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Elevated plasma vitamin B12 levels and cancer prognosis: A population-based cohort study

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ABSTRACT

Background: Elevated plasma vitamin B12 levels (cobalamin, Cbl) are associated with increased short-term cancer risk among patients referred for this laboratory measurement. We aimed to assess prognosis in cancer patients with elevated plasma Cbl.

Methods: We conducted a population-based cohort study using data from Danish medical registries during 1998–2014. The study included 25,017 patients with a cancer diagnosis and Cbl levels of 200–600 pmol/L (reference/normal range), 601–800 pmol/L and >800 pmol/L measured up to one year prior to diagnosis, and a comparison cohort of 61,988 cancer patients without a plasma Cbl measurement. Patients treated with Cbl were excluded. Survival probability was assessed using Kaplan–Meier curves. Mortality risk ratios (MRR) were computed using Cox proportional hazard regression, adjusted for age, sex, calendar year, cancer stage and comorbidity, scored using the Charlson comorbidity index.

Results: Survival probabilities were lower among patients with elevated Cbl levels than among patients with normal levels and among members of the comparison cohort [(1-year survival,%) Cbl: 200–600 pmol/L: 69.3%; 601–800 pmol/L: 49.6%; >800 pmol/L: 35.8%; comparison cohort: 72.6%]. Thirty-day mortality was elevated for patients with Cbl levels of 601–800 pmol/L or >800 pmol/L, compared to patients with levels of 200–600 pmol/L [(MRR (95% confidence interval): 601–800 pmol/L vs. 200–600 pmol/L: 1.9 (1.6–2.2); >800 pmol/L vs. 200–600 pmol/L: 2.7 (2.4–3.1)]. This association remained robust for 31–90-day and 91–365-day mortality, showing similar dose-response patterns.

Conclusion: Cancer patients with elevated Cbl levels had higher mortality than those with normal Cbl levels. These findings may have clinical significance for assessing the prognosis of cancer patients.

1. Introduction

Low circulating vitamin B12 levels (cobalamin, Cbl) are associated with conditions such as anemia and neuropsychiatric disorders [1], while high Cbl levels have been linked to a number of other diseases, including cancer [2]. In a large population-based study, we recently showed that elevated plasma Cbl levels were associated with increased cancer risk among patients referred for this laboratory test [3]. This study and others [4–8] have enhanced awareness of the clinical implications of high Cbl levels.

A few previous studies have focused on the prognostic impact of elevated Cbl levels. Some have shown that high Cbl levels were associated with increased mortality risk, both among patients with cancer [9–14] and among patients without cancer [11,15–19]. Other studies do not support these findings [20–22]. Previous studies on mortality among cancer patients with elevated Cbl levels were limited by small sample size (61 to 329 patients) [9–14], and only one was a multi-center study [12]. Also, all patients were diagnosed with cancer prior to plasma Cbl measurement, thereby restricting the study populations to hospitalised cancer patients.

Abbreviations: ATC, anatomical therapeutic chemical; AUPD, Aarhus University Prescription Database; Cbl, cobalamin, vitamin B12; CCI, Charlson comorbidity index; CI, confidence interval; CPR, Civil Personal Registration; DCR, Danish Cancer Registry; DNRP, Danish National Registry of Patients; ICD-10, international classification of diseases, 10th revision; LABKA, clinical laboratory information system research database; MRR, mortality risk ratio; NPU, nomenclature, properties and units.

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To assess the prognostic significance of pre-diagnostic Cbl levels, we conducted a population-based cohort study to investigate survival among cancer patients with a plasma Cbl measurement prior to cancer diagnosis. We hypothesised that elevated Cbl levels would be associated with poorer survival.

2. Materials and methods

2.1. Design and data sources

This population-based cohort study was based on data from Northern Denmark from medical registries during the period from January 1 1998 through December 31 2014. The following data sources were used: the Clinical Laboratory Information System Research Database (LABKA) [23], the Aarhus University Prescription Database (AUPD) [24], the Danish Cancer Registry (DCR) [25] and the Danish National Registry of Patients (DNRP) [26]. The Danish Civil Registration System, established in 1968, assigns a Civil Personal Registration (CPR) number to all residents, allowing unambiguous individual-level linkage of data among all Danish registries [27]. The Danish Civil Registration System also maintains data on vital status and migration. It provided dates of death for this study until December 31 2014. For a detailed description of the registries and databases and the codes used in this study, please see Supplementary data.

For the current study we used the DCR [25] to retrieve data on cancer diagnosis and cancer stage for patients diagnosed from January 1 2001 through November 30 2013 in Northern Denmark.

We identified all patients in the LABKA [23] database with a plasma Cbl measurement of 200 pmol/L or more (271 pg/mL, the lower reference limit in Northern Denmark [28]) from January 1 2000 through November 30 2013. Thus, the Cbl measures were derived from routine clinical testing performed upon request from the clinician at the time of testing.

The AUPD [24] provided data on prescriptions for Cbl drugs. Patients were classified as having received Cbl therapy if they had one or more prescriptions for Cbl therapy drugs recorded in the AUPD up to two years prior to measurement of their plasma Cbl levels. In Denmark, treatment with Cbl drugs at doses >0.5 mg [29] is only available by prescription.

