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In pancreatic ductal adenocarcinoma blood concentrations of some organochlorine compounds and coffee intake are independently associated with *KRAS* mutations

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While *KRAS* activation is a fundamental initiating event in the aetiopathogenesis of pancreatic ductal adenocarcinoma (PDA), environmental factors influencing the occurrence and persistence of *KRAS* mutations remain largely unknown. The objective was to test the hypothesis that in PDA there are aetiopathogenic relationships among concentrations of some organochlorine compounds (OCs) and the mutational status of the *KRAS* oncogene, as well as among the latter and coffee intake. Incident cases of PDA were interviewed and had blood drawn at hospital admission ($N = 103$). OCs were measured by high-resolution gas chromatography with electron capture detection. Cases whose tumours harboured a *KRAS* mutation had higher concentrations of *p,p′*-dichlorodiphenyltrichloroethane (DDT), *p,p′*-dichlorodiphenyldichloroethene (DDE) and polychlorinated biphenyls (PCBs) 138, 153 and 180 than cases with wild-type *KRAS*, but differences were statistically significant only for *p,p′*-DDT and PCBs 138 and 153. The association between coffee intake and *KRAS* mutations remained significant (P-trend < 0.015) when most OCs where accounted for. When *p,p′*-DDT, PCB 153, coffee and alcohol intake were included in the same model, all were associated with *KRAS* ($P = 0.042$, 0.007, 0.016 and 0.025, respectively). *p,p′*-DDT, *p,p′*-DDE and PCB 138 were significantly associated with the two most prevalent *KRAS* mutations (Val and Asp). OCs and coffee may have independent roles in the aetiopathogenesis of PDA through modulation of *KRAS* activation, acquisition or persistence, plausibly through non-genotoxic or epigenetic mechanisms. Given that *KRAS* mutations are the most frequent abnormality of oncogenes in human cancers, and the lifelong accumulation of OCs in humans, refutation or replication of the findings is required before any implications are assessed.

Introduction

Over three decades of research has yielded a wealth of knowledge on the biology of *ras* effects, but very little on their environmental influences. Today, *ras* genes are deemed the most commonly activated oncogenes in human cancer, and alterations in *KRAS* are the object of intense scientific scrutiny (1–3). Yet, a complete understanding of *ras* function is still to come (1) and 'concepts that once seemed on the verge of comprehension have evolved such that elegant complexity coupled with hopeless confusion better defines our current state of knowledge' (2).

Mutation of oncogenes in the *ras* family has been associated with exposure to environmental carcinogens (3). In the early 1980s, *ras* genes were discovered to be activated by point mutation, *ras* mutations were identified in experimental tumours of rodents exposed to chemical or physical carcinogens and evidence accrued on the involvement of the mutations in the initiation of carcinogenesis; the mutations were later shown to be essential for tumour development and maintenance (1–4). Once the oncogenic nature and properties of *ras* genes were established, basic research on the environmental causes of their mutations waned. Fortunately, molecular pathology and molecular epidemiological studies contributed additional evidence on the potential role of genetic, clinical and environmental interactions in the occurrence and persistence of *ras* mutations in several human cancers, most notably, pancreatic, colon and lung adenocarcinomas (5–28).

Some evidence suggests that pancreatic intraepithelial neoplasia (PanIN) lesions are precursors of pancreatic ductal adenocarcinoma (PDA). While several dimensions are being tested of a PanIN–PDA progression model, most studies indicate that *KRAS* mutations precede genetic alterations at the *p16INK4A* or *Tp53* loci (4,29). *KRAS* mutations are an initiating step in PDA pathogenesis (4). However, knowledge is still limited on factors—particularly, environmental factors—that influence the occurrence of *KRAS* mutations, the expansion of mutant cells and the initiation and progression of PDA (4). Eventually, such knowledge should both clarify biological mechanisms and enable a more effective primary prevention of PDA (5).

Ten years ago, we published two reports suggesting that some organochlorine compounds (OCs) (7) and coffee intake (8) might play a role in the pathogenesis of pancreatic cancer through modulation of *KRAS* activation or maintenance. While we stressed that findings required replication, their potential mechanistic relevance received considerable attention (13,15,20–23,30). First, findings on OCs (7) were deemed to show 'for the first time that a mutation of a *ras* gene was associated with serum concentrations of OCs in any group of cancer patients' (31). Second, 'following recent conflicting but intriguing results thrown up by rodent and cell culture research about the effects of caffeine and coffee' (32), the association between coffee intake and *KRAS* (8) was considered to be 'shown for the first time in people suffering from pancreatic cancer' (32).

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OCs are of concern because of their persistence, prevalence in humans and potential role in contributing to increase risks of some diseases, including PDA and other cancers (5,7,9,20,33–48). OCs have a variety of effects on physiological, cellular and molecular processes that are relevant in cancer aetiopathogenesis: induction of enzymes that activate procarcinogens, interference with DNA repair, provision of a growth advantage to mutated cells, disruption of cell-cycle checkpoints and apoptosis and probably epigenetic effects (5,7–9,12,20,33–43,48–53). However, research on OCs, *KRAS* and pancreatic cancer has been limited, and conclusions on mechanistic, clinical or public health implications would be premature (5,7,25).

