Timing of toenail collection and concentrations of metals in pancreatic cancer

Evidence against disease progression bias

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Abbreviations: BMI, body mass index; CI, confidence interval; CT, computerized axial tomography; ERCP, endoscopic retrograde cholangiopancreatography; GM, geometric mean; iSB, interval from first symptom of pancreatic cancer to blood extraction; iSD, interval from first symptom of pancreatic cancer to diagnosis of pancreatic cancer; iST, interval from first symptom of pancreatic cancer to toenail collection; OCs, organochlorine compounds; PDAC, pancreatic ductal adenocarcinoma; SD, standard deviation; SRM, standard reference material; TNM, tumor-node-metastasis system.

Declarations

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Not applicable.

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The Ethics Committees of participating hospitals approved the study protocol.

Consent to participate (include appropriate statements)

Patients gave informed consent to be included in the study.

Abstract

Trace elements such as cadmium, arsenic, zinc or selenium increase or decrease risk of a wide range of human diseases. Their levels in toenails may provide a measure of mid-term intake of trace elements for studies in humans. However, in biologically and clinically aggressive diseases as pancreatic cancer, the progression of the disease could modify such concentrations and produce reverse causation bias. The aim was to analyze the influence of specific time intervals between several clinical events and the collection of toenails upon concentrations of trace elements in patients with pancreatic cancer. Subjects were 118 incident cases of pancreatic adenocarcinoma prospectively recruited in eastern Spain. Toenails were collected at cancer diagnosis, and soon thereafter interviews were conducted. Information on cancer signs and symptoms was obtained from medical records and patient interviews. Levels of 12 trace elements were determined in toenail samples by inductively coupled plasma mass spectrometry. General linear models adjusting for potential confounders were applied to analyze relations between log-concentrations of trace elements and the time intervals, including the interval from first symptom of cancer to toenail collection (iST). Toenail concentrations of the 12 trace elements were weakly or not influenced by the progression of the disease or the diagnostic procedures. Concentrations of aluminum were slightly higher in subjects with a longer iST (age, sex and stage adjusted geometric means: 11.44 vs. 7.75 μg/g for iST >120 days vs. ≤40 days). There was a weak inverse relation of iST with concentrations of zinc and selenium (maximum differences of about 20 and 0.08 μg/g, respectively). Conclusions: concentrations of the trace elements were weakly or not influenced by the development of the disease before toenail collection. Only concentrations of aluminum increased slightly with increasing iST, whereas levels of zinc and selenium decreased weakly. Even in an aggressive disease as pancreatic cancer, toenail concentrations of trace elements may provide a valid measure of mid-term intake of trace elements, unaffected by clinical events and disease progression.

Introduction

Trace elements (in particular chromate, nickel, cadmium and arsenic compounds) are well-known carcinogens, and dietary contaminants [1]. They act by diverse mechanisms, including oxidative stress, they inhibit zinc-finger DNA repair machinery, and induce polyadenylation of canonical histones [2-4]. While these metals are best known for induction of lung, liver, colon and prostate cancer, their contribution to other cancers is under scrutiny.

Increases in pancreatic cancer incidence have raised substantial concern about environmental contributions, and suggest opportunities for prevention [5-7]. While multiple studies have examined occupational exposures as risks, particularly for cadmium [8], few have examined exposures in general populations, which are subject to chronic, low-level exposure due to ubiquitous contamination.

Previous studies suggest an association between pancreatic cancer risk and concentrations of trace metals such as cadmium, lead, arsenic, and selenium measured in toenails [9-11]. However, the influence of clinical events (signs and symptoms, time until diagnosis, diagnostic tests, tumor stage) on body concentrations of trace elements has not been evaluated in pancreatic cancer, other neoplasms, or other diseases.

Information on concentrations of trace elements in nails has long been used in humans to cost-effectively measure exposure and internal dose. In healthy and physically stable individuals, and compared to blood, urine, or hair samples, levels in toenails of mercury, manganese, and arsenic appear to reflect better the corresponding concentrations in the organism for a time frame from a few months to a year preceding sample collection [11-22].

Little is known about the toxicokinetic and toxicodynamic profiles of trace elements during tumor formation and progression, particularly about the methodological implications for research on their possible etiologic roles in cancer [23,24].

Studies that employ biomarkers of exposure in individuals who already have (asymptomatic or symptomatic) cancer may be biased by metabolic processes inherent to the disease. This has been demonstrated for organochlorine compounds (OCs) in blood samples of individuals diagnosed with pancreatic cancer, in whom the time interval between the first symptom of the disease and blood extraction (iSB) was associated with serum concentrations of total lipids and OCs [25-27]. Concentrations of the highly lipophilic OCs were also associated with signs and symptoms of the disease and with tumor's stage at diagnosis. Hence, during the progression of pancreatic cancer and other diseases, patients experience weight loss, cholestasis, and other clinical and pathophysiological changes that alter concentrations of lipids, lipophilic nutrients, and lipophilic environmental compounds. Disease progression bias is thus a form of reverse causation. It results in a lack of etiologic significance of the disease-altered exposure biomarkers [25-32]. This conceptual and empirical framework provides the rationale to hypothesize that pancreatic cancer progression might also modify concentrations of trace elements in toenails [9-11,33].

Therefore, the aim of the present study was to analyze the influence of specific time intervals between several clinical events and the collection of toenails upon concentrations of trace elements in patients with pancreatic cancer.

Materials and methods

Study population

Methods of the PANKRAS II study have been previously described [9-11,25-27,30,33-37]. Briefly, subject recruitment took place between 1992 and 1995 at five general hospitals in the Mediterranean part of Spain, where 185 incident cases of PDAC were prospectively identified. The present report is based on 118 incident cases of PDAC with toenail samples obtained and metal concentrations analyzed [11]. There were no significant differences between the 118 patients with and the 67 patients without available trace elements concentrations for a broad range of sociodemographic and clinical variables, including age, sex, social class, education, occupation, smoking, coffee consumption, duration of interview, interval from first symptom of pancreatic cancer to diagnosis, and interval from first symptom to blood extraction [9,10,34] (Supplementary Table 1). The Ethics Committees of participating hospitals approved the study protocol, and patients gave informed consent to be included in the study.

Personal interviews and information on symptoms

A structured form was used to collect clinicopathological information from medical records, including details on semiology, diagnostic procedures, laboratory results and follow-up [36, 37]. Follow-up extended for 17.5 years [34]. Hospital discharge diagnoses and the tumor clinical stage were also recorded. The tumor's clinical stage at diagnosis was classified according to the tumor-node-metastasis (TNM) system. Diagnostic tests included ultrasound scan, computerized axial tomography (CT), fibrogastroscopy, endoscopic retrograde cholangiopancreatography (ERCP), gammagraphy, laparoscopy, and exploratory laparotomy [25]. When a diagnostic procedure was performed more than once, the physician who abstracted the data chose the more informative result. Over 88% of the PDAC patients were interviewed face-to-face by trained monitors during their hospital stay, close to the time of diagnosis [10,11,34].

Detailed information on the first symptom of cancer, including the date, and signs and symptoms of the disease was obtained from two sources: medical records (where they were registered by the attending physician at hospital admission), and interviews with patients [30,33,36]. The information was reviewed by two experienced oncologists and checked for consistency. If data elicited from the interview contradicted data abstracted from the medical record, the latter was taken as the consensus data [30]. Pathology of all cases was independently reviewed by the study reference pathologists, who were unaware of the original diagnosis. A panel of clinical and surgical experts in gastrointestinal diseases reviewed hospital discharge diagnoses of all patients and, based on all clinical and pathological information available, including follow up, reached a consensual clinicopathological diagnosis [37].

