Clinical implications and biochemical understanding of high plasma vitamin B12 levels

PhD dissertation

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Preface

The work presented in this thesis was conducted from February 2011 to August 2016, during which I was employed as an MD/PhD-student at Department of Clinical Biochemistry, Aarhus University Hospital. The employment consisted of a four year period from February 2011 to January 2015, during which the PhD studies were conducted in parallel with the studies for an MD degree. From February 2015 to August 2016, the employment was a fulltime PhD position. During the entire period, I have been closely affiliated to Department of Clinical Epidemiology, Aarhus University Hospital. Part of the work for study I was conducted during my research year at Department of Clinical Biochemistry, Aarhus University Hospital, from February 2010 to January 2011.

I recently read the first few pages of a book on successful modern leadership. It stated that genuine interest in the individual employee was the key component for becoming a successful modern leader. I don't know if Ebba Nexø ever read books like this, but I have always felt her genuine and sincere interest in my personal and professional development and well-being. I cannot thank you enough for what you have taught me and for what you have done for me.

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Thesis papers

Paper I.

Arendt JF, Nexo E. Cobalamin Related Parameters and Disease Patterns in Patients with Increased Serum Cobalamin Levels. PLoS ONE. 2012;7(9):e45979.

Paper II.

Arendt JF, Pedersen L, Nexo E, Sorensen HT. Elevated Plasma Vitamin B12 Levels as a Marker for Cancer: A Population-based Cohort Study. J Natl Cancer Inst 2013;105:(23):1799-805.

Paper III.

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Abbreviations (alphabetical order)

ATC: Anatomical Therapeutic Chemical

AUPD: Aarhus University Prescription Database

Cbl: vitamin B12, cobalamin

CCI: Charlson Comorbidity Index

CI: confidence interval

CPR: Civil Personal Registration

DCR: Danish Cancer Registry

DNPR: Danish National Patient Registry

eGFR: estimated glomerular filtration rate

HC: haptocorrin

HoloTC: cobalamin-saturated transcobalamin

ICD: International Classification of Diseases

LABKA database: Laboratory Information Systems Research Database

MMA: methylmalonic acid

MRR: mortality risk ratio

NPU: Nomenclature, Properties and Units

OR: odds ratio

PRR: prevalence rate ratio

sCD320: soluble transcobalamin receptor

SIR: standardized incidence ratio

TC: transcobalamin

1. Introduction

Vitamin B12 (cobalamin, Cbl) is an essential micronutrient involved in mitosis and one-carbon and odd-chain fatty acid metabolism (1). In a clinical context, risk factors for Cbl deficiency include a vegetarian diet, high age, existing autoimmune or gastrointestinal diseases, or previous gastrointestinal surgery (2, 3), and Cbl deficiency can cause anemia and gastrointestinal and neurological symptoms. Therefore, these factors and symptoms are the indications for measuring total plasma Cbl levels, and low Cbl levels mark deficiency (4). However, recent studies suggest that these indications are often not present in patients referred for plasma Cbl measurement (2, 3) and that in these patients, an elevated Cbl level is not an uncommon laboratory finding (5). Based on earlier literature, elevated Cbl levels can be found in patients with a variety of diseases, including diseases of the liver, kidney, and immune system as well as several different malignancies. The pathogenesis underlying these associations is only partly understood, as are the clinical implications of elevated plasma Cbl levels (5).

This thesis attempts to add further knowledge to this area and includes three published studies. Study I was a cross-sectional study of hospital patients, examining the alterations in Cbl metabolism and the disease prevalence underlying elevated Cbl levels. Study II evaluated the association between elevated Cbl levels and cancer in a cohort study, using population-based Danish health registries. In study III, the prognostic bearing of elevated Cbl levels in cancer patients was assessed, also using registry-based data in a cohort design.

The present thesis takes the reader through a background section on Cbl, including a brief summary of its research history, metabolism, and functional role in the human body. A more

extensive outline of the clinical context of plasma Cbl measurements is followed by a review of the existing literature on elevated plasma Cbl, including the limitations and areas of uncertainty in the literature. The literature review forms the basis for the hypotheses and aims of the three studies, and subsequently, the methods of the studies are outlined. The main results are presented and strengths and limitations are discussed extensively in the context of methodology, validity, and the existing literature. In the last section, the implications and perspectives for future research are discussed.

The three studies are appended (Appendices I–III), including the supplementary data that were published with each study. In this thesis, results from the studies are referenced according to their study and/or appendix number.

2. Background

2.1. Cobalamin

A brief look into research on Cbl reveals that several Nobel laureates have conducted research within this field. First, in 1934, William P. Murphy, George Minot, and George H. Whipple were awarded the Nobel Prize in Physiology or Medicine for discovering the therapeutic effect of liver in patients with the autoimmune disease pernicious anemia (6, 7). In 1948, the active treatment compound in liver therapy was found to be Cbl (8). The same year, Karl Folkers and colleagues made the first crystal structure of Cbl (9), and later Nobel winner Alexander R. Todd unraveled the complex structure of Cbl. The definitive structure was identified by Dorothy C. Hodgkin, who was awarded the Nobel Prize in Chemistry in 1964 for her work on the crystallographic chemical structures (10). In 1965, Robert Burns Woodward was awarded the Nobel Prize in Chemistry, and he was the first to synthesize the compound (11). Soon thereafter, the intracellular role of Cbl as a co-enzyme was discovered by Barker and colleagues (12, 13).
The dietary sources containing Cbl consist of meat, fish, eggs, and dairy products (14). The dietary reference value for adults was recently set to 4 μg/day by the European Food Safety Authority (15). The vitamin has a complex path from ingestion through absorption to cellular uptake. Proteins in the food bind Cbl, and during digestion, it is liberated and then bound to the protein haptocorrin (HC), which presumably protects Cbl from degradation in the gastric juice. This protein is present in many tissues and body fluids, but its physiologic function is not fully known (16). The HC–Cbl complex is degraded in the acid gastric juice, and Cbl then binds to intrinsic factor, a protein secreted in the gastric mucosa. The intestinal absorption of Cbl is mediated in the distal ileum through the brush-border membrane complex, called cubam. In the human circulation, newly ingested Cbl is predominantly bound to transcobalamin (TC). The TC–

Cbl complex, also known as holoTC, facilitates cellular uptake through the TC receptor, a membrane protein called CD320 (17).

The cellular role of Cbl includes the methylation of homocysteine to methionine and the transformation of methylmalonyl-CoA into succinyl-CoA (1). These reactions are crucial for cellular methylation reactions and for odd-chained fatty acid and amino acid metabolism. Hence, mitosis and mitochondrial functions are affected when the cells are Cbl deficient (1). From a physiological perspective, this in turn means that rapidly growing cells, such as hematopoietic cells and gastrointestinal epithelium, and also neurons, are vulnerable to Cbl deficiency. The clinical manifestations are therefore often gastrointestinal complaints, anemia, and neurological symptoms. The gastrointestinal symptoms most frequently associated with Cbl deficiency are glossitis and symptoms related to general malabsorption, including diarrhea and deficiency of other micronutrients (4). The red blood cells are commonly enlarged in anemia caused by Cbl deficiency; hence, Cbl deficiency anemia is in the group of macrocytic anemias. Other hematopoietic cell lines can also be affected (4). Macrocytic anemias can additionally be caused by folate deficiency, alcoholism, liver disease, side effects of various drugs, and diseases of the bone marrow, including reticulocytosis and malignant hematological diseases (18).

Approximately 25% of patients with pernicious anemia, the most severe cause of Cbl deficiency, have no hematological manifestations (19). They show neurological symptoms, most often peripheral paresthesia and loss of motor function, but severe demyelinating nervous system disease can also be seen (4). Furthermore, changes in mental and cognitive state can be seen in Cbl-deficient patients, and general malaise and fatigue are often also attributed to neurological dysfunction. The clinical spectrum of Cbl deficiency spans from mild subclinical disease, where only biomarkers show impaired Cbl status and no symptoms are present (20), to severe disabling

and even life-threatening conditions (4). The many symptoms and the variety in symptom severity mean that Cbl deficiency can mimic several other diseases. Hence, the diagnosis and treatment of Cbl deficiency can be challenging.

Since the late 1940s, measurement of plasma Cbl levels has been a cornerstone in diagnosing Cbl deficiency, although this biomarker has a high rate of both false-negative and false-positive results for diagnosing Cbl deficiency (20). Other biomarkers have been established, namely measurement of methyl malonic acid (MMA), homocysteine, and holoTC (4). However, the costs, limitations, and availability of these markers as well as clinical tradition hinder their full integration into routine clinical practice. Therefore, the measurement of plasma Cbl remains the first-choice biomarker in the routine diagnosis of Cbl deficiency.

The total concentration of Cbl is measured when measuring plasma Cbl levels, so it is the combined levels of Cbl-saturated HC and holoTC that are measured (21). In the human circulation, 90% of TC is unsaturated, and TC binds approximately 20% of circulating Cbl (22) while HC binds the remaining 80%. Inactive Cbl forms, the so-called Cbl analogues, also bind to HC, and the protein mainly circulates saturated with either Cbl or Cbl analogues (16). Total HC, total TC, and holoTC can be measured separately, but they are not measured in routine clinical practice (22-24).

In clinical practice, biomarkers are used as tests for diagnosis, prognosis, screening, and monitoring diseases (25). In this context, measurement of plasma Cbl is requested to diagnose (or rule out) Cbl deficiency in patients with the symptoms described above of the hematological, gastrointestinal, and neurological kind. Such symptoms can also be caused by numerous other diseases, so the differential diagnoses for Cbl deficiency are many. Hence, it is often indicated to request other biomarkers together with plasma Cbl, depending on the symptoms. According to

current online guidelines for Danish physicians (26), these other tests could include hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin concentration, counts of reticulocytes, white blood cells, and thrombocytes, and plasma levels of folate, alanine transaminase, lactate dehydrogenase, alkaline phosphatase, bilirubin, coagulation factors II, VII, and X, albumin, creatinine, C-reactive protein, sodium, and potassium. A diagnostic biomarker algorithm of Cbl deficiency was introduced in 2003 at Aarhus University Hospital (27) and is now widely used in Denmark (personal communication). The algorithm is as follows: with a reference interval of 200–600 pmol/L, a patient value of <125 pmol/L marks deficiency; a patient value of >250 pmol/L marks repleteness; and a patient value of 125–250 pmol/L should be further investigated by measuring plasma MMA. When MMA is >0.75 μmol/L, the patient is Cbl deficient; if MMA is <0.28 μmol/L, the patient is Cbl replete; and if the MMA is 0.28–0.75 μmol/L, the patient should be re-examined with the same algorithm one year later. The initiation of treatment, either oral or parenteral, is indicated if biomarkers show Cbl deficiency or if symptoms strongly suggest Cbl deficiency. Plasma Cbl measurement is not recommended for monitoring treatment effect with Cbl drugs, unless poor compliance with oral treatment is suspected (28).

Furthermore, some patients are at risk of developing Cbl deficiency, namely, vegetarians, the elderly, patients with gastrointestinal diseases or who have undergone gastrointestinal surgery, and patients with other autoimmune diseases because they have an elevated risk of pernicious anemia. Treatment with proton pump inhibitors (29) and metformin is also associated with low Cbl levels (30), but it is controversial whether the latter treatment also leads to Cbl deficiency (31). Measurement of plasma Cbl in patients with these risk factors is done as a screening procedure. Other biomarkers may also be relevant in such a screening procedure, and in addition to those listed above, could include markers related to the specific risk factor, i.e., malabsorption

with different etiology or insufficient diet. The diagnosis of Cbl deficiency in the screening procedure follows the same biomarker algorithm. No studies have assessed adherence among Danish physicians to the published (27) and online guidelines (26).

A recent publication reported that the indications mentioned above were present only in a third of patients admitted at an internal medicine ward in whom plasma Cbl levels were requested (2). In addition, another study found that up to 70% of requested plasma Cbl measurements could be categorized as “inappropriate” (3). In conclusion, there is a lack of resolution around the indications for measuring plasma Cbl levels, the diverse and complex clinical presentation of patients with Cbl deficiency, and the apparently excessive testing for plasma Cbl levels. In this unresolved area, the clinical implications of elevated Cbl levels are unclear, but the proportion of patients with elevated Cbl levels is not negligible.

2.2. A literature review on elevated plasma cobalamin levels

The existing literature on plasma Cbl levels and the associations of interest to this thesis is roughly divided into two categories:

1. Studies describing the diseases that affect plasma Cbl levels, causing elevated Cbl levels.

2. Studies assessing whether Cbl causes or is a risk factor for the disease, and measurement of plasma Cbl levels is used as a biomarker of nutritional Cbl status. These studies most often focus on low plasma Cbl levels and thereby examine whether Cbl deficiency potentially cause disease.

I have included literature on plasma Cbl levels according to three different topics as separated in the sections below:

2.2.1. Studies of certain diagnoses in patients with elevated Cbl levels and studies of patients with certain diagnoses where elevated Cbl levels can be found.

2.2.2. The association between plasma Cbl levels and cancer. Studies assessing whether Cbl causes disease are included only if they studied plasma Cbl levels in association with cancer. The studies are presented separately for each type of cancer.

2.2.3. The association between plasma Cbl levels and mortality in patients with cancer. I included studies with study populations not exclusively consisting of cancer patients and studies with mortality as a secondary outcome.

The literature search was performed in April 2016 using PubMed and Embase. Literature on the associations between elevated plasma Cbl levels and diseases, plasma Cbl and cancer, and plasma Cbl and mortality was sought. A priori, it was decided to exclude studies with the following features: addressing only nutrient intake of Cbl, effects of Cbl treatment, or where the effect of Cbl treatment on plasma Cbl levels could not be distinguished; those not providing abstracts in English or a Scandinavian language or available online; studies published before 1985; and publications that consisted only of a conference abstract. The following search strings were used:

PubMed: 1: ("Vitamin B 12"[Majr] OR "Transcobalamins"[Majr] OR "cobalamin" OR "vitamin B12") AND (elevated OR high). 2: ("Vitamin B 12"[Majr] OR "Transcobalamins"[Majr] OR "cobalamin" OR "vitamin B12") AND (cancer) 3: ("Vitamin B 12"[Majr] OR "Transcobalamins"[Majr] OR "cobalamin" OR "vitamin B12") AND (mortality OR survival OR prognosis).

Embase: 1: 'vitamin b12'/exp OR 'vitamin' AND b12 OR 'transcobalamin' OR ‘cobalamin’ AND ('high' OR 'elevated'). 2: 'vitamin b12'/exp OR vitamin AND b12 OR 'transcobalamin' AND 'cancer'. 3: 'vitamin b12'/exp OR vitamin AND b12 OR 'transcobalamin' OR 'cobalamin' AND ('mortality' OR 'prognosis' OR 'survival').

A total of 6,605 search hits were found on PubMed and 6,900 search hits on Embase using the above search terms. Ultimately, 144 studies were included.

2.2.1. Elevated plasma cobalamin levels and disease associations

Six studies assessed the prevalence of certain diagnoses in patients with elevated Cbl levels, and 58 assessed whether elevated Cbl levels are found in certain diagnoses.

Table 1 presents the conclusions of the six studies evaluating the prevalence of certain diagnoses in patients with high plasma Cbl levels. All populations consisted of patients referred for plasma Cbl measurement, were cross-sectional, and had sample sizes ranging from 135–16,497 patients; however, three studies (32-34) focused only on a subset of patients with elevated Cbl levels. Overall, the studies reported associations between elevated Cbl levels and renal, liver, and autoimmune diseases and cancer, both hematological and solid tumor cancers. Carmel et al. (35) were the only authors to describe the underlying alterations in Cbl-binding proteins, reporting that both saturated and unsaturated HC and TC were elevated. Jeffery et al. (33) and Remacha et

al. (34) found that between 18% (34) and 25% (33) of patients with elevated Cbl levels had an immune complex with Cbl or Cbl-binding proteins, although the majority of the patients in the study were treated with high-dose Cbl (33, 34). There was not full consensus across the studies on Cbl level cut-off points, prevalence of elevated Cbl levels, and which diseases were highly prevalent in patients with elevated Cbl levels. Only Brah et al. (36) conducted a multicenter study, and none of the studies used direct measurement of Cbl-binding proteins.

Table 1

Study	N	Prevalence of elevated Cbl levels	Cut-off (pmol/L)	Other biomarkers	Disease associations
Carmel et al. (35)	135	14%	664	HC and TC, both Cbl-saturated and unsaturated; serum creatinine and albumin	Renal failure
Chiche et al. (32)	411	18.5%	701	Not measured	Hematological and solid tumor cancer, liver and renal diseases
Jeffery et al. (33)	16,497	9% (0.5% when excluding Cbl-treated patients)	664	IgG immune complex	Not assessed
Jammal et al. (37)	2,943	18%	605	Not measured	Hematological and solid tumor cancer, liver and renal diseases
Brah et al. (36)	380	18%	Above upper reference limit	Not measured	Hematological cancer, liver and renal diseases
Remacha et al. (34)	10,325	1.3% (0.2% when excluding Cbl-treated patients)	2,500	Immune complex with IgG or IgA + Cbl-binding protein (only in some patients)	Hematological and solid tumor cancer, liver and autoimmune diseases

The 58 studies assessing the prevalence of elevated Cbl levels in certain diagnoses are presented below.

Elevated Cbl levels have most consistently been associated with diseases of the liver, both alcoholic liver disease (38-46) and non-alcoholic liver disease (43, 47-52). Despite different etiology, the pathogenesis leading to high Cbl levels is thought to be high HC levels because of impaired clearance together with high holoTC levels because of Cbl release from hepatocytes (38, 41, 44, 45, 50). Several studies have suggested Cbl levels and/or levels of Cbl-binding

proteins as markers for disease progression, treatment response, and prognosis (41, 46, 49, 53, 54). Also, alcoholism without apparent liver disease seems to be associated with high Cbl levels (55), but liver disease linked with intestinal failure was not associated with high Cbl levels in one study (56).

Chronic renal failure (57-59), acute renal failure (57, 60-64), and diabetic renal disease (65, 66) have been associated with high Cbl levels. However, low Cbl levels and other markers showing Cbl deficiency have also been reported in patients with renal disease (58, 67, 68). The pathogenesis causing high Cbl levels in renal disease patients is not well characterized. Areekul et al. (57, 60-64) showed elevated TC levels in patients with acute renal disease due to malaria and typhus, and Carmel et al. (35) reported high HC levels in renal disease patients. One author group speculated that impaired renal tubular reabsorption of holoTC causes high holoTC levels (69).

In autoimmune diseases, elevated plasma Cbl levels and levels of Cbl-binding proteins have been studied in some detail. For rheumatoid arthritis (70, 71) and systemic lupus erythematosus (72, 73), elevated unsaturated TC levels have been identified, but without concurrent elevated Cbl levels. Another form of arthritis, Still's disease, has been associated with elevated Cbl levels (74). Macrophage activation is a central component of this disease, and TC correlates with macrophage activity (75). Thus, elevated Cbl levels in Still's disease could be caused by high TC release related to macrophage activity. Elevated Cbl levels are a part of the diagnostic criteria for autoimmune lymphoproliferative syndrome (76) and are caused by high HC production from proliferating leukocytes (77). Also, high Cbl levels have been reported in autoimmune neutropenia (78).

Different infectious diseases have been associated with elevated Cbl levels or elevated levels of Cbl-binding proteins in studies other than those on viral hepatitis (49, 53, 54, 79) and by Areekul et al., noted above (57, 60-64). Scrub and murine typhus have been linked to elevated Cbl levels (80, 81), and patients with HIV have been reported to have elevated levels of Cbl, TC, and HC (82-85). In HIV and AIDS, a high prevalence of low Cbl and holoTC has also been reported (86-88).

Finally, high Cbl levels can be seen because of interference with laboratory analyses, as has been reported for patients with very high HC levels (89) and among patients with autoantibodies against intrinsic factor (90-92). However, not all analytical platforms are vulnerable to interference from these autoantibodies (93).

