Association between coffee drinking and K-*ras* mutations in exocrine pancreatic cancer

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Accepted for publication 16 March 1999

Abstract

***Study objective*—To analyse the relation between coffee consumption and mutations in the K-*ras* gene in exocrine pancreatic cancer.**

***Design*—Case-case study. Consumption of coffee among cases with the activating mutation in the K-*ras* gene was compared with that of cases without the mutation.**

***Setting and patients*—All cases of pancreatic cancer newly diagnosed at five hospitals in Spain during three years were included in the PANKRAS II Study (n=185, of whom 121 whose tissue was available for molecular analysis are the object of the present report). Over 88% were personally interviewed in hospital. DNA was amplified from paraffin wax embedded tissues, and mutations in codon 12 of K-*ras* were detected by the artificial RFLP technique.**

***Main results*—Mutations were found in tumours from 94 of 121 patients (77.7%). Mutations were more common among regular coffee drinkers than among non-regular coffee drinkers (82.0% *v* 55.6%, p=0.018, n=107). The odds ratio adjusted by age, sex, smoking and alcohol drinking was 5.41 (95% CI 1.64, 17.78). The weekly intake of coffee was significantly higher among patients with a mutated tumour (mean of 14.5 cups/week *v* 8.8 among patients with a wild type tumour, p<0.05). With respect to non-regular coffee drinkers, the odds ratio of a mutated tumour adjusted by age, sex, smoking and alcohol drinking was 3.26 for drinkers of 2–7 cups/week, 5.77 for drinkers of 8–14 cups/week and 9.99 for drinkers of ⩾15 cups/week (p<0.01, test for trend).**

***Conclusions*—Pancreatic cancer cases without activating mutations in the K-*ras* gene had drank significantly less coffee than cases with a mutation, with a significant dose response relation: the less they drank, the less likely their tumours were to harbour a mutation. In exocrine pancreatic cancer the K-*ras* gene may be activated less often among non-regular coffee drinkers than among regular drinkers. Caffeine, other coffee compounds or other factors with which coffee drinking is associated may modulate K-*ras* activation.**

(*J Epidemiol Community Health* 1999;**53**:702–709)

Experimental studies in rodents and in vitro provide inadequate evidence that coffee is carcinogenic in the pancreas or in other tissues.[1] Coffee and caffeine cannot be definitely categorised as mutagen or non-mutagen.[1–4] The effects of coffee intake on the activity of known mutagens and carcinogens vary from clear enhancement to clear inhibition of the occurrence of tumours.[1 2] Caffeine can affect a wide variety of biological processes related to carcinogenesis, including multiple pathways involved in the cellular response to DNA damage.[5]

Epidemiological evidence does not indicate any significantly increased risk of pancreatic cancer with coffee intake,[1 6–17] although a weak association with higher levels of consumption remains a possibility.[1 10–17] None the less, from a mechanistic perspective it is important to note that coffee could play a modulating part in a subgroup of patients with pancreatic cancer, an effect that would be diluted in the entire population of subjects with the disease.

Progress in knowledge of the genetic mechanisms of human cancer provides a new basis for its molecular classification, and the integration of molecular and epidemiological approaches is helping to identify pathogenic clues.[17–22] Exocrine pancreas cancer shows the highest frequency of K-*ras* gene mutations of any human neoplasm; the reasons are unknown.[15 17 23 24] In particular, no association between such mutations and lifestyle or environmental factors has been firmly established. Molecular pathology studies suggest that wild type K-*ras* carcinomas of the pancreas may arise through a genetic pathway distinct from carcinomas that harbour a K-*ras* mutation.[24] There is a dearth of information on the relation between coffee consumption and K-*ras* mutations in human cancers.

The aim of this study was to analyse whether a relation exists between coffee drinking and the presence of K-*ras* mutations in patients with exocrine pancreas cancer.