In order to assess possible confounding from comorbidities, we retrieved data from the DNRP [26] on all diagnoses prior to the cancer diagnosis. We also retrieved data on hospital treatment with Cbl drugs.

2.2. Study cohorts

Patients in Northern Denmark with a first cancer diagnosis recorded in the DCR from January 1 2001 through November 30 2013 and a plasma Cbl record in the LABKA database within one year prior to the cancer diagnosis date (index date) were included in the study as the patient cohort. For patients with more than one recorded plasma Cbl level, we used the record closest to the index date. Patients were excluded if they received Cbl therapy within two years prior to measurement of plasma Cbl levels ($n = 3009$).

A comparison cohort from Northern Denmark was sampled from the DCR and matched to patients with a Cbl measurement by sex, age (10-year intervals), calendar period of diagnosis (5-year intervals) and cancer type. Each patient with a Cbl measurement was matched with up to 3 persons in the comparison cohort. Members of the patient cohort and the comparison cohort were followed from the index date until death, emigration or 31 December 2014, whichever came first.

2.3. Statistical analyses

We disaggregated patients with Cbl measurements into three groups according to plasma Cbl levels: 200–600 pmol/L (271–813 pg/mL, population reference range [28], normal Cbl levels), 601–800 pmol/L (814–1084 pg/mL) and >800 pmol/L (>1084 pg/mL). Using the Cochran–Armitage test [30,31], we tested for trends in distribution of sex, cancer types, cancer stages and comorbidity across the three Cbl level groups. We focused specifically on patients with elevated Cbl levels and examined a possible dose–response association. We fitted a cubic spline curve using plasma Cbl levels in 5%-percentiles to further assess any dose–response association with 1-year survival.

TNM stage for solid tumors and Ann Arbor stage for lymphomas were divided into two stage categories: localised and non-localised. Stage was not defined for lymphatic leukemia and malignant myeloid diseases. Therefore, these two cancers were analysed separately and not including stage, and also not included in the imputation model for missing stage (see below). Overall survival was assessed using Kaplan–Meier curves. We stratified the Kaplan–Meier curves according to cancer stage. We computed survival probability estimates at 30 days, 90 days and 365 days, 2 years, 5 years and 10 years after cancer diagnosis. Using log-rank tests, we compared survival among patients in the three Cbl level groups and also compared the survival between members of the comparison cohort and the patient cohort. Cox proportional hazards regression was used to evaluate mortality risk for patients in the three Cbl level groups, using the group with Cbl levels of 200–600 pmol/L as reference. Mortality risk ratios (MRR) with corresponding 95% confidence intervals (CI) were computed by comparing mortality risks among the three groups. The regression analyses were adjusted for the following potential confounding factors: sex, age (continuous), calendar year, cancer stage and Charlson Comorbidity Index (CCI) score [32]. Cancer and cancer stage were omitted from the CCI score. We classified patients in each of the three Cbl level groups according to three CCI categories: low = score of 0, medium = score of 1–2 and high = score of $\geq$3. We also stratified patients in each of the three Cbl level groups according to age (0–40, 41–60, 61–80 and $\geq$81 years), calendar year of cancer diagnosis, sex and cancer stage (not adjusting for the variable used for stratification). All results from the Cox regression analyses were also disaggregated according to length of follow-up: 30 days, 31–90 days, 91–365 days, 366 days-2 years, 3–4 years and $\geq$5 years. We also computed MRRs for specific cancer types, adjusted for all of the above covariates. We tested for equality between MRR estimates for 601–800 pmol/L vs. 200–600 pmol/L and MRR estimates for >800 pmol/L vs. 200–600 pmol/L by using Wald chi-square test. The proportional hazards assumption was fulfilled based on visual evaluation of log–log plots.

Cancer stage was missing in 23% of the patient and the comparison cohorts combined. To account for this, we used multiple imputations with chained equations and created multiple different complete datasets. This approach has been shown to produce estimates with less bias and higher precision [33], under the assumption of data being missing at random. This has also been shown to be valid for missing cancer stage in medical registries [34] and in prognostic studies [35]. This yielded a model consisting of the following covariates, used for prediction of cancer stage: sex, age (continuous), calendar year, cancer type, plasma Cbl levels (continuous), CCI score, length of follow-up and death (yes/no). We then computed 30 complete datasets, and performed the analyses described above for each dataset. The estimates were then combined into one single estimate with corresponding 95% confidence intervals using the Rubin's rule [33]. To validate the model, we compared estimates between the complete case analyses and the imputed model.

The statistical analyses were performed using SAS version 9.2 (SAS Institute Inc., Cary, NC, USA). All tests were two-sided and considered statistically significant if $p < 0.05$. The study was approved by the Danish Data Protection Agency (record number: 2013-41-1924). The use of registry data for research in Denmark does not require ethical approval.