The present paper improves our previous reports (7,8,54) in five directions, as follows: (i) we double the number of patients with results on OCs (from 51 to 103); (ii) we adjust the relationship between coffee intake and *KRAS* mutations by OC concentrations; (iii) we also adjust the relationship between alcohol intake and *KRAS* mutations by OC concentrations; (iv) we adjust the relationships between the two main exposures (OCs and coffee) and *KRAS* by PDA symptoms and (v) when assessing the relationships between the two main exposures and *KRAS*, we also take into account the influence of past medical conditions such as diabetes, pancreatitis and peptic ulcer. The objective of the present analyses was to test the hypothesis that in PDA mechanistic relationships exist among concentrations of some OCs and the prevalence at diagnosis of mutations in the *KRAS* oncogene, as well as among the latter and coffee intake.

Materials and Methods

Study population

Methods and strategies of analysis of the PANKRAS II study have been described in detail (7,8,12,19,24–26,54–68). Briefly, subject recruitment took place between 1992 and 1995 at five general hospitals in the eastern Mediterranean part of Spain, where 185 incident cases of PDA were prospectively identified. The present report is based on 103 PDA patients: they stem from patients in whom *KRAS* status was determined ($N = 121$), who had results on serum concentrations of OCs ($N = 144$) and who had information on potential confounders (obtained from patient interviews and medical records, $N = 165$). There were no significant differences between the 103 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, energy intake, diet, consumption of coffee, tobacco and alcohol and duration of the interview), except that the included cases were slightly younger (8,12,19,54,57,58,61–64,68). All cases were reviewed by a panel of experts and by the study reference pathologists, blinded to the original diagnoses and to molecular results (55,60,65). Twenty-nine conventional hospital controls were recruited at one of the study hospitals among subjects admitted for benign, non-digestive disorders unrelated to tobacco and alcohol consumption (7,24,26,54,68). The ethics committees of participating hospitals approved the study protocol, and patients gave informed consent to participate.

Clinicopathological information and personal interviews

More than 94% of the 103 cases were interviewed face-to-face by trained monitors during hospital stay, close to the time of diagnosis. The respondent was the patient itself in 96.9% of the cases and a relative alone in 3.1%. Interviews included questions about past clinical history, signs and symptoms of pancreatic cancer, occupation, diet and coffee, alcohol and tobacco consumption (7,8,59,61–64,68). A structured form was used to collect clinicopathological information from medical records, including details on diagnostic procedures, signs and symptoms of pancreatic cancer, tumour stage, laboratory results and follow-up (55,60,61,67). Histological type was classified according to the International Classification of Diseases for Oncology. All items concerning the presence of signs and symptoms were further reviewed by two experienced oncologists and checked for consistency (59). Cholestatic syndrome involved jaundice, hypocholia (clay-coloured stools) and choluria (dark brown urine). The constitutional syndrome comprised fatigue, anorexia and weight loss (59,61,65). Lower lipid-corrected concentrations of six of the seven OCs analyzed [*p*,*p*′-dichlorodiphenyldichloroethene (DDE), three polychlorinated biphenyls (PCBs), hexachlorobenzene (HCB) and β-hexachlorocyclohexane (β-HCH)] were observed in patients with cholestatic syndrome (61); the constitutional syndrome increased only *p*,*p*′-dichlorodiphenyltrichloroethane (DDT) (61). When symptoms were considered, stage had only weakly inverse relationships with OC levels (61). The effects of symptoms on *p*,*p*′-DDE, *p*,*p*′-DDT and the three PCBs remained significant after adjusting by the interval from first symptom of PDA to blood extraction (ISE) and even when further adjusting by stage (61,65).

Detection of KRAS mutations

Details of laboratory protocols have also been described elsewhere (7,8,12,19). Briefly, mutations in codon 12 of *KRAS* oncogene were studied using DNA extracted from paraffin-embedded tumour tissue. Amplifications were done in two steps by nested polymerase chain reaction; an artificial BstNI restriction endonuclease site was introduced to discriminate between wild-type and mutated *KRAS* codon 12 sequences. Products were analyzed by acrylamide gel electrophoresis and ethidium bromide staining. This technique was able to detect one homozygous mutated cell in the presence of 10^2 normal cells. To characterize the nucleotide substitution in codon 12, all mutated samples were further analyzed using a similar restriction fragment length polymorphism-based approach. Interpretation of digestion products' electrophoresis was performed independently by two investigators to confirm the results. The spectrum of *KRAS* mutations was available for 40 of the 81 mutated cases (7,8,12,19).