Methods

SELECTION OF PATIENTS AND CLINICOPATHOLOGICAL REVIEW

The PANKRAS II study was conducted at five general hospitals in the eastern part of Spain. Between February 1992 and February 1995 the study prospectively included patients in whom one of the following diagnoses were suspected at admission: cancer of the exocrine pancreas, chronic pancreatitis, pancreatic cysts and pseudocysts, and cancer of the extrahepatic biliary system. The broad eligibility criteria respond to one of the study primary aims,

namely, to assess the clinical usefulness of detecting mutations in K-*ras* for the diagnosis of cancers of the exocrine pancreas and the extrahepatic biliary system.[25–29]

A structured form was used to collect detailed information from medical records on presenting symptoms, physical examination at admission, past medical history, findings made through ancillary procedures, and laboratory results.

The discharge diagnosis of all patients was reviewed by a panel of two surgeons and two gastroenterologists. Blinded to molecular results, they analysed all the clinical and pathological information available, including follow up.[30] Overall, the PANKRAS II study included 602 subjects. Their diagnoses were: exocrine pancreatic cancer (n=185), cancer of the gall bladder and the extrahepatic bile ducts (n=128); non-malignant diseases of the pancreas (n=166, including 119 patients with chronic pancreatitis), benign diseases of the gall bladder and the extrahepatic bile ducts (n=54), other benign pathologies (n=22), and other neoplasms (n=47). In addition, one of the study hospitals recruited a conventional control group: 29 subjects admitted for benign, non-digestive surgical conditions unrelated to tobacco and alcohol were individually matched to pancreatic cancer cases by age and sex.

The tumour's clinical stage at diagnosis was classified according to the tumour-node-metastasis (TNM) system.[31 32] All cases were independently reviewed by the study reference pathologists, who were unaware of the original diagnosis. Histological type was classified according to the International Classification of Diseases for Oncology.[33 34] The present report is based on 121 patients with pancreatic cancer from whom cytological or histological material could be analysed for K-*ras* mutations (see below). The tumours of 106 of them (88%) were classified as adenocarcinomas, including adenocarcinoma NOS (n=96), mucinous adenocarcinoma (4), papillary adenocarcinoma (2), cystadenocarcinoma (2) and adenosquamous carcinoma (2). In addition, there was one case of anaplastic carcinoma, one case of squamous carcinoma and seven cases of carcinoma NOS; a definite morphological diagnosis of cancer could not be achieved in six cases.

The study design was approved by the ethics committee of the participating hospitals, and patients gave informed consent to be included in the study.

PATIENT INTERVIEWS

Trained monitors conducted interviews with patients during the hospital stay. Questions focused on past clinical history, lifestyle, and occupation. Detailed information was obtained on tobacco and alcohol consumption for each period of life, including changes in the type and amount of products. The first question concerning consumption of coffee was: "Do you drink or did you drink coffee habitually?"; an affirmative answer was recorded only if the subject reported drinking ≥ 2 cups/week for one year or more up to the year before the first symptom of the current illness —a definition of regular coffee drinker previously used by epidemiological studies in Spain[35 36] and elsewhere,[9] which accords with consumption patterns in the older cohorts. The average number of cups/week, and the ages of initiation and discontinuation of coffee drinking were then elicited as well.

To assess the reliability of interviews, a sample of relatives was concurrently and separately interviewed about the patient's clinical history and habits, and agreement between the two sets of responses was compared (n=110 pairs). For coffee consumption, positive agreement was 87% and overall agreement was 79%.[37]

Interviews were completed for 107 of the 121 subjects (88%) who are the object of the present report. The respondent was the patient himself in 96% of the cases and a relative alone in 4%. There were no significant differences in variables related to the interview between patients with a tumour carrying a K-*ras* mutation and those with the wild-type K *ras* gene.