Analyses of trace element concentrations

Nail clippings from the larger toe were collected once per patient during the hospital stay when the cancer was diagnosed, and were stored at room temperature until the time of the analyses. Trace elements analyzed were cadmium, arsenic, selenium, nickel, lead, chromium, manganese, aluminum, iron, vanadium, copper, and zinc [9-11,34]. After careful cleaning and washing to remove external contaminants, trace elements were quantified at the Trace Element Analysis Core (Dartmouth College, Hanover, New Hampshire, USA), using inductively coupled plasma mass spectrometry. Toenails were acid digested with Optima nitric acid (Fisher Scientific, St Louis, Missouri, USA) at 105ºC followed by the addition of hydrogen peroxide and further heating of 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 content (GBW 07601, powdered human hair) and six analytical blanks, along with the study samples [9-11,34].

The within-assay coefficients of variation for SRM replicates were <15% for aluminum, arsenic, copper, lead, manganese, selenium, and zinc; and 15-40% for cadmium, chromium, iron, nickel, and vanadium. The between-assay coefficients of variation for SRM replicates were <15% for arsenic, lead, manganese, selenium, and zinc; 15-30% for aluminum, cadmium, copper, and nickel; and >30% for chromium, iron, and vanadium (Supplementary Table 2). The amount of SRM used ranged from less than 10 to 50 mg to mimic the mass of toenails. This small SRM sample mass may be the cause of some of the variability seen in the within- and between-batch SRM results.

Eight of the 12 trace elements were detected in *all* 118 subjects (i.e., the percentage of detection was 100%), whereas the other four elements were detected in 99.2%, 98.3%, 98.3%, and 92.4% of subjects (Supplementary Table 2). Therefore, the corresponding percentage of participants *below* the detection limit was 0% for eight of the 12 trace elements, and 0.8%, 1.7%, 1.7%, and 7.6% for the other four.

The lowest concentration of each element detected in a given subject is also shown in Supplementary Table 2. Given the very high percentages of detection, such concentration is the most meaningful information; it reflects the limit of detection of the instrumental technique for the individual metals.

Statistical analysis

Univariate statistics were computed as customary to describe key patient and trace metal variables [38,39]. All time intervals were calculated using the date of the clinical or diagnostic event as reference. As an example, the interval between the first symptom of pancreatic cancer and the date of toenail collection is the time elapsed from the first symptom to toenail collection (iST). We analyzed the time intervals between toenail collection and: hospital admission, performance of diagnostic tests, blood extraction, diagnosis, treatment onset, and interview (Supplementary Tables 3 and 4).

Among patients with an iST ≤40 days 48% of patients were in tumor stage I and 21% in tumor stage IV, while among patients with iST >120 days there were 16% of patients in tumor stage I and 37% of patients in stage IV. The median time of the interval from hospital admission to toenail collection was 15 days, and to diagnosis and blood extraction 0 days. The median iST was 70.5 days, with a range from one week to more than two years (11 to 763 days) (Figure 1), and is similar to the median time interval between the first symptom of the disease and blood extraction (iSB), 72 days (167 patients); the distribution of these two intervals (iST and iSB) is virtually identical (Figure 1). When only patients with both samples, blood and toenails, were considered (N=117), the median iST and iSB were 71 and

70 days, respectively; in 84 such patients (72%) the blood and toenails were collected on the same day.

Supplementary Figure 1 shows the iST for each patient (all represented on the ‘y’ axis), with patients classified from shorter to longer interval. The color of the lines shows the number of diagnostic tests performed before the toenail collection. We collected about 60% of toenail samples during the first three months after symptom onset; at 6 months 80% of the samples had been collected. In >75% of patients the toenail collection was performed during the first month following hospital admission (Supplementary Table 3). This timing is a remarkable logistic achievement for a study of a severe disease as pancreatic cancer, and it may be a benchmark for other studies. A total of 315 diagnostic tests were performed in the hospitals before the collection of toenails (median of 3 diagnostic tests per patient); over 60% of patients with iST ≤40 days underwent three diagnostic tests before the collection (Supplementary Figure 1).

Kruskal-Wallis’ test and ANOVA’s test were used to assess differences between metal concentrations by iST categorized as ≤40 days, 41 to 120 days, and >120 days [25]; and Mann-Whitney’s *U* test and Student’s *t*-test to assess differences between participants with and without available toenails by sociodemographic characteristics. When a trend was observed, the Jonckheere-Terpstra test for trend was used. Spearman’s rank correlation coefficient (ρ) was used to evaluate the correlations between the concentrations of trace elements and the time intervals. For the correlations we only considered clinical events that happened previous to or on the day of the nail sample collection. Correlations without the time intervals equal to zero were also calculated. To analyze the relative influence of time intervals on trace concentrations, general linear models were used. Values of trace elements were normalized by natural logarithmic transformation [10,39]. Age, gender, tumor stage, and cholestatic syndrome were treated as potential confounding factors. Results were expressed as adjusted geometric means (GMs) with the corresponding 95% confidence intervals (CIs). The level of statistical significance was set at 0.05, and all tests are two-tailed. Statistical significance, the precision of the estimates, and the magnitude of the associations were all taken into consideration to assess the significance of the results [39-41]. Based on previous work and hypotheses [11,25-27,33,34], we analyzed a limited number of predictors and outcomes, many times lower than the millions of SNPs that are analyzed in an agnostic GWAS, for instance. Furthermore, techniques to adjust for the number of comparisons have been shown to have low efficiency or poor accuracy in studies as ours [40]. Thus, adjustment for the number of comparisons was not appropriate. Analyses were conducted using SPSS v22 (IBM SPSS Statistics, Armonk, NY, USA, 2013) and Stata 8.0.

Results

Median toenail concentrations of the trace elements were weakly or not influenced by iST (Table 1). Crude concentrations of aluminum were slightly higher in subjects with a longer iST: the maximum difference between the medians shown in Table 1 was 5.4 µg/g. Concentrations of zinc and selenium were slightly lower in subjects with a longer iST: the maximum difference between the same medians (Table 1) was 8.2 µg/g and 0.04 µg/g, respectively. The cloud of data points shows how weak the relationship with zinc is (Figure 2), and so does the R^2: only 1.5% of the variability in concentrations of zinc is statistically explained by the iST.

After adjustment for age and sex, concentrations of aluminum continued to increase weakly with iST (maximum difference, 3.7 µg/g) (Table 2, Model 1). When models were further adjusted for tumor stage (Model 2) and/or the cholestatic syndrome (Table 3, Models 3 and 4), results for aluminum did not materially change.

For zinc and selenium, the inverse relations observed with iST became slightly stronger after adjustment for age, sex, cholestatic syndrome, and tumor stage (Tables 2 and 3). The difference between the fully adjusted GM of zinc concentrations for iST≤40 days and iST>120 days was of 20.4 µg/g (GMs=127.1 and 106.7 µg/g, respectively) (Table 3, Model 4). In the fully adjusted models, zinc concentrations were inversely associated also with the cholestatic syndrome and tumor stage; i.e., patients with partial or complete cholestatic syndrome, and patients in more advanced tumor stages had lower zinc toenail concentrations than patients with no cholestatic syndrome or the tumor at stage I (differences of about 20 µg/g or less) (Table 3, Model 4). Cholestatic syndrome and tumor stage were not associated with concentrations of the other elements analyzed.

Age and sex-adjusted concentrations of selenium, as well as concentrations of selenium further adjusted for tumor stage, decreased weakly with increasing iST (difference of 0.08 µg/g for iST>120 days vs. ≤40 days) (Table 2); selenium concentrations were not influenced by stage nor by cholestatic syndrome (Table 3).