3. Results

3.1. Descriptive data

The study included 25,017 patients with a Cbl measurement within one year prior to their cancer diagnosis. Among these, 3443 (14%) had high Cbl levels (>600 pmol/L). A total of 61,988 persons with cancer were included in the comparison cohort. The sex distribution was 50.6% male among patients with a Cbl measurement and 51.3% male among persons in the comparison cohort. Median time in days (interquartile range) from Cbl measurement to cancer diagnoses for the patient cohort was as follows: 200–600 pmol/L: 57 (16–168); 601–800 pmol/L 32 (8–112) and >800 pmol/L: 16 (3–57). Table 1 shows the major types of cancer in the patient cohort with Cbl measurements and in the comparison cohort. In the comparison cohort, cancer stage was missing or unknown in 23% of the persons, while the proportion ranged from 26% to 42% in the patient cohort. A higher proportion of non-localised cancer was found with higher Cbl levels. Variation in CCI scores among the three Cbl level groups reached statistical significance, but with no clear trend.

3.2. Cancer survival

The Kaplan–Meier curve in Fig. 1A depicts the overall survival of members of the comparison cohort and patients in the three Cbl level groups. The figure shows that patients with a Cbl measurement had a significantly lower survival probability than members of the comparison cohort ($p < 0.0001$), and that survival decreased substantially with higher Cbl levels ($p < 0.0001$ comparing survival between different Cbl level groups). The difference in survival remained similar when stratifying according to cancer stage (Fig. 1B and C). The spline regression analysis is shown in Fig. S1 (see Supplementary material). The analysis showed that 1-year survival was decreasing at Cbl levels near the upper reference limit of 600 pmol/L and above.

Table 2 shows the survival probability at 30 days, 90 days and 365 days after cancer diagnosis among patients in the three Cbl level groups and among members of the comparison cohort. When testing for trend in survival rates between the three Cbl level groups, we observed trends toward lower survival with higher Cbl

Table 1
Characteristics of cancer patients with a Cbl measurement and of the comparison cohort, Northern Denmark, 2001–2013.

	Cohort with plasma Cbl measurements (pmol/L)			*P* for trend[a]	Comparison cohort
	200–600	601–800	>800		
Number of patients, *n*	21,574	1,795	1,648		61,988
Sex (male), %	51.3	46.5	45.6	<0.0001	51.3
Age at diagnosis, years					
Median (range)	71 (3–100)	71 (3–104)	71 (4–100)		70 (3–102)
Year of diagnosis, *n* (%)					
2001–2005	4,179 (19.4)	323 (18.0)	367 (22.3)	0.0513	13,692 (22.1)
2006–2010	9,951 (46.1)	773 (43.1)	727 (44.1)	0.0156	28,667 (46.2)
2011–2013	7,444 (34.5)	699 (38.9)	554 (33.6)	0.3629	19,629 (31.7)
Cancer type, *n* (%)[b]					
Gastric	430 (2.0)	29 (1.6)	38 (2.3)	0.75	1,194 (1.9)
Colorectal	3,088 (14.3)	190 (10.6)	163 (9.9)	<0.0001	8,103 (13.1)
Liver	181 (0.8)	66 (3.7)	80 (4.9)	<0.0001	551 (0.9)
Pancreas	567 (2.6)	121 (6.7)	147 (8.9)	<0.0001	1,817 (2.9)
Lung	2,636 (12.2)	300 (16.7)	225 (13.7)	0.0002	8,148 (13.1)
Breast	1,470 (6.8)	102 (5.7)	78 (4.7)	0.0003	4,942 (8.0)
Prostate	2,348 (10.9)	91 (5.1)	60 (3.6)	<0.0001	7,292 (11.8)
Kidney	487 (2.3)	33 (1.8)	37 (2.2)	0.62	1,343 (2.2)
Urinary bladder	468 (2.2)	35 (1.9)	38 (2.3)	0.94	1,528 (2.5)
Non-Hodgkin lymphoma	1,230 (5.7)	79 (4.4)	70 (4.2)	0.0016	2,539 (4.1)
Lymphatic leukemia	396 (1.8)	26 (1.4)	24 (1.5)	0.14	824 (1.3)
Malignant myeloid diseases	694 (3.2)	143 (8.0)	256 (15.5)	<0.0001	872 (1.4)
Brain and other CNS tumors	325 (1.5)	10 (0.6)	4 (0.2)	<0.0001	836 (1.3)
Other cancers	7,254 (33.6)	570 (31.8)	428 (26.0)	<0.0001	21,999 (35.5)
Cancer stage, *n* (%)					
Localised	9,082 (42.1)	539 (30.0)	302 (18.3)	<0.0001	28,195 (45.5)
Non-localised	6,887 (31.9)	695 (38.7)	655 (39.7)	<0.0001	20,764 (33.5)
Unknown/missing[c]	5,605 (26.0)	561 (31.3)	691 (41,9)	<0.0001	13,029 (21.0)
CCI score, *n* (%)[b]					
CCI = 0 (Low)	11,951 (55.4)	871 (48.5)	848 (51.5)	<0.0001	39,562 (63.8)
CCI = 1-2 (Medium)	7,476 (34.7)	677 (37.7)	585 (35.5)	0.09	18,713 (30.2)
CCI ≥ 3 (High)	2,147 (10.0)	247 (13.8)	215 (13.0)	<0.0001	3,713 (6.0)

Abbreviations: Cbl: cobalamin; CCI: Charlson comorbidity index.
[a] Cochran–Armitage test for trend for age, sex, cancer type, cancer stage and CCI score across the three Cbl level groups.
[b] Percentages do not total 100% due to rounding.
[c] Also includes lymphatic leukemia and malignant myeloid diseases where stage was not defined.