Analysis of serum concentrations of OCs

The 10-ml screw-capped Pyrex centrifuge tubes capped with Teflon septa were used to keep and digest the samples (7,57,58,62,69). About 50 μl of the surrogate solution [0.36 μg/l of 1,2,4,5-tetrabromobenzene (TBB) and 0.52 μg/l of PCB 209] was added to 2 ml aliquots of serum in the same Pyrex centrifuge tubes where the samples were stored. Acid digestion of the mixture was performed by addition of 3 ml of *n*-hexane and 2 ml of concentrated sulphuric acid. Gas chromatography analyses were performed with a Hewlett–Packard model 5890A provided with an electron capture detection and a 30-m × 0.25-mm i.d. DB-5 column (J & W Scientific, Folsom, CA; film thickness 0.25 μm). A fused silica precolumn of 2 m × 0.32 mm i.d. was used and renewed every 30 samples. Selected samples were analyzed by negative-ion chemical ionization gas chromatography–mass spectrometry with a Fisons MD 800. The linear range of the detector was determined from injection of standard mixtures. Calibration lines were performed for all OCs mentioned above. These compounds were then quantitated in the samples by the external standard method after replicate analysis. The concentrations of HCB and β-HCH were corrected for volatility losses using TBB as internal standard. The recoveries of TBB and PCB 209 were 102% (±4.2) and 93% (±1.8), respectively. The recoveries of spiked serum samples were 74–86% for pentachlorobenzene and HCB, 92–93% of DDTs, 81–95% for HCHs and 77–110% for PCBs. Statistical analyses were limited to compounds that were detected above the detection limit in >85% of cases: *p*,*p*′-DDT was detected in 87.4% of the 103 PDA patients and PCB 138 in 95.2%, whereas *p*,*p*′-DDE, PCBs 153 and 180, HCB and β-HCH were detected in all cases.

Analysis of serum concentrations of lipids

Total cholesterol and triglycerides' levels were determined enzymatically (CHOD-PAP and GPO-PAP methods, respectively) using serum obtained at the same time than the serum used for the organochlorine analyses. Total serum lipids were calculated by the standard short formula (or Standard formula 2) of Phillips *et al.* (70,71). Organochlorine concentrations were individually corrected for total lipids and are expressed in nanograms per gram lipid (7,62–64,66). Cases and controls had similar concentrations of total cholesterol, triglycerides and total lipids (63). Correlations among OCs have also been published (62).

Statistical analysis

In this case–case study (72), we compared serum concentrations of OCs and coffee intake in 81 cases of PDA with a *KRAS*-mutated tumour and 22 cases of PDA whose tumours did not harbour such mutations. Cases with and without *KRAS* mutations did not differ significantly in age, sex, education, hospital, duration of the interview, tumour stage, signs and symptoms of pancreatic cancer, ISE, energy intake and smoking (8,12,19,25,54–68). To estimate the direction of the case–case association (7,24,26,72), case–control analyses compared all 103 cases with the 27 controls with OCs determined. Univariate statistics were computed as customary (73). For comparisons between continuous variables, Mann–Whitney's *U*-test was used. In contingency tables, Fisher's exact test for homogeneity or independence was applied to assess the

relationship between two categorical variables. OC concentrations were categorized in tertiles based on all cases with OC results. Receiver operator characteristic (ROC) curves were constructed to dichotomize OC concentrations. To estimate the magnitude of the associations, multivariate-adjusted odds ratios (ORs) and their corresponding 95% confidence intervals (CIs) were calculated by unconditional logistic regression. Categorical ordinal variables were analyzed for a linear dose–response relation through the multivariate analogue of Mantel's extension test; when a linear trend was not apparent, the probability test was used. Age, sex, tumour stage and ISE were assessed in all models as potential confounders (63). Because of findings mentioned above (61,65), the constitutional syndrome and the cholestatic syndrome were assessed for inclusion in all models. Other possible confounding variables were also retained in the models when they materially altered the estimates, such confounders included alcohol, pancreatitis, peptic ulcer and diabetes mellitus (the latter four if diagnosed $\geq$2 years before PDA) (54,67). Final models were chosen coherently with the nature of the variables and the study objectives. The level of statistical significance was set at 0.05, and all tests are two tailed. Analyses were performed using SPSS, version 12.0 (SPSS Inc., Chicago, IL, 2003).

Results

Cases with a *KRAS* mutation had higher concentrations of *p*,*p*′-DDT, *p*,*p*′-DDE and PCBs 138, 153 and 180 than cases with wild-type *KRAS*, but differences were statistically significant only for *p*,*p*′-DDT and for PCBs 138 and 153 (Table I). A dose–response pattern was approximately linear only for PCB 138 (ORs of 2.8 and 5.1 for the second and third tertiles, *P*-trend = 0.012). Patients in the mid-tertile of *p*,*p*′-DDT were over 29 times more likely to have a *KRAS*-mutated PDA than patients in the lower tertile, whereas the OR was only 2.8 in the upper tertile (Table I, Model 1). Most ORs increased slightly when adjusted by symptoms (Table I, Model 2). There was no association between the mutation status of the *KRAS* gene and the DDT:DDE ratio nor with concentrations of HCB and β-HCH.

OCs and coffee intake had statistically independent effects on the probability of having a *KRAS*-mutated PDA. *p*,*p*′-DDT and PCBs 138 and 153 each remained statistically significant after adding coffee to their respective models (Table II Models 1, 3, 4). Furthermore, the positive association between coffee intake and *KRAS* mutations remained statistically significant when four of the five OCs where in the models, and the ORs significantly increased with increasing coffee consumption (all four *P*-trend $<$ 0.01) (Table II, Models 2–5). Further adjusting by diabetes tended to decrease slightly the association between coffee and *KRAS* and to not materially affect the association with OCs (Table II). The results were virtually unaltered when pancreatitis or peptic ulcer was included in the models instead of diabetes.