TISSUE SPECIMENS

Twenty sections of 5 μm thickness were cut from each specimen and placed on glass slides. The first, 10th, and 20th sections were stained with haematoxylin and eosin and used for histological evaluation. The two study reference pathologists independently defined tumour areas by microscopic examination, as well as the percentage of neoplastic cells therein. Pancreatic cytohistological material was obtained from 150 of the 185 patients with exocrine pancreatic cancer (81%). For 10 patients only fresh, frozen material was available, and these were not analysed. Of 140 subjects with paraffin wax embedded samples from primary and/or metastatic lesions, pathologists deemed that the sample was unrepresentative of the tumour in seven cases, and from non-tumoural pancreas in 10 cases, and these cases were also excluded. DNA amplification was not achieved for samples from two subjects. Thus, results from 121 subjects are included in this report (86% of the 140 subjects with paraffin wax embedded samples and 65% of the 185 subjects with exocrine pancreatic cancer). The analysis includes three cases with histological confirmation of pancreatic cancer based on the analysis of the block used to obtain tissue sections for molecular analysis; in the latter, however, the pathologists were unable to identify tumour cells conclusively. There were no statistically significant differences between the 121 subjects and the remaining 64 patients with respect to gender, education, study site, tumour stage, duration of the interview, and consumption of coffee, tobacco and alcohol, except that the former were slightly younger.

DETECTION OF K-*RAS* MUTATIONS

Careful measures were taken to avoid contamination during all steps of amplification and analysis. The detailed method for detection of K-*ras* mutations has been described elsewhere.[38–40] Briefly, DNA was extracted and amplified in two steps by nested polymerase chanin reaction; in the second amplification reaction, an artificial BstNI restriction

Table 1 Selected patient characteristics and prevalence of K-ras mutations

Characteristic	Total	K-ras		OR	p value (OR 95% CI)
		Mutated	Wild type		
Total	121 (100)	94 (77.7)	27 (22.3)		
Gender					
Female	51 (42.1)	39 (76.5)	12 (23.5)	1.00	0.784†
Male	70 (57.8)	55 (78.6)	15 (21.4)	1.13	(0.48, 2.67)
Age (y)					
All patients*	64.5 (12.4)	64.8 (12.1)	63.5 (13.6)	—	0.651‡
Women*	68.6 (13.1)	69.1 (12.3)	66.7 (16.0)	—	0.574‡
Men*	61.5 (10.9)	61.7 (11.0)	61.0 (11.3)	—	0.845‡
⩽60 years	45 (37.2)	34 (75.6)	11 (24.4)	1.00	0.665†
>60 years	76 (62.8)	60 (78.9)	16 (21.1)	1.21	(0.51, 2.91)
Study site					
Mallorca	34 (28.1)	25 (73.5)	9 (26.5)	1.00	0.945¶
Elche	21 (17.4)	16 (76.2)	5 (23.8)	1.15	(0.28, 5.20)
Barcelona 1	31 (25.6)	24 (77.4)	7 (22.6)	1.23	(0.34, 4.57)
Barcelona 2	16 (13.2)	13 (81.2)	3 (18.7)	1.56	(0.31, 10.41)
Barcelona 3	19 (15.7)	16 (84.2)	3 (15.8)	1.92	(0.39, 12.54)
Clinical stage					
I	25 (20.7)	18 (72.0)	7 (28.0)	1.00	0.795§
II	21 (17.4)	19 (90.5)	2 (9.5)	3.69	(0.68, 20.19)
III	15 (12.4)	12 (80.0)	3 (20.0)	1.56	(0.33, 7.23)
IV	60 (49.6)	45 (75.0)	15 (25.0)	1.17	(0.41, 3.34)

*Values are means (SD). Otherwise, figures refer to the number of subjects (figures within parentheses are the corresponding percentages). The first category of each variable is the reference category (OR=1.00). †Pearson's χ^2. ‡Student's *t* test. ¶Fisher's exact test (two tail). §Mantel-Haenszel χ^2 test for linear trend.

endonuclease site was introduced to discriminate between wild type and mutated K-*ras* codon 12 sequences. Products were analysed 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 characterise the nucleotide substitution in codon 12, all mutated samples were further analysed using a similar RFLP-based approach, as described elsewhere.[38–40] DNA from oral mucosal scrapings was used as normal control and DNA from pancreas cancer cell lines or tumours were used as controls for the Val, Asp, Arg, Cys and Ser mutations. Interpretation of digestion products' electrophoresis was performed independently by three 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.[39 40]