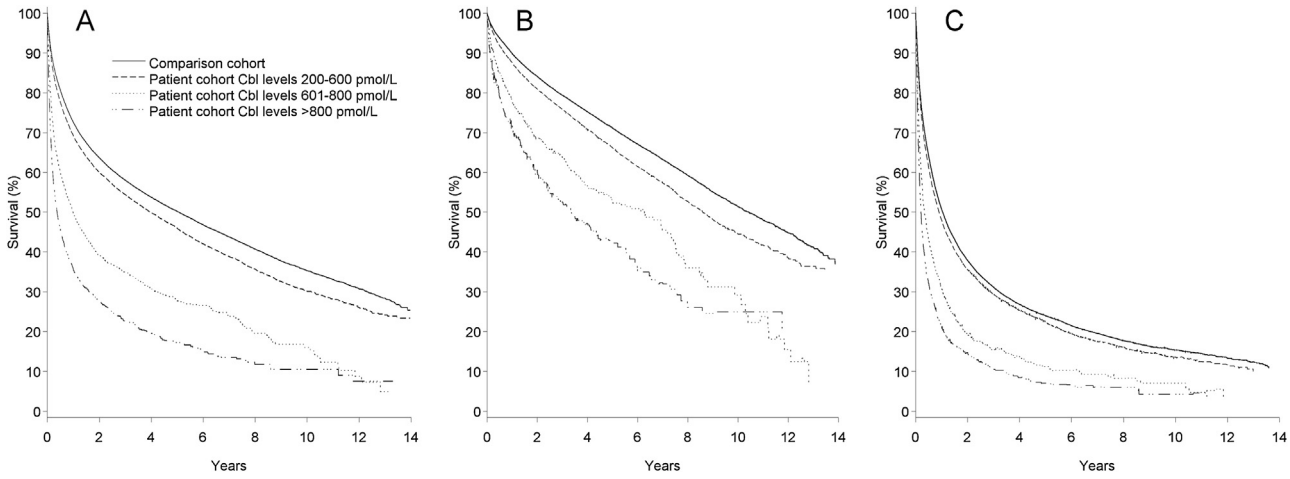

Fig. 1. Kaplan–Meier curves showing survival as a percentage in the comparison cohort (—) and in the patient cohort, disaggregated according to Cbl levels of 200–600 pmol/L (- - -), 601–800 pmol/L (···) and >800 pmol/L (·-·). These figures are based on multiple imputations to account for missing cancer stage. Lymphatic leukemia and malignant myeloid diseases were not included in the analyses. Fig. 1A: overall survival; Fig. 1B: survival for patients with localised cancer; Fig. 1C: survival for patients with non-localised cancer.

levels for all three follow-up periods for both patients with localised and non-localised cancer. The differences in survival with higher Cbl levels were similar after 2, 5 and 10 years (data not shown). When assessing survival using complete case analysis, we observed slighter lower survival probabilities and minor differences in survival with increasing plasma Cbl levels, compared to imputed data. For survival probabilities based on complete case analysis, please see Supplementary Table S2.

Table 2
Survival probability in percentages (with 95% CIs) for cancer patients with different Cbl levels and for the comparison cohort, disaggregated according to follow-up time. Estimates for both overall survival and survival according to cancer stage are shown. These results are based on multiple imputations to account for missing cancer stage (not including lymphatic leukemia and malignant myeloid diseases).

	Survival probability by follow-up, % (95% CI)		
	30 days	90 days	365 days
Comparison cohort			
Overall	94.0 (93.8–94.2)	86.8 (86.5–87.1)	72.6 (72.2–72.9)
Localised cancer	98.2 (98.1–98.4)	96.0 (95.8–96.3)	89.8 (89.4–90.2)
Non-localised cancer	88.6 (88.1–89.0)	75.1 (74.5–75.7)	50.7 (49.8–51.5)
Patient cohort			
Overall			
Plasma Cbl levels (pmol/L)			
200–600	93.2 (92.8–93.5)	84.7 (84.2–85.2)	69.3 (68.7–70.0)
601–800	84.9 (83.1–86.6)	70.1 (67.7–72.3)	49.6 (47.1–52.1)
>800	76.8 (74.4–79.0)	56.9 (54.2–59.6)	35.8 (33.2–38.4)
P for trend[a]	<0.0001	<0.0001	<0.0001
Localised cancer			
Plasma Cbl levels (pmol/L)			
200–600	97.9 (97.6–98.2)	94.9 (94.4–95.4)	87.5 (86.7–88.2)
601–800	95.4 (93.5–97.3)	90.1 (87.5–92.6)	77.5 (74.0–81.0)
>800	92.0 (88.7–95.3)	85.3 (81.2–89.5)	71.6 (66.6–76.6)
P for trend[a]	<0.0001	<0.0001	<0.0001
Non-localised cancer			
Plasma Cbl levels (pmol/L)			
200–600	87.6 (86.9–88.3)	72.6 (71.5–73.7)	47.9 (46.4–49.4)
601–800	77.8 (75.0–80.6)	56.4 (53.0–59.8)	30.6 (27.3–34.0)
>800	70.5 (67.5–73.5)	45.2 (41.8–48.6)	21.0 (18.1–23.9)
P for trend[a]	<0.0001	<0.0001	<0.0001

Abbreviations: Cbl: cobalamin; CI: confidence interval.
[a] Log rank test for trend for survival across the three Cbl level groups.