When *p*,*p*′-DDT, PCB 153 and coffee intake were included in the same model (along with age, sex, alcohol and cholestatic syndrome), all three exposures were statistically significant. Again, only the mid-tertile of *p*,*p*′-DDT was strongly associated with *KRAS* mutation status. The ORs for the mid-tertile and upper tertile of PCB 153 were $>$7 and 22, respectively (*P*-trend = 0.007), and the ORs for coffee intake were 3, 8 and 12 (*P*-trend = 0.016) (Table III, Model 1). Coffee and PCB 138 were both significantly associated with *KRAS* status when adjusted by each other and further adjusted by *p*,*p*′-DDE (Table III, Model 2). When models were adjusted by pancreatitis rather than diabetes, the associations decreased only slightly, e.g. in Model 2, the *P*-values for PCB 138 and coffee intake became 0.027 and 0.045, respectively. Alcohol was also significantly associated with *KRAS* status, but the low number of patients prevented further analyses (e.g. with more than two categories of alcohol consumption).

All five OCs were statistically significantly associated with the Asp mutation, but CIs were wide (Table IV). *p*,*p*′-DDT and *p*,*p*′-DDE were also similarly associated with the mutation to Val. Five of six *P*-values for trend for PCBs were $<$0.05, but models are adjusted by many factors and, again, precision was low.

Concentrations of *p*,*p*′-DDT and *p*,*p*′-DDE were significantly higher among the 103 cases of pancreatic cancer than among the 27 controls (*P* = 0.014 and 0.002, respectively). The ORs for the mid and upper tertiles of *p*,*p*′-DDT were 4.2 and 7.3, respectively (*P* = 0.003), whereas for *p*,*p*′-DDE, they were 1.8 and 4.5, respectively (*P* = 0.029). Levels of the two compounds were statistically significantly higher in *KRAS*-mutated cases than in controls: the ORs for the mid and upper tertiles of *p*,*p*′-DDT were 7.7 and 10.2, respectively (*P* = 0.002), whereas for *p*,*p*′-DDE they were 4.2 and 8.4, respectively (*P* = 0.007). With ORs for the upper tertile ~3, they were slightly higher (but statistically non-significant) in *KRAS* wild-type cases than in controls. Concentrations of PCBs were slightly (and again statistically non-significantly) higher in cases (particularly, *KRAS*-mutated cases) than controls. Analyses did not unveil any significant interaction between OCs and age, gender, coffee, alcohol or tobacco consumption, diabetes or other diseases or among OCs themselves, but sample sizes were often small.

Discussion

We found that PDA patients whose tumours harboured a *KRAS* mutation had higher concentrations of *p*,*p*′-DDT, *p*,*p*′ DDE and PCBs 138, 153 and 180 than cases with wild-type *KRAS*, but differences were statistically significant only for *p*,*p*′-DDT and PCBs 138 and 153, and the dose–response pattern was approximately linear only for congener 138. These patterns may be deemed to suggest that the associations are spurious. Although it is biologically plausible that DDT shows non-linear carcinogenic effects at low doses (50,51), interactions between OC and unmeasured factors are likely and may result in non-linear patterns; they need to be explored in large studies. Our use of ROC curves to dichotomize OC concentrations is just a contribution to the exploration of such response patterns.

The positive relationship between coffee intake and *KRAS* mutations remained significant when four of the five OCs where in the models, and ORs increased with increasing coffee consumption. When two OCs (*p*,*p*′-DDT and PCBs 138 or 153) and coffee intake were included in the same model, both compounds and coffee remained strongly associated to *KRAS* mutations. *p*,*p*′-DDT, *p*,*p*′ DDE and PCBs 138 and 153 were similarly associated with the two most prevalent mutations, to valine and to aspartic acid. Adjusting by past medical conditions (notably, diabetes and pancreatitis) tended to decrease slightly the association between coffee and *KRAS* mutation status and not to materially affect the association between OCs and *KRAS*. These results were not confounded by alcohol and tobacco consumption.

The study size was small and many of our estimates were statistically imprecise. Size also limited the number of variables that models could account for. However, data had enough power to detect associations of relevant magnitude and scientific relevance. Furthermore, this is the largest study on OCs and genetic alterations in pancreatic cancer and one of the largest with analyses on OCs and tumour DNA in any type of cancer (9,20,21,40). Findings hence deserve to encourage

Table I. Serum concentrations of OCs in cases of PDA with and without a mutation in the *KRAS* oncogene[a]