2.2.2. Plasma cobalamin levels and cancer

A total of 75 studies identified had assessed the association between plasma Cbl levels and risk of cancer. A large cohort study by Ryg et al. (94) found that plasma Cbl levels >1200 pmol/L were associated with an elevated risk of several types of cancer, including hematological cancers and different solid tumors. They found 490 patients with Cbl levels >1200 pmol/L, of whom 54 were diagnosed with cancer. Because 33 of the cancer patients were diagnosed within 6 months of plasma Cbl measurement, the authors argued that cancer caused the elevated Cbl levels. The authors also concluded that cancer was suspected in 87% of these patients at the time of plasma Cbl measurement, for the following reasons: 69% had either anemia, weight loss, general malaise, or abnormal liver biomarkers or the doctor raised concern of malignancy in the patients' medical record, and 77.8% had undergone one or more scans or biopsies within 3 weeks of

plasma Cbl level measurement. However, the authors did not assess any cancer suspicion for the 2,546 patients diagnosed with cancer who had Cbl levels <1200 pmol/L.

Several studies have addressed the association between specific cancer types and plasma Cbl levels. As is well known, elevated Cbl levels are seen in patients with hematological malignancies, and most studies have focused on chronic myeloid leukemia (95-97), in which the Cbl levels are caused by high production of HC from proliferating leukocytes. Several other hematological diseases have been associated with elevated Cbl levels, including acute myeloid leukemia, chronic neutrophilic leukemia (98-102), eosinophilia (103), polycythemia vera (95), and multiple myeloma (101). The underlying mechanism is also thought to be high production of HC but has not been studied to the same extent as in chronic myeloid leukemia. Conflicting results have been reported in lymphatic leukemia, with Gimsing et Nexo (95) reporting high TC levels in adult patients with lymphatic leukemia but others reporting low Cbl levels in pediatric patients with this disease (104-106).

Levels of Cbl and Cbl-binding protein in liver cancer patients have been studied in several publications since 1985, and different cancer types show different characteristics. The tumor in fibrolamellar hepatocellular carcinoma produces high amounts of HC through increased gene expression of the protein; thus, plasma HC is a biomarker for the disease (107-110). This liver cancer subtype is rare, and for the more common liver cancers, the pathogenesis is not fully understood. Several studies have reported high Cbl and HC levels in liver cancer patients (111-116). Despite this, two recent studies demonstrated that the elevated levels of Cbl and HC were not suitable as markers to separate chronic liver disease from liver cancer (50, 117). In turn, this overlap could imply that the pathogenesis underlying elevated Cbl levels in liver cancer and chronic liver disease is similar.

All of the following studies on specific cancer types examined if Cbl (or Cbl deficiency) is a causal risk factor for cancer. However, some of the studies provide evidence that cancer may cause elevated Cbl levels because plasma Cbl levels were measured when cancer was prevalent or possibly occult. The studies are outlined below, and several other studies are also included in which HC was found in different cancer tissues.

Early immunohistochemical studies showed that HC was highly prevalent in cancers of the upper gastrointestinal tract (118, 119) and circulating in high concentrations in gastric cancer patients (119). More recent case–control studies have shown conflicting results; two studies showed no association between esophageal cancer and Cbl levels (120) and lower gastric cancer risk with higher Cbl levels (121), while Chang et al. (116) reported the opposite pattern of an elevated risk of both esophageal and gastric cancer with higher Cbl levels. This discrepancy could trace to different methods applied in the studies. Two studies (120, 121) chose cases and controls that were nested within the EPIC cohort, and controls were matched with cases according to the date of blood sampling. One of the studies reported unaltered results when excluding cases diagnosed within 2 years from Cbl measurement (121) whereas the other excluded all prevalent cancer cases (120). In this way, the authors attempted to preclude the possibility of finding that cancer affect Cbl levels. Chang et al. also concluded that their results indeed described this latter association. None of these three studies explicitly assessed high Cbl levels.

The presence of HC in malignant lung tissue was identified by Ogawa et al. (122), but results are conflicting on the association between lung cancer and plasma Cbl levels (123-125). Hartman et al. (123) found no association but provided no estimates for short-term follow-up <1 year, potentially blurring the association between high Cbl levels and occult lung cancer. Interestingly, the positive association between plasma Cbl levels and lung cancer risk reported by Johansson et

al. (124) remained robust in a subanalysis excluding patients diagnosed within one year after plasma Cbl level measurement, but the association was found only for current and former smokers and not for never smokers. Both studies excluded participants with prevalent cancer at the time of blood sampling, and the recent cross-sectional study by Tastekin et al. reported no difference in Cbl levels between lung cancer patients and healthy controls (125).

The results also are not congruent for prostate cancer. None of the studies provided odds ratios (ORs) for short-term cancer risk, e.g., within one year from plasma Cbl measurement. Thus, the potential association between plasma Cbl levels and occult prostate cancer was not assessed. Three studies reported null associations, all for incident prostate cancer (126-128). Three studies reported an association between higher Cbl levels and prostate cancer, and one of them excluded prevalent cancer cases (129) while the two others included incident prostate cancer diagnoses (130, 131). A very recent nested case-control study combined six different populations, and found an OR for prostate cancer of 1.12 when comparing the lower and upper quintiles of plasma Cbl, but the authors provided no information on whether cases with prevalent cancer at the time of blood sampling were excluded (132). Otherwise, the methods employed in the studies were very similar, making the discrepancy between their results more obscure.

Most studies report no association between colorectal cancer risk and plasma Cbl levels (133-139). These studies either excluded prevalent cancers at the time of blood sampling or matched controls according to age and to the time of blood sampling for cases. However, Dahlin et al. (140) reported a higher risk of left-sided colon cancer with higher Cbl levels but a lower risk of rectal carcinoma with higher Cbl levels. The latter was later supported by the results from Gylling et al. (141). Two other cross-sectional studies (142, 143) reported higher plasma Cbl levels in colorectal cancer patients than in healthy controls, and one of them also reported an

association between cancer stage and increasing plasma Cbl levels (143). These two groups obtained blood samples when patients were already diagnosed with cancer; therefore, higher plasma Cbl could be a marker for occult colorectal cancer.

The association between plasma Cbl levels and the following cancers have been studied, and no association with elevated plasma Cbl levels were reported: cancers of the pancreas (144-146), breast (147-151), kidney (152, 153), and cervix uteri (154-164) and head-and-neck cancers (120, 165-167). All of the studies (120, 144-167) that reported no association between a specific cancer and plasma Cbl levels assessed the potentially causal association that Cbl affects cancer risk. The studies have differences in methodology for time between plasma Cbl measurement and cancer diagnosis, censoring of events within a short time span from plasma Cbl measurement and follow-up, and stratified results. Still, they consistently report no association between levels of Cbl and the cancer types under study, although none of the studies specifically assessed elevated Cbl levels. However, immunohistochemical studies have shown the presence of HC in breast cancer, renal cancer, and cancer of the salivary glands (168-170).

2.2.3. Plasma cobalamin levels and mortality in cancer patients

Six cohort studies and one nested case–control study have examined whether plasma Cbl levels are associated with poor survival in cancer patients (94, 153, 171-175). Not all of these studies specifically studied cancer patients but included elderly patients both with and without cancer (173, 175). Interestingly, these studies also showed higher mortality in elderly patients without cancer. For specific cancer types, renal cancer was not associated with mortality in the nested case–control study by Johansson et al. (153), but they excluded prevalent renal cancer cases. In patients with liver cancer, high plasma Cbl levels were associated with more severe cancer and

with poorer survival (174), and in a small cohort study on 93 colorectal cancer patients, Byström et al. (171) showed that plasma Cbl ≥300 pmol/L was associated with lower survival and an unfavorable treatment response.

2.2.4. Summary of existing literature

The literature reviewed above presents a number of interesting issues but leaves several questions unanswered. First, from the literature presented in 2.2.1., the prevalence of patients with elevated Cbl levels is generally quite high. There was some but not full congruence in the six studies assessing disease prevalence in patients with high Cbl levels. Furthermore, none of the studies made direct measurements of Cbl-binding proteins and did not assess potential dose-response associations between disease prevalence and Cbl levels. However, the studies showed some concordance when assessing prevalence of elevated Cbl levels in specific diseases. These disease associations suggest that the prevalent diseases give rise to elevated Cbl levels, but the pathogenesis underlying these associations is not fully elucidated.

As presented in 2.2.2., the majority of the included studies attempted to assess if Cbl (or Cbl deficiency) is a causal risk factor for cancer, and these studies report null findings. However, some of the studies showed that elevated Cbl levels can mark prevalent cancer. Elevated Cbl levels were most consistently reported to mark prevalent hematological malignancies and liver cancer, while results on cancers of the lung, prostate, and gastrointestinal tract were less clear. The study by Ryg et al. (94) showed elevated short-term cancer risk, but some issues were not addressed: the high cut-off that was applied precluded any assessment of the association at lower Cbl levels; a potential dose-response pattern in the association was not assessed; and the study reported no risk estimates for specific cancers or groups of cancers.

The studies on the association between mortality and elevated Cbl levels in cancer patients were evaluating either cancer patients without regard to cancer type (94, 172, 173, 175) or only patients with one specific cancer type (153, 171, 174). Furthermore, most studies had small sample sizes (94, 171-175), did not obtain plasma Cbl levels prior to diagnosis (171-175), and did little confounder assessment (94, 171, 173, 175). Ultimately, the use of plasma Cbl levels in estimating survival in cancer patients is left unresolved.

Thus, there is a need for a better understanding of which diseases are prevalent in patients with high Cbl levels compared to patients with normal Cbl levels, as well as a need for understanding the alterations in Cbl-binding protein in patients with elevated Cbl levels. In addition, scrutinizing the association between prevalent or occult cancer is required if we are to come any closer to understanding the clinical implications of elevated Cbl levels in early cancer diagnosis. Finally, the prognostic value of pre-diagnostic Cbl levels has not been elucidated in a large group of unselected cancer patients.

2.3. Hypotheses and aims

Study I

Hypothesis: Elevated Cbl levels are associated with changes in Cbl metabolism, and disease prevalence differs according to plasma Cbl levels because some diseases cause elevated Cbl levels.

Aim: To examine the alterations in Cbl-related biomarkers and the disease prevalence in patients with elevated serum Cbl levels.

Study II

Hypothesis: Elevated Cbl level can mark occult cancer because some cancers cause elevated Cbl levels.

Aim: To assess the cancer risk and incidence in patients with a measurement of plasma Cbl levels.

Study III

Hypothesis: Elevated Cbl levels are caused by aggressive cancer, and therefore, elevated Cbl levels are associated with poor survival in cancer patients.

Aim: To examine the survival probability and the relative mortality risk among cancer patients with a measurement of plasma Cbl levels prior to cancer diagnosis.

3. Methods

3.1. Setting

The Danish national health care system is tax paid and offers free health care to all Danish residents. Hospital care is provided by five geographical administrative regions. The studies in this thesis were all conducted within the regions of Northern and Central Denmark. These two regions cover approximately one third of the Danish population. The majority of provided health care is recorded at the individual level, such as hospital diagnoses and procedures, reimbursed prescriptions, and hospital laboratory analyses. The data produced are widely available for research purposes, and access to the data is regulated by the Danish Data Protection Agency under the Danish Act on Processing of Personal Data (176).

3.2. Data sources

3.2.1. The Danish Civil Registration System

The Danish Civil Registration System was instituted in 1968 (177) to register all Danish residents with a unique personal identification number, the Civil Personal Registration (CPR) number. This 10-digit number is assigned uniquely to each person at birth or immigration to Denmark. The first six digits contain the date, month, and year of birth, so the age of the person can be determined at all calendar times. The remaining four digits are derived from the first six, with the last digit identifying the sex of the person; males have odd numbers, females have even numbers.

We used the CPR number to identify persons and to link the different data sources, as outlined below. Furthermore, age and sex of all study individuals were derived from the CPR number.

3.2.2. LABKA and the Laboratory Information System Research Database (LABKA database)

The software LABKA integrates all the biomarker measurements from laboratory analytical platforms. It holds the following information: the patient's CPR number, a unique barcode for the tube in which the biological specimen was collected, a code for the requesting physician/department, the biomarker name, code, unit, and reference range (see below for biomarker codes), and the test results. We used LABKA for study I to include information on serum Cbl levels (pmol/L) and serum creatinine levels (μmol/L) from hospital-treated patients from November 1, 2009, to December 31, 2010.

The LABKA database is short for the Laboratory Information System Research Database (178), a name originating from the LABKA software, as described above. The content of LABKA is transferred electronically to the LABKA database. However, the LABKA database is not a mirror image of the LABKA software because it holds less detailed information and also data from laboratory software used prior to the introduction of LABKA in the study regions. The LABKA database holds information on analyses done at the departments of clinical biochemistry in the two study regions. The requesting physician or department covers the study area, and the requesters are public hospital departments, general and specialist practitioners, and private hospitals. The variables recorded are the patient's CPR number, the biomarker name, Nomenclature, Properties, and Units (NPU) code or a local biomarker identification code, the unit and reference range, and the test result (or that it is missing). The requester or the laboratory performing the analyses requested is not included in the database, so the patients cannot directly be traced to the requester, and information on the laboratory platforms performing the individual

analysis cannot be obtained. The LABKA database provided data on plasma Cbl measurements for studies II and III.

3.2.3. Electronic medical files

We used electronic medical files in the form of E-record (E-journal in Danish) to obtain information for study I about diagnoses (179). The E-record is an electronic copy of the individual patient's medical file and includes only the actual medical file notes of the patient but no test results or prescription/medication details. This format was developed to make medical files electronically available for clinicians from hospitals and departments outside their own. Currently, it is used for giving the individual patients online access to their own medical files.

3.2.4. The Aarhus University Prescription Database

Aarhus University Prescription Database (AUPD) (180) is hosted by the Department of Clinical Epidemiology, Aarhus University Hospital. It contains data on prescription reimbursements from pharmacies in the Northern and Central regions of Denmark from the year 1989 and is considered chronologically complete from 1998 and onwards for the entire study area. Most prescription costs are reimbursed to patients, except for some sedatives, oral contraceptives, and over-the-counter drugs. The AUPD records reimbursed prescription with the following information: patient's CPR number, date of sale, prescriber identification, and information about the drug including Anatomic Therapeutic Chemical (ATC) code, name, dose, pack size, and manufacturer. The AUPD provided data on Cbl treatment for all three studies.

3.2.5. The Danish National Patient Registry

As stated above, the Danish health care system records many of its services in registries and databases. The Danish National Patient Registry (DNPR) (181) is an administrative register, used for remuneration of hospital departments according to defined rates allocated to diagnoses and certain procedures, mainly surgical procedures. It was established in 1977, and from 1995 it also included outpatient visits and psychiatric ward and emergency ward visits. The DNPR contains the following patient variables: CPR number, date and type of admission, date of discharge or outpatient clinic contact, a primary diagnosis indicating the main cause for hospital contact, up to 20 secondary diagnoses, and codes for the procedures performed. All codes are assigned by hospital physicians. Diagnoses are coded according to International Classification of Diseases (ICD)-8 (1977–1993) or ICD-10 (1994–), and procedures are coded according to Danish or Nordic classification systems (181). The registry was recently reviewed and found to be overall complete and with good validity (182). The DNPR provided data on hospital treatment with Cbl drugs and data on diagnoses used for comorbidity assessment.

3.2.6. The Danish Cancer Registry

The registration of cancers to the Danish Cancer Registry (DCR) began in 1943. The registration includes variables related to the patient and to the cancer disease. Together, these data include patient's CPR number, region and municipality of the patient, date of diagnosis, cancer type (ICD-10 codes), and cancer stage and histology. The DCR provided data on cancer diagnosis for study II and data on cancer diagnosis and cancer stage for study III.

3.3. Study designs and periods

Study I is a cross-sectional study on hospital patients at Aarhus University Hospital from November 1, 2009, to December 31, 2010. Studies II and III are population-based cohort studies using registry data from the regions of Northern and Central Denmark from January 1, 1998, to December 31, 2010 (study II) and January 1, 1998, through December 31, 2014 (study III).

3.4. Study populations

Study I

We included hospital patients (either admitted or treated at hospital outpatient clinics) with a plasma Cbl measurement measured at the Department of Clinical Biochemistry, Aarhus University Hospital. Patients were identified directly in LABKA, where their status as hospital patients was listed. Prior to collection, we aimed to include 200 patients in each of the following Cbl level groups: <200 pmol/L, 200–600 pmol/L, 601–1000 pmol/L, and >1000 pmol/L; thus, the study period was defined by the aim for the study population size. Levels of 200–600 pmol/L are the population reference range (183). We excluded patients who provided less than 1 mL of surplus serum, patients below 18 years of age, and patients with incomplete or inaccessible medical files (thus also excluding patients from general practice and private hospitals). On the basis of 40,047 serum Cbl analyses performed during the study period, nearly 30% were requested for hospital patients. We did not collect data on the number of samples discarded due to too little surplus serum, so we have no information on these patients.

Study II

We included patients with a first-time plasma Cbl measurement in the LABKA database from January 1, 1998, to December 31, 2009. Patients were excluded if they had a cancer diagnosis recorded in the DCR prior to plasma Cbl measurement or Cbl treatment up to 2 years before plasma Cbl measurement (see section 3.4.1.).

Study III

We included two cohorts as the study population. The patient cohort consisted of patients with a first-time cancer diagnosis and a plasma Cbl measurement within one year prior to cancer diagnosis (index date) from January 1, 2001, through November 30, 2013. In case of multiple tests in that period, we used the one closest to the index date. Patients were excluded if they were Cbl-treated up to 2 years before plasma Cbl measurement (see section 3.4.1.). We created a comparison cohort by matching patients from the patient cohort to patients with the same cancer type, age (in 10-year intervals), sex, and diagnosed in the same calendar period (in 5-year intervals).

3.4.1. Cobalamin treatment

For study I, patients were classified as Cbl-treated if they had any information about oral or injection treatment in their medical file or any filled prescription in the AUPD before blood sampling. For studies II and III, patients were classified as Cbl-treated if they had one or more filled prescriptions on Cbl drugs recorded in the AUPD or any record of Cbl hospital treatment up to 2 years prior to plasma Cbl measurement.

3.5. Exposure

3.5.1. Plasma cobalamin

From an etiological point-of-view, the exposure is the disease causing the elevated Cbl levels, thereby making Cbl levels the outcome of interest. However, for all three studies, the main inclusion criterion was a plasma or serum Cbl measurement, and all statistical analyses were based on the levels of plasma Cbl as the predictor variable. Therefore, we describe plasma/serum Cbl measurement as the exposure and the diseases under study as the outcomes in the following sections.

In study I, serum Cbl was analyzed at the routine hospital laboratory at Aarhus University Hospital on the platform Cobas 6000 E (Roche Diagnostics, www.roche.com). Samples were diluted and reanalyzed if results were above the upper detection limit of 1476 pmol/L or if the unsaturated Cbl binding capacity could interfere with the assay (89) (defined as: total TC + total HC – total serum Cbl >6000 pmol/L).

For studies II and III, plasma Cbl measurements were obtained from the LABKA database (see section 3.2.2.). Nine different codes were used to identify plasma Cbl measurements, and we evaluated comparability across calendar years and the different laboratory codes. Two tables in Appendix IV show that the comparability was satisfactory. One code showed approximately 150 pmol/L higher median Cbl values, but this code corresponded to only 0.3% of the Cbl measurements and 0.4% of the patients.

3.5.2. Cobalamin-related biomarkers

The Cbl-related parameters in study I constituted the total concentration of the two Cbl-binding proteins, total TC and total HC, Cbl-saturated TC (holoTC), the soluble TC receptor sCD320,

and the metabolic marker for cellular Cbl status, MMA. For study I, we used established in-house ELISAs for the measurement of total TC (22), holoTC (24), total HC (23), and sCD320 (184). The analyses were performed at the Department of Clinical Biochemistry, Aarhus University Hospital, from March 2010 to January 2012. Measurements of MMA on samples from non-Cbl treated patients were done using an LC-MS/MS method (185) at the Department of Clinical Biochemistry, Rigshospitalet, University of Copenhagen. Serum creatinine was measured on a Cobas 6000 E platform (Roche Diagnostics; www.roche.com), and results were obtained through LABKA.