STATISTICAL ANALYSES

In this case-case study[18–22 41 42] all results refer to the 121 patients whose K-*ras* mutational status was determined (that is, cases with a mutation and cases without a mutation). In contingency tables, comparison of two qualitative or categorical variables was performed with Pearson's χ^2 test for homogeneity or independence; alternatively, when ⩾20% of cells had expected counts less than five, Fisher's exact test was applied. For ordered categorical variables the Mantel-Haenszel χ^2 test for linear trend was used.[43] Odds ratios were used to estimate the magnitude of associations between variables; if the observed number of cases in one cell of the contingency table was zero, the Woolf-Haldane correction was applied.[44] The logit estimator of the OR was calculated with precision-based confidence intervals (CI).[44] Multivariate adjusted odds ratios and their corresponding 95% CI were estimated by unconditional logistic regression. Student's *t* test or Mann-Whitney's U test were used to analyse the relation between a categorical variable with two levels, and a normally or non-normally distributed quantitative variable, respectively.[43]

The number of cups of coffee/week was analysed both as a continuous variable and as an ordered categorical variable. Categories were defined as follows: non-regular drinkers (<2 cups/week), drinkers of 2–7 cups/week, of 8–14 cups/week, and of ⩾15 cups/week. This categorical variable was analysed for a linear dose response relation between coffee intake and K-*ras* activation through the multivariate analogue of Mantel's extension test, the χ^2 test of an ordered categorical variable in the logistic regression model.[45] The level of statistical significance was set at 0.05, and all statistical tests are two tailed.

In the ensuing analyses, to adjust for smoking the cumulative number of years smoked (estimated on the basis of every individual period of active smoking) was used; highly similar odds ratios for the association between coffee and K-*ras* were obtained when adjusting by cumulative lifetime number of cigarettes, and by smoking as a dichotomous variable (ever/never). To adjust by alcohol consumption, a variable with five categories was used: non-drinker, occasional (subject reported occasional drinking for all types of alcoholic beverages), low consumption (for women, <168 g/week; for men, <280 g/week), high consumption (for women, 168–280 g/week; for men, 280–560 g/week), and heavy drinker (for women, more than 280 g/week; for men, more than 560 g/week). Again, very similar odds ratios were obtained when adjusting by cumulative lifetime grams of alcohol, and by total number of years of alcohol drinking.

Results

K-*ras* codon 12 mutations were detected in tumours from 94 of the 121 subjects (77.7%) (table 1). There were no statistically significant differences in the prevalence of mutations according to sex, age, years of education, study site, clinical stage of the tumour at diagnosis, and origin of the sample used for the molecular analyses.

Of 107 subjects for whom interview data were available, 89 (83.2%) were regular coffee drinkers (82.6% of women and 83.6% of men). Ninety eight per cent of regular coffee drinkers drank ⩾7 cups/week. At the time of diagnosis regular coffee drinkers were slightly younger than non-drinkers (mean of 63.8 years *v* 66.2 years, p=0.434). The frequency of "ever smokers" was 58.4% among regular coffee drinkers and 50.0% among non-coffee drinkers (p=0.604). Alcohol drinking was also only slightly more common among regular coffee drinkers (non-linear, statistically non-significant relation).

Mutations were significantly more common in tumours of regular coffee drinkers than in those of non-regular coffee drinkers (82.0% *v* 55.6%, age adjusted odds ratio 3.71) (table 2). The association was somewhat stronger among men (age adjusted odds ratio 6.13, 95% CI 1.35, 27.77), among ever smokers

Table 2 Coffee drinking among pancreatic cancer cases with and without K-ras mutation