Virtually all correlations between time intervals and metal concentrations were modest, with all Spearman's ρ <0.35 (from –0.33 to 0.32) (Table 4). Concerning toenail levels of aluminum, future studies may consider their associations with iST (ρ = 0.19), with the interval from diagnosis to toenail collection (ρ = 0.25), and with the interval from the exploratory laparotomy to toenail collection (ρ = 0.31); in the analysis of these correlations we took into account only clinical events that took place before or the same day as toenail collection. Also worth noting were the associations between concentrations of iron and the interval from diagnosis to toenail collection (ρ = 0.29), and between cadmium levels and the interval from treatment onset to toenail collection (ρ = –0.33) (Table 4).

Discussion

Toenail concentrations of the 12 trace elements studied were weakly or not influenced by the progression of the disease or the diagnostic process. Levels of aluminum increased slightly with increasing iST, whereas levels of zinc and selenium decreased weakly.

Adjustment for multiple factors did not materially change the results. Thus, when adjusting for age and sex, concentrations of aluminum continued to increase weakly and statistically non-significantly with iST. Further adjusting for tumor stage or cholestatic syndrome [27,30] did not change these findings. For zinc and selenium, the inverse relation observed with iST became slightly stronger after further adjusting for cholestatic syndrome, and tumor stage. Moreover, mutually adjusted models showed that all iST, cholestatic syndrome, and stage were weakly but statistically significantly related to levels of zinc; the relations with cholestatic syndrome and tumor stage were not observed for selenium or the other trace elements. Thus, for instance, future studies could confirm whether at diagnosis concentrations of zinc are lower in patients with more disseminated than with more localized tumors. It remains to be seen whether the weak magnitude of the changes in concentrations that we observed (about 20 µg/g or less) is applicable to other studies; if it is, it might not be efficient to measure the chronology of symptoms as comprehensively as we did [30], and simpler alternatives could be considered [42-44]. It may be more feasible to measure intervals between other clinical events (such as diagnosis, clinical procedures as laparotomy, treatment onset) and toenail collection. For future studies, our findings warrant checking the possible effects of disease progression on toenail levels of at least aluminum, zinc, and selenium. Findings also suggest that, with the mentioned checks, it may be valid to use toenail samples collected at diagnosis.

We previously reported that pancreatic cancer patients with cholestatic syndrome had significantly lower serum concentrations of OCs than patients with other signs and symptoms [27]. When symptoms were taken into account, tumor stage had only weak (and inverse) relationships with all OCs. Overall, the prevailing direction was for most signs and symptoms to lower serum OCs concentrations, even when the latter were lipid-corrected [26]. These relationships with symptoms were not observed in the present study with trace elements measured in toenails; most likely, because concentrations of such biomarkers, which are not lipophilic, are not influenced by the pathophysiological processes inherent to the progression of pancreatic cancer [45].

Variations in concentrations of aluminum according to iST suggest that accumulation of this element in pancreatic cancer patients could be enhanced, maybe due to an increased intestinal absorption and decreased excretion of the metal [46]. Lower toenail concentrations of selenium and zinc during the development of pancreatic cancer could be due to nutritional needs of the tumor itself.

Disease progression bias [28] may occur when the biological samples where the exposure biomarkers will be analyzed are collected just before or after the diagnosis of the disease, and when disease-related conditions experienced during the development of the disease and around its diagnosis cause a change in the biomarkers. One type of such conditions are the disease-induced pathophysiological processes that we already mentioned in the Introduction and in previous papers [25-32]. Related but different are the metabolic requirements of the tumor [12-20]. A third mechanism can operate as follows: symptoms (such as tiredness, loss of appetite, weight loss, abdominal pain, nausea) caused by the still subclinical disease can cause changes in the diet of the subject that change the intake of some nutrients and subsequently their body concentrations. Naturally, other changes in behaviors promoted by the clinically-emerging but yet undiagnosed disease can affect other lifestyle factors (e.g., smoking) and the corresponding exposures. The common effect of all these processes is that the disease-altered exposure biomarkers measured around the time of diagnosis lack etiologic significance: they do not reflect the causally-relevant exposures that took place in the more distant past [28].

In a previous case-control study we found that individuals with the lowest levels of selenium in toenails at diagnosis presented a higher risk of pancreatic cancer [11]. Also, lower levels of zinc were associated with a higher risk of a KRAS mutated pancreatic cancer [9]. In the present study, the inverse associations of the concentrations of zinc and selenium with iST were not explained by tumor stage. Thus, the development of the disease [28] could partly explain the lower levels of selenium observed in the case-control study [11].

Concentrations of the other trace elements analyzed were not associated with time intervals from clinical events to toenails collection. Such elements include cadmium, lead, and arsenic, the three elements previously found positively associated with pancreatic cancer risk [11]. Thus, even in a biologically and clinically aggressive disease as pancreatic cancer, toenail concentrations of trace elements may provide a measure of mid-term intake of trace elements unaffected by clinical events and disease progression.

In our study participants the toenail concentrations of aluminium, selenium, zinc and the other elements were similar to concentrations in the homogenized published literature in the same matrix [11,13-15].

Problems in using toenails as a matrix for measurement of biomarkers include variability in growth rate, external contamination, and inconsistent protocols for collection and analysis [12-15,47]. Because in the present study sample collection was conducted in-hospital, collection of toenails was similar for all study patients, and the external contamination of nails was minimized by having toenails collected immediately after bathing and scraping, and by sonicating nails in the laboratory prior to analysis [48]. All nails were analyzed simultaneously in a laboratory with state-of-the art procedures [9-11,49]. In some instances a small SRM sample mass may have been the cause of some of the variability seen in the within- and between-batch SRM results (Supplementary Table 2).

In our study, patients were screened for eligibility early during the hospital admission, and selected, if eligible [10]. Epidemiological studies of risk factors rarely include information on the health care process that patients undergo before inclusion. In some health care systems, computerized medical records shared by primary health care centers and hospitals include information on the duration of symptoms, clinical procedures, and disease progression [27, 42,43,50]. Our approach integrates concepts and methods from clinical, environmental, and molecular epidemiology [51].

Limitations of our study include its relatively small sample size, and unmeasured factors potentially related to concentrations of trace elements, such as, perhaps, body mass index (BMI) [12], and, likely, size of the toe; however, our clippings were from the larger toe, all sample preparation steps were recorded gravimetrically, and state-of-the-art methods were used in a reference laboratory. Signs and especially symptoms are by nature difficult to measure, although our methods on these aspects are among the most reliable [30,42-44], and this information is unlikely to be available from other studies. The present study did not aim at identifying the dietary or environmental sources of the trace elements found.

Repeated toenail samples collected during years from individual cohort members would allow to more directly assess individual longitudinal changes over time (even before the subclinical onset of the disease), and intraindividual variability [12], but such samples are seldom available in large studies. The aims and methodological frameworks that are relevant to assess reproducibility over time of measurements of metals in toenails and other matrixes in healthy and stable individuals [12] are different from ours [25,28,29] (see Introduction); notably, the former rarely consider the influence of the subclinical or clinical disease of interest; they may also lack valid data on other relevant changes (BMI, lifestyles, environmental and social conditions). Nevertheless, there are obvious relationships.

As expected [27,29], a substantial number of clinical and epidemiological studies continue to be based on diagnosed cases; hence, they collect post-diagnostic biological samples from cases, rather than collecting the samples from the entire cohort at baseline and conducting nested case-control studies after cases are ascertained during follow-up [31,32]. Among other strengths, the latter approach has considerable advantages with regard to timing of the biological sample collection; notably, avoidance of disease progression bias.