Results from the Cox regression analyses based on multiple imputations are presented in Table 3. Patients with high Cbl levels had significantly higher mortality than patients with normal Cbl levels, after adjusting for potential confounders. The difference was most pronounced for 30-day mortality, comparing mortality risks among the three Cbl level groups. The difference in MRR estimates remained statistically significant for 31–90-day and 91–365-day mortality. The associations remained similar after stratifying by sex, age and calendar year (Table 3). The overall 1-year MRRs (95% CIs) were: 601–800 pmol/L vs. 200–600 pmol/L: 1.7 (1.6–1.8); >800 pmol/L vs. 200–600 pmol/L: 2.3 (2.1–2.5). The MRRs attenuated with longer follow-up. For MRR estimates on longer follow-up, please see Supplementary Table S3.

The analyses stratified by cancer stage also yielded robust results. As expected, patients with non-localised disease had poorer survival (Fig. 1B and C), with the same trend for lower survival probability with higher Cbl levels within strata for cancer stage (Table 2). Further, MRRs comparing patients with high Cbl levels to patients with normal Cbl levels were similar for those with localised versus non-localised cancer for all follow-up periods, but attenuated with longer follow-up time (Table 3 and Supplementary Table S3). The MRRs based on multiple imputations were very comparable to those based on complete case analysis (for results based on complete case analysis, please see Supplementary Table S4). For both overall MRRs and stratified according to age, sex, calendar year and cancer stage the results were essentially similar.

Looking at cancer type, we found that patients with elevated Cbl levels had higher mortality risks for some specific cancer types, while other cancers showed null associations (Table 4). The highest MRR estimates were seen for gastric, colorectal, liver, breast, prostate and urinary bladder cancer. The estimates were most predominant in the first 30 days when comparing patients with Cbl >800 pmol/L to those with Cbl levels of 200–600 pmol/L. Most elevated MRRs estimates attenuated with longer follow-up and when comparing patients with Cbl levels of 601–800 pmol/L to those with Cbl levels of 200–600 pmol/L, but we also observed some elevated MRRs for specific cancer types for patients with Cbl levels of 601–800 pmol/L and in follow-up intervals of 31–90 and 91–365 days. None of the cancer types showed a lower mortality with higher Cbl levels. We also observed some variation in MRR estimates and wide CIs among the different cancer types and follow-up intervals. Survival estimates for specific cancers based

Table 3
Mortality risk ratios and 95% CIs computed by comparing mortality risks for patients with Cbl levels of 601–800 pmol/L and >800 pmol/L, using those with 200–600 pmol/L as reference. The Cox regression analyses were adjusted for age, sex, calendar year, CCI score and cancer stage, except when the variable was used for stratification. These results are based on multiple imputations to account for missing cancer stage. Lymphatic leukemia and malignant myeloid diseases were not included in the analyses.

	30 days			31–90 days			91–365 days		
	601–800 pmol/L vs. 200–600 pmol/L	>800 pmol/L vs. 200–600 pmol/L	P[a]	601–800 pmol/L vs. 200–600 pmol/L	>800 pmol/L vs. 200–600 pmol/L	P[a]	601–800 pmol/L vs. 200–600 pmol/L	>800 pmol/L vs. 200–600 pmol/L	P[a]
Overall	1.9 (1.6–2.2)	2.7 (2.4–3.1)	0.0003	1.7 (1.5–2.0)	2.4 (2.1–2.7)	0.0005	1.6 (1.4–1.8)	1.9 (1.7–2.2)	0.0179
Sex									
Male	1.8 (1.5–2.2)	3.0 (2.5–3.5)	0.0001	1.9 (1.6–2.3)	2.2 (1.8–2.8)	0.2398	1.7 (1.4–2.0)	1.9 (1.6–2.3)	0.2768
Female	2.0 (1.6–2.4)	2.5 (2.1–2.9)	0.0644	1.5 (1.3–1.9)	2.4 (2.0–2.9)	0.0003	1.4 (1.2–1.7)	1.8 (1.6–2.2)	0.0330
Age at diagnosis									
0–40	0.0 (.-.)	4.2 (0.9–21.0)	0.9994	0.0 (.-.)	5.2 (0.7–36.5)	0.9984	1.5 (0.4–5.4)	1.8 (0.4–7.6)	0.8880
41–60	3.3 (2.2–4.9)	3.5 (2.3–5.1)	0.8819	1.9 (1.3–2.8)	2.5 (1.7–3.5)	0.3074	1.3 (1.0–1.9)	2.8 (2.1–3.7)	0.0004
61–80	1.8 (1.5–2.2)	2.9 (2.4–3.4)	0.0001	1.8 (1.5–2.1)	2.6 (2.2–3.0)	0.0014	1.6 (1.4–1.8)	1.8 (1.5–2.1)	0.1946
≥81	1.6 (1.3–2.1)	2.2 (1.8–2.7)	0.0566	1.5 (1.1–2.0)	1.7 (1.3–2.3)	0.4061	1.6 (1.3–2.1)	1.5 (1.1–2.0)	0.6182
Year of diagnosis									
2001–2005	1.7 (1.2–2.2)	2.2 (1.7–2.8)	0.1445	1.8 (1.4–2.4)	2.4 (1.9–3.1)	0.1240	1.9 (1.5–2.4)	1.7 (1.3–2.3)	0.6489
2006–2010	2.0 (1.7–2.5)	3.0 (2.5–3.6)	0.0011	1.8 (1.5–2.2)	2.3 (1.9–2.8)	0.0855	1.5 (1.3–1.8)	1.9 (1.6–2.3)	0.0798
2011–2013	1.8 (1.4–2.3)	2.8 (2.2–3.5)	0.0098	1.5 (1.2–2.0)	2.4 (1.9–3.1)	0.0055	1.4 (1.2–1.8)	2.0 (1.6–2.5)	0.0203
Cancer stage									
Localised	2.0 (1.3–3.1)	3.6 (2.3–5.5)	0.0452	1.7 (1.2–2.5)	2.1 (1.3–3.2)	0.4952	1.7 (1.3–2.2)	2.0 (1.5–2.7)	0.3790
Non-localised	1.9 (1.6–2.2)	2.6 (2.3–3.0)	0.0002	1.7 (1.5–2.0)	2.4 (2.1–2.7)	0.0007	1.5 (1.3–1.7)	1.9 (1.6–2.2)	0.0222