	Total	K-ras		Age adjusted OR	p value (OR 95% CI)
		Mutated	Wild type		
Regular coffee drinkers					
No	18 (16.8)	10 (55.6)	8 (44.4)	1.00	0.018†
Yes	89 (83.2)	73 (82.0)	16 (18.0)	3.71	(1.26, 10.93)
Cups per week*‡	13.2 (12.3)	14.5 (13.1)	8.8 (8.1)	1.00	0.043§
				1.06	(1.01, 1.12)
Non-regular drinkers	18 (17.1)	10 (55.6)	8 (44.4)	1.00	0.038¶
2 to 7 cups/week	28 (26.7)	22 (78.6)	6 (21.4)	2.93	(0.80, 10.71)
8 to 14 cups/week	27 (25.7)	22 (81.5)	5 (18.5)	3.62	(0.93, 14.06)
⩾15 cups/week	32 (30.5)	27 (84.4)	5 (15.6)	4.45	(1.16, 17.11)
Years of drinking*‡	35.4 (22.4)	36.8 (20.9)	30.6 (26.7)	—	0.234††
median	37	37	28.5	—	0.761‡‡
⩾50 years	36 (34.3)	29 (35.8)	7 (29.2)	—	0.547§§

*Values are means (SD). Otherwise, figures refer to the number of subjects (figures within parentheses are the corresponding percentages). †Fisher's exact test. ‡Information on the number of cups of coffee per week and on years of coffee drinking missing for two additional subjects. §Mann-Whitney's U test. ¶Multivariate analogue of Mantel's extension test. ††Student's *t* test. ‡‡Median two sample test (normal approximation). §§Pearson's χ^2 test.

(age adjusted odds ratio 5.69, 95% CI 1.25, 25.98) and among subjects less than 60 years old (crude odds ratio 7.00, 95% CI 0.95, 51.50). Similarly, the association between coffee intake and K-*ras* mutation was moderately stronger when the analysis was restricted to adenocarcinomas (age adjusted odds ratio 4.43, 95% CI 1.44, 13.63). By contrast, it did not vary significantly at different levels of alcohol consumption.

The weekly intake of coffee was significantly higher among patients with tumours carrying a K-*ras* mutation (mean of 14.5 cups/week *v* 8.8 among patients with a wild type tumour). There was evidence of a dose response relation: with respect to non-regular coffee drinkers, the likelihood of a mutated tumour for each of the three upper categories of coffee intake was 2.93, 3.62 and 4.45, respectively (p=0.038, test for linear trend) (table 2). There were only nine subjects who reported drinking more than 21 cups/week; their tumours were all mutated.

On average, patients with a mutated tumour reported drinking coffee during 6.2 more years than patients with a wild type tumour (table 2). The mean age at which patients started drinking coffee was almost identical in the two groups: 19.9 years in the mutated group (SD 10.4) and 20.7 in the wild type group (SD 16.5) (p=0.867).

Tumours of regular coffee drinkers were over five times more likely to harbour a codon 12 K-*ras* mutation than tumours of non-coffee drinkers when age, gender, smoking, and alcohol consumption were taken into account (table 3, model 1). Adjustment by tumour stage, study site, and years of education yielded virtually identical results (data not shown). The dose response relation was also strengthened in the multivariate analyses (table 3, model 4), whereas years of coffee drinking approached statistical significance (model 5). The interactions of coffee drinking with smoking and with alcohol consumption were not statistically significant. Alcohol was essentially unrelated to the mutation, whereas smoking was slightly more frequent among cases with wild type tumours (as the PANKRAS II study collected detailed information on tobacco and alcohol consumption, these results will be reported separately; none the less, let us note that smoking negatively confounded the association between coffee drinking and K-*ras* mutations).

Table 3 Multivariate analysis of the association between K-ras mutations and coffee drinking. All estimates adjusted by age, gender, smoking and alcohol consumption†*

Model	Patients (n)	OR‡ (95% CI)	p value§
Regular coffee drinkers v non-regular coffee drinkers			
1 All subjects	107	5.41 (1.64, 17.78)	0.005
2 Adenocarcinomas only	95	6.30 (1.81, 21.99)	0.004
Number of cups of coffee per week			
3 As a continuous variable¶	105	1.10 (1.03, 1.18)	0.007
4 As a categorical ordinal variable	105		0.005††
Non-regular drinkers		1.00	
2 to 7 cups/week		3.26 (0.83, 12.75)	
8 to 14 cups/week		5.77 (1.30, 25.59)	
⩾15 cups/week		9.99 (2.03, 49.22)	
Number of years of coffee drinking			
5 As a continuous variable¶	105	1.02 (0.99, 1.05)	0.062

*Cumulative number of years smoked. †Five categories (see Methods). ‡OR: multivariate adjusted odds ratio. §p Value derived from the corresponding regression coefficients in the logistic model. ¶Risk increase per unit increase. ††Multivariate analogue of Mantel's extension test.