3.6. Outcomes

3.6.1. Diagnoses from medical files

Data on diagnoses for study I were obtained from E-records. We included diagnoses made either during the admission or outpatient clinic visit or, in case of chronic diseases, prior to inclusion date. Based on previous literature (see section 2.2.), we pre-specified the following diseases to be included and grouped accordingly: alcoholism, liver disease (analyzed as both alcoholic and non-alcoholic liver disease and in one category), cancer (analyzed as myeloid malignancies, lymphatic malignancies, and solid tumor cancer and in one category), renal disease, and autoimmune diseases. Furthermore, we included diabetes mellitus type II and cardiovascular, psychiatric, bronchopulmonary, neurologic, and gastrointestinal diseases. I reviewed all medical files.

3.6.2. Cancer

Diagnoses of cancer in study II were obtained from the DCR. Incident first-time cancer diagnosis was the outcome of interest. We grouped the cancer types into four categories: smoking and alcohol-related, hematological, immune-related, and (sex) hormone-related cancers (Appendix II, Table 1). We also analyzed 10 different cancer types separately.

For study III, DCR provided data on cancer type for both the patient and comparison cohorts. We analyzed all cancers and 11 different specific cancers (Appendix III, Table 4 and Supplementary Tables S5 and S6).

3.6.3. All-cause mortality

All-cause mortality was the outcome of interest for study III. We used the Danish Civil Registration System for information on vital status including date of death.

3.7. Covariates and confounders

3.7.1. Demographics

Sex and age at the date of serum/plasma Cbl measurement were obtained using the CPR number for all three studies. Because of the geographical restriction of the LABKA database (178) and the AUPD (180), residence location was obtained from the Danish Civil Registration System.

3.7.2. Cancer stage

Data on cancer stage was obtained from the DCR, except for lymphatic leukemia and malignant myeloid diseases that were not staged according to TNM or Ann Arbor classifications. We classified cancer stage into localized or non-localized. In case of missing or unknown cancer

stage, we used multiple imputations with chained equations to provide less biased and more precise estimates (186-188) (see section 3.8.4).

3.7.3. Comorbidity

We used the DNPR to obtain data on comorbid diseases of the cancer patients included in studies II and III. In study II, we computed risk estimates for patients with pre-existing disease recorded in the DNPR and grouped according to ICD-10. This step was done to assess if underlying morbidity influenced the risk estimates. In study III, the comorbid diseases were categorized according to the Charlson Comorbidity Index (CCI) (189), omitting cancer and cancer stage from the score. We divided patients according to their CCI scores, as follows: low=0; medium=1–2; high ≥3.

3.8. Statistical analyses

For study I, we used GraphPad Prism® version 4.0 for Windows (GraphPad Software, San Diego, CA, USA), Stata® 11 (StataCorp LP, College Station, TX, USA), and Microsoft® Excel 2003 (Microsoft Corporation, Redmond, WA, USA) for statistical analyses. For studies II and III, we used SAS version 9.2 (SAS Institute Inc., Cary, NC, USA).

3.8.1. Cobalamin-related biomarkers (study I)

Patients who were classified as Cbl-treated were excluded from analysis of MMA and analyzed separately for total plasma Cbl, total TC, holoTC, total HC, and sCD320. Furthermore, 129 were excluded from analyses of MMA due to reduced kidney function based on estimated glomerular filtration rate (eGFR) <60 mL/min/1.73 m^2. The eGFR was calculated as follows: 175 × (serum

creatinine (μmol/L)/88.4)$^{-1.154}$ × (age in years)$^{-0.203}$ × 0.742 (if patient was female), with no racial correction (190). Gaussian distribution of all Cbl-related biomarkers could not be obtained through transformation, so the Kruskal-Wallis test was used to compare median levels of these biomarkers across the four Cbl level groups. The Mann-Whitney U test was used to compare the median levels of the Cbl-related biomarkers between Cbl-treated and non-treated patients. For the non-treated patients, we also analyzed correlations between the different Cbl-related biomarkers using Spearman's rank correlation (191).

The Department of Clinical Biochemistry, Aarhus University Hospital, makes use of a laboratory algorithm for diagnosing Cbl deficiency (27, 28). This algorithm specifies that plasma Cbl <125 pmol/L indicates Cbl deficiency; plasma Cbl >250 pmol/L indicates replete Cbl status; plasma Cbl 125–250 pmol/L is inconclusive; and MMA is automatically measured as a more sensitive marker for Cbl deficiency. To assess possible Cbl deficiency in patients otherwise categorized as Cbl replete, we defined borderline impaired Cbl status in patients with Cbl levels >250 pmol/L as either MMA >0.28 μmol/L (27, 28) or holoTC <40 pmol/L (24).

3.8.2. Disease associations (study I)

Patients classified as Cbl-treated were excluded from analysis of diagnosis (n=186). Two different analyses were performed: in the published paper (Appendix I), we used logistic regression analyses to assess the association between high Cbl levels and the selected diagnoses. The patients with Cbl levels <200 pmol/L were used as the reference category, and crude and adjusted ORs with corresponding 95% confidence intervals (CIs) were computed. We adjusted for two covariates, age and sex, because both plasma Cbl (192) and disease prevalence differ with age and sex. The ORs served as an adjusted estimate of the prevalence rate ratios (PRRs).

For this thesis, we also analyzed PRR with corresponding 95% CIs in recognition of the fact that the disease prevalence was high; therefore, ORs could overestimate disease risk.

3.8.3. Cancer risk (study II)

We assessed cancer risk with both relative and absolute risk estimates. The relative risk estimates were computed as standardized incidence ratios (SIRs) with corresponding 95% CIs, assuming a Poisson distribution of observed cancers in the study period (193). The SIRs were computed as the ratio of the observed to expected cancers. The expected cancers were calculated based on national cancer incidence rates for persons with the same age, sex, and year of diagnosis as the patients with plasma Cbl measurement, assuming that the patients had the same cancer incidence as the background population. Then person-years of follow-up were multiplied by the expected cancer incidence. For computing 95% CIs, Byar's approximation was used. If fewer than 10 cancer cases were observed, exact 95% CIs were computed (194). All SIR estimates were calculated overall and according to plasma Cbl, in three grouped categories (pmol/L): 200–600, 601–800, and >800. The SIR estimates were further stratified into sex, age, calendar year, years of follow-up, cancer groups (as above), and specific cancer types. The one-year SIR estimates were also assessed in smaller plasma Cbl level intervals of 100–200 pmol/L. In addition, patients were classified as hospital patients if they had a hospital contact in the DNPR within 30 days before or 7 days after the plasma Cbl measurement. If no contact was recorded, patients were classified as general practitioner patients.

The absolute risk estimates were computed as cumulative incidence proportions divided according to plasma Cbl and according to follow up (≤1 year and >1 year). This step was

performed to evaluate whether elevated Cbl levels were an early marker of occult cancer even more than a year before the cancer was diagnosed.

3.8.4. Mortality (study III)

All-cause mortality was also assessed with both relative mortality estimates and absolute survival estimates using time-to-event analyses. For the patient cohort, all analyses were stratified according to plasma Cbl levels in three pre-defined groups: 200–600 pmol/L, 601–800 pmol/L, and >800 pmol/L. We assessed absolute mortality by computing Kaplan-Meier curves for overall survival, both overall and stratified according to the two cancer stage groups. Cumulative survival proportions were computed at different follow-up intervals: 30, 90, and 365 days, and 2, 5, and 10 years after index date. We used the log-rank test to compare survival estimates overall and at 30, 90, and 365 days in the three Cbl level groups and to compare the overall survival between the entire patient cohort and the comparison cohort. We also computed a cubic spline curve (195, 196) using plasma Cbl in 5% percentiles to elucidate any dose-response association in detail between plasma Cbl levels and 1-year survival.

We used Cox proportional hazards regression to compute mortality risk ratios (MRRs) with corresponding 95% CIs and used patients with plasma Cbl levels of 200–600 pmol/L as reference. These analyses were adjusted for the potential confounding effect of age, sex, calendar period, comorbidity (CCI score), and cancer stage and stratified according to follow-up: 30 days, 31–90 days, 91–365 days, 366 days–2 years, 3–4 years, and ≥5 years. Furthermore, we also stratified on sex, calendar period (three categories), age (four categories), and cancer stage. We visually evaluated log–log plots and ensured that the proportional hazards assumption was met.

Information on cancer stage was missing for 23% of the study population. Thus, we used multiple imputations with chained equations to provide less biased and more precise estimates (186-188). We computed 30 complete datasets using the following variables in the imputation model: sex, age (continuous), calendar year, cancer type, plasma Cbl levels (continuous), CCI score, length of follow-up, and death (yes/no). The statistical analyses were performed on each dataset separately, and estimates, including 95% CIs, were combined into one using Rubin's rule (188). Complete case analysis, where patients with missing stage were excluded, was also performed, and estimates were then compared with the estimates based on the multiple imputation. All analyses on lymphatic leukemia and malignant myeloid diseases were performed separately.

3.9. Ethics

The collection of blood samples, information from medical files, and Cbl prescriptions from AUPD for study 1 was approved by the Danish Data Protection Agency (record nr.: 2010-331-0378 and 2010-41-4559) and by the Regional Ethics Committee of Central Jutland, Denmark (record nr.: 20090187), including the permission to collect serum samples without informed consent from patients with surplus serum (Appendix I).

For studies II and III, the Danish Data Protection Agency approved the use of registry data (record nrs: study II: 2009-41-3866; study III: 2013-41-1924). The use of registry data for research is permitted without patient informed consent and ethical evaluation and permission (Appendices II and III).

Table 2. Study overview

	Study I	Study II	Study III
Aim	Examine alterations in Cbl-related biomarkers and disease prevalence in hospital patients with a serum Cbl measurement	Examine the cancer risk and incidence in patients with a plasma Cbl measurement	Examine the survival and relative mortality among cancer patients with a pre-diagnostic plasma Cbl measurement
Design	Cross-sectional	Population-based cohort study	Population-based cohort study
Data sources	E-record, LABKA, AUPD, laboratory analyses	LABKA database, AUPD, DCR, DNPR, Civil Registration System	LABKA database, AUPD, DCR, DNPR, Civil Registration System
Study area and period	Aarhus University Hospital, Nov 1 2009–Dec 31 2010	Regions of Northern and Central Denmark, Jan 1 1998– Dec 31 2010	Regions of Northern and Central Denmark, Jan 1 1998– Dec 31 2014
Study population	818 hospital patients (Cbl treated: n = 186)	333,667 patients with plasma Cbl measurement and no prior cancer	Patient cohort: 25,017 cancer patients with plasma Cbl measurement Comparison cohort: 61,988 cancer patients without plasma Cbl measurement
Exposure	Serum Cbl (categorized: <200, 200–600, 601–1000, and >1000 pmol/L)	First-time plasma Cbl measurement and no Cbl treatment within 2 years from measurement (categorized: 200–600, 601–800, and >800 pmol/L)	Plasma Cbl measurement ≤1 year prior to cancer diagnosis and no Cbl treatment within 2 years from measurement (categorized: 200–600, 601–800, and >800 pmol/L)
Outcome	Total TC, holoTC, total HC, sCD320, MMA, disease prevalence	Diagnosis of cancer	All-cause mortality
Covariates	Cbl treatment, age, sex, eGFR	Follow-up time, age, sex, calendar year, cancer group and type, previous morbidity	Follow-up time, age, sex, calendar year, CCI, cancer type and stage
Statistical analyses	Non-parametric tests for comparing outcome biomarkers and covariates, adjusted odds ratios using logistic regression for comparing disease prevalence,	Standardized incidence ratio using Poisson regression, cumulative cancer incidence	Cumulative survival using Kaplan-Meier, mortality risk ratios using multivariate Cox regression
Confounder control	Restriction, age, sex	Restriction, standardization, stratification	Restriction, multivariate adjustment, stratification
Sensitivity analyses	Prevalence rate ratio, Spearman's rank correlation	1-year SIRs in 100–200 pmol/L intervals, SIRs according to hospital or general practitioner patient status and according to previous morbidity	Complete case analysis, multiple imputations for missing cancer stage, cubic spline for 1-year cumulative survival

4. Results

4.1. Cobalamin-related biomarkers (study I)

In the study period, the distribution of Cbl levels in the hospital patients was as follows: <200 pmol/L, 9%; 200–600 pmol/L, 71%; 601–1000 pmol/L, 13%; >1000 pmol/L, 7%. The corresponding distribution in the general practitioner patients was as follows: <200 pmol/L, 11%; 200–600 pmol/L, 77%; 601–1000 pmol/L, 9%; >1000 pmol/L, 3%.

We included 818 patients, of whom 186 were classified as Cbl-treated. The proportion of Cbl-treated patients was higher with higher levels of plasma Cbl, and 39% of patients with elevated Cbl levels were Cbl-treated. Furthermore, total TC, holoTC, total HC, and sCD320 were all higher with higher plasma Cbl levels, while MMA was lower. We observed borderline impaired Cbl status in 62 patients (20%) with serum Cbl >250 pmol/L.

We found higher HC levels in non-treated patients and higher holoTC levels for Cbl-treated patients (see Appendix I for specific comparison). More patients had Cbl-related biomarkers levels above the upper reference limit in the groups with high serum Cbl than in the groups with low or normal serum Cbl. Most notably, high HC was seen in non-treated patients with serum Cbl >1000 pmol/L.

In correlation analyses, we found that sCD320 correlated positively with holoTC, serum Cbl levels correlated positively with holoTC and total HC, and MMA correlated negatively with total Cbl, holoTC, and total HC levels (see Appendix I for correlation coefficients).

Table 3. (Summary of Appendix I, Tables 1 and 2)

	Groups according to serum Cbl				
	Patients not Cbl-treated				
	<200 pmol/L (n=189)	**200–600 pmol/L (n=190)**	**601–1000 pmol/L (n=159)**	**>1000 pmol/L (n=94)**	
Age, mean (range)	54 (18–92)	58 (18–97)	56 (18–91)	62 (21–93)	[a]p=0.003
Sex, male (%)	67 (35%)	104 (55%)	73 (46%)	42 (45%)	
Serum Cbl, pmol/L [200–600]	170 (149–186)	329 (262–429)	716 (652–830)	1328 (1165–2113)	
Total TC, pmol/L [600–1500]	970 (800–1140)	960 (840–1180)	1070 (820–1320)	1100 (880–1420)	[b]p=0.0002
HoloTC, pmol/L [40–150]	41 (31–52)	64 (46–96)	150 (100–220)	180 (77–410)	[b]p<0.0001
Total HC, pmol/L [240–680]	510 (400–610)	620 (490–760)	730 (600–920)	1300 (900–2200)	[b]p<0.0001
sCD320, arb.u. [12–97]	17 (14–21)	18 (16–22)	21 (17–33)	24 (19–36)	[b]p<0.0001
MMA[c], µmol/L [<0.28]	0.24 (0.19–0.34)	0.19 (0.15–0.27)	0.16 (0.13–0.20)	0.17 (0.13–0.24)	[b]p<0.0001
Patients with measured MMA	n=155	n=160	n=120	n=68	
Impaired Cbl status[d]	–	41/124 (33%)	7/120 (6%)	14/68 (21%)	
	Patients Cbl-treated				
	<200 pmol/L (n=11)	**200–600 pmol/L (n=12)**	**601–1000 pmol/L (n=58)**	**>1000 pmol/L (n=105)**	
Age, mean (range)	48 (22–82)	47 (25–90)	65 (18–91)	68 (21–99)	[a]p=0.0003
Sex, male (%)	5 (45%)	2 (17%)	19 (33%)	32 (30%)	
Serum Cbl, pmol/L [200–600]	179 (148–181)	414 (258–484)	757 (673–884)	1358 (1129–2420)	
Total TC, pmol/L [600–1500]	1250 (1090–1400)	920 (780–1090)	980 (820–1380)	1300 (940–1880)	[b]p=0.005
HoloTC, pmol/L [40–150]	57 (44–66)	94 (79–116)	207 (145–290)	720 (370–1130)	[b]p<0.0001
Total HC, pmol/L [240–680]	480 (370–680)	500 (450–550)	590 (490–770)	680 (550–840)	[b]p=0.0005
sCD320, arb.u. [12–97]	20 (16–29)	18 (15–21)	23 (18–29)	32 (24–43)	[b]p<0.0001

Table 3. Characteristics and Cbl-related biomarkers of patients included in study I (n=818), divided according to Cbl treatment or no Cbl treatment. Cbl-related biomarkers are displayed with medians (interquartile ranges) [reference ranges]. [a]One-way analysis of variance for testing for mean age across Cbl level groups. [b]Kruskal-Wallis test for comparison of medians across Cbl groups. [c]Only measured in patients not Cbl treated with eGFR >60 mL/min/1.73 m^2. [d]Numbers and percentages of patients with impaired Cbl status among those with serum Cbl >250 pmol/L and eGFR >60 mL/min/1.73 m^2.

4.2. Disease associations (study I)

High serum Cbl levels were associated with alcoholism, liver disease, and cancer in a dose-response manner – the higher the Cbl levels the higher ORs we observed (Table 4). The prevalence of high Cbl levels for these three diseases combined was as follows: <200 pmol/L, 19.6%; 200–600 pmol/L, 28.9%; 601–1000 pmol/L, 35.2%; and >1000 pmol/L, 67.0%. The crude PRR showed an elevated risk of bronchopulmonary diseases in patients with Cbl levels >1000 pmol/L, while the CI of the adjusted OR included zero. Overall, the PRRs and ORs showed similar associations between disease prevalence and serum Cbl levels, although the ORs were farther away from the null than the PRRs.

We obtained similar results when disaggregating liver disease into alcoholic and non-alcoholic and cancer into malignant myeloid diseases, malignant lymphatic diseases, and solid tumors (see Appendix I Supplementary Data). Patients with serum Cbl levels of 200–600 pmol/L had no higher risk of any of the diseases included.

The levels of Cbl-related biomarkers across the different diseases are shown in Figure 1. The median levels of holoTC were highest in patients with alcoholism, liver disease, renal disease, and autoimmune diseases but stayed within reference range for all diseases, although wide interquartile ranges were seen for some diseases. For total HC, we found median levels above the upper reference limit in patients with alcoholism, liver disease, cancer, and renal, autoimmune, and bronchopulmonary diseases. The highest HC levels were seen in patients with cancer. Total TC and sCD320 showed little variation, except for higher total TC in autoimmune disease and higher sCD320 in renal disease.

Figure 1 (data derived from Appendix I, Table 4).