Compound	All cases (N = 103)	*KRAS*			Model 1[b] (N = 103)			Model 2[c] (N = 103)		
		Mutated (N = 81)	Wild-type (N = 22)	P	OR	95% CI	P[d]	OR	95% CI	P[d]
p,p′-DDT										
Mean ± SD	626.6 ± 1029.7	692.0 ± 1108.9	385.7 ± 623.2	0.016[e]						
Median	354.7	414.8	114.9							
Tertiles										
≤224	41 (39.8%)	26 (32.1%)	15 (68.2%)	0.001[f]	1.0		0.007	1.0		0.009
225–614	34 (33.0%)	33 (40.7%)	1 (4.5%)		29.8	3.3–270.0		30.0	3.2–283.7	
>614	28 (27.2%)	22 (27.2%)	6 (27.3%)		2.8	0.8–9.4		2.8	0.8–9.9	
Dichotomous										
≤190	34 (33.0%)	19 (23.5%)	15 (68.2%)	<0.001[f]	1.0		<0.001	1.0		<0.001
>190	69 (67.0%)	62 (76.5%)	7 (31.8%)		9.1	2.9–28.4		9.2	2.8–30.1	
p,p′-DDE										
Mean ± SD	4078.1 ± 4417.6	4368.0 ± 4827.5	3010.7 ± 2119.1	0.342[e]						
Median	2706.8	2721.4	2548.3							
Tertiles										
≤1652	31 (30.1%)	22 (27.2%)	9 (40.9%)	0.429[f]	1.0		0.146[g]	1.0		0.190[g]
1653–4384	39 (37.9%)	31 (38.3%)	8 (36.4%)		1.8	0.6–5.8		1.7	0.5–5.4	
>4384	33 (32.0%)	28 (34.6%)	5 (22.7%)		2.6	0.7–9.7		2.4	0.6–9.5	
Dichotomous										
≤4750	74 (71.8%)	55 (67.9%)	19 (86.4%)	0.112[f]	1.0		0.081	1.0		0.084
>4750	29 (28.2%)	26 (32.1%)	3 (13.6%)		3.4	0.9–13.7		3.7	0.8–16.2	
PCB 138										
Mean ± SD	255.1 ± 203.5	268.1 ± 172.1	207.6 ± 291.8	0.014[e]						
Median	221.0	260.8	152.2							
Tertiles										
≤167	40 (38.8%)	27 (33.3%)	13 (59.1%)	0.073[f]	1.0		0.012[g]	1.0		0.004[g]
168–299	26 (25.2%)	21 (25.9%)	5 (22.7%)		2.8	0.8–10.0		3.0	0.8–11.3	
>299	37 (35.9%)	33 (40.7%)	4 (18.2%)		5.1	1.4–18.5		7.2	1.8–28.8	
Dichotomous										
≤302	67 (65.0%)	48 (59.3%)	19 (86.4%)	0.023[f]	1.0		0.020	1.0		0.007
>302	36 (35.0%)	33 (40.7%)	3 (13.6%)		4.9	1.3–18.5		6.9	1.7–27.9	
PCB 153										
Mean ± SD	297.3 ± 200.5	314.1 ± 209.6	235.3 ± 150.7	0.087[e]						
Median	233.5	244.1	181.0							
Tertiles										
≤187	37 (35.9%)	25 (30.9%)	12 (54.5%)	0.045[f]	1.0		0.003[g]	1.0		0.001[g]
188–313	31 (30.1%)	24 (29.6%)	7 (31.8%)		2.4	0.8–7.9		2.7	0.8–9.5	
>313	35 (34.0%)	32 (39.5%)	3 (13.6%)		9.2	2.0–42.3		15.4	2.9–82.3	
Dichotomous										
≤305	67 (65.0%)	48 (59.3%)	19 (86.4%)	0.023[f]	1.0		0.009	1.0		0.004
>305	36 (35.0%)	33 (40.7%)	3 (13.6%)		6.7	1.6–27.50		9.3	2.1–41.6	
PCB 180										
Mean ± SD	314.1 ± 224.2	328.7 ± 240.7	260.4 ± 140.0	0.489[e]						
Median	239.6	251.9	229.1							
Tertiles										
≤186	35 (34.0%)	29 (35.8%)	6 (27.3%)	0.185[f]	1.0		0.222	1.0		0.220
187–322	34 (33.0%)	23 (28.4%)	11 (50.0%)		0.5	0.1–1.6		0.5	0.2–1.8	
>322	34 (33.0%)	29 (35.8%)	5 (22.7%)		1.4	0.3–5.5		1.6	0.4–6.7	
Dichotomous										
≤475	85 (82.5%)	64 (79.0%)	21 (95.5%)	0.111[f]	1.0		0.074	1.0		0.053
>475	18 (17.5%)	17 (21.0%)	1 (4.5%)		7.0	0.8–59.3		8.5	1.0–74.6	

[a]Values of OCs are corrected by total lipids and expressed in nanograms per gram lipid.
[b]Model 1: ORs adjusted by age, sex and alcohol consumption.
[c]Model 2: ORs adjusted by age, sex, alcohol consumption and the appropriate signs and symptoms: *p,p′*-DDT adjusted by the constitutional syndrome; *p,p′*-DDE, PCB 138, PCB 153 and PCB 180 adjusted by the cholestatic syndrome (61).
[d]Unless otherwise specified, *P*-value derived from Wald test.
[e]Mann–Whitney *U*-test.
[f]Fisher's exact test.
[g]Test for linear trend (multivariate analogue of Mantel's extension test).