When multivariate analyses were restricted to subjects with the adenocarcinoma histological type, regular coffee drinkers were over six times more likely to harbour a codon 12 K-*ras* mutation than non-coffee drinkers (table 3, model 2). As compared with adenocarcinomas of non-regular coffee drinkers, the multivariate odds ratio of a mutated tumour for each of the three upper categories of coffee intake was 4.05, 5.78 and 11.33 (p=0.0047, test for linear trend). Again, adjustment by tumour stage, study site or education did not change these results.

The spectrum of mutations, which could be determined for 48 of the 94 mutated tumours, was as follows: Val (24 cases, 50%), Asp (22 cases, 46%), Arg (7 cases, 15%), and Cys (3 cases, 6%). A double mutation was detected in 8 of the 48 patients (17%). Among patients with an Asp substitution, 84% were regular coffee drinkers. The corresponding figure for patients with a Val substitution was 91% and, thus, they were over four times more likely to be regular coffee drinkers than cases with wild type tumours (odds ratio 4.75, p=0.077). All patients with a double mutation had drank coffee regularly: they were over six times more likely to have done so than subjects with the wild-type K-*ras* gene (odds ratio 6.69, p=0.155).

Discussion

This case-case study suggests that an association may exist in pancreatic cancer between K-*ras* mutations and regular coffee intake. As this is the first time that such a finding is reported, additional studies are clearly needed to either confirm it or refute it. One possibility is to conduct an improved case-case study, a design that constitutes a valid and efficient option to explore gene-environment interactions.[18 21 22 41 42] The additional effort required by case-case-control studies may be justified if case-case studies confirm the association. One or more control groups may eventually need to be selected, based on the genetic and metabolic hypotheses outlined later in this section. We deemed premature to recruit a conventional hospital control group in all our five study hospitals because the K-*ras* gene is never or

KEY POINTS
- Pancreatic cancer cases without activating mutations in the K-*ras* gene drank significantly less coffee than cases with a mutation, with a "dose response" relation.
- Coffee compounds or other factors with which coffee drinking is associated could modulate K-*ras* activation in pancreatic cancer.
- Pancreatic cancers with and without a mutation in the K-*ras* gene may result from different genetic-environment interactions.
- Results support need to assess if coffee modifies the effects of other exposures on the risk of cancers with *ras* mutations.
- While results may have a mechanistic and pathogenic interest, they lack immediate clinical or health policy implications.

extremely rarely mutated in subjects eligible to be part of such control group, because it would be unethical to obtain pancreatic tissue samples from healthy controls and, most importantly, because our primary aim was to test the interaction of K-*ras* with lifestyle and environmental factors in pancreatic cancer.[25–27]

None the less, other groups of the PANKRAS II study can tentatively be used as referents to estimate the "direction" of the association, and the resulting figures follow. As we saw, the proportion of regular coffee drinkers among K-*ras* mutated pancreatic cancer cases was 88% (73 of 83) (table 2). This figure is remarkably similar in other groups of the PANKRAS II study: 87% of patients with cancer of the extrahepatic biliary system were regular coffee drinkers, as were 85% of subjects with benign biliary disorders, and 83% of the conventional hospital controls. By contrast, the corresponding figure for K-*ras* wild type pancreas cancer cases was 67% (16 of 24) (table 2). Accordingly, K-*ras* activation would be less common in coffee abstainers, rather than higher among regular coffee drinkers.

The proportion of regular coffee drinkers in our entire series of patients with pancreatic cancer was 83%, a figure that is not statistically significantly different from that observed in any of the three above mentioned referent groups. Thus, our results agree with studies indicating that no overall association exists between coffee drinking and pancreatic cancer.