Abbreviations: Cbl: cobalamin; CCI: Charlson comorbidity index; CI: confidence interval.
[a] Wald chi-square test for equality in MRR estimates.

on results using multiple imputations are provided in Supplementary Table S5. Survival estimates for lymphatic leukemia and malignant myeloid diseases (that were analysed separately and without cancer stage) showed the same dose-response pattern as the other cancer types in all follow-up strata (Supplementary Table S5). The MRRs for these two cancer types also showed essentially the same, although the risk estimates for malignant myeloid diseases attenuated less with increasing follow-up and were generally lower than for lymphatic leukemia.

For MRRs for specific cancers based on complete case analysis, please see Supplementary Table S6. The magnitude of the association between high Cbl levels and mortality for some specific cancers was slighty attenuated using multiple imputation, but overall, the results were very comparable. In addition, the use of multiple imputation

Table 4
Mortality risk ratios and 95% CIs computed by comparing mortality risks for patients with Cbl levels of 601–800 pmol/L and >800 pmol/L, using those with 200–600 pmol/L as reference. The Cox regression analyses were adjusted for age, sex, calendar year, CCI score and cancer stage. Results are shown according to different follow-up periods and different cancer types. These results are based on multiple imputations to account for missing cancer stage.

Cancer type	30 days			31–90 days			91–365 days		
	601–800 pmol/L vs. 200–600 pmol/L	>800 pmol/L vs. 200–600 pmol/L	P[a]	601–800 pmol/L vs. 200–600 pmol/L	>800 pmol/L vs. 200–600 pmol/L	P[a]	601–800 pmol/L vs. 200–600 pmol/L	>800 pmol/L vs. 200–600 pmol/L	P[a]
Gastric	1.5 (0.6–3.7)	2.4 (1.1–5.0)	0.4337	0.8 (0.3–2.2)	2.3 (1.2–4.4)	0.0702	1.3 (0.7–2.5)	1.2 (0.6–2.5)	0.9047
Colorectal	1.6 (1.0–2.5)	2.8 (1.9–4.1)	0.0570	2.1 (1.5–3.1)	2.6 (1.7–3.9)	0.4736	1.6 (1.1–2.2)	2.4 (1.8–3.4)	0.0663
Liver	1.2 (0.6–2.5)	3.0 (1.7–5.3)	0.0134	1.5 (0.8–2.6)	1.6 (0.9–2.9)	0.7650	1.4 (0.8–2.4)	1.4 (0.8–2.5)	0.8530
Pancreas	1.4 (0.9–2.1)	1.7 (1.2–2.5)	0.3570	1.2 (0.9–1.8)	1.3 (0.9–1.8)	0.9345	1.3 (0.9–1.9)	1.2 (0.8–1.7)	0.6799
Lung	1.4 (1.1–1.8)	1.9 (1.4–2.4)	0.1322	1.2 (0.9–1.5)	1.9 (1.5–2.5)	0.0052	1.2 (1.0–1.5)	1.4 (1.1–1.8)	0.3339
Breast	3.6 (1.3–10.0)	3.9 (1.3–11.7)	0.9180	1.5 (0.4–4.9)	6.6 (3.1–14.3)	0.0248	1.3 (0.6–2.8)	0.9 (0.3–2.5)	0.5598
Prostate	1.9 (0.7–5.2)	2.8 (1.3–5.9)	0.5335	1.1 (0.3–4.7)	1.4 (0.4–4.5)	0.8303	2.0 (1.0–3.8)	2.2 (1.2–4.4)	0.8018
Kidney	2.9 (1.1–7.5)	0.9 (0.3–3.1)	0.1325	1.0 (0.3–3.4)	2.2 (1.1–4.1)	0.2703	1.4 (0.5–3.8)	2.1 (1.0–4.0)	0.5354
Urinary bladder	0.6 (0.1–2.4)	3.0 (1.3–6.8)	0.0371	0.7 (0.3–2.0)	1.5 (0.6–3.5)	0.2813	1.3 (0.7–2.4)	1.5 (0.8–2.9)	0.7165
Non-Hodgkin lymphoma	3.2 (1.8–5.7)	1.7 (0.8–3.7)	0.1723	1.6 (0.7–3.5)	1.4 (0.6–3.0)	0.7814	1.0 (0.5–1.9)	1.2 (0.7–2.3)	0.5740
Lymphatic leukemia[b]	1.6 (0.2–12.6)	5.1 (1.4–18.7)	0.3260	2.1 (0.5–9.8)	5.3 (1.1–25.5)	0.3904	3.0 (1.1–8.1)	1.8 (0.4–7.5)	0.5199
Malignant myeloid diseases[b]	1.3 (0.6–2.5)	2.2 (1.4–3.6)	0.1137	1.2 (0.7–2.2)	1.2 (0.7–1.9)	0.8334	1.1 (0.7–1.6)	1.4 (1.1–2.0)	0.2382
Brain and other CNS tumors	10.0 (1.9–53.1)	8.3 (0.9–77.2)	0.8906	4.3 (0.9–19.5)	0.0 (.-.)	0.9834	1.2 (0.3–5.3)	1.8 (0.4–7.8)	0.7065