further studies, e.g. on the relationships between OCs and somatic mutations in sporadic cancers. Large prospective studies that collected blood many years before diagnosis could refute or replicate our findings if they are able to avoid selection biases caused by low retrieval of tumour specimens, a requirement for *KRAS* and other (but not all) genetic analyses (56,61). The timing of blood draw and pathophysiologic changes common in PDA (e.g. lipid mobilization and cholestasis) could not bias comparisons between *KRAS*-mutated and wild-type cases because: (i) there were only small differences between the two types of cases in symptoms at presentation (e.g. constitutional and cholestatic syndromes), in tumour stage or in other clinical variables (54,56,61,68) and (ii) based on previous research (61,63,65), such factors were

Table II. The joint effect of each OC and coffee consumption on the probability of a *KRAS*-mutated (versus *KRAS* wild-type) tumour[a]

Model	Exposures	OR[b]	95% CI	*P*[c]	OR[d]	95% CI	*P*[c]
1	*p,p′*-DDT						
	≤224	1.0		0.011	1.0		0.009
	225–614	33.9	3.3–353.7		44.0	3.8–512.8	
	>614	3.0	0.7–12.2		3.8	0.8–17.5	
	Coffee intake						
	Non-regular drinkers	1.0		0.074[e]	1.0		0.097[e]
	1–7 cups/week	3.8	0.7–19.8		3.6	0.7–19.6	
	8–14 cups/week	3.0	0.5–18.8		2.5	0.4–16.0	
	≥15 cups/week	5.2	1.0–26.7		5.0	0.9–27.8	
2	*p,p′*-DDE						
	≤1652	1.0		0.169[e]	1.0		0.181[e]
	1653–4384	2.2	0.6–8.2		2.2	0.6–8.0	
	>4384	2.6	0.6–10.7		2.5	0.6–10.5	
	Coffee intake						
	Non-regular drinkers	1.0		0.008[e]	1.0		0.010[e]
	1–7 cups/week	4.6	1.0–20.9		4.6	1.0–21.1	
	8–14 cups/week	6.4	1.3–31.5		6.2	1.2–31.1	
	≥15 cups/week	9.5	1.9–47.6		9.4	1.8–48.0	
3	PCB 138						
	≤167	1.0		0.005[e]	1.0		0.006[e]
	168–299	3.8	0.9–16.6		3.8	0.9–16.5	
	>299	8.1	1.8–36.9		8.0	1.8–36.6	
	Coffee intake						
	Non-regular drinkers	1.0		0.009[e]	1.0		0.011[e]
	1–7 cups/week	2.8	0.6–13.6		2.8	0.6–13.9	
	8–14 cups/week	6.9	1.3–35.5		6.4	1.2–33.2	
	≥15 cups/week	8.2	1.5–44.1		8.1	1.5–44.4	
4	PCB 153						
	≤187	1.0		0.001[e]	1.0		0.001[e]
	188–313	5.0	1.1–21.4		5.3	1.2–23.5	
	>313	22.1	3.4–143.6		27.3	3.8–196.7	
	Coffee intake						
	Non-regular drinkers	1.0		0.007[e]	1.0		0.010[e]
	1–7 cups/week	3.8	0.7–20.3		3.9	0.7–21.8	
	8–14 cups/week	9.5	1.7–55.0		8.4	1.4–48.6	
	≥15 cups/week	11.1	1.9–66.6		10.5	1.7–65.1	
5	PCB 180						
	≤186	1.0		0.240	1.0		0.180
	187–322	0.6	0.2–2.2		0.6	0.2–2.2	
	>322	2.0	0.4–9.0		2.3	0.5–11.1	
	Coffee intake						
	Non-regular drinkers	1.0		0.009[e]	1.0		0.013[e]
	1–7 cups/week	4.5	1.0–20.5		4.6	1.0–21.9	
	8–14 cups/week	7.4	1.5–36.7		6.9	1.4–35.1	
	≥15 cups/week	8.5	1.8–40.7		8.5	1.7–42.7	

[a]Values of OCs are corrected by total lipids and expressed in nanograms per gram lipid ($N = 95$).
[b]ORs for the OC and for coffee are mutually adjusted for; ORs are also adjusted by age, sex, alcohol consumption and the appropriate signs and symptoms: *p,p′*-DDT adjusted by constitutional syndrome; *p,p′*-DDE, PCB 138, PCB 153 and PCB 180 adjusted by cholestatic syndrome.
[c]Unless otherwise specified, *P*-value derived from Wald test.
[d]Further adjusted by diabetes.
[e]Test for linear trend (multivariate analogue of Mantel's extension test).

