatment	1.00	-	<0.001
	Radical surgery	0.24	(0.11, 0.51)	
	Palliative surgery	0.51	(0.31, 0.86)	
	Symptomatic surgery	0.67	(0.35, 1.27)	
	Chemotherapy or other	0.13	(0.05, 0.35)	
2	*KRAS*			
	Wild-type	1.00	–	0.087
	Mutated	1.52	(0.94, 2.46)	
	Tumour stage			
	Stage I	1.00	–	0.089
	Stage II	0.72	(0.36, 1.45)	
	Stage III	1.06	(0.54, 2.09)	
	Stage IV	1.49	(0.81, 2.76)	
	Treatment intention			
	Radical	1.00	–	<0.001
	Palliative	1.38	(0.71, 2.69)	<0.001[c]
	Symptomatic	3.16	(1.62, 6.18)	

[a] In each Cox's proportional-hazards model the three variables are mutually adjusted for, as well as adjusted for age and gender. Model 1, N = 116; Model 2, N = 114. There were 121 patients with available results on *KRAS* mutational status, of whom 5 and 7 had missing values for treatment or treatment intention, respectively.

[b] Unless otherwise specified, *p*-value derived from Wald's test.

[c] Test for linear trend (multivariate analogue of Mantel's extension test).

Table 3.

Influence upon survival of each persistent organic pollutant.[a]

		Unadjusted model			Adjusted model[c]		
Compound	**Median[b] (months)**	**Hazard ratio**	**(95% CI)**	***p*-value**	**Hazard ratio**	**(95% CI)**	***p*-value**
p,p'-DDT							
≤224	4.2	1.00	-	0.121[d]	1.00	-	0.219[d]
225 – 614	3.1	1.03	(0.68, 1.55)		0.83	(0.53, 1.31)	
>614	2.5	1.39	(0.93, 2.07)		0.75	(0.47, 1.19)	
p,p'-DDE							
≤1652	3.4	1.00	-	0.183[d]	1.00	-	0.200[d]
1653 – 4384	2.1	1.09	(0.72, 1.65)		0.86	(0.55, 1.36)	
>4384	3.4	1.32	(0.88, 1.99)		0.74	(0.47, 1.17)	
PCB 138							
≤167	2.4	1.00	-	0.211	1.00	-	0.052
168 – 299	5.0	0.77	(0.52, 1.17)		0.61	(0.39, 0.96)	
>299	2.9	1.12	(0.74, 1.69)		0.97	(0.62, 1.53)	
PCB 153							
≤187	2.4	1.00	-	0.250[d]	1.00	-	0.239
188 – 313	3.4	1.09	(0.72, 1.65)		0.71	(0.45, 1.11)	
>313	3.1	1.28	(0.84, 1.95)		0.97	(0.63, 1.50)	
PCB 180							
≤186	3.4	1.00	-	0.482[d]	1.00	-	0.698
187 – 322	3.1	1.11	(0.74, 1.67)		0.96	(0.61, 1.50)	
>322	3.8	1.16	(0.77, 1.74)		1.15	(0.74, 1.77)	
HCB							
≤997	3.1	1.00	-	0.324	1.00	-	0.866
998 – 1950	4.2	0.90	(0.60, 1.37)		0.90	(0.56, 1.43)	
>1950	2.2	1.22	(0.81, 1.85)		0.88	(0.52, 1.48)	
β-HCH							
≤625	3.1	1.00	-	0.717	1.00	-	0.199
626 – 1217	4.2	0.97	(0.65, 1.47)		0.65	(0.41, 1.05)	
>1217	2.5	1.14	(0.76, 1.72)		0.73	(0.46, 1.18)	

Unadjusted model, N = 144; adjusted model, N = 137. There were 144 patients with available results on POP concentrations, of whom 7 had missing values for tumour stage or treatment.

[a] Concentrations of each persistent organic pollutant in ng/g of lipid.

[b] Differences were statistically non-significant in all compounds.

[c] Hazard ratios for each POP in Cox's proportional-hazards models are adjusted for age, gender, tumour stage and treatment.

[d] Test for linear trend (multivariate analogue of Mantel's extension test).

Table 4.

Influence upon survival of *KRAS* mutational status and each persistent organic pollutant.[a]

Model	Exposures	Hazard ratio[b]	(95% CI)	*p*-value
1	*KRAS*			
	Wild-type	1.00	-	0.098
	Mutated	1.79	(0.90, 3.58)	
	p,p'-DDT			
	≤224	1.00	-	0.260
	225 – 614	0.62	(0.35, 1.10)	
	>614	0.75	(0.43, 1.31)	
2	*KRAS*			
	Wild-type	1.00	-	0.266
	Mutated	1.43	(0.76, 2.68)	
	p,p'-DDE			
	≤1652	1.00	-	0.422
	1653 – 4384	0.70	(0.40, 1.22)	
	>4384	0.91	(0.52, 1.60)	
3	*KRAS*			
	Wild-type	1.00	-	0.573
	Mutated	1.21	(0.63, 2.33)	
	PCB 138			
	≤167	1.00	-	0.042
	168 – 299	0.55	(0.30, 1.00)	
	>299	1.18	(0.69, 2.02)	
4	*KRAS*			
	Wild-type	1.00	-	0.416
	Mutated	1.32	(0.68, 2.55)	
	PCB 153			
	≤187	1.00	-	0.381
	188 – 313	0.75	(0.43, 1.31)	
	>313	1.08	(0.63, 1.87)	
5	*KRAS*			
	Wild-type	1.00	-	0.335
	Mutated	1.40	(0.71, 2.75)	
	PCB 180			
	≤186	1.00	-	0.311
	187 – 322	1.38	(0.79, 2.42)	
	>322	1.49	(0.87, 2.55)	
6	*KRAS*			
	Wild-type	1.00	-	0.255
	Mutated	1.45	(0.77, 2.74)	

Model	Exposures	Hazard ratio[b]	(95% CI)	*p*-value
	HCB			
	≤997	1.00	-	0.777
	998 – 1950	1.01	(0.59, 1.73)	
	>1950	1.24	(0.64, 2.40)	
7	*KRAS*			
	Wild-type	1.00	-	0.355
	Mutated	1.34	(0.72, 2.49)	
	β-HCH			
	≤625	1.00	-	0.290
	626 – 1217	0.65	(0.38, 1.11)	
	>1217	0.80	(0.45, 1.41)	

[a] Concentrations of each persistent organic pollutant in ng/g of lipid.