The previous considerations do not rule out the possibility of an interaction between coffee drinking and other risk factors for pancreatic cancer, such as cigarette smoking. The coffee-smoking interaction is supported by some epidemiological studies on pancreatic cancer[11–15] and by research on the genetic polymorphisms of caffeine metabolic enzymes. The latter indicates that smokers accumulate less caffeine in the body.[46] This line of mechanistic evidence might eventually contribute to explain our observation of a negative confounding by smoking of the association between coffee drinking and K-*ras* mutations.

Like case-control studies, the case-case design we used is able to study only prevalent genetic alterations. Despite the inherent ethical, clinical and logistic difficulties, longitudinal studies are clearly needed, preferably of inception cohorts and with repeated measures over time of exposures and of intermediate genetic events. Such need has been emphasised by the finding that K-*ras* mutations are not uncommon in putative preneoplastic lesions in subjects with pancreatic cancer, in the pancreas from patients with other gastrointestinal tumours or with chronic pancreatitis, and even in the macroscopically normal pancreas.[23 47–51] Factors that influence the progression of precursor lesions remain to be determined, including the precise role of mutations in *K*-ras and other genes (for example, *p53, p16, DPC4*), the sequence in which they occur, and defective DNA mismatch repair[5 47 48 52–56] among other.[17 23] From our results coffee would emerge as a candidate for study.

Several hundred compounds have been identified in roasted coffee.[1 4 57] Some of these substances may act as direct mutagens (for example, methylglyoxal), may modulate the effects of carcinogens through metabolic and other pathways (for example, caffeine, theobromine)[1–3 58] and may affect other processes relevant to malignant transformation and tumour progression. Considering that coffee is consumed worldwide in large quantities, that it has not been strongly linked with human cancer in epidemiological studies, and the results of carcinogenicity assays in experimental animals, it is unlikely that strong mutagens are present in this beverage.[2 4 59] On the other hand, experimental evidence indicates that caffeine can affect DNA repair, modify the apoptotic response and perturb cell cycle checkpoint integrity.[2 4 5 52–56] Modification of *p53* expression by caffeine may interfere with normal induction of *p53* in response to DNA damage.[52] The lack of data on the relation between coffee and *ras* mutations in human cancers is also noteworthy because coffee drinking has been implicated (either as a beneficial or as a harmful habit) in cancers where *ras* mutations are common, such as colon and bladder cancer.[1 7 8]

Caffeine exerts a large variety of behavioural effects, which may affect exposure to factors that can either promote or inhibit cancer.[4 59] Perhaps the association reported here reflects different environmental exposures between patients whose tumours harbour or not a mutation at diagnosis. Coffee would hence be just associated with a factor able to modulate K-*ras* activation. It is also possible that, under equal conditions of exposure to the putative activator of K-*ras*, coffee abstainers might have a better capacity to repair the K-*ras* mutation than coffee drinkers. Perhaps these processes are influenced by dietary factors; for instance, coffee abstainers may[60] (or may not[61]) eat more fruits and vegetables than heavy coffee drinkers. While knowledge is beginning to accrue on possible mechanisms for the dietary modulation of pancreatic carcinogenesis,[6 7 17 62] further research is needed to establish what part diet plays in the activation of *ras* genes. Whatever the mechanistic scenario, the findings reported here support the notion that K-*ras* mutated and

K-*ras* wild-type pancreatic cancer may be characterised by aetiological heterogeneity, which can be attributable to different causal pathways or merely reflect a different magnitude of effect via the same mechanism.[42]

The odds of a mutated tumour increased in an approximately linear fashion with increasing levels of coffee consumption. Our analyses of linear trend were complemented by the values of the odds ratios for different strata, which convey the shape of the exposure-response relation. The inclusion of a wholly unexposed group in the analyses for linear trend has been criticised.[59 63] Our reference category included patients who did not drink coffee at all along with subjects who drank ⩽1 cups/week (or ⩾2 cups/week for less than a year); therefore, the reference group probably included some sporadic coffee drinkers.[35] Ninety eight per cent of regular coffee drinkers drank at least 7 cups/week; the dose is not extremely low for Spain, where consumption of coffee is lower than in northern Europe.[64]

The findings do not seem to be the result of "multiple testing". Firstly, the only variables analysed were tobacco, coffee and alcohol. Secondly, these variables were selected before the study initiation based on findings from previous studies of pancreatic cancer. Thirdly, the association between K-*ras* mutations and coffee drinking is evident at the simplest, crudest level of analysis. And fourthly, a "dose response" pattern is even less likely to arise simply by chance.