Abbreviations: Cbl: cobalamin; CCI: Charlson comorbidity index; CI: confidence interval.
[a] Wald chi-square test for equality in MRR estimates.
[b] Lymphatic leukemia and malignant myeloid diseases were not staged, and therefore not analysed using multiple imputations.

allowed for computing MRRs for some cancer types where the model based on complete case analysis failed.

4. Discussion

This study of more than 80,000 cancer patients demonstrated that those with elevated plasma Cbl levels prior to diagnosis had higher mortality, indicating more advanced and aggressive cancers. These results could not be explained by cancer type, sex, age, comorbidity or presence of non-localised disease. We speculate that these associations reflect underlying alterations in the Cbl metabolism caused by the cancer.

Our study has several advantages over earlier studies that yielded results consistent with ours [9–14]. We examined a large cohort and were able to adjust for key potential confounders. In addition, our study can be considered population-based, with mandatory registration of cancers ensuring complete information on cancer diagnoses. Comparable Cbl blood test results were available across the hospital laboratories and during the study period (data not shown). Further, due to the study's population-based design, we were able to identify all cancer patients diagnosed in Northern Denmark during the study period who had a Cbl measurement prior to diagnosis. We also included a large comparison cohort of persons with cancer from Northern Denmark. Our results remained robust in the stratified analyses, most importantly when disaggregated according to cancer stage and when adjusted for comorbidity. We observed some differences in age, sex, cancer stage and CCI score, both between the comparison cohort and the patient cohort and between patients with different plasma Cbl levels within the patient cohort. This could provide confounded crude survival estimates. However, all stratified analyses showed robust results; that cancer patients with high plasma Cbl levels had a poorer survival than those with normal plasma Cbl levels. Further, in the comparative analyses, we adjusted for the differences in these covariates.

Several issues must be considered when interpreting our results. First, use of medical registries to investigate research hypotheses requires accurate and complete coding of information stored in the registries. Data on diagnoses and prescribed medications in Danish registries are considered complete and of high quality [24–27], nearly eliminating the risk of misclassification due to information bias. This is substantiated further by the high comparability of Cbl test results across the study region. Only cancer stage was not registered completely and was unknown or missing for 23% of persons in the comparison cohort and for 28–43% of those in the patient cohort. The incomplete registration of cancer stage is a study limitation, but multiple imputations were used to reduce bias in the estimates due to missing cancer stage. In addition, results were very comparable between complete case analyses and imputed datasets and did not change the association found between high Cbl levels and mortality. Risk of selection bias is also a potential concern. We found the number of patients included in the study to increase during the study period. While this could introduce selection bias, analyses stratified by calendar year yielded robust results, indicating no substantial bias.

We found that survival among cancer patients with normal Cbl levels was lower than that among persons with cancer and no pre-diagnostic Cbl measurement. This indicates that the difference in survival could be confounded by the indication leading to physicians' requests for Cbl measurements. While this can explain differences in survival between the patient and the comparison cohorts, we consider it unlikely to be the cause of differences in survival between patients with high Cbl levels compared to those with normal levels. We do not believe that the clinical indication for a requisition is related to a test result showing high Cbl levels. If a high Cbl test result should make the physician more alert of possible cancer, this would, in turn lead to earlier cancer diagnosis in patients with high Cbl levels and have driven the association between high Cbl levels and mortality toward the null. Hence, we conclude that confounding by indication did not influence the association between high Cbl levels and mortality in cancer patients which we found when comparing cancer patients with different Cbl levels. Ultimately, the actual indication for requesting a plasma Cbl measurement for the individual patient can only be speculated, but as outlined, we doubt it could explain the results of the present study.