[b] In the Cox's proportional-hazards models the two variables are mutually adjusted for, as well as adjusted for age, gender, tumour stage and treatment (N = 98). There were 103 patients with available results on *KRAS* mutational status and POP concentrations, of whom 5 had missing values for tumour stage or treatment.

Table 5.

Influence upon survival of each trace element.

		Unadjusted model			Adjusted model[a]		
Trace element	**Median (months)**	**Hazard ratio**	**(95% CI)**	***p*-value**	**Hazard ratio**	**(95% CI)**	***p*-value**
Lead							
≤0.32	2.1	1.00	-	0.039[b]	1.00	-	0.057
0.33–0.66	3.1	0.66	(0.36, 1.20)		0.76	(0.40, 1.46)	
>0.66	4.1	0.55	(0.32, 0.94)		0.53	(0.30, 0.94)	
Cadmium							
≤0.01	1.6[c]	1.00	-	0.010[b]	1.00	-	0.032[b]
0.01–0.02	3.1	1.05	(0.57, 1.92)		0.88	(0.47, 1.65)	
>0.02	5.0	0.58	(0.35, 0.97)		0.60	(0.35, 1.02)	
Arsenic							
≤0.06	2.2[c]	1.00	-	0.016[b]	1.00	-	0.011
0.07–0.09	3.1	0.99	(0.63, 1.58)		1.23	(0.75, 2.01)	
>0.09	5.0	0.59	(0.38, 0.92)		0.58	(0.35, 0.96)	
Selenium							
≤0.55	2.5	1.00	-	0.437	1.00	-	0.432
0.56–0.65	4.4	0.75	(0.48, 1.16)		0.72	(0.44, 1.19)	
>0.65	4.5	0.94	(0.52, 1.70)		0.85	(0.44, 1.61)	
Zinc							
≤98.23	2.0[c]	1.00	-	0.035[b]	1.00	-	0.116[b]
98.24–117.6	2.7	0.76	(0.48, 1.21)		0.74	(0.45, 1.21)	
>117.6	5.8	0.60	(0.37, 0.96)		0.65	(0.39, 1.10)	
Vanadium							
≤0.01	2.7	1.00	-	0.079[b]	1.00	-	0.015
0.02–0.03	2.5	0.91	(0.58, 1.42)		0.49	(0.29, 0.82)	
>0.03	5.5	0.66	(0.40, 1.06)		0.55	(0.33, 0.92)	
Manganese							
≤0.17	3.1	1.00	-	0.224[b]	1.00	-	0.318[b]
0.18–0.44	2.7	0.80	(0.52, 1.23)		0.92	(0.58, 1.44)	
>0.55	3.7	0.76	(0.47, 1.22)		0.77	(0.46, 1.28)	
Aluminium							
≤7.10	3.8	1.00	-	0.547	1.00	-	0.074[b]
7.11–16.75	2.7	1.06	(0.70, 1.60)		0.75	(0.47, 1.18)	
>16.75	5.1	0.80	(0.49, 1.33)		0.61	(0.34, 1.07)	
Chromium							
≤0.21	2.1	1.00	-	0.097[b]	1.00	-	0.844

		Unadjusted model			Adjusted model[a]		
Trace element	**Median (months)**	**Hazard ratio**	**(95% CI)**	***p*-value**	**Hazard ratio**	**(95% CI)**	***p*-value**
0.22–0.69	3.4	0.68	(0.42, 1.08)		0.84	(0.47, 1.51)	
>0.69	4.7	0.65	(0.40, 1.04)		0.92	(0.53, 1.60)	
Iron							
≤9.92	2.7	1.00	-	0.198	1.00	-	0.225[b]
9.93–21.20	3.1	1.14	(0.72, 1.78)		0.93	(0.57, 1.51)	
>21.20	5.1	0.75	(0.46, 1.24)		0.73	(0.43, 1.24)	
Nickel							
≤0.23	2.3	1.00	-	0.340	1.00	-	0.908
0.24–0.64	4.0	0.73	(0.48, 1.12)		1.00	(0.63, 1.59)	
>0.64	5.5	0.95	(0.57, 1.58)		1.13	(0.64, 1.98)	
Copper							
≤3.03	2.1[c]	1.00	-	0.089[b]	1.00	-	0.712
3.04–3.89	2.9	0.99	(0.63, 1.56)		1.22	(0.76, 1.97)	
>3.89	5.5	0.68	(0.43, 1.08)		1.14	(0.69, 1.88)	

Concentrations of trace elements (μg/g) in tertiles. Unadjusted model, N = 118; adjusted model, N = 114.

[a] Hazard ratios for each trace element in each model are adjusted for age, gender, tumour stage and treatment.

[b] Test for linear trend (multivariate analogue of Mantel's extension test).

[c] *p*-value <0.05 (Tarone trend test). There were 118 patients with available results trace elements, of whom 4 had missing values for tumour stage or treatment.