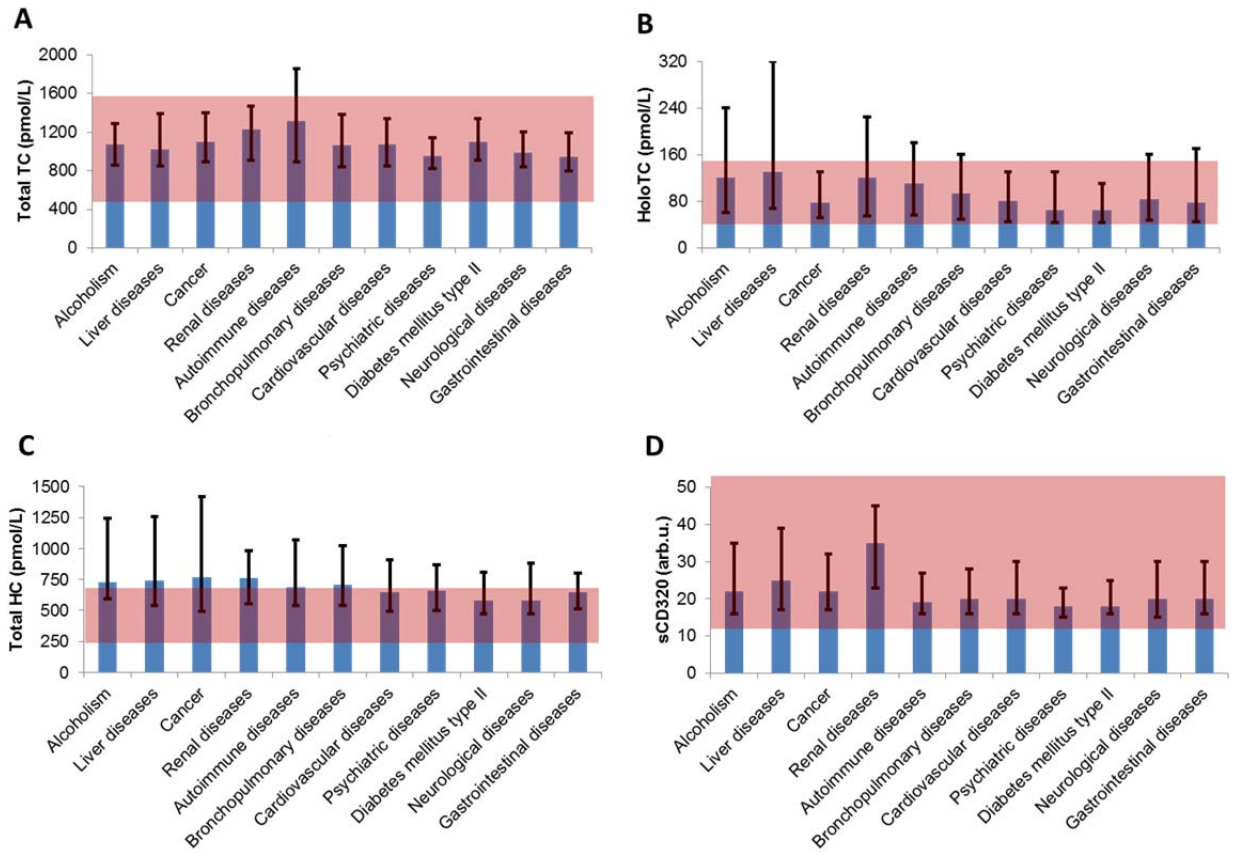

Figure 1. Median levels (bars) and interquartile ranges (error bars) of Cbl-related biomarkers in patients with the different included diagnoses irrespective of plasma Cbl levels. Reference ranges are marked in red. Numbers of patients with each disease are found in Table 4.

Table 4. (Appendix I, Table 3)

	Groups according to serum cobalamin (pmol/L)			
	<200	**200–600**	**601–1000**	**>1000**
Diagnoses	**n=189**	**n=190**	**n=159**	**n=94**
Alcoholism, n (%)	13 (6.9)	20 (10.5)	36 (22.6)	28 (29.8)
PRR (95% CI)	Ref	1.53 (0.78–2.99)	3.29 (1.81–5.97)	4.33 (2.35–7.97)
Adjusted[a] OR (95% CI)	Ref	1.35 (0.64–2.82)	3.73 (1.88–7.39)	5.74 (2.76–11.96)
Liver diseases, n (%)	8 (4.2)	13 (6.8)	22 (13.8)	24 (25.5)
PRR (95% CI)	Ref	1.62 (0.69–3.81)	3.27 (1.50–7.14)	6.03 (2.81–12.91)
Adjusted[a] OR (95% CI)	Ref	1.64 (0.66–4.09)	3.65 (1.57–8.50)	8.53 (3.59–20.23)
Cancer, n (%)	18 (9.5)	26 (13.7)	21 (13.2)	38 (40.4)
PRR (95% CI)	Ref	1.44 (0.82–2.53)	1.39 (0.77–2.51)	4.24 (2.57–7.02)
Adjusted[a] OR (95% CI)	Ref	1.24 (0.64–2.39)	1.30 (0.66–2.58)	5.48 (2.85–10.55)
Renal diseases, n (%)	11 (5.8)	13 (6.8)	19 (11.9)	7 (7.4)
PRR (95% CI)	Ref	1.18 (0.54–2.56)	2.05 (1.01–4.18)	1.28 (0.51–3.19)
Adjusted[a] OR (95% CI)	Ref	1.07 (0.46–2.48)	2.08 (0.95–4.56)	1.07 (0.40–2.88)
Autoimmune diseases, n (%)	15 (7.9)	12 (6.3)	12 (7.5)	9 (9.6)
PRR (95% CI)	Ref	0.80 (0.38–1.65)	0.95 (0.46–1.97)	1.21 (0.55–2.65)
Adjusted[a] OR (95% CI)	Ref	0.86 (0.39–1.91)	1.00 (0.45–2.21)	1.28 (0.53–3.11)
Bronchopulmonary diseases, n (%)	24 (12.7)	31 (16.3)	31 (19.5)	25 (26.6)
PRR (95% CI)	Ref	1.28 (0.78–2.10)	1.53 (0.94–2.50)	2.09 (1.27–3.46)
Adjusted[a] OR (95% CI)	Ref	1.16 (0.63–2.13)	1.58 (0.86–2.91)	1.89 (0.98–3.66)
Cardiovascular diseases, n (%)	35 (18.5)	37 (19.5)	31 (19.5)	26 (27.7)
PRR (95% CI)	Ref	1.05 (0.69–1.59)	1.05 (0.68–1.63)	1.49 (0.96–2.33)
Adjusted[a] OR (95% CI)	Ref	0.82 (0.46–1.45)	0.93 (0.51–1.68)	1.14 (0.60–2.16)
Psychiatric diseases, n (%)	46 (24.3)	53 (27.9)	47 (29.6)	18 (19.1)
PRR (95% CI)	Ref	1.15 (0.82–1.61)	1.21 (0.86–1.72)	0.79 (0.48–1.28)
Adjusted[a] OR (95% CI)	Ref	1.26 (0.78–2.02)	1.36 (0.84–2.22)	0.86 (0.46–1.62)
Diabetes mellitus type II, n (%)	29 (15.3)	22 (11.6)	13 (8.2)	12 (12.8)
PRR (95% CI)	Ref	0.75 (0.45–1.26)	0.53 (0.29–0.99)	0.83 (0.45–1.56)
Adjusted[a] OR (95% CI)	Ref	0.58 (0.31–1.08)	0.42 (0.21–0.86)	0.61 (0.29–1.28)
Neurological diseases, n (%)	29 (15.3)	23 (12.1)	30 (18.9)	22 (23.4)
PRR (95% CI)	Ref	0.79 (0.47–1.31)	1.23 (0.77–1.96)	1.53 (0.93–2.50)
Adjusted[a] OR (95% CI)	Ref	0.68 (0.37–1.23)	1.21 (0.68–2.13)	1.40 (0.74–2.64)
Gastrointestinal diseases, n (%)	50 (26.5)	47 (24.7)	52 (32.7)	24 (25.5)
PRR (95% CI)	Ref	0.94 (0.66–1.32)	1.24 (0.89–1.71)	0.97 (0.63–1.47)
Adjusted[a] OR (95% CI)	Ref	1.02 (0.64–1.64)	1.46 (0.91–2.34)	1.12 (0.63–1.99)

Table 4. Logistic regression analyses and prevalence rate ratios of disease associations with high serum Cbl, treating patients with Cbl levels <200 pmol/L as reference. Patients were allowed to have more than one diagnosis.

[b]Adjusted for age and sex.

4.3. Cancer risk (study II)

In total, we included 333,667 patients with Cbl levels >200 pmol/L. Furthermore, we excluded 19,164 patients treated with Cbl drugs prior to plasma Cbl measurement. A total of 19,665 patients had plasma Cbl levels >600 pmol/L, and 276,229 patients (83%) were classified as general practitioner patients. In the study population, there were 1,421,512 person-years of follow-up (median: 3.5 years) and 22,652 patients who were diagnosed with cancer. The overall cumulative incidences for the different Cbl level groups were as follows: 200–600 pmol/L, 6.7%; 601–800 pmol/L, 7.8%; and >800 pmol/L, 11.0%. When disaggregated according to follow-up intervals, the incidences were as follows for ≤1 year: 200–600 pmol/L, 2.3%; 601–800 pmol/L, and 3.7%; >800 pmol/L, 6.6%; for >1 year, they were as follows: 200–600 pmol/L, 4.4%; 601–800 pmol/L, 4.1%; and >800 pmol/L, 4.4%.

In Table 5, the SIR estimates can be seen. The overall SIR for all cancers was 1.26 (1.24–1.28). We found higher cancer risk with higher plasma Cbl levels for all ≤1 year strata of sex, age, and cancer group, except for immune-related cancers. In the four cancer groups, we found the strongest association for <1 year risk of hematological cancers and smoking and alcohol-related cancers. These two cancer groups also showed a clear dose-response relation with increasing Cbl levels (Figure 2).

Figure 2 (Appendix II, Figure 1).

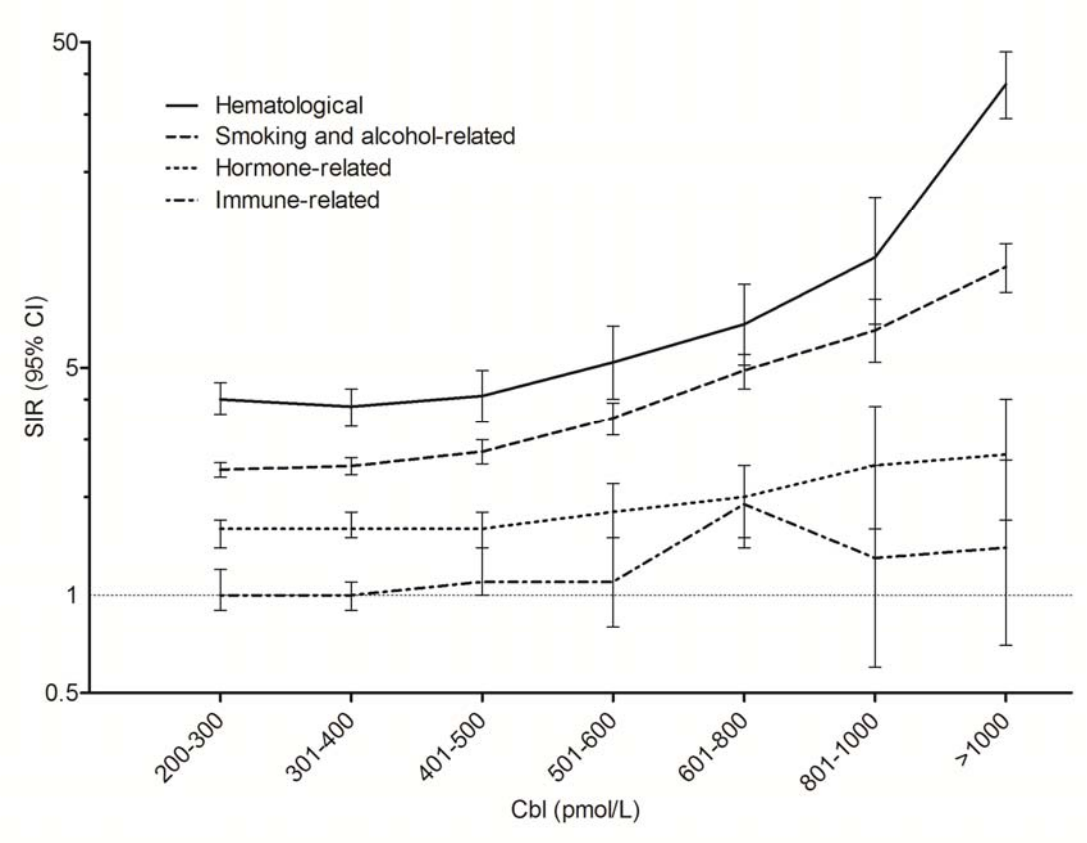

Figure 2. ≤1-year SIRs (vertical bars are 95% CIs) for the four different cancer groups, disaggregated according to plasma Cbl levels in 100–200 pmol/L intervals. The scale on the vertical axis is logarithmic.

Table 5 (Appendix II, Table 2).

				Plasma Cbl levels		
	Persons, n	**Incident cancers, n**	**Overall SIR (95% CI)**	**200–600 pmol/L (n=314,002)**	**601–800 pmol/L (n=12,909)**	**>800 pmol/L (n=6,756)**
All Cancers	333,667	22,652	1.26 (1.24–1.28)	1.23 (1.21–1.24)	1.61 (1.51–1.71)	2.38 (2.22–2.56)
<1 year SIR (95% CI)		8,103	2.17 (2.13–2.22)	2.04 (1.99–2.09)	3.44 (3.14–3.76)	6.27 (5.70–6.88)
>1 year SIR (95% CI)		14,549	1.02 (1.00–1.04)	1.01 (1.00–1.03)	1.09 (1.00–1.18)	1.24 (1.10–1.39)
Men	135,485	10,815	1.36 (1.34–1.39)	1.32 (1.29–1.35)	1.93 (1.75–2.12)	2.94 (2.64–3.27)
Women	198,182	11,837	1.18 (1.16–1.20)	1.15 (1.13–1.17)	1.43 (1.31–1.55)	2.05 (1.86–2.27)

Age						
0–50 years	142,000	2,411	1.29 (1.24–1.34)	1.26 (1.20–1.31)	1.63 (1.32–2.00)	2.99 (2.36–3.72)
<1 year SIR (95% CI)		752	2.26 (2.10–2.43)	2.14 (1.98–2.30)	3.23 (2.24–4.52)	9.04 (6.46–12.31)
>1 year SIR (95% CI)		1,659	1.08 (1.03–1.14)	1.07 (1.01–1.12)	1.28 (0.97–1.64)	1.77 (1.26–2.42)
≥51 years	191,667	20,241	1.26 (1.24–1.27)	1.22 (1.21–1.24)	1.61 (1.50–1.71)	2.33 (2.15–2.51)
<1 year SIR (95% CI)		7,351	2.17 (2.12–2.22)	2.03 (1.98–2.08)	3.46 (3.14–3.79)	6.09 (5.51–6.71)
>1 year SIR (95% CI)		12,890	1.01 (1.00–1.03)	1.01 (0.99–1.03)	1.07 (0.97–1.17)	1.19 (1.05–1.34)
Cancer groups						
Smoking and alcohol-related		9,501	1.46 (1.43–1.49)	1.40 (1.37–1.43)	2.13 (1.95–2.33)	3.05 (2.74–3.39)
<1 year SIR (95% CI)		3,799	2.75 (2.67–2.84)	2.56 (2.47–2.65)	4.89 (4.30–5.54)	8.37 (7.31–9.55)
>1 year SIR (95% CI)		5,702	1.11 (1.08–1.14)	1.09 (1.07–1.12)	1.33 (1.17–1.52)	1.44 (1.20–1.71)
Hematological		1,748	1.85 (1.76–1.94)	1.72 (1.63–1.81)	2.27 (1.79–2.85)	7.96 (6.66–9.44)
<1 year SIR (95% CI)		912	4.52 (4.23–4.82)	4.03 (3.75–4.32)	6.82 (5.08–8.97)	24.14 (19.51–29.54)
>1 year SIR (95% CI)		836	1.12 (1.05–1.20)	1.10 (1.02–1.18)	0.94 (0.60–1.40)	2.99 (2.12–4.11)
Immune-related		3,565	0.93 (0.90–0.96)	0.92 (0.89–0.95)	1.17 (1.00–1.37)	0.97 (0.74–1.23)
<1 year SIR (95% CI)		834	1.08 (1.01–1.16)	1.04 (0.97–1.12)	1.89 (1.42–.2.46)	1.37 (0.84–2.12)
>1 year SIR (95% CI)		2,731	0.89 (0.85–0.92)	0.88 (0.85–0.92)	0.97 (0.80–1.18)	0.85 (0.62–1.14)
Hormone-related		5,116	1.10 (1.07–1.13)	1.10 (1.06–1.13)	1.12 (0.96–1.29)	1.19 (0.96–1.45)
<1 year SIR (95% CI)		1,555	1.62 (1.54–1.70)	1.59 (1.51–1.67)	1.96 (1.53–2.47)	2.61 (1.92–3.47)
>1 year SIR (95% CI)		3,561	0.96 (0.93–0.99)	0.97 (0.94–1.00)	0.88 (0.72–1.05)	0.78 (0.57–1.03)

Table 5. Risk of cancer diagnosed after a plasma Cbl measurement according to sex, follow-up interval, age, cancer group, and plasma Cbl levels.

We assessed the risk of 10 specific cancer types and found the same dose-response pattern for ≤1 year SIRs (Figure 4A). We found the strongest association for myeloid malignancies and liver cancer, and these two cancer types also showed elevated cancer risk when exceeding the first year of follow-up (Figure 4B). The estimates that were disaggregated according to existing morbidity showed results similar to the overall results (data not shown).

Figure 3 (Appendix II, Figure 2 and Supplementary Table 3)

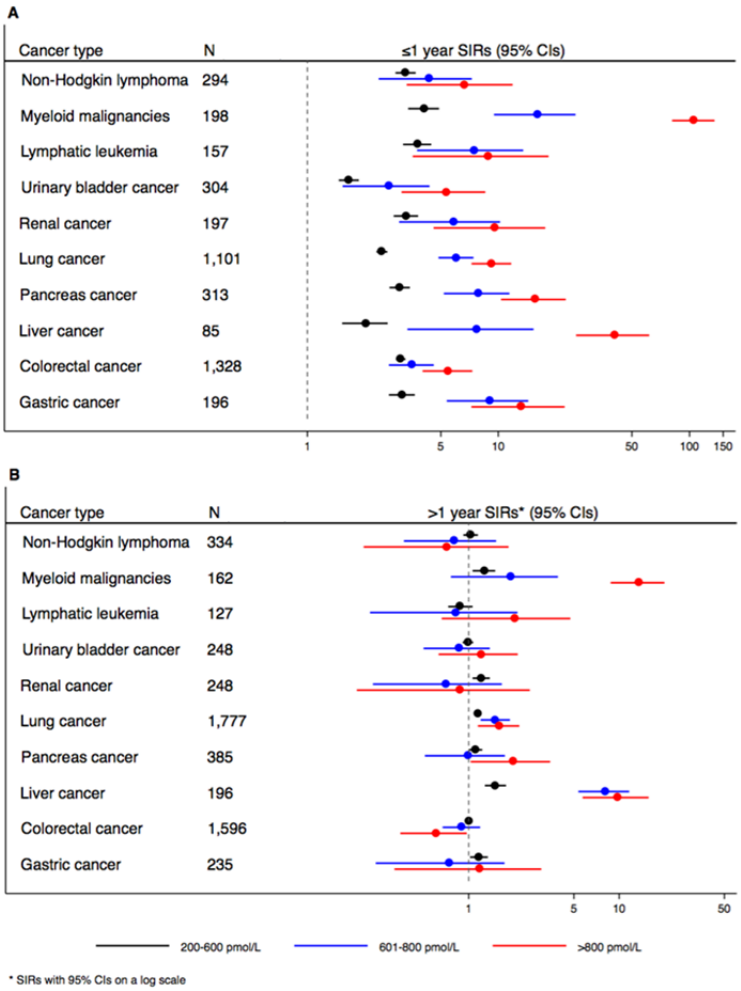

Figure 3. SIRs (95% CIs) and numbers for specific cancer types diagnosed ≤1 year (A) and >1 year (B) after plasma Cbl measurement. SIRs were disaggregated according to Cbl levels: black lines and dots: 200–600 pmol/L; blue lines and dots: 601–800 pmol/L; and red lines and dots: >800 pmol/L. The vertical dotted line indicates SIR=1. The scale on the horizontal axis is logarithmic.

4.4. Mortality (study III)

The populations in study III consisted of 25,017 cancer patients with a plasma Cbl measurement up to one year prior to diagnosis (patient cohort) and a matched comparison cohort of 61,988 cancer patients with no prior plasma Cbl measurement. Patients with higher Cbl were more often females, more often had non-localized or unknown cancer stage, and had higher CCI score compared to patients with normal Cbl levels and compared to the comparison cohort. The distribution of different cancer types was not similar across the three Cbl level groups (Appendix III, Table 1).

We observed a lower survival in the patient cohort compared to the comparison cohort (Figure 4). Patients in the two groups with high Cbl levels had lower survival than patients with reference range/normal values, both overall (Figure 4A) and when stratifying according to cancer stage, localized (Figure 4B) or non-localized (Figure 4C). The same dose-response pattern was observed when follow-up intervals were disaggregated (30 days, 31–90 days, and 91–365 days). In all follow-up strata, the comparison cohort had higher cumulative survival proportions (Appendix III, Table 2).