Our original approach and findings may be of use when designing and monitoring the conduct of studies (e.g., intervals between first symptom of the disease, first contact with carers or first hospital admission and collection of toenails, diagnostic procedures performed before nails collection). They also have implications for the analysis and interpretation of future studies of pancreatic cancer and possibly other cancers. The findings warrant checking the possible effects of disease progression on toenail levels of at least aluminum, zinc, and selenium. Findings also suggest that with the mentioned checks it may be valid to measure trace elements in toenail samples collected at diagnosis, not necessarily years before diagnosis.

FIGURE LEGENDS

Figure 1 Distributions of the time interval from first symptom of pancreatic cancer to toenails collection, and time interval from first symptom of pancreatic cancer to blood extraction.
Footnote: Blue broken line indicates the median days of the time interval from first symptom of pancreatic cancer to toenails collection (iST), and red broken line indicates the median days of time interval from first symptom to blood extraction (iSB).

Figure 2 Scatterplot of zinc concentrations against the time interval from first symptom of pancreatic cancer to toenails collection.
Footnote: ρ: Spearman's rho coefficient.

Supplementary Figure 1 Chronological process from first symptom of pancreatic cancer to toenails collection by the number of diagnostic tests previous to toenails collection.
Footnote: Each individual pancreatic cancer patient is represented by one line, and patients are sorted from shorter to longer interval from first symptom to toenails collection (iST). Colors indicate the number of diagnostic tests performed to patient previous to toenails collection. Two patients had an iST over 420 days (N = 118).

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Table 1 Metal concentrations by time from first symptom of pancreatic adenocarcinoma to toenail collection

Metal concentrations (µg/g)	Interval from first symptom to toenails collection: ≤40 days N (%)	41 – 120 days N (%)	>120 days N (%)	P-value[a]
Number of subjects	36 (30.5)	45 (38.1)	37 (31.4)	
Aluminum				
GM (95% CI)	8.02 (6.1-10.6)	9.27 (7.2-11.9)	11.73 (8.9-15.5)	0.159
Median	6.96	8.62	12.35	0.080[b]
Nickel				
GM (95% CI)	0.22 (0.16-0.32)	0.25 (0.18-0.34)	0.24 (0.17-0.34)	0.929
Median	0.23	0.24	0.22	0.773[c]
Zinc				
GM (95% CI)	124.8 (114-137)	113.5 (104-123)	108.0 (98.5-118)	0.085
Median	115.0	107.1	106.8	0.023[b]
Arsenic				
GM (95% CI)	0.07 (0.06-0.09)	0.08 (0.07-0.10)	0.07 (0.06-0.09)	0.525
Median	0.07	0.08	0.06	0.435[c]
Selenium				
GM (95% CI)	0.54 (0.49-0.59)	0.51 (0.47-0.55)	0.46 (0.42-0.50)	0.039
Median	0.54	0.51	0.50	0.055[b]
Cadmium				
GM (95% CI)	0.03 (0.02-0.05)	0.02 (0.01-0.04)	0.03 (0.02-0.05)	0.677
Median	0.03	0.03	0.03	0.812[c]
Lead				
GM (95% CI)	0.87 (0.61-1.24)	0.86 (0.63-1.18)	0.89 (0.63-1.26)	0.986
Median	0.80	0.91	0.86	0.899[c]
Vanadium				
GM (95% CI)	0.02 (0.02-0.03)	0.02 (0.02-0.03)	0.03 (0.02-0.03)	0.753
Median	0.02	0.02	0.02	0.761[c]
Chromium				
GM (95% CI)	0.37 (0.25-0.54)	0.54 (0.38-0.76)	0.49 (0.34-0.72)	0.306
Median	0.33	0.52	0.59	0.240[b]
Manganese				
GM (95% CI)	0.29 (0.20-0.40)	0.23 (0.17-0.32)	0.27 (0.19-0.37)	0.679
Median	0.27	0.21	0.22	0.634[c]
Iron				
GM (95% CI)	16.41 (12.4-21.7)	15.30 (11.9-19.7)	17.91 (13.6-23.6)	0.707
Median	14.88	11.49	12.68	0.443[c]
Copper				
GM (95% CI)	3.81 (3.3-4.4)	3.65 (3.2-4.1)	3.69 (3.2-4.2)	0.892
Median	3.36	3.53	3.50	0.915[c]

Total number of subjects: 118. GM: geometric mean. CI: confidence interval.
[a] Unless otherwise specified, *p* value derived from ANOVA.
[b] Jonckheere-Terpstra test for trend. [c] Kruskal-Wallis test (two-tailed).

Table 2 Influence of the interval from first symptom of pancreatic adenocarcinoma to toenail collection (iST) on concentrations of trace elements (µg/g)

	Aluminum		**Nickel**		**Zinc**	
	GM	(95% CI)	GM	(95% CI)	GM	(95% CI)
Model 1 (N = 118)						
iST						
≤40 days	8.05	(6.04, 10.74)	0.23	(0.16, 0.34)	124.7	(113.6, 136.8)
41 – 120 days	9.25	(7.18, 11.92)	0.24	(0.17, 0.33)	112.9	(104.1, 122.6)
>120 days	11.72	(8.85, 15.52)	0.24	(0.17, 0.34)	108.7	(99.31, 119.1)*
Model 2 (N = 116)						
iST						
≤40 days	7.75	(5.77, 10.40)	0.22	(0.15, 0.33)	122.8	(111.6, 135.2)
41 – 120 days	9.18	(7.11, 11.84)	0.24	(0.17, 0.34)	113.1	(104.1, 122.8)
>120 days	11.44	(8.58, 15.24)	0.24	(0.16, 0.34)	109.6	(99.82, 120.2)
Tumor stage						
Stage I	10.71	(7.58, 15.14)	0.25	(0.16, 0.40)	128.2	(114.7, 143.4)
Stage II	8.20	(5.38, 12.51)	0.26	(0.15, 0.45)	110.1	(96.01, 126.2)
Stage III	9.39	(6.21, 14.21)	0.21	(0.12, 0.37)	107.5	(94.03, 122.9)*
Stage IV	9.12	(7.27, 11.44)	0.23	(0.17, 0.31)	112.9	(104.9, 121.5)

iST: Interval from first symptom of pancreatic cancer to toenails collection. GM: Geometric mean. CI: Confidence Interval.
All models are adjusted for age and sex. Furthermore, in Model 2 iST and stage are also mutually adjusted.
* p value < 0.05 (vs. iST ≤40 days or vs. tumor stage I).

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Table 2 (continued)

	Arsenic		Selenium		Cadmium	
	GM	(95% CI)	GM	(95% CI)	GM	(95% CI)
Model 1 (N = 118)						
iST						
≤40 days	0.078	(0.063, 0.097)	0.54	(0.50, 0.59)	0.032	(0.019, 0.054)
41 – 120 days	0.084	(0.070, 0.102)	0.51	(0.47, 0.55)	0.023	(0.015, 0.037)
>120 days	0.070	(0.057, 0.087)	0.46	(0.42, 0.50)*	0.029	(0.018, 0.049)
Model 2 (N = 116)						
iST						
≤40 days	0.079	(0.063, 0.099)	0.54	(0.49, 0.59)	0.029	(0.017, 0.050)
41 – 120 days	0.085	(0.070, 0.103)	0.51	(0.47, 0.55)	0.024	(0.015, 0.038)
>120 days	0.070	(0.056, 0.087)	0.46	(0.42, 0.50)*	0.030	(0.018, 0.051)
Tumor stage						
Stage I	0.071	(0.054, 0.092)	0.53	(0.48, 0.59)	0.037	(0.019, 0.070)
Stage II	0.092	(0.067, 0.128)	0.49	(0.43, 0.55)	0.034	(0.016, 0.074)
Stage III	0.076	(0.055, 0.104)	0.48	(0.43, 0.55)	0.029	(0.014, 0.062)
Stage IV	0.079	(0.066, 0.094)	0.50	(0.47, 0.53)	0.022	(0.014, 0.033)

iST: Interval from first symptom to toenails collection. GM: Geometric mean. CI: Confidence Interval.
All models are adjusted for age and sex. Furthermore, in Model 2 iST and stage are also mutually adjusted.
* p value < 0.05 (vs. iST ≤40 days or vs. tumor stage I).