We were unable to explore further the association between elevated Cbl levels and non-localised cancer. Geissbühler *et al.* [10] suggested that hepatic metastases in particular are associated with high Cbl levels. Unfortunately, the registry-based design of our study precluded collection of information on the exact localisation of metastases, and the lack of detailed information about cancer stage is an obvious limitation. Further, when using registry data, we were unable to identify mortality as cancer specific. Deaths from other causes are, however, unlikely to bias the results, given the fact that the association between mortality and high Cbl levels revealed mainly an elevated risk in the short term and was adjusted for comorbidity. Also, the registry data precluded the assessment of possible confounding from life-style factors, since such data are not recorded. While smoking does not affect Cbl metabolism [36], alcohol and alcohol-related liver disease is known to cause high Cbl levels [4]. Thus, both liver metastases and benign liver disease could influence the results. Additionally, we did not include results from other biochemical tests in our study. While inclusion of such test results possibly could have helped to identify other potential prognostic biomarkers, it would also have increased the probability of confounding by indication, since the laboratory tests found in the LABKA database are performed only at the request of a physician. Furthermore, data on ethnicity are not available from the registries. While racial differences in plasma Cbl levels have been reported [37], the Danish population is fairly homogenous and consists mainly of Caucasians.

The underlying pathogenesis leading to high Cbl levels in cancer patients is not fully understood. Previous studies have shown elevated non-cancer mortality [11,15–19], pointing to a pathogenesis related not only to the cancer itself. Our results are unlikely to be related to diet, since the intestinal absorptive capacity for a regular diet and low-dose vitamin supplements is not thought to give rise to high Cbl levels [38]. This also justifies why we chose not to include data on medication considered to lower Cbl levels, such as metformin or proton pump inhibitors, because these drugs are thought to affect Cbl absorption and metabolism in the long term, and thus are unlikely to affect the short-term associations in this study [39,40]. High-dose Cbl drugs can induce elevated Cbl levels [29], but patients treated with such drugs were excluded from our study.

Altered Cbl metabolism is likely to be an underlying factor for our results. We speculate that cancer causes changes in the Cbl metabolism which then give rise to high plasma Cbl levels. Our interpretation is thus that the cancer somehow induces high Cbl levels, not that high Cbl levels cause cancer or promote a more aggressive cancer. This assumption is based both on the current results and on our previous observations that cancer risk was elevated mainly within the first year after plasma Cbl measurement [3] and that one particular Cbl-binding protein, haptocorrin, was found to be elevated in patients with high Cbl levels, including cancer patients [4]. Since circulating Cbl is exclusively protein-bound, and haptocorrin is metabolised solely in the liver, alterations in the Cbl metabolism may involve the liver. The finding of higher mortality also in patients with localised cancer implies that other mechanisms could underlie the association. It is known that both circulating and tissue-resident inflammatory cells

can produce Cbl-binding proteins, including haptocorrin [2]. Thus, the association between high Cbl levels and aggressive cancer could involve a pronounced inflammatory response to the cancer. We observed that some cancer types showed a stronger association than others between high Cbl levels and mortality. However, no protective effect of high Cbl levels was observed for any of the cancer types. The statistically imprecise estimates for specific cancers were difficult to interpret, also precluding stage-stratified analyses for each cancer type. Ultimately, further studies are warranted that could also elucidate the possible alterations in the Cbl metabolism and help to identify particular clinical settings, in which evaluation of Cbl levels is relevant for cancer patients. One way of evaluating plasma Cbl as a prognostic marker, and at the same time come closer to an understanding of the alterations in Cbl metabolism in cancer patients, would be to set up prospective studies. In this way, patients could undergo continous measurements of plasma Cbl levels and the levels of Cbl binding proteins. This would make it possible to evaluate the levels during the course of disease, and to assess the association with treatment response and markers of disease activity and any possible inflammatory response.

Our study lends strong support to earlier studies demonstrating that elevated Cbl levels were associated with lower cancer survival compared to those with normal Cbl levels. We found the association to be particularly pronounced for short-term mortality, and it showed a dose-response pattern. Our study suggests that high Cbl levels may be a potential biomarker for cancer prognosis. However, further prospective studies are needed to establish the clinical applicability of plasma Cbl levels as a prognostic biomarker.

Authorship contribution

DKF had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. DKF and LP conducted and are responsible for the data analysis. JFHA, DKF, LP, EN and HTS initiated, planned and designed the conduct of the study; DKF and LP conducted data acquisition, management and analysis; JFHA, DKF, LP, EN and HTS interpreted the study results; JFHA drafted the manuscript; JFHA, DKF, LP, EN and HTS wrote and approved the final manuscript, and approved the decision to submit the manuscript.

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Conflict of interest

The sponsors of this study had no role in the initiation, planning, design or conduct of the study, data acquisition, management and analyses, interpretation of results, writing and approval of the manuscript, or the decision to submit the manuscript for publication.

The researchers involved in this study declare their independence from the sponsors of the study and have no conflicts of interests to declare.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.canep.2015.12.007.

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