Figure 4 (Appendix III, Figure 1).

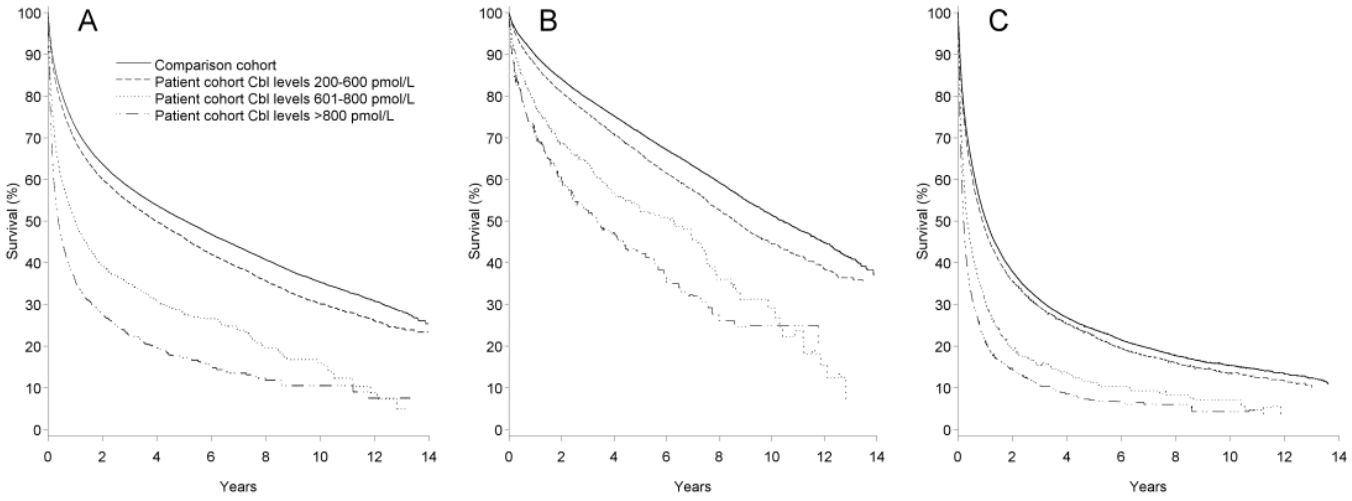

Figure 4. Kaplan-Meier curves for overall survival in percentage, for all patients (A) and when disaggregated according to localized cancer (B) or non-localized cancer (C). We used multiple imputations in this model to account for missing cancer stage. Lymphatic leukemia and malignant myeloid diseases were not included panels B and C.

We estimated relative mortality risk using a Cox regression model and found a dose-dependent association between higher Cbl and higher mortality risk. The overall one-year MRRs (95% CIs) were as follows: 601–800 pmol/L vs. 200–600 pmol/L, 1.7 (1.6–1.8) and >800 pmol/L vs. 200–600 pmol/L, 2.3 (2.1–2.5). We found the highest MRR estimates for follow-up of 30 days, but the estimates attenuated with longer follow-up time (Table 6). The elevated MRRs persisted for up to 4 years after the index date and remained similar for patients with localized and non-localized cancer (Appendix III, Supplementary Table S3). We observed similar results when stratified according to sex, age, calendar year of diagnosis, and cancer stage (Table 6).

Table 6 (Appendix III, Table 3).

	Mortality risk ratios (95% CIs)								
	30 days			31–90 days			91–365 days		
	601–800 vs. 200–600	>800 vs. 200–600	P[a]	601–800 vs. 200–600	>800 vs. 200–600	P[a]	601–800 vs. 200–600	>800 vs. 200–600	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
≥94	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									
Localized	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-localized	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

Table 6. MRRs with corresponding 95% CIs computed by Cox regression analyses using patients with Cbl levels of 200–600 as the reference group. The analyses were adjusted for age, sex, calendar year, CCI score, and cancer stage (not when the variable was used for stratification). We used multiple imputations in this model to account for missing cancer stage. [a] Wald chi-square test for equality in MRR estimates.

We also assessed cumulative survival proportions and MRRs for 11 specific cancer types. We observed the same dose-response pattern of lower cumulative survival proportions with higher Cbl levels for all cancers within 30 days, at 31–90 days, and 91–365 days of follow-up (Appendix III, Supplementary Data Table S5). In patients with high Cbl levels, we found higher MRRs for gastric, colorectal, liver, breast, prostate, and urinary bladder cancer, and the results were highest within 30 days from the index date and for the Cbl level group of >800 pmol/L. Most estimates attenuated with follow-up time exceeding 30 days and for patients with Cbl levels of 601–800 pmol/L.

We assessed the validity of the multiple imputations model by comparing the estimates obtained by multiple imputations with the estimates obtained by complete case analysis, and the estimates were essentially comparable (see Study III, Supplementary Data Tables S2, S4, and S6). The imputed model enabled the computation of estimates for some specific cancer types that the complete case analysis could not produce.

5. Discussion

5.1. Conclusions

The main findings in study I were as follows. First, Danish patients had a relatively high prevalence of elevated Cbl levels, with 20% in hospital patients and 12% in general practitioner patients, and only a proportion was attributable to high-dose Cbl treatment. Second, the elevated Cbl levels could be ascribed to altered Cbl metabolism, namely, elevated HC levels and to a lesser degree, high holoTC levels. The main diseases associated with high Cbl levels were alcoholism, liver disease, and cancer, and high HC was also found in these diseases. In the clinical setting, these three diseases could be relevant to assess in patients with elevated Cbl levels, especially if levels are very high, because these diseases are prevalent in two thirds of hospital patients with Cbl levels >1000 pmol/L. The potential of HC as a biomarker for these diseases warrants further examination.

The association between cancer and elevated Cbl levels prompted us to investigate this relation further in a large population-based cohort study. Here, we demonstrated a dose-response relation between cancer risk and plasma Cbl levels, a risk that was high mainly within the first year of plasma Cbl measurement. The main cancer types were hematological cancers and solid tumor cancers related to smoking and alcohol, such as cancers of the stomach, liver, pancreas, colon and rectum, lung, kidney, and urinary bladder. We conclude that elevated Cbl levels can mark prevalent or occult cancer and therefore can be an early sign of clinical importance. Further studies will need to examine whether elevated Cbl levels can lead to earlier cancer diagnosis, which in turn could have major clinical implications.

We also found that elevated pre-diagnostic plasma Cbl levels were associated with higher mortality in cancer patients. This association was also dose-response dependent and was

strongest within a short time span from cancer diagnosis. Thus, elevated Cbl levels mark aggressive cancer with high mortality and measuring plasma Cbl levels in cancer patients may provide an easily accessible tool for estimating prognosis at the time of cancer diagnosis. Future research will determine if this is the prognostic meaning of elevated Cbl levels in cancer patients.

5.2. Potential bias and data accuracy

Several methodological considerations are required to correctly interpret our findings. First, the internal validity is considered in a discussion of the effects of random error and systematic error in our studies, and systematic error is further discussed separately for selection bias, information bias, and confounding. Last, the external validity, or generalizability, is discussed.

5.2.1. Random error

Random error can occur if risk estimates are computed using a small study population or subgroup and manifests as wide CIs for the corresponding risk estimates. In study I, the ORs were used to estimate the PRR of diseases between patients with low and increasing levels of Cbl. The computed ORs had wide 95% CIs and may have overestimated the disease due to the relatively high disease prevalence in the population. The imprecision and potentially inflated ORs in turn hamper the inference made from these estimates because the associations could have occurred only by chance. This possibility cannot be precluded, but the consistency with previous studies and the dose-response association support that our findings were not merely due to chance. Furthermore, the elevated HC levels in patients with these diseases are also consistent with earlier findings. Finally, we failed to demonstrate an association between elevated Cbl levels and renal and autoimmune disease and found only a borderline association with

bronchopulmonary disease. These results could be because of the small sample size, and if these associations were indeed true, our findings might lead to a type II error.

For studies II and III, the large study populations minimized random errors, although subgroup analyses yielded imprecise estimates in some cases.

5.2.2. Selection bias

Selection bias can arise if study population sampling is subject to systematic error. In cross-sectional studies like study I, selection bias may arise if patients are selected differently according to the exposure and/or outcome. Thus, selection bias would occur if the probability of having a plasma Cbl measurement correlated with the disease prevalence. In the extreme, the inclusion of only hospital patients could have introduced such Berkson's bias (197), a type of selection bias that create associations between the exposure and the outcome merely because both frequently occur in a hospital setting. Study I share this potential bias with previous studies (32-37). In the ideal setting, we would include patients before the relevant disease became incident (the exposure), and the alterations in levels of plasma Cbl and/or Cbl binding-proteins could be measured over time until the disease became incident. We chose to focus on the implications of elevated Cbl levels for the diseases under study when these diseases were prevalent.

In study I, we deliberately oversampled patients with Cbl levels below and above the reference range to investigate the hypothesis that different diseases are associated with low, normal, or high plasma Cbl levels. Therefore, the set number of 200 patients in each of the four Cbl level groups was achieved earlier during the study period for the group with normal levels (183) than for the other three groups. This difference in timing could introduce bias if either the exposure

(plasma Cbl levels in the four different groups) or the outcomes (the diseases under study) had different prevalence during the study period. Such variation is unlikely; therefore, we consider the risk of this bias to be minimal. In subanalyses in studies II and III, we classified patients as hospital-treated if they had a record of hospital contact within a short period of the plasma Cbl measurement. Those with no contact were considered as patients treated by general practitioners. The use of hospital records to classify patients is, indeed, a crude proxy, and we did not distinguish between the hospital patients who were admitted and those who were treated at outpatient hospital clinics. However, we observed very similar results for the two patient populations and retrieved the dose-response association between short-term cancer risk and increasing Cbl levels. These outcomes imply that such potential selection bias had little effect on our risk estimates.

We had no information on the underlying reason for requesting plasma Cbl levels, which is also a limitation in studies II and III. Without knowing the indication, it is difficult to assess whether this selection correlated with disease prevalence or whether it was unequally distributed across Cbl levels. In this context, the selection based on the indication for measuring plasma Cbl can introduce bias because of confounding by indication. We can only speculate what the actual indications were for measuring plasma Cbl levels. If the indication was diagnosis of symptoms potentially caused by Cbl deficiency, e.g., megaloblastic anemia, then it is likely that other diseases also associated with megaloblastic anemia would be highly prevalent. This could have introduced bias away from the null in the association we demonstrated between elevated Cbl levels and alcoholism, liver disease, and hematological cancers. In addition, nonspecific fatigue, malaise, or other symptoms associated with Cbl deficiency could also be early symptoms of severe disease, including cancer. However, if the indication was unspecific screening for Cbl

deficiency, then the confounding effect is likely less dominant. Studies on the request pattern for plasma Cbl measurement show that the indication is not restricted only to patients with symptoms or risk factors for Cbl deficiency (2, 3), which could suggest that the indication could be nonspecific or opportunistic screening and therefore not necessarily a strong confounding factor. In opposition to this, Ryg et al. (94) argued that the majority of patients with Cbl levels >1200 pmol/L were already suspected of cancer at the time of Cbl measurement, although they did not assess if such cancer was also suspected in patients with lower Cbl levels or patients who were not diagnosed with cancer. We did not include information on the use of other diagnostic procedures (scans, other biomarkers, etc.) in our studies. Thus, we did not assess whether these were more frequently performed in patients with high Cbl levels. Furthermore, other biomarker abnormalities could have been detected simultaneously with elevated Cbl levels. If the current online guidelines were followed (26), these could include abnormal hematological parameters and biomarkers for inflammation, liver disease, and renal function. Consequently, the use of several diagnostic procedures among the patients in the study populations could have biased our results because the indication for measuring plasma Cbl would then related to these procedures. As an example, in study II, we reported that the <1 year SIRs were elevated even for patients with Cbl levels within the reference range, as found for hematological, smoking, and alcohol-related and hormone-related cancers. Moreover, we observed a lower risk of hormone-related and immune related cancers after the first year of follow-up. We interpret this as a compensatory deficit – that suspicion of cancer could be underlying the indication for measuring plasma Cbl levels, possibly due to a concurrent request for other diagnostic procedures or other biomarkers abnormalities. Thus, measurement of plasma Cbl levels is probably not associated with the prevalence of these cancers but potentially with more extensive diagnostic testing. However, it is

implausible that the observed dose-response association between cancer risk and elevated Cbl levels and the robustness in the stratified estimates was merely caused by confounding by indication. If so, such confounding should follow a similar dose-response pattern and have equal effect across the strata.

In study III, we sampled a matched comparison cohort of cancer patients. They differed by not having a plasma Cbl measurement. As seen in Figure 4, there was a difference in survival between the comparison cohort and the patient cohort with measured plasma Cbl. But the numerical difference was larger between patients with normal and high Cbl levels than between the comparison cohort and the patients with normal Cbl levels, both for patients with localized and non-localized cancer. It is unlikely that these results were fully attributable to confounding by indication. Only the difference in survival between the patient and the comparison cohort was attributable to the effect of indication. Other aspects of confounding are discussed in 5.2.4.

In studies II and III, we used only population-based registries that are considered complete and with virtually complete follow-up. In general terms, the use of population-based registries within the context of a universal health care system, such as that in Denmark, will minimize selection bias (177). However, other than the confounding by indication (outlined above and in 5.2.4), there is a risk of immortal person time bias in the selection of our study populations. In study I, patients had to be alive with their prevalent disease(s) to have a serum Cbl measurement. Thus, death could not have occurred in the time between disease incidence and serum Cbl measurement. The same applies to studies II and III, except the immortal time was from plasma Cbl measurement to cancer diagnosis. Because mortality is associated with elevated Cbl levels (both in our study and those of others (94, 171-175, 198-202)), we may have underestimated the true associations in all three studies because we might not have included the most severely ill

patients. Immortal person time could also have influenced the results in study III, but in a different direction. We found that the median time from Cbl measurement to cancer diagnosis was lower in patients with elevated Cbl levels, although there was some overlap between the three Cbl level groups. Thus, the potential immortal time from plasma Cbl measurement to cancer diagnosis was longest for patients with normal Cbl levels. This difference could have biased our results, especially for short-term survival. We chose to include the plasma Cbl measurement closest to cancer diagnosis to have the levels that most likely represented prevalent cancer, but at the potential expense of bias due to immortal person time.

5.2.3. Information bias

As explained in section 3.5., the exposures in all three studies are etiologically the diseases under study. Nonetheless, the studies were designed using serum/plasma Cbl as exposure and the diseases as outcomes. Therefore, in the following assessment of information bias, serum/plasma Cbl levels are referred to as the exposure and the outcomes are the diagnoses.

5.2.3.1. Misclassification of exposure

In study I, we used a validated, automated platform for measuring serum Cbl. This platform is not vulnerable to interference by intrinsic factor autoantibodies (93), and we used a cobinamide-sepharose pre-analysis treatment to analyze plasma Cbl in patients with high HC levels (89). Furthermore, patient samples with concentrations above the upper measurement limit were diluted to obtain a more accurate concentration.

We were unable to assess the specific analytical platforms providing measurements of plasma Cbl for studies II and III because this information was not available in the LABKA database

(178). However, the included measurements showed a high degree of comparability across the different codes used for identifying plasma Cbl measurement in the database for the study periods (Appendix IV).

5.2.3.2. Misclassification of outcome

The diagnoses included in study I were not validated according to strict diagnostic criteria but were included if they were found in the medical files of the patient. Thus, we trusted the validity of the diagnosis to the physician. We deliberately avoided using our own inference of, e.g., other biomarkers to diagnose patients.

The outcome in study II was cancer, and the mandatory registration and the unified Danish health care system ensures high accurateness and completeness of the DCR. The DCR has been validated to confirm this (203, 204). Hence, the risk of misclassification of cancer diagnosis in studies II and III is minimal. Only cancer stage was not completely registered, and to avoid bias due to missing cancer stage, we used multiple imputations in our analyses in study III. This approach has been shown to provide less biased estimates than other strategies for handling missing information on covariates (186-188). The comparison with the complete case analyses supported this analytical approach.

For all three studies, we cannot preclude that detection bias could have been introduced. This bias might arise if physicians were more alert towards the diseases under study in the patients and if the awareness differed according to different Cbl levels. If they were more alert towards alcoholism, liver disease (study I), and cancer (study I+II) in patients with elevated Cbl levels, the bias would be away from a null association and the opposite for patients with low or normal plasma Cbl levels. For study III, detection bias due to increased cancer awareness would have

introduced bias towards the null because it would have led to earlier cancer diagnosis and potentially better prognosis. However, the awareness of the diseases associated with high Cbl levels is generally very low, although this argument is based on personal communications with physicians and other scientists during the period where the studies were undertaken.

5.2.4. Confounding

Confounding is defined as a factor associated with the exposure and causing the outcome, thereby mixing or confusing the effect measure with something other than the exposure, i.e., confounding the effect estimates. The confounding factor cannot be a part of the causal path. Confounding is inherent to observational research because the possible confounding factors may not be evenly distributed in the study population as they would be in randomized studies.

We studied plasma Cbl levels as a diagnostic and prognostic marker for disease, not as a causal risk factor. In the following, I argue that mainly the unmeasured confounding effect of alcohol and alcohol-related diseases could have biased our results.

In study I, we adjusted for age and sex in the logistic regression model to eliminate their effect on disease prevalence. The proportion of females was higher, plasma Cbl levels show a mild decline in the elderly (192), and the disease prevalence differs according to sex and age. We chose not to adjust mutually for the other diseases and allowed patients to be categorized with more than one disease because our study was not powered for multiple adjustment. In essence, study I was conducted to get a better understanding of the biomarker alterations in patients with elevated Cbl levels and secondarily to crudely estimate the disease prevalence in patients with elevated Cbl levels.

To minimize confounding in study III, we adjusted for age, sex, calendar year of diagnosis, cancer stage, and comorbidity in the Cox regression analysis. We used the CCI to adjust for comorbidity (189). Thus, we adjusted for the overall confounding effect of comorbidity on mortality risk; however, we did not adjust specifically for diseases that cause elevated Cbl levels, e.g., liver disease. We could have created a model to assess whether the association between mortality and elevated Cbl levels was independent of the specific diseases other than cancer that cause elevated Cbl levels.

For all three studies, we lacked information on lifestyle factors, e.g., smoking, alcohol consumption, diet, and exercise. Only excessive alcohol consumption was included in study I, where we assessed alcoholism and alcoholic liver disease as an outcome. Thus, we cannot exclude the possibility that alcoholism and alcoholic liver disease might have confounded the association between cancer and high plasma Cbl levels in study I, between alcohol-related cancers and high plasma Cbl levels in study II, and between mortality in cancer patients and high plasma Cbl levels in study III. For all three associations, alcoholism and alcoholic liver disease are strong risk factors for these cancers and for mortality, and alcoholism and alcoholic liver disease prior to Cbl measurement could induce elevated Cbl levels. Furthermore, Simonsen et al. (50) showed that Cbl and HC levels were very similar in patients with chronic liver disease and liver cancer.

The association between smoking and Cbl levels is not clear-cut. Some studies report lower Cbl levels among smokers (205-206) while others report no association between the two (207-209). A large cohort study by Ulvik et al. (210) found no long- or short-term effects of smoking on plasma Cbl levels. With regard to diet, it is not thought that a regular diet can increase plasma

Cbl levels substantially because absorption under physiologic conditions is saturated at 6 µg/day (211).

Treatment with high-dose Cbl gives rise to elevated Cbl levels, but high-dose B vitamin treatment (both with and without Cbl) is not associated with cancer risk, according to a recent review (212). Nonetheless, we chose not to analyze diagnostic associations in study I for Cbl-treated patients, and we excluded these patients from studies II and III. The rationale for this was that the plasma Cbl levels would reflect the treatment rather than any effect that the disease may have on Cbl levels.