Table 3 Influence of time interval from first symptom to toenails collection (iST) on toenail concentrations of metals (µg/g)

	Aluminum		Nickel		Zinc	
	GM	(95% CI)	GM	(95% CI)	GM	(95% CI)
Model 3 (N = 118)						
iST[a]						
≤40 days	8.08	(5.95, 10.99)	0.24	(0.16, 0.35)	127.9	(116.0, 140.9)
41 – 120 days	9.21	(7.13, 11.90)	0.24	(0.17, 0.33)	112.7	(103.9, 122.2)
>120 days	11.74	(8.77, 15.73)	0.24	(0.16, 0.34)	106.4	(97.01, 116.7)*
Cholestatic syndrome[b]						
No syndrome	9.12	(6.72, 12.38)	0.24	(0.17, 0.36)	126.1	(114.4, 138.9)
Partial syndrome	10.82	(7.83, 14.95)	0.23	(0.15, 0.35)	107.8	(97.30, 119.4)*
Complete syndrome	9.23	(7.25, 11.75)	0.24	(0.17, 0.32)	112.1	(103.9, 121.1)
Model 4 (N = 116)						
iST[a]						
≤40 days	8.00	(5.84, 10.95)	0.22	(0.15, 0.34)	127.1	(115.1, 140.3)
41 – 120 days	9.07	(7.01, 11.74)	0.24	(0.17, 0.34)	112.4	(103.7, 121.9)
>120 days	11.25	(8.36, 15.14)	0.24	(0.16, 0.35)	106.7	(97.23, 117.2)*
Cholestatic syndrome[b]						
No syndrome	9.55	(6.96, 13.11)	0.24	(0.16, 0.37)	129.1	(116.8, 142.6)
Partial syndrome	10.63	(7.64, 14.80)	0.22	(0.14, 0.34)	108.6	(97.86, 120.4)*
Complete syndrome	8.62	(6.68, 11.12)	0.24	(0.17, 0.33)	109.4	(101.0, 118.5)*
Tumor stage[c]						
Stage I	11.01	(7.70, 15.75)	0.25	(0.16, 0.40)	131.5	(117.5, 147.1)
Stage II	8.06	(5.25, 12.37)	0.26	(0.15, 0.45)	108.1	(94.49, 123.7)*
Stage III	9.60	(6.28, 14.66)	0.22	(0.12, 0.38)	110.8	(96.96, 126.6)*
Stage IV	9.00	(7.14, 11.35)	0.23	(0.17, 0.31)	111.3	(103.5, 119.7)*

iST: Interval from first symptom to toenails collection. GM: Geometric mean. CI: Confidence Interval.
All models are adjusted for age and sex.
[a] Reference category is ≤40 days of interval. [b] Reference category is no cholestatic syndrome.
[c] Reference category is tumor stage I. * *p* value <0.05.

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Table 3 (continued)

	Arsenic		Selenium		Cadmium	
	GM	(95% CI)	GM	(95% CI)	GM	(95% CI)
Model 3 (N = 118)						
iST[a]						
≤40 days	0.082	(0.065, 0.103)	0.55	(0.50, 0.61)	0.035	(0.020, 0.061)
41 – 120 days	0.083	(0.068, 0.101)	0.51	(0.47, 0.55)	0.022	(0.014, 0.036)
>120 days	0.068	(0.055, 0.085)	0.45	(0.41, 0.49)*	0.028	(0.016, 0.047)
Cholestatic syndrome[b]						
No syndrome	0.084	(0.067, 0.106)	0.54	(0.49, 0.59)	0.029	(0.017, 0.049)
Partial syndrome	0.083	(0.065, 0.106)	0.48	(0.44, 0.53)	0.043	(0.024, 0.077)
Complete syndrome	0.072	(0.060, 0.086)	0.49	(0.46, 0.53)	0.021	(0.014, 0.033)
Model 4 (N = 116)						
iST[a]						
≤40 days	0.082	(0.064, 0.104)	0.56	(0.51, 0.61)	0.034	(0.019, 0.060)
41 – 120 days	0.084	(0.069, 0.103)	0.51	(0.47, 0.55)	0.023	(0.014, 0.036)
>120 days	0.068	(0.054, 0.086)	0.45	(0.41, 0.49)*	0.028	(0.016, 0.048)
Cholestatic syndrome[b]						
No syndrome	0.083	(0.065, 0.106)	0.55	(0.50, 0.60)	0.032	(0.018, 0.057)
Partial syndrome	0.083	(0.064, 0.107)	0.49	(0.44, 0.54)	0.045	(0.025, 0.081)
Complete syndrome	0.073	(0.060, 0.089)	0.48	(0.44, 0.52)	0.019	(0.012, 0.030)
Tumor stage[c]						
Stage I	0.072	(0.055, 0.096)	0.54	(0.49, 0.61)	0.042	(0.022, 0.080)
Stage II	0.091	(0.065, 0.127)	0.48	(0.42, 0.54)	0.031	(0.014, 0.068)
Stage III	0.078	(0.056, 0.108)	0.50	(0.44, 0.57)	0.032	(0.015, 0.070)
Stage IV	0.077	(0.065, 0.093)	0.49	(0.46, 0.53)	0.021	(0.014, 0.031)

iST: Interval from first symptom to toenails collection. GM: Geometric mean. CI: Confidence Interval.
All models are adjusted for age and sex.
[a] Reference category is ≤40 days of interval. [b] Reference category is no cholestatic syndrome.
[c] Reference category is tumor stage I. * p value <0.05.

Table 4 Spearman's correlations (ρ) between concentrations of trace elements (µg/g) and time intervals (days) from clinical events to collection of toenails