Table III. The statistically independent effect of two OCs and coffee on the probability of a *KRAS*-mutated (versus *KRAS* wild-type) tumour[a]

Exposures	OR[b]	95% CI	*P*
Model 1			
p,p′-DDT			
≤224	1.0		0.042[c]
225–614	16.2	1.6–167.0	
>614	0.8	0.2–4.1	
PCB 153			
≤187	1.0		0.007[d]
188–313	7.5	1.2–45.8	
>313	22.1	2.3–213.6	
Coffee intake			
Non-regular drinkers	1.0		0.016[d]
1–7 cups/week	3.1	0.5–20.7	
8–14 cups/week	8.3	1.0–70.5	
≥15 cups/week	11.9	1.6–88.9	
Alcohol consumption			
No and occasional	1.0		0.025[c]
Moderate and heavy	6.7	1.3–35.6	
Model 2			
p,p′-DDE			
≤1652	1.0		0.693[c]
1653–4384	1.8	0.5–7.3	
>4384	1.3	0.3–7.0	
PCB 138			
≤167	1.0		0.011[d]
168–299	3.9	0.9–17.3	
>299	7.5	1.5–36.8	
Coffee intake			
Non-regular drinkers	1.0		0.007[d]
1–7 cups/week	3.0	0.6–14.8	
8–14 cups/week	6.9	1.3–36.7	
≥15 cups/week	10.0	1.7–58.9	
Alcohol consumption			
No and Occasional	1.0		0.025[c]
Moderate and Heavy	5.1	1.2–21.1	

[a]Values of OCs are corrected by total lipids and expressed in nanograms per gram lipid ($N = 95$).
[b]OR adjusted by age, sex and cholestatic syndrome.
[c]Wald test.
[d]Test for linear trend (multivariate analogue of Mantel's extension test).

accounted for in the analyses. Thus, ORs for OCs increased slightly when controlling for symptoms (Table I, Model 2) (61). Results shown do not adjust for smoking because it is weakly or not associated with *KRAS* and OCs and, therefore, it did not alter estimates (24); the same applies to a history of peptic ulcer (67).

Having a small control group from a single hospital clearly limits the case–control component of the analyses, but is unlikely to bias the main conclusions. The primary purpose of comparing the two types of PDA cases with the controls was to explore the direction of the case–case association (7,8,72,74); such results suggest that mutated cases had higher levels than controls, whereas wild-type cases and controls had similar concentrations. Patients from the different hospitals did not differ in their clinical and sociodemographic characteristics (68); they also had very similar mutation rates and levels of OCs.

This report should not be seen as an independent replication of our previous findings on OCs (7) since half of the patients were the same. However, the improvement over the initial report in methods and analyses is highly relevant. With improved methods (56), the present results largely confirm previous findings on OCs, coffee intake and alcohol consumption (7,8,54).

The results are compatible with the hypothesis that in PDA OCs help activate *KRAS* or provide a growth advantage to *KRAS*-mutated cells. DDT and the more oestrogenic PCBs are established animal carcinogens. However, as previously hypothesized (5,7,8), the association between OCs and *KRAS* need not be through a directly mutagenic mechanism. First, OCs are enzyme inducers and may thus enhance enzymes that

Table IV. Concentrations of OCs and *KRAS* mutation spectra

	Valine[a] ($N = 17$)	Aspartic acid[a] ($N = 17$)	Valine versus wild-type[b]			Aspartic acid versus wild-type[c]		
			OR	95% CI	P[d]	OR	95% CI	P[d]
p,p′-DDT								
≤224	3 (17.7%)	3 (17.6%)	1.0		0.032	1.0		0.028
225–614	9 (52.9%)	8 (47.1%)	1026.6	5.7–UH		260.4	3.6–19 015	
>614	5 (29.4%)	6 (35.3%)	20.3	0.7–618.5		17.1	1.4–210	
p,p′-DDE								
≤1652	3 (17.6%)	3 (17.6%)	1.0		0.049[e]	1.0		0.022[e]
1653–4384	6 (35.3%)	5 (29.4%)	4.1	0.5–31.6		2.6	0.3–21.2	
>4384	8 (47.1%)	9 (53.0%)	11.6	1.0–133.9		18.0	1.6–208	
PCB 138								
≤167	3 (17.6%)	4 (23.5%)	1.0		0.045	1.0		0.026[e]
168–299	8 (47.1%)	4 (23.5%)	47.4	1.9–1182.5		2.5	0.3–22.3	
>299	6 (35.3%)	9 (53.0%)	29.4	1.3–670.4		8.2	1.3–52.5	
PCB 153								
≤187	5 (29.4%)	2 (11.8%)	1.0		0.049[e]	1.0		0.003[e]
188–313	7 (41.2%)	5 (29.4%)	2.2	0.3–14.4		6.0	0.6–64.2	
>313	5 (29.4%)	10 (58.8%)	20.1	0.8–496.4		42.7	3.4–531	
PCB 180								
≤186	7 (41.2%)	3 (17.6%)	1.0		0.440	1.0		0.038[e]
187–322	6 (35.3%)	4 (23.5%)	0.3	0.0–2.4		1.0	0.1–8.5	
>322	4 (23.5%)	10 (58.8%)	1.0	0.1–11.5		8.3	0.8–82.1	

Comparison of cases mutated to valine and to aspartic acid against wild-type cases. Values of OCs are corrected by total lipids and expressed in nanograms per gram lipid. UH indicates unquantifiably high.

[a]*KRAS* codon 12 wild-type: glycine (GGT); mutation to valine: GTT (transversion); mutation to aspartic acid: GAT (transition).

[b]ORs adjusted by age, sex, coffee intake, alcohol consumption and the appropriate signs and symptoms: *p,p′*-DDT adjusted by constitutional syndrome; *p,p′*-DDE, PCB 138, PCB 153 and PCB 180 adjusted by cholestatic syndrome.