5.2.5. External validity

The results obtained in study I were for hospital patients and cannot be readily extrapolated to patients treated at their general practitioner's office. These latter patients will more likely have a lower burden of morbidity and cancer risk factors than hospital patients. The association may, however, very well also be found in this generally healthier patient population, given the biologically plausible mechanisms underlying the associations demonstrated. We further support this plausible biological explanation by showing the specific alterations in Cbl-related biomarkers underlying the associations. The elevation of HC, and in part also holoTC, is also consistent with previous literature. Therefore, it is plausible that elevated Cbl levels are present in patients with alcoholism outside the hospital; it is plausible that liver disease also produces high Cbl levels, even though it is not diagnosed in the hospital setting; and it is plausible that hematological and other cancers are also associated with elevated Cbl levels regardless of the health care setting.

Furthermore, in study II, we identified hospital patients to separate them from general practitioner patients. Several characteristics may differ between the two populations, including the burden of morbidity, lifestyle, medication use, and indication for plasma Cbl measurement. Nonetheless, the cancer risk estimates were essentially the same regardless of whether the patients were classified as hospital-treated or not.

5.3. In the context of existing literature

5.3.1. Study I

Reviewing the literature, only six studies were found that assessed the overall disease associations with high plasma Cbl levels (32, 34-37). They reported that high Cbl levels were associated with cancer and liver, renal, and autoimmune diseases. However, their findings were not fully consistent. Study I confirmed some of these previous finding. Hence, elevated plasma Cbl levels were associated with alcoholism, liver disease, and cancer, but not with renal and autoimmune diseases. Study I was also the first to report measured concentrations of Cbl-related biomarkers in a population of patients referred for plasma Cbl measurement, showing that overall the diseases that were associated with elevated plasma Cbl levels were also related to elevated HC levels and borderline high holoTC levels. This result is in accordance with most studies on Cbl levels and Cbl metabolism in specific disease entities. We supported that in liver disease, the predominantly elevated Cbl-related biomarker is HC (38, 47, 50), while an increase in holoTC can mark acute liver damage (50). Furthermore, our results reinforce that both holoTC and HC are high in renal disease, as demonstrated by previous studies (35, 57, 60-64, 69). In hematological cancers (97, 101) and liver cancer (50, 111, 113-115), high HC levels give rise to

high Cbl levels, and high HC levels in cancer patients were also demonstrated in study I. Finally, our novel biomarker, sCD320 (184), was not associated with any diseases in our study.

5.3.2. Study II

The study of cancer risk in patients with Cbl measurements demonstrated that elevated Cbl levels were strongly associated with hematological cancers and liver cancer. Most previous studies on these cancer types were cross-sectional (50, 95, 97-102, 111-117). Study II contributed to existing knowledge by demonstrating that Cbl levels are high even before the diagnosis is made and that the risk of these cancer types remains elevated after the first year. The risk of hematological cancers was elevated even 5 years after the measurement of high Cbl levels, suggesting that malignant hematological cells influence Cbl metabolism many years before the cancer is clinically manifest.

For liver cancer, the study by Chang et al. (116) is the only one that provided relative cancer risk estimates by comparing liver cancer risk across Cbl levels. Their study also measured plasma Cbl levels in prevalent cancer cases. However, plasma Cbl levels were divided into quartiles, and their upper quartile was ≥324 pmol/L, hindering them from demonstrating an association with high Cbl levels and hampering a comparison of their results with ours. Still, in concordance with our findings, they also demonstrated an association between increasing plasma Cbl levels and gastric and esophageal cancers with their relatively low upper quartile cut-off.

The majority of studies on plasma Cbl levels and risk of cancers (120, 121, 123, 124, 126-128, 130, 131, 133-141, 144-167) have assessed plasma Cbl as a causal risk factor. We studied the opposite, i.e., whether plasma Cbl levels can mark an as-yet-undiagnosed cancer, and we demonstrate an association with several of the cancer types where the other studies reported null

associations. Several of these other studies performed sensitivity analyses or excluded prevalent cancers to avoid mixing associations with different causal directions. In contrast to our study, most of the previous studies (120, 121, 123, 124, 126-128, 130, 131, 133-141, 144-167) disaggregated plasma Cbl levels according to percentiles, and did not employ reference range cut-offs to specifically define elevated Cbl levels. These issues taken together explain why they reported null associations.

5.3.3. Study III

In study III, we demonstrated an association between elevated plasma Cbl levels and low survival in cancer patients. Geissbühler et al. (172), Salles et al. (175) and Byström et al. (171) applied cut-offs at lower plasma Cbl levels than in the present study. We validated our chosen cut-off using a cubic spline curve (Appendix III, Supplementary Figure S1). Salles et al. reported combined estimates for patients with and without cancer using a cut-off of 400 pmol/L, and their cited 90-day cumulative survival was higher than ours (175). Byström et al. (171) employed a cut-off of 300 pmol/L but did not provide specific 90-day survival estimates. Based on visual inspection of their Kaplan–Meier curve, they too demonstrated better survival for colorectal cancer patients than in our study. Lin et al. (174) used higher cut-offs than ours and showed better overall survival but reported higher hazard ratios than we did. In their study, Ryg et al. (94) reported a one-year survival of 31% with a cut-off of 1200 pmol/L, an estimate very similar to our own finding of 35.8% (95% CI: 33.2–38.4). The different cut-off for plasma Cbl may explain the slightly higher estimates in our study.

Our study further substantiated this association and provided additional insights. We included a large cohort of cancer patients and could provide results for several specific cancer types.

Opposite the other studies (94, 171-175), we used plasma Cbl measurements performed prior to cancer diagnosis. Furthermore, we obtained information on several confounding factors and included it in a regression model, yielding confounder-adjusted estimates.

6. Implications

The implications of this thesis are intriguing but offer more perspectives than definitive conclusions. First, we confirmed that several diseases are highly prevalent among hospital patients with high plasma Cbl levels. Thus, high Cbl levels should not be considered a harmless chance finding on the laboratory sheet but instead should lead to critical evaluation of the patient's condition and other diagnostic tests. Further investigation could mean earlier diagnosis of these diseases, with the potential for earlier intervention and treatment. In particular, our results are interesting for early cancer diagnosis. The finding of elevated Cbl levels may help direct attention towards cancer but may also be a guide to which cancers are relevant to suspect and which are not. For some cancers, namely hematological cancers and liver cancer, the elevated risk exceeding the first year of follow-up implies that elevated Cbl levels could perhaps shorten any diagnostic delay of these cancer types.

High Cbl levels do not equal a cancer diagnosis, however, and more harm than benefit can be done by focusing on the high comparative cancer estimates without paying attention to the relatively low absolute cancer risk estimates that we report. Only 6.6% of patients with Cbl levels >800 pmol/L were diagnosed with cancer within one year of plasma Cbl measurement.

The very robust association between high mortality and elevated Cbl levels can have direct implications for the assessment of prognosis for cancer patients in the near future. The existing literature on this topic also lends support to the use of plasma Cbl levels as a prognostic biomarker. Estimating prognosis for cancer patients is essential for clinical decision-making in terms of deciding treatment modalities and intensity. Moreover, it can have social and psychological consequences for the individual patient if prognosis can be estimated precisely and reliably.

7. Perspectives

The three-step structure of the studies included in this thesis provides interesting perspectives and generates several new questions for future research. The associations between elevated Cbl levels and alcoholism and liver disease warrants new studies with larger study populations because the estimates were imprecise. Also, the results were not produced in the setting of general practice, and given the large patient population in general practice, there can be major implications for assessing elevated Cbl levels for early diagnosis for these diseases. The interesting finding of an association between plasma Cbl levels and prevalent or occult cancer provides openings for a number of future studies. The lack of information about the indication for requesting plasma Cbl measurement obviously deserves attention. If a suspicion of cancer is not already present at the time of Cbl measurement, then that could have major implications for earlier cancer diagnosis. More knowledge is also needed, however, to establish which patients with elevated Cbl levels are at high risk of cancer and which are not. These data could potentially be obtained if future studies included information on other abnormal biomarkers or the underlying symptoms leading to plasma Cbl measurement. Also, future studies should include more exact information on the population under study to disaggregate hospital patients from patients in general practice. The alterations in Cbl metabolism found in cancer patients imply that measurement of Cbl-related biomarkers may give a better understanding of the pathogenesis underlying the association between cancer and elevated Cbl levels, and also potentially help elucidate the application of these biomarkers in early diagnosis of specific cancer types. If diagnostic delay could be shortened by findings confirming that elevated Cbl levels or levels of Cbl binding proteins are early signs of cancer, the implications would be major.

There are still unresolved issues around the association between elevated Cbl levels and mortality. First, prospective studies with systematic measurements of plasma Cbl levels in cancer patients are needed to better assess the implications of high plasma Cbl levels in these patients. Other studies have found that patients without cancer also have a lower survival with higher plasma Cbl levels (198-202). This association implies that any underlying causal explanation may involve factors other than those related to the cancer itself. Thus, future studies should also examine the association between mortality and elevated Cbl levels in patients without cancer, preferably in large populations and with sufficient confounder assessment.

We have recently found that venous thromboembolism, a known complication of cancer, is associated with high Cbl levels in cancer patients. This finding supports the hypothesis that elevated Cbl levels are associated with more aggressive cancer. We are also planning studies to better understand how the association between Cbl levels and cancer is affected by any differences in indication for measuring plasma Cbl levels. We want to come closer to identifying the group of patients with high cancer risk by better characterizing the population of patients with plasma Cbl measurements and by examining how various clinical characteristics may differ in patients with different Cbl levels. Here, the addition of other information could provide an answer. This information could include other biomarker results, patient health care–seeking behavior, and physician request pattern near cancer diagnosis. Thus, our research on elevated plasma Cbl levels continues with the aim of improving knowledge about the clinical implications of this common laboratory abnormality.

8. English summary

The clinical indication for measuring plasma Cbl levels is to diagnose or rule out Cbl deficiency. Cbl deficiency can result in anemia, gastrointestinal complaints, and neurological symptoms, and risk factors include vegetarian diet, advanced age, and prior surgery or existing disease in the gastrointestinal tract. However, many patients who are measured show elevated Cbl levels, which have been associated with diseases of the liver and kidney and with cancer, but the clinical implications and the understanding of the underlying pathogenesis are still unclear.

We conducted three studies with the overall aim of improving knowledge about the clinical implications of elevated Cbl levels. First, we examined disease prevalence and biomarker alterations in a cross-sectional study of hospital patients (study I). Then we assessed the cancer risk in patients with plasma Cbl measurement in a cohort design using regional and national health registries (study II). Last, we studied the mortality in cancer patients with a pre-diagnostic plasma Cbl measurement, also in a registry-based cohort design (study III). For all three studies, we focused on patients with elevated Cbl levels, defined as >600 pmol/L (reference range 200-600 pmol/L).

Study I included 818 hospital patients, and during the study period, the prevalence of elevated Cbl levels was 20% in hospital patients and 12% in general practitioner patients. In 39% of the included hospital patients with high Cbl levels, the reason was Cbl treatment. In the remaining 61 %, elevated Cbl levels were associated with alcoholism, liver disease, and cancer, and elevated haptocorrin was the major determinant of elevated Cbl levels in these patients and was also elevated in the diseases mentioned above.

In study II, we included 333,667 patients with Cbl levels >200 pmol/L with no prior cancer diagnosis. Of these, a total of 19,665 had elevated plasma Cbl levels, and 22,652 were diagnosed

with cancer. The cancer risk was mainly elevated within the first year from plasma Cbl measurement. The absolute risks were as follows, for ≤1 year: 200–600 pmol/L, 2.3%; 601–800 pmol/L, 3.7%; >800 pmol/L, 6.6%; for >1 year: 200–600 pmol/L, 4.4%; 601–800 pmol/L, 4.1%; and >800 pmol/L: 4.4%. Using Poisson regression, the relative risk estimates with ≤1 year of follow-up yielded a dose-response association between elevated Cbl levels and both hematological cancers and smoking and alcohol-related cancers.

Study III included a patient cohort of 25,017 cancer patients (plasma Cbl measurement up to one year prior to diagnosis) and a matched comparison cohort of 61,988 cancer patients (no prior plasma Cbl measurement). The patient cohort had a higher mortality than the comparison cohort, and the mortality increased in a dose-dependent manner with higher Cbl levels. The one-year survival in both cohorts was as follows: Cbl, 200–600 pmol/L, 69.3%; 601–800 pmol/L, 49.6%; >800 pmol/L, 35.8%; comparison cohort, 72.6%. Using Cox regression, we computed adjusted MRRs of 30-day mortality, treating patients with Cbl levels of 200–600 pmol/L as reference: 601–800 pmol/L vs. 200–600 pmol/L, MRR 1.9 (95% CI: 1.6–2.2); >800 pmol/L vs. 200–600 pmol/L, MRR 2.7 (95% CI: 2.4–3.1).

Based on these three studies, we conclude that elevated Cbl levels are highly prevalent among Danish patients. The biomarker underlying elevated Cbl levels is haptocorrin. Patients with elevated Cbl levels have a high prevalence of several severe diseases. Most important, elevated Cbl levels can be a marker of occult or prevalent cancer, and elevated Cbl levels in cancer patients mark an aggressive cancer with high mortality.

9. Dansk resume

Måling af plasma cobalamin (Cbl, vitamin B12) anvendes i klinisk praksis til at diagnosticere eller udelukke Cbl-mangel. Denne mangeltilstand kan give blodmangel og symptomer fra mave-tarmkanalen og nervesystemet. Veganere, ældre og patienter med sygdomme eller tidligere operationer på mave-tarmsystemet er i særlig risiko for at udvikle Cbl-mangel, der normalt vil ses ved et lavt plasma Cbl-niveau. Men blandt patienter der får målt plasma Cbl er der mange der har et forhøjet niveau. Dette har tidligere været forbundet med sygdomme i leveren eller nyrerne samt med cancer, men der eksisterer en ufuldstændig forståelse af både den kliniske betydning og den underlæggende patologiske årsag til forhøjet plasma Cbl.

Det overordene formål med denne ph.d.-afhandling er at forbedre vores viden om den kliniske betydning af forhøjet Cbl og den underlæggende patogenese. Afhandlingen indeholder tre studier, hvoraf det første undersøgte abnormiteter i biomarkørkoncentrationer og sygdomsprævalensen hos hospitalspatienter i et tværsnitsdesign. I det andet studie, et kohortestudie, anvendte vi data danske sundhedsregistre til at undersøge kræftrisikoen hos patienter med en plasma Cbl-måling. Det tredje studie undersøgte dødeligheden hos kræftpatienter med plasma Cbl målt før kræftdiagnosen, og anvendte ligeledes registerdata i et kohortedesign. Fælles for alle tre studier, valgte vi at fokusere på patienter med forhøjet plasma Cbl, defineret som >600 pmol/L (referenceinterval 200-600 pmol/L).

Det første studie involverede 818 hospitalspatienter. I løbet af studieperioden fandt vi en prævalens af forhøjet plasma Cbl på 20 % blandt hospitalspatienter og 12 % blandt patienter behandlet i almen praksis. Blandt de inkluderede patienter med forhøjet Cbl kunne 39 % forklares ved at de modtog højdosis Cbl-behandling. Hos de resterende 61 % var forhøjet Cbl forbundet med alkoholisme, leversygdom og kræft, og høje koncentrationer af plasmaproteinet

haptocorrin var den vigtigste underliggende forklaring på forhøjet Cbl og dette fandtes også forhøjet hos patienter med de ovennævnte sygdomme.

I studie II inkluderede vi 333.667 patienter med plasma Cbl >200 pmol/L og uden en tidligere kræftdiagnose. Heraf havde 19.665 forhøjet Cbl, og 22.652 fik diagnosticeret kræft efter plasma Cbl-målingen. Vi fandt at kræftrisikoen primært var forhøjet indenfor det første år fra plasma Cbl-målingen. Den absolutte kræftrisiko var: ≤1 år: 200-600 pmol/L: 2,3 %; 601-800 pmol/L: 3,7 %; >800 pmol/L: 6,6 %; og >1 år: 200-600 pmol/L: 4,4 %; 601-800 pmol/L: 4,1 %; >800 pmol/L: 4,4 %. Vi undersøgte kræftrisikoen i studiepopulationen relativt i forhold til den danske baggrundsbefolkning. Vi fandt at jo højere plasma Cbl-niveauer jo højere var risikoen for hæmatologisk kræft og ryge- og alkoholrelateret kræft, især indenfor det første år fra målingen.

Det tredje studie bestod af en patientkohorte på 25.017 kræftpatienter med plasma Cbl målt indenfor ét år før kræftdiagnosetidspunktet, og en sammenligningskohorte med i alt 61.988 kræftpatienter uden tidligere plasma Cbl-måling. Patientkohorten havde højere dødelighed end sammenligningskohorten, og jo højere plasma Cbl-niveauer jo højere var dødeligheden. Andelen der overlevede det første år med kræft var i patientkohorten: plasma Cbl: 200-600 pmol/L: 69,3 %; 601-800 pmol/L: 49,6 %; >800 pmol/L: 35,8 %; sammenligningskohorten: 72,6 %. Vi undersøgte den relative dødelighed indenfor 30 dage fra diagnosen i patientkohorten. Dødeligheden var øget 1,5-2 gange for patienter med plasma Cbl 601-800 pmol/L og 2,5-3 gange for patienter med plasma Cbl >800 pmol/L relativt til patienter med plasma Cbl 200-600 pmol/L.

Disse tre studier viser tilsammen at forhøjet plasma Cbl er hyppigt blandt danske patienter der får det målt. Proteinet haptocorrin er forhøjet og flere alvorlige sygdomme er hyppige blandt disse patienter. Centralt blandt disse sygdomme, fandt vi at forhøjet Cbl kan være tegn på endnu ikke diagnosticeret kræft, og kræftpatienter med forhøjet Cbl havde øget dødelighed blandt.

10. References

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11. Appendices

The appendices consist of the three published papers together with the supplementary material.

Appendix I.

Arendt JF, Nexo E. Cobalamin Related Parameters and Disease Patterns in Patients with Increased Serum Cobalamin Levels. PLoS ONE. 2012;7(9):e45979. Including Supplementary Data.

Appendix II.

Arendt JF, Pedersen L, Nexo E, Sorensen HT. Elevated Plasma Vitamin B12 Levels as a Marker for Cancer: A Population-based Cohort Study. J Natl Cancer Inst 2013;105:(23):1799-805. Including Supplementary Data.

According to journal guidelines, this is a pre-copyedited, author-produced PDF of the article accepted for publication in the Journal of the National Cancer Institute following peer review. The version of record is available online at:

http://jnci.oxfordjournals.org/content/105/23/1799.full

Appendix III.

Arendt JF, Farkas DK, Pedersen L, Nexo E, Sorensen HT. Elevated plasma vitamin B12 levels and cancer prognosis: A population-based cohort study. Cancer Epidemiol. 2016;40:158-165. Including Supplementary Data.

Appendix IV.

Comparability of plasma Cbl measurements in the LABKA database.

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PLOS ONE

Cobalamin Related Parameters and Disease Patterns in Patients with Increased Serum Cobalamin Levels

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Abstract

Background: Measurement of serum cobalamin levels is routinely used to diagnose cobalamin deficiency. Surprisingly, approximately 15% of patients have high cobalamin levels and no consensus exists regarding the clinical implications.

Methods: Hospital-treated patients above 18 years of age referred for serum cobalamin measurement were included in groups of patients [percentage cobalamin supplemented] with low (<200 pmol/L, n = 200 [6%]), normal (200–600, n = 202 [6%]) high (601–1000, n = 217 [27%]) and very high (>1000, n = 199 [53%]) cobalamin levels. Total and cobalamin-saturated (holo) transcobalamin, total haptocorrin, soluble TC receptor, sCD320, and methylmalonic acid were analyzed. Data on diagnoses and medical prescriptions was obtained through medical files and the Aarhus University Prescription Database.