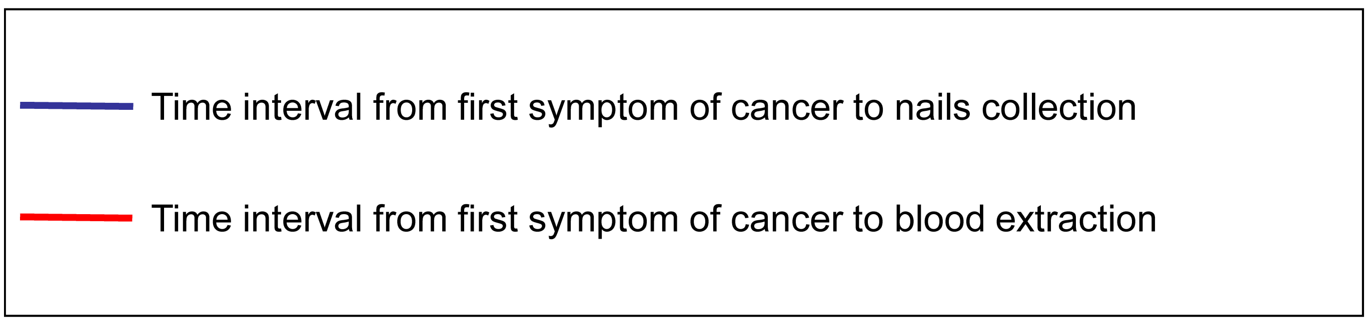
	Clinical events related to pancreatic cancer									
	First symptom	**Hospital admission**	**Interview**	**Diagnostic**	**Treatment onset**	**Ultrasound scan**	**CT**	**Fibrogas-troscopy**	**ERCP**	**Exploratory laparotomy**
	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
Total number	118 (100)	115 (97.5)	97 (82.2)	68 (57.6)	36 (30.5)	101 (85.6)	82 (69.5)	30 (25.4)	41 (34.7)	37 (31.4)
Aluminum	0.186**	0.087	-0.121	0.253**	0.090	0.063	0.089	-0.144	0.054	0.311*
Nickel	0.001	0.082	-0.109	0.201	-0.009	0.138	0.162	0.052	0.207	0.143
Zinc	-0.112	-0.017	-0.020	-0.075	-0.216	0.084	-0.025	0.084	0.043	-0.092
Arsenic	-0.032	0.084	-0.033	0.022	-0.102	0.048	0.129	0.321*	-0.006	-0.152
Selenium	-0.117	-0.002	0.089	0.062	0.026	0.120	0.124	-0.014	0.045	-0.028
Cadmium	-0.012	0.061	-0.159	-0.063	-0.327*	-0.074	-0.020	-0.110	-0.129	-0.166
Lead	-0.001	0.073	-0.128	0.049	-0.247	0.041	0.144	0.073	-0.080	-0.061
Vanadium	0.079	0.005	-0.128	0.125	-0.051	0.013	0.059	-0.192	-0.072	0.062
Chromium	0.065	0.013	0.097	0.019	-0.064	0.030	0.094	-0.094	-0.071	0.003
Manganese	-0.061	-0.045	-0.131	0.092	-0.225	-0.096	-0.078	-0.225	-0.130	-0.108
Iron	0.010	-0.052	-0.143	0.292**	0.084	0.028	-0.041	-0.038	-0.098	-0.171
Copper	0.054	0.046	-0.058	0.121	-0.004	0.139	-0.069	0.149	0.104	-0.054

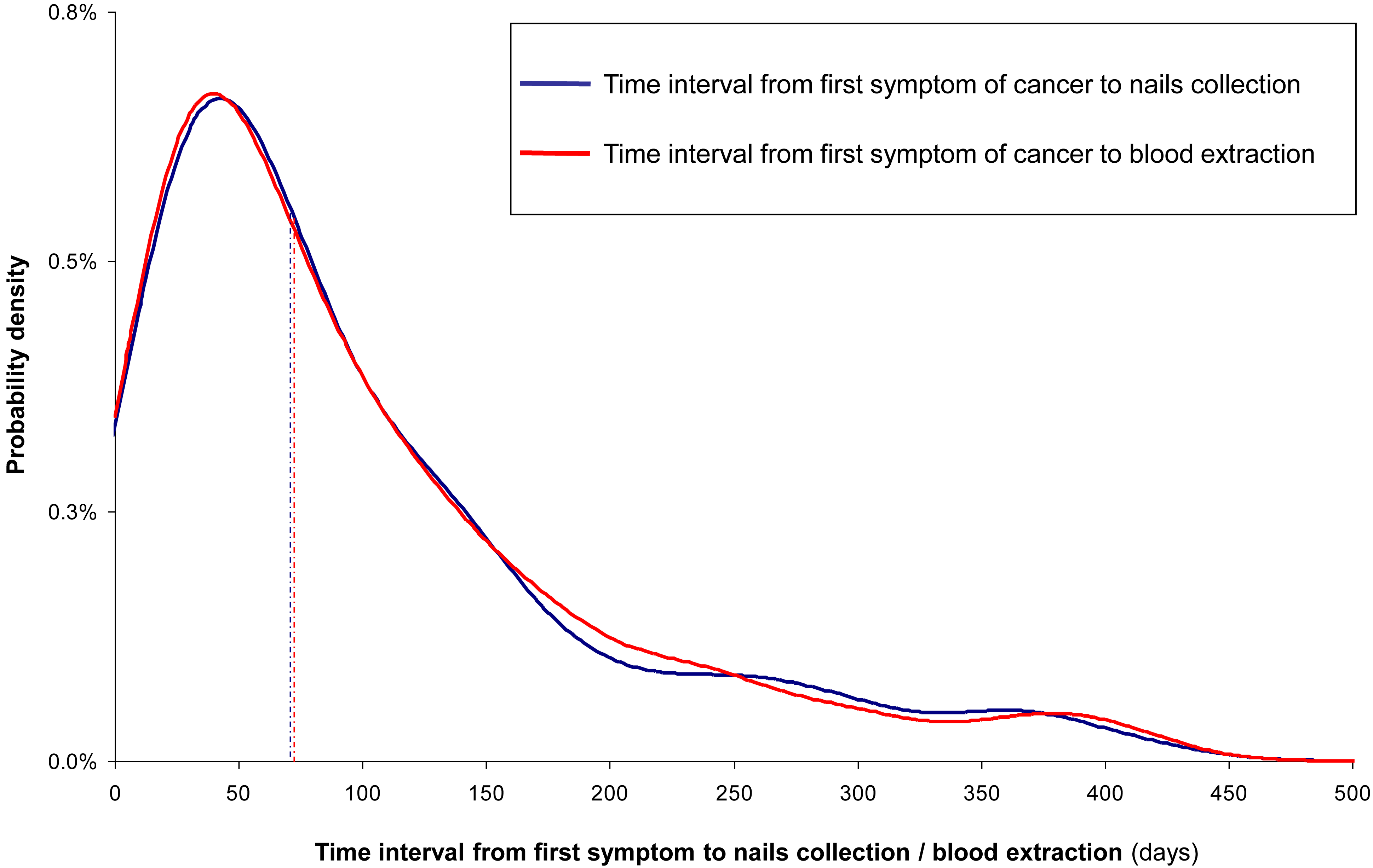
Total number of subjects: 118.
CT: Computerized axial tomography. ERCP: Endoscopic retrograde cholangiopancreatography.
* 0.05 <*p* value ≤0.10.
** *p* value ≤0.05.

Figure 1. Distribution of the interval from first symptom of cancer to toenails collection and the interval from first symptom of cancer to blood extraction in patients with pancreatic cancer

To be published as a footnote to the figure:

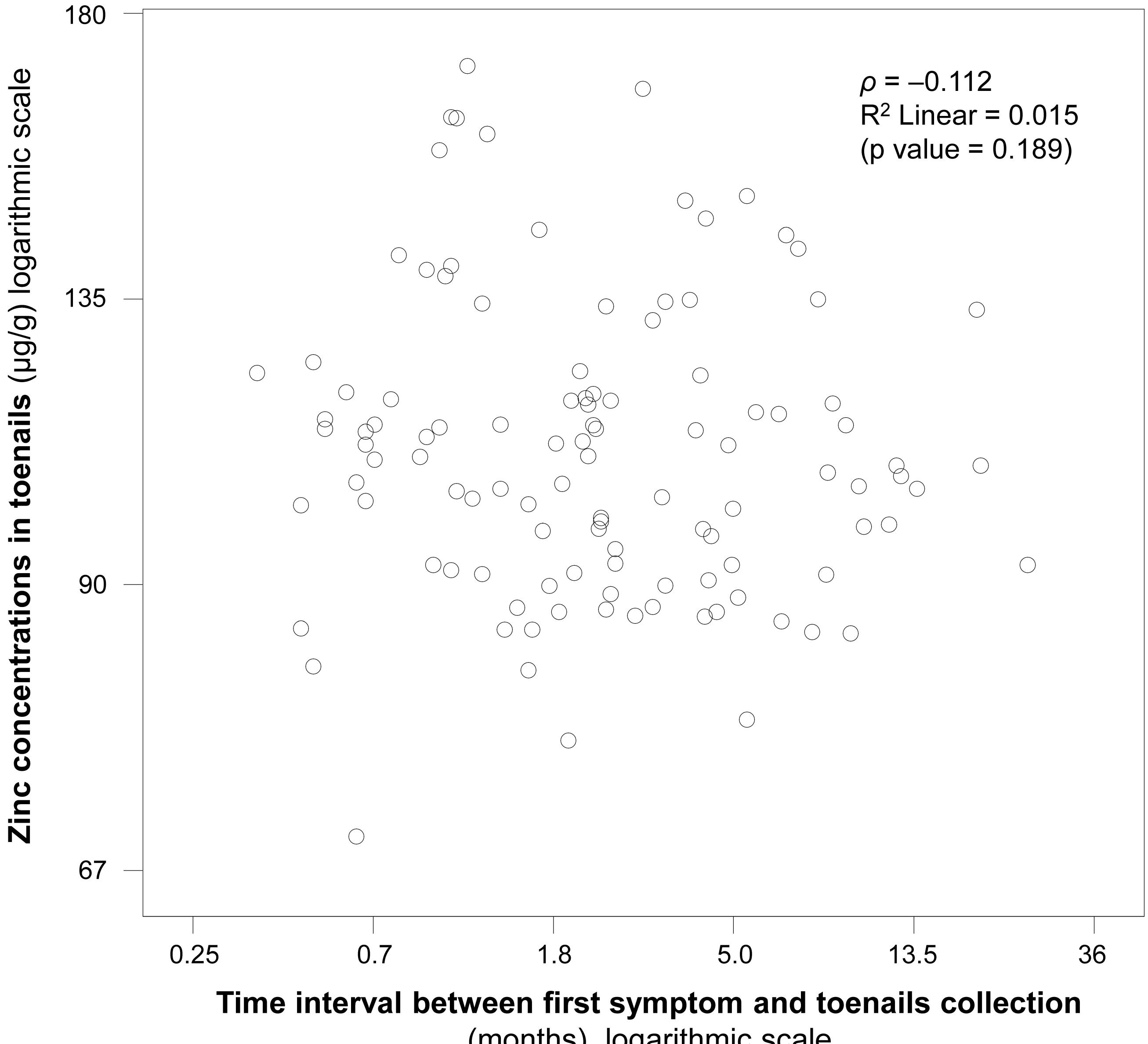
Note: The blue broken line indicates the median (in days) of the interval from first symptom of cancer to toenails collection, and the red broken line indicates the median (in days) of the interval from first symptom of cancer to blood extraction in patients with pancreatic cancer.





Time interval from first symptom of cancer to nails collection
Time interval from first symptom of cancer to blood extraction
Probability density
0.8%
0.5%
0.3%
0.0%
0 50 100 150 200 250 300 350 400 450 500
Time interval from first symptom to nails collection / blood extraction (days)

Figure 2. Scatterplot of toenail concentrations of zinc against the interval between first symptom of pancreatic cancer and toenails collection (iST).



Supplementary Table 1 Characteristics of pancreatic cancer patients with and without available toenail samples

Characteristics	Toenails available		
	Yes	No	
	N (%)	N (%)	P-value
Number of patients	118 (63.8)	67 (36.2)	
Age (years)	66.1 ± 12.5	68.0 ± 12.9	0.320 [a]
Sex (males)	75 (61.9)	37 (55.2)	0.437 [b]
Social class			0.967 [b]
I – II	12 (10.5)	6 (12.0)	
III	30 (26.3)	13 (26.0)	
IV – V	72 (63.2)	31 (62.0)	
Education			0.758 [b]
Illiterate	15 (13.2)	5 (10.0)	
Can only read and write	32 (28.1)	12 (24.0)	
Up to 10 years of schooling	58 (50.9)	27 (54.0)	
>10 years of schooling	9 (7.9)	6 (12.0)	
Smoking			
Ever-smokers (%)	65 (56.5)	27 (54.0)	0.865 [b]
Pack-years (median)	9.1	13.5	0.627 [c]
Coffee drinking			
Regular coffee drinkers (%)	98 (86.0)	42 (84.0)	0.811 [b]
Cups per week (median)	2.0	1.0	0.320 [c]
Alcohol drinking			0.050 [b]
Non-drinker or occasional	31 (27.2)	12 (24.0)	
Regular drinker	64 (56.1)	21 (42.0)	
Heavy drinker	19 (16.7)	17 (34.0)	
iSD			
Days (median)	71.0	68.0	0.473 [c]
iSB			
Days (median)	70.0	78.0	0.917 [c]
Cholestatic syndrome[d]			
No syndrome	35 (29.7)	29 (43.3)	0.164 [b]
Partial syndrome	28 (23.7)	11 (16.4)	
Complete syndrome	55 (46.6)	27 (40.3)	
Constitutional syndrome[e]			
No syndrome	5 (4.2)	7 (10.4)	0.003 [b]
Partial syndrome	33 (28.0)	6 (9.0)	
Complete syndrome	80 (67.8)	54 (80.6)	
Tumor stage at diagnosis			
Stage I	25 (21.6)	20 (29.9)	0.345 [b]
Stage II	17 (14.7)	6 (9.0)	
Stage III	17 (14.7)	6 (9.0)	
Stage IV	57 (49.1)	35 (52.2)	

iSD: Interval from first symptom of pancreatic cancer to diagnosis.
iSB: Interval from first symptom of pancreatic cancer to blood extraction.
Plus-minus values are mean ± standard deviation.
[a] Student's *t*-test (two-tailed). [b] Fisher's exact test (two-tailed). [c] Mann-Whitney's *U* test (two-tailed). [d] Cholestatic syndrome involved jaundice, hypocholia, and choluria. [e] Constitutional syndrome involved asthenia, anorexia, and weight loss.

Supplementary Table 2 Within- and between-assay coefficients of variation, percentage of detection, and lowest concentration detected.[a]

Trace element	Within-assay coefficient of variation (%)	Between-assay coefficient of variation (%)	Percentage of detection (%) [b,c]	Lowest concentration detected (µg/g)
Aluminum (Al)	10	16	100	1.35
Arsenic (As)	7	9	98.3	0.024
Cadmium (Cd)	15	25	92.4	0.001
Chromium (Cr)	40	96	100	0.010
Copper (Cu)	11	21	100	1.24
Iron (Fe)	20	32	100	4.15
Lead (Pb)	6	8	98.3	0.106
Manganese (Mn)	5	6	100	0.040
Nickel (Ni)	19	25	100	0.011
Selenium (Se)	8	12	99.2	0.134
Vanadium (V)	16	40	100	0.023
Zinc (Zn)	5	8	100	35.54

[a] Trace elements were quantified at the Trace Element Analysis Core (Dartmouth College, Hanover, New Hampshire, USA), using inductively coupled plasma mass spectrometry (see Methods).

[b] Percentage of the 118 subjects who had the trace element detected.

[6] When geometric means were computed, for subjects with concentrations undetected the half-value of the lowest concentration detected was assigned.