The abundance of experimental studies on caffeine contrasts with the paucity of genetic studies assessing actual coffee consumption in patients.[59 61 65] The latter must bear several factors in mind. Firstly, there is no standardised measure for a cup of this beverage.[1 64] None the less, self reported coffee intake has been found to be significantly correlated with salivary and plasma concentrations of caffeine and paraxanthine, thereby providing qualified support for the use of questionnaires to estimate patterns of caffeine consumption.[65–68] In our study, the reliability of information obtained through patient interviews was assessed with a sample of proxy, next of kin respondents, and agreement was high.[37] The proportion of eligible patients that were interviewed is also among the highest of all studies on pancreas cancer; the success stems from the prospective identification of potential cases. Because we used a case-case design, misclassification is more likely to have been non-differential than in other studies. Secondly, the type of coffee beans, roasting and brewing vary across geographical areas. In Spain, it is estimated that the average caffeine content of a cup of coffee is 95–115 mg, based on a 1:1 arabica to robusta ratio, the use of espresso and mocha coffee, and a usual cup size of 35–50 ml.[1 64 69 70] Thirdly, contaminants or products added to coffee, such as sugar and saccharine, might act as confounders. Consumption of other methylxanthine containing beverages may also play a part. Yet, use of artificial sweeteners is very low in the older Spanish cohorts, and cola beverages and tea account for an extremely low fraction of the daily caffeine intake.[35 64 65 70]

Epidemiological studies of pancreatic and other types of cancer have largely treated coffee either as a primary exposure or as confounder, but seldom as an effect modifier. Our results provide a new rationale to assess whether coffee modifies previously reported effects (both null and positive) of other exposures on the risk of cancers with *ras* mutations.

To our knowledge, this is the first report on the relation between coffee consumption and *ras* mutations for any human cancer, as well as the first molecular study of pancreatic cancer in which detailed information on environmental factors was collected through personal interviews with patients. It is also the largest case series published to date on K-*ras* mutations in this neoplasm. The broad eligibility criteria, and the prospective collection of cytohistological material represent a step forward, with respect to previous reports, in the attempt to reduce selection bias.[27]

As all patients included in the core analyses had pancreatic cancer, our findings may not have immediate clinical or health policy implications. They do suggest, however, that studies on the mechanisms of pancreatic carcinogenesis should consider to integrate the analysis of K-*ras* mutations and coffee consumption. If extended by other studies, the results could open a new avenue towards a better understanding of the pathogenesis of exocrine pancreatic cancer.

We are indebted to the following colleagues and members of our supporting staff who were associated with the study: A Serrat, E Carrillo, J Alguacil, M Soler, S Costafreda, D J MacFarlane, J Gomez, P Barbas and L Español. Scientific advise was provided by H Vainio, C Malaveille, F Bolúmar, R de la Torre, J Segura, J Sunyer and T Thomson. We also gratefully acknowledge valuable criticisms of earlier versions of this manuscript made by H O Adami, J Rodés, E Fernandez, D Trichopoulos, A Escolar, G Lopez-Abente and J Grimalt.

Funding: Fondo de Investigación Sanitaria (92/0007, 95/0017 and 97/1138), Fundación Salud 2000, Generalitat de Catalunya (CIRIT/1995 SGR 434), and MSD Spain.
Conflicts of interest: none.

Results of this study were presented to the Gruppo di Cooperazione in Cancerologia, Università di Torino (Torino, Italy, 22 January 1997), to the European Regional Meeting of the International Epidemiological Association (Münster, Germany, 4–6 September 1997), and to the 15th Scientific Meeting of the Spanish Society of Epidemiology (Oviedo, Spain, 24–26 September 1997).

Appendix

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