Results: Among patients not cobalamin supplemented median total haptocorrin and holo transcobalamin levels were markedly higher in the groups with high/very high cobalamin levels compared to groups with low/normal cobalamin levels. Median total transcobalamin and sCD320 levels were similar across the groups. A number of diagnoses were significantly associated to very high Cbl levels (odds ratio (95% confidence interval)): alcoholism (5.74 (2.76–11.96)), liver disease (8.53 (3.59–20.23)), and cancer (5.48 (2.85–10.55)). Elevated haptocorrin levels were seen in patients with alcoholism, cancer, liver-, renal-, autoimmune-, and bronchopulmonary disease. No clinical associations to sCD320 and total and holo transcobalamin levels were found.

Conclusion: In non-supplemented patients, high cobalamin levels were associated to high haptocorrin levels, and several diagnoses, including alcoholism, liver disease and cancer. Our study emphasizes that clinicians should take high serum cobalamin levels into consideration in the diagnostic process.

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Introduction

Measurement of serum cobalamin (vitamin B12, Cbl) is routinely used to diagnose or rule out a suspected Cbl deficiency. Therefore, we expect to obtain either a low or a normal value for serum Cbl. Surprisingly, a high fraction of patients display Cbl levels well above the upper limit of the reference interval [1,2]. In our laboratory, we find that almost 15% of the patients referred for Cbl measurement have values above the reference range of 200–600 pmol/L (271–813 pg/mL), while only about 11% have values below the reference range (data not shown). The possible underlying causes for these findings are unclear, as are their clinical relevance.

Elevated serum Cbl levels are most consistently found in some types of myeloproliferative disorders, such as chronic myeloid leukemia, polycythemia vera, and hypereosinophilic syndrome. This is due to increased concentrations of haptocorrin (HC), one of the two circulating Cbl binding proteins [3–7]. Several studies have been conducted to link a number of other diseases or group of diseases to high Cbl levels and/or high levels of Cbl binding proteins [1;2;8–18]. These include different malignancies and hepatic-, renal-, infectious-, and autoimmune diseases. However, the studies are limited by a relatively small sample size and must be interpreted with caution. Moreover, the underlying alterations in Cbl related markers are not fully understood. Consequently, a lack of consensus exists regarding the clinical assessment of high serum Cbl levels.

This study presents a systematic evaluation of Cbl related parameters and the disease patterns in patients for whom routine measurements of serum Cbl were requested. We report that high Cbl levels are associated to an increased level of haptocorrin and to a number of diseases, most notably alcoholism, liver disease and cancer.

Materials and Methods

Blood Sample Collection and Patients

All patient serum samples were obtained as part of routine analysis on hospitalized patients, and only surplus serum was used for this study. Informed consent was not obtained from the

Table 1. Age and gender distribution of study population.

	Groups according to serum cobalamin				
Patients not in cobalamin supplementation therapy					
	Low (n = 189)	**Normal (n = 190)**	**High (n = 159)**	**Very high (n = 94)**	
Age, mean (95% CI)	54 (51–56)	58 (55–60)	56 (53–59)	62 (59–66)	*p = 0.003
Sex, male (%)	67 (35%)	104 (55%)	73 (46%)	42 (45%)	
Patients in cobalamin supplementation therapy					
	Low (n = 11)	**Normal (n = 12)**	**High (n = 58)**	**Very high (n = 105)**	
Age, mean (95% CI)	48 (37–60)	47 (35–59)	65(60–70)	68 (64–72)	*p = 0.0003
Sex, male (%)	5 (45%)	2 (17%)	19 (33%)	32 (30%)	

Basic characteristics of patients referred for measurement of serum Cbl levels and included in the study (n = 818). Age is displayed with means and corresponding 95% confidence intervals. Sex is displayed as number and fractions (%) of males.

*P-values were obtained by one-way analysis of variance when testing for difference in mean age across Cbl levels groups. Cbl: cobalamin, vitamin B12; 95% CI: 95% confidence interval.

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patients, as approved by the Regional Ethics Committee of Central Jutland, Denmark (record nr.: 20090187). The committee reviewed and approved the entire study protocol, specifically the consent procedure. The study was approved by the Danish Data Protection Agency (record nr.: 2010-331-0378 and 2010-41-4559).

Blood sample collection was conducted between 1st of November 2009 and 31st of December 2010 at the Department of Clinical Biochemistry, Aarhus University Hospital. Aarhus University Hospital covers all major medical fields in a region of approximately 400,000 inhabitants. During the study period of 14 months the laboratory performed 40,047 serum Cbl measurements. Approximately 30% were from hospital-treated patients and the remaining were from general practitioner (GP) treated patients.

Exclusion criteria were the following: patients <18 years of age or incomplete medical files (see below for details on clinical data) and a surplus blood sample containing <1 mL serum. The latter criteria led to exclusion of a large number of patients. Samples requested from GPs or private hospitals/clinics were excluded because medical files were not available.

The samples were divided into four groups according to Cbl levels: low: 55–199 pmol/L normal: 200–600 pmol/L (reference range in the local routine laboratory), high: 601–1000 pmol/L and very high: >1000 pmol/L. A priori, we decided to stop the collection when each of these groups contained approximately 200.

Blood samples were collected in test tubes without any additives, centrifuged at 2,300 g and analyzed for serum Cbl within 4 hours of sample collection. The surplus serum was removed and frozen at -20°C on the same day and stored until further analyzed.

We assessed the distribution of serum Cbl levels, age, and gender by withdrawing all Cbl measurements in the study period from the electronic laboratory information system.

Biochemical Analyses

The serum samples were analyzed for total serum Cbl levels on Cobas 6000 E, Roche Diagnostics (www.roche.com) in the routine laboratory. All samples with Cbl levels >1476 pmol/L (upper scale limit of the routine assay) or unsaturated B12 binding capacity >6000 pmol/L (interferes with the routine assay [19]) were reanalyzed for total serum Cbl levels after diluting serum in 0.025 mmol/L sodium phosphate buffer containing 115 mmol/L NaCl and 1 g/L bovine albumin, pH 8.0 (Sigma Aldrich, St. Louis, MO, USA, catalog #: A7030, www.sigmaaldrich.com).

All blood samples were analyzed for serum concentrations of Cbl binding proteins, total transcobalamin (TC), Cbl-saturated TC (holoTC) and total HC according to the established in-house ELISA protocols [20–22]. We also analyzed all samples for the soluble form of the TC receptor, soluble CD320 (sCD320), according to our newly described ELISA protocol [23]. All serum samples from non-Cbl supplemented patients (see below) were analyzed for serum methylmalonic acid (MMA) levels at the Department of Clinical Biochemistry, Rigshospitalet, University of Copenhagen, Denmark. These analyses were performed with an optimized LC-MS/MS-version of the HILIC-based method [24] using a Waters Micromass LC-MS/MS system (www.waters.com). All samples from non-Cbl supplemented patients were also analyzed for serum creatinine on Cobas 6000 E, Roche Diagnostics (www.roche.com). From this, estimated glomerular filtration rate (eGFR) was calculated using the formula: $175 \times (\text{serum creatinine } (\mu\text{mol/L})/88{,}4)^{-1{,}154} \times (\text{age in years})^{-0{,}203} \times 0{,}742$ (if patient is female), with no racial correction [25].

All biochemical analyses were performed between March 2010 and January 2012.

Clinical Data

Information on diagnoses of chronic or acute disease was obtained from the Electronic Patients Medical Chart at Aarhus University Hospital. All medical charts were examined by the same investigator. The data was collected according to the time of blood sampling. Hence, the diagnoses were related to the admission or outpatient procedure where the Cbl measurement was requested or, in case of prior chronic disease, diagnosed before the blood sampling. The diagnoses from the clinician(s) were considered valid without validation according to diagnostic criteria. Data on medical prescriptions were collected from the medical charts and from the Aarhus University Prescription Database [26]. Data collection was conducted between October 2010 and March 2011.

We analyzed disease patterns according to the defined Cbl level groups. Additionally, we analyzed levels of Cbl related parameters in the different diagnostic categories. This was done to examine whether specific alterations in the Cbl parameters was associated to a particular disease. In selecting the diagnostic categories we paid attention to diseases previously suggested to be associated

Table 2. Cobalamin related parameters.

	Groups according to serum cobalamin				
	Low	**Normal**	**High**	**Very high**	
	$n_{non\text{-}supplemented}$ = 189	$n_{non\text{-}supplemented}$ = 190	$n_{non\text{-}supplemented}$ = 159	$n_{non\text{-}supplemented}$ = 94	
Cobalamin related parameters	$n_{supplemented}$ = 11	$n_{supplemented}$ = 12	$n_{supplemented}$ = 58	$n_{supplemented}$ = 105	
Total serum cobalamin, pmol/L [200–600]					
- Not Cbl supplemented	170 (149–186)	329 (262–429)	716 (652–830)	1328 (1165–2113)	
- Cbl supplemented	179 (148–181)	414 (258–484)	757 (673–884)	1358 (1129–2420)	
Total transcobalamin, pmol/L [600–1,500]					
- Not Cbl supplemented	970 (800–1140)	960 (840–1180)	1070 (820–1320)	1100 (880–1420)	p = 0.0002
- Cbl supplemented	1250 (1090–1400)	920 (780–1090)	980 (820–1380)	1300 (940–1880)	p = 0.005
Holo transcobalamin, pmol/L [40–150]					
- Not Cbl supplemented	41 (31–52)	64 (46–96)	150 (100–220)	180 (77–410)	p<0.0001
- Cbl supplemented	57 (44–66)	94 (79–116)	207 (145–290)	720 (370–1130)	p<0.0001
Total haptocorrin, pmol/L [240–680]					
- Not Cbl supplemented	510 (400–610)	620 (490–760)	730 (600–920)	1300 (900–2200)	p<0.0001
- Cbl supplemented	480 (370–680)	500 (450–550)	590 (490–770)	680 (550–840)	p = 0.0005
Serum sCD320, arb.u. [12–97]					
- Not Cbl supplemented	17 (14–21)	18 (16–22)	21 (17–33)	24 (19–36)	p<0.0001
- Cbl supplemented	20 (16–29)	18 (15–21)	23 (18–29)	32 (24–43)	p<0.0001
MMA[a], μmol/L [<0.28]					
- Not Cbl supplemented	0.24 (0.19–0.34)	0.19 (0.15–0.27)	0.16 (0.13–0.20)	0.17 (0.13–0.24)	p<0.0001
	n = 155	n = 160	n = 120	n = 68	
Impaired cobalamin status in patients with Cbl >250 pmol/L[b]	–	41 (33%)	7 (6%)	14 (21%)	
	–	n = 124	n = 120	n = 68	

Medians (interquartile ranges) [reference ranges] are shown for Cbl related parameters in patients referred for measurement of serum Cbl divided according to Cbl supplementation (n = 818).

[a]Only non-supplemented patients with normal kidney function (eGFR ≥60 mL/min/1.73 m^2, n = 503).

[b]Numbers and percentages of non-supplemented patients with normal kidney function and serum Cbl >250 pmol/L [31] showing biochemical Cbl deficiency, defined as holoTC <40 pmol/L and/or MMA >0.28 μmol/L. P-values were obtained by comparing medians across Cbl groups using Kruskal-Wallis test. Abbreviations: Cbl: cobalamin, vitamin B12; holo transcobalamin: Cbl-saturated transcobalamin; sCD320: soluble transcobalamin receptor CD320; MMA: methylmalonic acid; eGFR: estimated glomerular filtration rate.

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with elevated serum Cbl and/or Cbl binding proteins (alcoholism, liver disease, myeloid diseases, lymphatic diseases, solid tumor cancer, renal disease and autoimmune diseases) [1–18]. Additionally, we added diagnoses of diabetes mellitus type II, cardiovascular (except patients with only hypertension), psychiatric, bronchopulmonary, neurologic, and gastrointestinal disease. If an organ-specific disease was malignant it was only included in the solid tumor cancer category. For simplicity, we pooled all malignant diagnoses (myeloid diseases, lymphatic diseases, and solid tumor cancer) into one category; cancer. Patients were allowed to have more than one diagnosis.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism® version 4.0 for Windows (GraphPad Software, San Diego, California, USA, www.graphpad.com), Stata® 11 (StataCorp LP, College Station, Texas, USA, www.stata.com), and Microsoft® Excel 2003 (Microsoft Corporation, Redmond, Washington, USA, www.microsoft.com).

Patients in either oral or parenteral Cbl replacement therapy were analyzed separately for Cbl related parameters and excluded from analyses of diagnostic associations to Cbl levels. 129 non-supplemented patients with eGFR <60 mL/min/1.73 m^2 were excluded from analyses of MMA.

All data on Cbl related parameters were positively skewed and no suitable transformation was found. Thus, non-parametric statistical tests were used and levels of Cbl related parameters are reported as medians with interquartile ranges. Means for age were compared using one-way analysis of variance. Median levels of total TC, holoTC, total HC, sCD320 and MMA (MMA only for non-supplemented patients, see exclusion criteria) were compared by Kruskal-Wallis test. Mann-Whitney U-test was applied for testing the difference between Cbl-supplemented and non-supplemented patients for median levels of total TC, holoTC, total HC, and sCD320 levels. Correlations between the Cbl related parameters in non-supplemented patients were analyzed using Spearman's rank correlation. Logistic regression analyses were performed to determine the associations between high Cbl levels and the selected diagnoses, using the group with low levels as reference. Results are presented as crude odds ratios (OR) and ORs adjusted for age (reference age 56 years) and gender (female

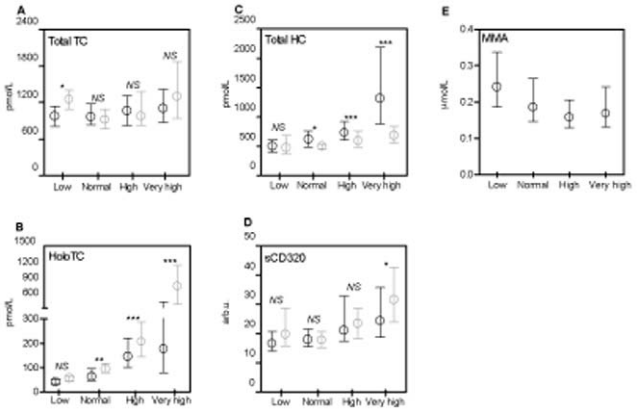

Figure 1. Medians and interquartile ranges of serum total transcobalamin(A), holo transcobalamin(B), total haptocorrin(C), sCD320(D), and methylmalonic acid(E). Black signature: patients not in Cbl therapy; grey signatures patients in Cbl therapy. Please note the split y-axis in figure 1B. NS: not significant ($p>0.05$), *:$p<0.01$, **:$p<0.005$, ***:$p<0.0001$, by comparing patients not in Cbl therapy to patients in Cbl therapy within same Cbl group using Mann-Whitney U-test. Abbreviations: Cbl: cobalamin, vitamin B12; TC: transcobalamin; holoTC: Cbl-saturated transcobalamin; HC: haptocorrin; sCD320: soluble transcobalamin receptor CD320; MMA: methylmalonic acid.
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as reference), with corresponding 95% confidence intervals (95% CI).

Results

Patient Characteristics

We collected blood samples from hospital-treated patients in order to analyze biochemical parameters and diagnostic patterns in patients with low (<200 pmol/L, normal (200–600 pmol/L), high (600–1000 pmol/L) and very high (>1000 pmol/L) Cbl levels. Samples from hospital-treated patients analyzed for Cbl during the study period (n = 12,070) showed a distribution amongst the four Cbl groups of 9% (low), 71% (normal), 13% (high) and 7% (very high). For comparison the distribution amongst Cbl requested from GPs (n = 27,977) was 11% (low), 77% (normal), 9% (high) and 3% (very high). The two populations were similar in age distribution, but the GP population covered more females (65% as compared to 56%).

We collected samples from a total of 825 patients. Six patients were excluded because of incomplete medical files. Notably, a 42-year old female patient with HIV (the only HIV positive patient) showed extremely high levels of Cbl binding proteins (total TC = 83,500 pmol/L, holoTC = 77,200 pmol/L and total HC = 23,700 pmol/L), but a normal sCD320 level (sCD320 = 22 arbitrary units (arb.u.)). HIV-infected patients have been suggested to have an altered Cbl metabolism, but reports show conflicting results [27–30]. This was not scrutinized further and the patient was excluded from further analyses of Cbl related parameters and diagnoses.

Table 1 displays the characteristics of the final study population of 818 patients. The mean age was 58.7 years (95% CI: 57.2–59.9, range: 18–99 years), and was significantly higher with higher Cbl levels. The gender distribution was 42% males and 58% females. The mean age of non-supplemented patients was 56.8 years (95% CI: 55.28–58.23, range 18–97 years).

A total of 22.7% of the patients (n = 186) were in Cbl supplementation therapy at the time of blood sampling. The distribution of Cbl supplemented patients across Cbl groups was: low: 5.5%, normal: 5.9%, high: 26.7%, very high: 52.8%. We were not able to elucidate why Cbl measurements were requested for these patients.

Biochemical Markers

Table 2 shows the Cbl related parameters (total and holoTC, total HC, sCD320, and MMA) in non Cbl-supplemented patients. By Kruskal-Wallis test, we observed significantly higher levels of all five parameters with higher levels of Cbl ($p<0.001$). Median levels of total TC ranged from 970 to 1,100 pmol/L, holoTC from 41 to 180 pmol/L, total HC from 510 to 1,300 pmol/L, sCD320 from 17 to 24 arb.u., and MMA from 0.24 to 0.16 µmol/L across the four Cbl groups.

Among Cbl-supplemented patients the levels of Cbl related parameters also differed between the Cbl groups (Kruskal Wallis test: $p<0.05$), but we found a broader range in median holoTC from 57 to 720 pmol/L and a narrower range in median total HC from 480 to 680 pmol/L compared to the non Cbl-supplemented (table 2).

A total of 352 (56% of total) non Cbl-supplemented patients had levels above the reference intervals in one or more of the Cbl related parameters (total and holoTC, total HC, sCD320). This was unequally distributed among the four Cbl groups: low: 35 (19%), normal: 92 (48%), high: 131 (82%) and very high: 94 (100%).

Regarding MMA, 22% of the non Cbl-supplemented patients with normal kidney function had levels above the reference range (upper limit: 0.28 µmol/L) with the following distribution in the four Cbl groups; low: 57 (37%), normal: 36 (23%), high: 5 (4%), and very high Cbl: 12 (18%). Only 11 patients (2% of total) showed extremely high MMA levels >0.75 µmol/L (table 2).

We compared levels of Cbl related parameters between Cbl-supplemented and non-supplemented patients (figure 1). In the group with low Cbl levels, the levels of total TC were higher among supplemented patients ($p<0.05$). Total HC levels were higher among non-supplemented, especially in patients with high and very high Cbl levels ($p<0.0001$). In the group with very high Cbl levels, sCD320 levels were higher in supplemented patients ($p<0.05$).

Correlation analyses between the Cbl related parameters in non Cbl-supplemented patients showed positive correlations between Cbl levels and holoTC (Spearman's rho: 0.74, 95% CI: 0.70–0.77) and total HC (Spearman's rho: 0.60, 95% CI: 0.55–0.65). We found a moderately strong positive association between levels of sCD320 and holoTC, the soluble receptor and the receptor ligand (Spearman's rho: 0.43, 95% CI: 0.36–0.49). MMA levels correlated negatively to total Cbl, holoTC and total HC levels ($p<0.001$).

We explored biochemical signs of impaired Cbl status in non-supplemented patients with normal kidney function and a plasma Cbl level >250 pmol/L (n = 312), the recommended decision level for ruling out Cbl deficiency [31]. Impaired Cbl status was defined as holoTC <40 pmol/L and/or MMA >0.28 µmol/L.Most of the patients meeting these criteria (n = 62) had Cbl levels between 250 and 600 pmol/L, but interestingly a relatively large group had Cbl levels >1000 pmol/L (table 2).

Diagnostic Distribution

Table 3 shows the diagnostic categories divided according to cobalamin level groups. We found significant associations between higher Cbl levels and alcoholism, liver disease, and cancer, the latter only significant in patients with Cbl levels >1000 pmol/L. The risk of alcoholism and cancer was higher for males than for

Table 3. Diagnostic associations to cobalamin levels.