Supplementary Table 3 Time intervals from first symptom of pancreatic adenocarcinoma, and from hospital admission, to collection of toenails in 118 patients with pancreatic cancer

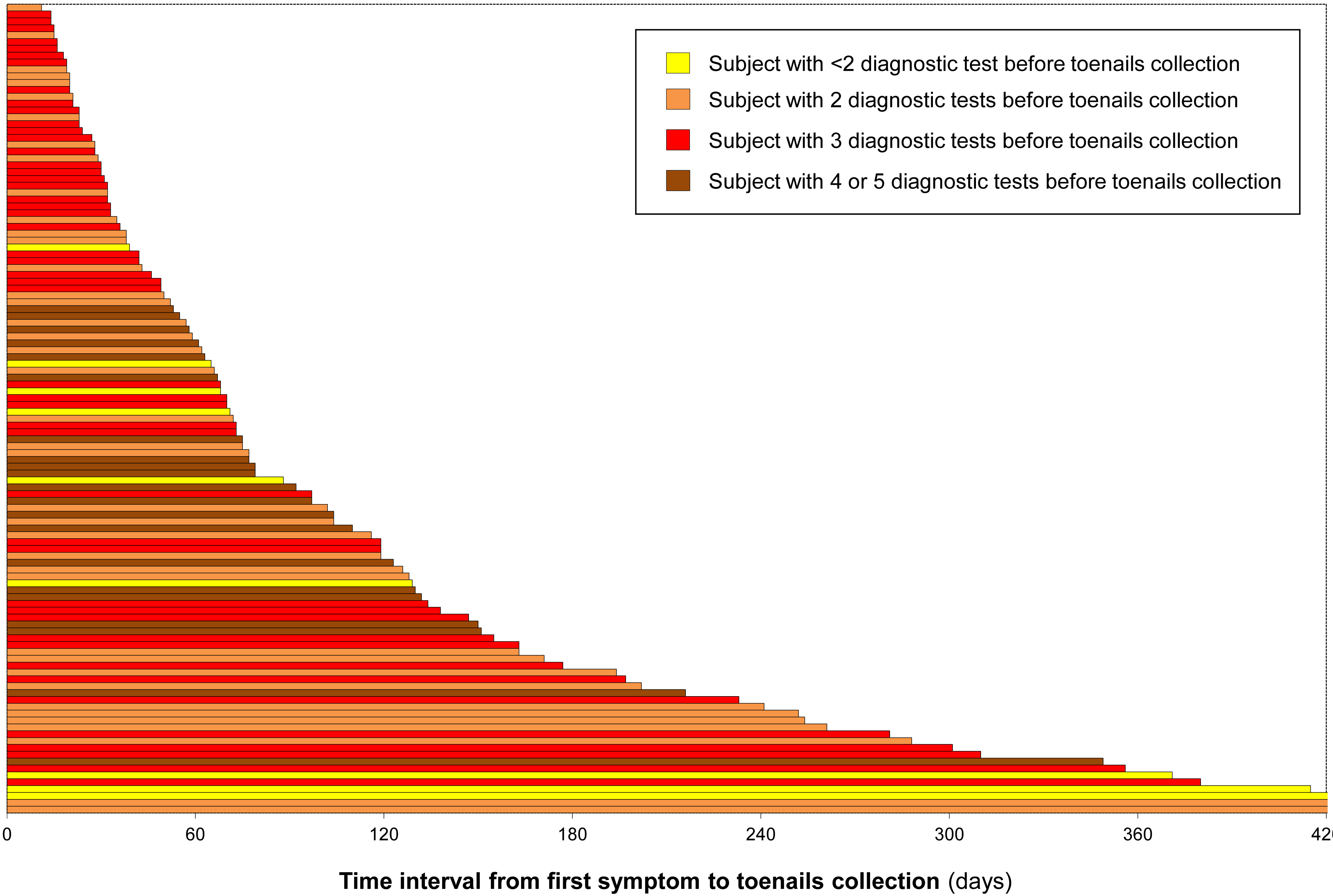
	Days	N	(%)	(Cumulative %)
Time from first symptom to toenails collection		118	(100)	
Mean	115.1			
Standard deviation	125.9			
25th percentile	32.8			
Median	70.5			
75th percentile	140.3			
Minimum	11			
Maximum	763			
Collection in the month following the first symptom		25	(21.2)	(21.2)
Collection in the 2nd following month		24	(20.3)	(41.5)
Collection in the 3rd following month		20	(16.9)	(58.5)
Collection in the 4th following month		12	(10.2)	(68.6)
Collection in the 5th following month		10	(8.5)	(77.1)
Collection in the 6th following month		6	(5.1)	(82.2)
Collection after the 6th following month		21	(17.8)	(100)
Time from hospital admission to toenails collection		118	(100)	
Mean	28.2			
Standard deviation	49.9			
25th percentile	10.0			
Median	15.0			
75th percentile	24.3			
Minimum	-32			
Maximum	349			
Collection of toenails shortly before or same day than hospital admission		5	(4.2)	(4.2)
Collection in the following week		11	(9.3)	(13.6)
Collection in the 2nd following week		40	(33.9)	(47.5)
Collection in the 3rd following week		26	(22.0)	(69.5)
Collection in the 4th following week		13	(11.0)	(80.5)
Collection after the first month		23	(19.5)	(100)

Supplementary Table 4 Time intervals from clinical events (performance of diagnostic tests, diagnosis, treatment onset and interview) to toenails collection

Time intervals	Ultrasound scan N (%)	CT N (%)	Fibrogastroscopy N (%)	ERCP N (%)
Total number of cases	103 (87.3)	93 (78.8)	35 (39.7)	47 (39.8)
Collection of toenails before or same day than the clinical event	3 (2.9)	18 (19.4)	6 (17.1)	7 (14.9)
Collection in the following week	17 (16.5)	33 (35.5)	3 (8.6)	24 (51.1)
Collection in the 2nd following week	27 (26.2)	19 (20.4)	7 (20.0)	5 (10.6)
Collection in the 3rd following week	20 (19.4)	7 (7.5)	3 (8.6)	4 (8.5)
Collection in the 4th following week	11 (10.7)	3 (3.2)	2 (5.7)	2 (4.3)
Collection after the first month	25 (24.3)	13 (14.0)	14 (40.0)	5 (10.6)

	Exploratory laparotomy N (%)	**Diagnosis N (%)**	**Treatment onset N (%)**	**Interview N (%)**
Total number of cases	69 (58.5)	118 (100)	73 (61.9)	115 (97.5)
Collection of toenails ≥15 days before the clinical event	9 (13.0)	13 (11.0)	13 (17.8)	2 (1.7)
Collection in the 2nd previous week	6 (8.7)	12 (10.2)	5 (6.8)	4 (3.5)
Collection in the previous week	17 (24.6)	25 (21.2)	19 (26.0)	12 (10.4)
Collection in the same day	4 (5.8)	12 (10.2)	6 (8.2)	67 (58.3)
Collection in the following week	13 (18.8)	25 (21.2)	13 (17.8)	23 (20.0)
Collection in the 2nd following week	8 (11.6)	14 (11.9)	7 (9.6)	4 (3.5)
Collection ≥ 15 following days	12 (17.4)	17 (14.4)	10 (13.7)	3 (2.6)

CT: Computerized axial tomography. ERCP: Endoscopic retrograde cholangiopancreatography.


Subject with <2 diagnostic test before toenails collection
Subject with 2 diagnostic tests before toenails collection
Subject with 3 diagnostic tests before toenails collection
Subject with 4 or 5 diagnostic tests before toenails collection
0
60
120
180
240
300
360
420
Time interval from first symptom to toenails collection (days)