[c]ORs adjusted by age, sex, coffee intake, diabetes and the appropriate signs and symptoms: *p,p′*-DDT adjusted by constitutional syndrome; *p,p′*-DDE, PCB 138, PCB 153 and PCB 180 adjusted by cholestatic syndrome.

[d]Unless otherwise specified, *P*-value derived from Wald test.

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

activate the carcinogens ultimately responsible for mutating *KRAS*. Second, as tumour promoters and possibly endocrine disruptors (34,52), OCs may also be relevant in other mechanistic scenarios involving interference with cell-cycle checkpoints and apoptosis, alterations in gene expression or other non-genotoxic and epigenetic effects (5,7,33–36,48–50,52,53). DDT and its metabolites, for instance, stimulate gene expression through various transcription factors and multiple response elements, including the mitogen-activated protein kinases (MAPKs) (49). Activated *KRAS* also engages multiple effecter pathways, notably MAPK (1–4).

We measured blood concentrations of only a few OC among the many that are commonly found in humans (38); furthermore, concentrations of environmental chemicals are often correlated, i.e. subjects with high levels of some substances are likely to have higher values of other compounds as well (58,62). Thus, unmeasured contaminants (OC or non-OC) could be partly responsible for the observed associations.

Increases in the risk of PDA have been associated with exposure to chlorinated organics but, overall, the evidence is limited (7,9,39–43,46,75,76). Surprisingly, only three studies have used biomarkers of exposure to OC (7,9,39,40); a positive association between serum DDE levels and pancreatic cancer risk remains to be published (D. Garabrant, personal communication). Studies that do not use biomarkers are likely to misclassify personal cumulative concentrations of OCs since OC exposure usually occurs inadvertently throughout the lifecourse (36,38,47,48,77–79). Serum levels provide accurate and specific estimates of individual internal dose at a time window that is aetiologically relevant for *KRAS* mutations in PDA (5,7,29). Background environmental exposure (mostly from foods contaminated at generally low doses) is usually a more important source of OC than occupation (25,47). We are conducting specific analyses to elucidate such sources in our study subjects.

In recent years, higher concentrations of several common OCs—including DDT, DDE and PCBs—have been associated with an increased prevalence of health disorders as diabetes, obesity and several other components of the metabolic syndrome (36,48,77–80); remarkably, all such disorders are also under scrutiny as risk factors for pancreatic cancer (42,43,81). Similarly, higher concentrations of persistent organic pollutants (POPs), especially OC pesticides, have been found positively associated with periodontal disease (82). Independently, periodontal diseases have also been associated with the risk of pancreatic and other cancers (83). Thus, our findings suggest that POPs might explain observed associations between some disorders (diabetes, obesity, metabolic syndrome and periodontal diseases) and pancreatic cancer risk. Findings are relevant not only for pancreatic cancer but also for research on other health effects of POPs as well.

The effects of OCs and coffee were not mutually confounded, i.e. they were statistically independent; they might also be biologically independent: in PDA the intake of caffeine or other coffee compounds may favour the occurrence or persistence of *KRAS* mutations through biological pathways different from OCs. Alcohol also independently increased the odds of a *KRAS*-mutated PDA (as always, versus that of a *KRAS* wild-type tumour), but detailed analyses of alcohol were hampered by sample size. Mechanistic scenarios for these

observations have been previously proposed (5,7,8,53,54,74). Ras proteins play a central role in conferring on tumour cells the capability of being self-sufficient in growth signals by virtue of their biochemical function as activators of intracellular effecter pathways that control transcription, cell survival and the actin cytoskeleton. These pathways form a signalling network that enables the cell to interpret growth-promoting signals in a context-dependent manner (2,30,84). Ras oncogenes facilitate neoplastic conversion by stimulating tumour cell growth, survival and motility. The role of *ras* in malignant transformation extends beyond these cell-intrinsic effects to include the establishment of a pro-tumorigenic host environment. *Ras*-induced secretion of the chemokine interleukin-8 elicits a local inflammatory reaction that is critical for neovascularization and sustained tumour growth. The *ras* oncogene hence promotes tumour–host interactions that are essential for cancer progression (30,85). Therefore, the study findings may have mechanistic implications for other neoplasms with a high frequency of *ras* mutations (28); they are relevant for scientists working on somatic mutations in human carcinogenesis, OCs or pancreatic cancer, and they contribute to the scant literature on the role of gene–environment interactions in the poorly understood aetiopathogenesis of PDA. Of course, replication is required before mechanistic or clinical implications, if any, are further considered (33–36,48,77).

In conclusion, *p*,*p*′-DDT, PCBs and coffee may have independent roles in the aetiopathogenesis of PDA by influencing *KRAS* activation, acquisition or persistence, perhaps through indirect, non-genotoxic or epigenetic mechanisms. Given that *KRAS* mutations are the most frequent abnormality of oncogenes in human cancers, and the common, lifelong accumulation of OCs, refutation or replication of the findings is required. If replicated by independent and valid studies, the results could open new paths to better understand the aetiology of pancreatic and other cancers. They might also help clarify other health effects of OCs.

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