		Groups according to serum cobalamin		
	<200 pmol/L[a]	200–600 pmol/L	601–1000 pmol/L	>1000 pmol/L
Diagnoses	**n = 189**	**n = 190**	**n = 159**	**n = 94**
Alcoholism, n	13	20	36	28
Crude OR (95% CI)	1.00	1.59 (0.77–3.30)	3.96 (2.02–7.78)	5.74 (2.81–11.75)
Adjusted[b] OR (95% CI)	1.00	1.35 (0.64–2.82)	3.73 (1.88–7.39)	5.74 (2.76–11.96)
Liver diseases, n	8	13	22	24
Crude OR (95% CI)	1.00	1.66 (0.67–4.11)	3.63 (1.57–8.41)	7.76 (3.33–18.08)
Adjusted[b] OR (95% CI)	1.00	1.64 (0.66–4.09)	3.65 (1.57–8.50)	8.53 (3.59–20.23)
Cancer, n	18	26	21	38
Crude OR (95% CI)	1.00	1.51 (0.80–2.85)	1.45 (0.74–2.82)	6.45 (3.41–12.19)
Adjusted[b] OR (95% CI)	1.00	1.24 (0.64–2.39)	1.30 (0.66–2.58)	5.48 (2.85–10.55)
Renal diseases, n	11	13	19	7
Crude OR (95% CI)	1.00	1.19 (0.52–2.72)	2.20 (1.01–4.77)	1.30 (0.49–3.47)
Adjusted[b] OR (95% CI)	1.00	1.07 (0.46–2.48)	2.08 (0.95–4.56)	1.07 (0.40–2.88)
Autoimmune diseases, n	15	12	12	9
Crude OR (95% CI)	1.00	0.78 (0.36–1.72)	0.95 (0.43–2.09)	1.23 (0.52–2.92)
Adjusted[b] OR (95% CI)	1.00	0.86 (0.39–1.91)	1.00 (0.45–2.21)	1.28 (0.53–3.11)
Bronchopulmonary diseases, n	24	31	31	25
Crude OR (95% CI)	1.00	1.34 (0.75–2.38)	1.67 (0.93–2.98)	2.49 (1.33–4.66)
Adjusted[b] OR (95% CI)	1.00	1.16 (0.63–2.13)	1.58 (0.86–2.91)	1.89 (0.98–3.66)
Cardiovascular diseases, n	35	37	31	26
Crude OR (95% CI)	1.00	1.06 (0.64–1.78)	1.07 (0.62–1.82)	1.68 (0.94–3.01)
Adjusted[b] OR (95% CI)	1.00	0.82 (0.46–1.45)	0.93 (0.51–1.68)	1.14 (0.60–2.16)
Psychiatric diseases, n	46	53	47	18
Crude OR (95% CI)	1.00	1.20 (0.76–1.90)	1.30 (0.81–2.10)	0.74 (0.40–1.36)
Adjusted[b] OR (95% CI)	1.00	1.26 (0.78–2.02)	1.36 (0.84–2.22)	0.86 (0.46–1.62)
Diabetes mellitus type II, n	29	22	13	12
Crude OR (95% CI)	1.00	0.72 (0.40–1.31)	0.49 (0.25–0.98)	0.81 (0.39–1.66)
Adjusted[b] OR (95% CI)	1.00	0.58 (0.31–1.08)	0.42 (0.21–0.86)	0.61 (0.29–1.28)
Neurological diseases, n	29	23	30	22
Crude OR (95% CI)	1.00	0.76 (0.42–1.37)	1.28 (0.73–2.25)	1.69 (0.91–3.13)
Adjusted[b] OR (95% CI)	1.00	0.68 (0.37–1.23)	1.21 (0.68–2.13)	1.40 (0.74–2.64)
Gastrointestinal diseases, n	50	47	52	24
Crude OR (95% CI)	1.00	0.91 (0.58–1.45)	1.35 (0.85–2.15)	0.95 (0.54–1.68)
Adjusted[b] OR (95% CI)	1.00	1.02 (0.64–1.64)	1.46 (0.91–2.34)	1.12 (0.63–1.99)

Diagnoses related to Cbl levels in groups in patients referred for measurement of serum Cbl levels and not in Cbl supplementation therapy (n = 632). Patients were allowed to have more than one diagnosis. Odds ratios and 95% confidence intervals were obtained by logistic regression analyses.

[a]Patients with Cbl levels ≤200 pmol/L were treated as reference group.

[b]Adjusted for age (reference age 56 years) and gender (female as reference). Abbreviations: Cbl: cobalamin, vitamin B12; OR: OR: Odds ratio; 95% CI: 95% confidence interval.

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females (data not shown). The risk of renal disease in patients with Cbl levels 601–1000 pmol/L was elevated, but this association was attenuated by adjusting for age and gender. In bronchopulmonary disease, the higher risk among patients with Cbl levels >1000 pmol/L was also attenuated by adjustment for age and gender. For diabetes mellitus type II, a small protective effect of higher Cbl levels was seen, although only significant for patients with Cbl levels 601–1000 pmol/L. When stratifying the diagnostic category cancer into the three subtypes, myeloid diseases, lymphatic diseases, and solid tumor cancers, patients with Cbl levels >1000 pmol/L had higher risks of all three subtypes, with the highest risk for myeloid cancers. When stratifying liver diseases into alcohol and non-alcohol related, both types of liver disease were associated to high Cbl levels, with the highest risk estimates for alcoholic liver disease (table S1).

Table 4 shows the median levels of Cbl related parameters in the different disease categories. For four disease categories; alcoholism, liver, renal, and autoimmune diseases, median holoTC levels were high, although within reference range. Median total HC levels were above reference range in six categories, and especially high in cancer and renal disease patients. Total TC and sCD320 levels showed no specific disease association, although the

Table 4. Cobalamin related parameters in diagnoses.

		Cobalamin related parameters		
	Total TC, pmol/L	**HoloTC, pmol/L**	**Total HC, pmol/L**	**sCD320, arb.u.**
Diagnoses	**[600–1500]**	**[40–150]**	**[240–680]**	**[12–97]**
Alcoholism, n = 97	1070 (860–1290)	120 (61–240)	730 (590–1240)	22 (16–35)
Liver diseases, n = 68	1020 (850–1390)	130 (68–320)	740(540–1260)	25 (17–39)
Cancer, n = 103	1100 (890–1400)	77 (52–130)	770 (490–1420)	22 (17–32)
Renal diseases, n = 50	1230 (910–1470)	120 (54–225)	760 (550–980)	35 (23–45)
Autoimmune diseases, n = 48	1310 (890–1860)	110 (56–180)	690 (540–1070)	19 (16–27)
Bronchopulmonary diseases, n = 111	1060 (840–1380)	93 (49–160)	710 (540–1020)	20 (16–28)
Cardiovascular diseases, n = 129	1070 (850–1340)	80 (44–130)	650 (490–910)	20 (16–30)
Psychiatric diseases, n = 164	950 (820–1140)	65 (43–130)	660 (500–870)	18 (15–23)
Diabetes mellitus type II, n = 76	1100 (910–1340)	65 (43–110)	580 (470–810)	18 (16–25)
Neurological diseases, n = 104	990 (840–1200)	83 (47–160)	580 (470–880)	20 (15–30)
Gastrointestinal diseases, n = 170	940 (800–1190)	77 (44–170)	650 (510–800)	20 (16–30)

Median (interquartile ranges) [reference ranges] levels of Cbl related parameters divided according to diagnoses in patients referred for measurement of serum Cbl levels and not in Cbl supplementation therapy (n = 632). Abbreviations: Cbl: cobalamin, vitamin B12; TC: transcobalamin; holoTC: Cbl-saturated transcobalamin; HC: haptocorrin; sCD320: soluble transcobalamin receptor CD320.

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latter was found higher in renal disease than in any of the other disease categories.

Discussion

This study was conducted to assess the possible clinical implications of elevated serum Cbl levels observed in hospital–treated patients referred for measurement of Cbl. In addition, we wanted to study Cbl levels and diagnoses in relation to the Cbl binding proteins, the soluble TC receptor, sCD320, and MMA.

When interpreting the results several issues must be taken into account. First, our focus was an examination of patients referred for analysis of Cbl, and thus the study do not necessarily represent the true distribution of patients with high Cbl levels. Nor does it allow us to conclude whether high Cbl levels can be used as a biomarker for specific diseases. Third, in order to focus on patients with high Cbl levels, this group, especially those with very high levels, was oversampled compared to the actual prevalence of patients with high Cbl levels in the group of patients referred to Cbl measurement. Fourth, the age of the patients was higher with higher Cbl levels. It is expected that older patients have more morbidities, and the disease associations might be related to the higher age, although the adjusted estimates did not alter the associations substantially. But since our underlying motive was to understand the unexpected high Cbl levels in patients suspected of Cbl deficiency, these issues are judged to be of minor importance for the interpretation of the results.

We used the local reference interval to make cut-offs for the Cbl level groups, thus defining high Cbl levels as above the upper reference limit. Reference intervals vary with the applied measurement methods and the relevant populations, so the exact cut-offs can not readily be applied in other settings.

Our comparison of the diagnostic patterns in patients with different Cbl levels showed several interesting features. We found that high Cbl levels were significantly associated to alcoholism and liver disease. This is consistent with earlier results [2;10;15]. In these diseases, a dominant finding in our study was a high level of HC.

Previous studies have described the association between high Cbl levels and different malignancies, possibly also related to high HC levels [3–7;9;11–12;16;18]. Our results show that cancer is significantly associated to high Cbl levels, and these cancer patients had high HC levels Although, we found only a small number of cases in each group, all three subgroups, myeloid, lymphatic, and solid tumors were associated to Cbl levels >1000 pmol/L.

Previously, renal disease has been reported associated to high Cbl levels and concurrent high holoTC and saturated HC levels [1]. In part, we confirmed such an association and showed that the high Cbl levels were caused by high levels of total HC and holoTC levels in the upper end of the reference range.

We report a novel (though not uniformly observed) association between bronchopulmonary diseases and high levels of Cbl. The high Cbl levels were associated to high HC levels. Earlier studies from our lab showed HC production in the bronchial mucosa [32], but whether this is related to the high circulating levels of HC in bronchopulmonary disease remains unresolved.

As mentioned above our results do not allow us to conclude whether high Cbl could be a biomarker for specific diseases. But our findings suggest further studies to clarify this issue. In particular, the high prevalence of cancer in our study population spurs to further study Cbl metabolism in cancer.

We excluded patients who were requested for Cbl measurement by their GPs, because clinical data and medical charts from these are not available for research purposes. Our crude analysis of all Cbl measurements performed during the study period showed that low levels of Cbl were more frequent (11% as compared to 9%) while very high levels were less frequent (3% as compared to 7%) in the GP population than in the hospital-treated population. While the age distribution was similar, the distribution between men and women differed. Taken together these data suggests that our results can not directly be transferred to GP-treated patients, but that the problem of unexpected high levels of Cbl may also exist for GP-treated patients.

Previous studies have compared Cbl levels to the concentration of other Cbl related parameters [33]. However, only few studies

include measurement of both total TC, holoTC and total HC, and to our knowledge this study is the first to include measurement of sCD320. We found that Cbl levels >600 pmol/L were related to high total HC levels rather than high total TC. Further studies are needed to clarify the disease specific associations to high HC levels.

Our data allowed us to examine the potential relations between the novel biomarker, sCD320, and other Cbl related parameters. While sCD320 correlated positively to the Cbl binding proteins, MMA and sCD320 levels did not correlate, suggesting that sCD320 levels are not influenced by cellular Cbl status. Interestingly, we found the strongest correlation between sCD320 and the receptor ligand, holoTC. This suggests that the metabolically active fraction, and possibly receptor activity, could influence receptor release from the cell surface and into the circulation. Further supporting this hypothesis, we found the highest median sCD320 levels in the Cbl supplemented patients with Cbl above 1000 pmol/L and extremely high holoTC levels. The possible underlying mechanisms demand further substantiation.

Finally, we explored the relation between sCD320 and diagnostic patterns. Earlier studies have shown increased receptor activity in proliferating cells and a possible cell-cycle regulation of the receptor gene expression [34–36]. Hence, diseases where cell proliferation is evident, e.g. cancer, infectious and inflammatory diseases, could be a reasonable suggestion for a possible association. We did not find any obvious clinical associations to sCD320 levels, nor did we find evidence to suggest sCD320 as a novel biomarker for Cbl deficiency.

In conclusion, our study strongly suggests that high Cbl levels in patients referred for measurement of Cbl should be taken into account in the diagnostic procedure as it may indicate underlying disease. Notably, alcoholism, liver disease and cancer were associated to high Cbl levels, and all of these diseases were associated to high HC levels.

Supporting Information

Table S1 Diagnostic associations to cobalamin levels, subgroup diagnoses. Diagnosis subgroups related to Cbl levels in groups in patients referred for measurement of serum Cbl levels and not in Cbl supplementation therapy (n = 632). Patients were allowed to have more than one diagnosis. Cancer was divided into three groups: myeloid, lymphatic and solid tumors. Liver disease was divided into alcoholic and other liver diseases. Odds ratios (OR) and 95% confidence intervals (CI) were obtained by logistic regression analyses. [a]Patients with Cbl levels ≤200 pmol/L were treated as reference group. [b]Adjusted for age (reference age 56 years) and gender (female as reference). Abbreviations: Cbl: cobalamin, vitamin B12; OR: OR: Odds ratio; 95% CI: 95% confidence interval.
(DOC)

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Author Contributions

Conceived and designed the experiments: JA EN. Performed the experiments: JA EN. Analyzed the data: JA EN. Contributed reagents/materials/analysis tools: JA EN. Wrote the paper: JA EN.

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Table S1.

	Groups according to serum cobalamin			
	< 200 pmol/L[a]	**200-600 pmol/L**	**601-1000 pmol/L**	**> 1000 pmol/L**
Diagnoses	**n = 189**	**n = 190**	**n=159**	**n = 94**
Alcoholic liver disease, n	2	4	15	13
Crude OR (95 % CI)	1.00	2.01 (0.36;11.11)	9.74 (2.19;43.27)	15.01 (3.31;68.02)
Adjusted[b] OR (95 % CI)	1.00	1.85 (0.33;10.31)	9.39 (2.11;41.90)	15.37 (3.35;70.57)
Other liver diseases, n	6	9	7	11
Crude OR (95 % CI)	1.00	1.52 (0.53;4.35)	1.40 (0.46;4.27)	4.04 (1.45;11.30)
Adjusted[b] OR (95 % CI)	1.00	1.61 (0.55;4.69)	1.47 (0.48;4.49)	4.64 (1.62;13.30)
Malignant myeloid diseases, n	1	5	5	9
Crude OR (95 % CI)	1.00	5.08 (0.59;43.91)	6.10 (0.71;52.80)	19.91 (2.48;159.62)
Adjusted[b] OR (95 % CI)	1.00	4.31 (0.49;37.55)	5.58 (0.64;48.46)	18.05 (2.23;145.95)
Malignant lymphatic diseases, n	6	14	3	12
Crude OR (95 % CI)	1.00	2.43 (0.91;6.45)	0.59 (0.14;2.38)	4.46 (1.62;12.30)
Adjusted[b] OR (95 % CI)	1.00	2.31 (0.86;6.21)	0.57 (0.14;2.32)	4.24 (1.52;11.85)
Solid tumor cancer, n	11	7	14	19
Crude OR (95 % CI)	1.00	0.62 (0.23;1.63)	1.56 (0.69;3.55)	4.10 (1.86;9.03)
Adjusted[b] OR (95 % CI)	1.00	0.46 (0.19;1.25)	1.38 (0.59;3.24)	3.16 (1.39;7.20)

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ARTICLE

Elevated Plasma Vitamin B12 Levels as a Marker for Cancer: A Population-Based Cohort Study

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Background A substantial proportion of patients referred for plasma vitamin B12 (cobalamin [Cbl]) measurement present with high Cbl levels, which have been reported in patients with different cancer types. However, the cancer risk among patients with newly diagnosed high Cbl levels has not been adequately examined.

Methods We conducted this cohort study using population-based Danish medical registries. Patients referred for Cbl measurement with levels greater than the lower reference limit (≥200 pmol/L) were identified from the population of Northern Denmark during the period from 1998 to 2009 using a database of laboratory test results covering the entire population. Data on cancer incidence (follow-up 1998–2010), Cbl treatment, and prior diagnoses were obtained from medical registries. Patients receiving Cbl treatment were excluded. Cancer risks were calculated as standardized incidence ratios (SIRs) with 95% confidence intervals (CIs), stratified by plasma Cbl levels. All statistical tests were two-sided.

Results We identified 333 667 persons without prevalent cancer and not receiving Cbl treatment. Six percent had Cbl levels greater than the upper reference limit (≥601 pmol/L). Cancer risk increased with higher Cbl levels and was highest during the first year of follow-up (Cbl 601–800 pmol/L: SIR = 3.44, 95% CI = 3.14 to 3.76; Cbl >800 pmol/L: SIR = 6.27, 95% CI = 5.70 to 6.88; both $P < .001$). The risks were particularly elevated for hematological and smoking- and alcohol-related cancers for persons with high Cbl levels.

Conclusions High Cbl levels were associated with the risk of subsequently diagnosed cancer, mostly within the first year of follow-up. This may have clinical implications for the interpretation of high Cbl levels.

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Vitamin B12 (cobalamin [Cbl]) is an essential nutrient involved in one-carbon metabolism and cell division. Daily intake of 2 to 5 μg, together with efficient absorption, transportation, and transformation, are needed to maintain health (1). In clinical practice, measurement of total plasma Cbl is requested widely for the biochemical assessment of Cbl deficiency (2). Three studies have shown that a substantial proportion of patients for whom Cbl measurement is requested have plasma Cbl levels greater than the upper limit of the reference range (3–5), and two of these studies have shown an association between high Cbl levels and cancer (3,5).

The association between elevated plasma Cbl levels and cancer risk is poorly understood. On one hand, a high prevalence of elevated Cbl levels has been reported in patients with liver cancer (6,7), other solid tumors (8,9), and hematological malignancies (10,11). On the other hand, some studies have indicated a high prevalence of cancer, both hematological and solid tumors, among patients with high Cbl levels (3,5). However, the latter studies are limited by their cross-sectional design, and only one study included a comparison group of patients with normal and low plasma Cbl levels (3). Most studies on normal or low Cbl levels in relation to cancer have been negative (12–16), except for some studies showing associations between increasing Cbl levels and lung and prostate cancer (17,18).

Elevated plasma Cbl levels have also been associated with several nonmalignant diseases, including liver diseases, alcoholism, and renal, autoimmune, and infectious diseases (19). Only a few patients with these diseases have high Cbl levels (19). Moreover, these diseases have been reported in a small proportion of patients with high Cbl levels (3–5).

To assess the possible clinical implications of elevated Cbl levels in diagnosing cancer, we conducted a population-based cohort study, examining the incidence of cancer diagnoses among patients with elevated plasma Cbl levels.

Methods

Design, study cohort, and data sources

We conducted this population-based cohort study using data from medical registries in Northern Denmark over the period from 1998 to 2010. Data were obtained from the Clinical Laboratory

Information System Research Database (LABKA) (20), the Aarhus University Prescription Database (AUPD) (21), the Danish Cancer Registry (DCR) (22), and the Danish National Registry of Patients (DNRP) (23). The Danish Civil Registration System, established in 1968, assigns a civil registration number to all residents, allowing for unequivocal individual-level data linkage among all Danish registries (24).

The LABKA database (20) contains all test results from routine tests performed in hospital laboratories in Northern Denmark, which has a total population of 2.2 million inhabitants. Results of more than 1700 different types of analyses are included in the database. For each analysis, the database stores the test result (or indicates that it is missing), civil registration number, date, and the international Nomenclature, Properties and Units code. For some analyses, a local analysis code is recorded. We identified all patients in the LABKA database with a plasma Cbl measurement of greater than 200 pmol/L [lower reference limit in Northern Denmark = 271 pg/mL (27)] recorded from 1998 through 2009. If a patient had more than one Cbl test result, only the first test was included in our analysis. (See Supplementary Table 1, available online, for all codes used in this study.)

The AUPD (21) collects data on all prescriptions reimbursed to patients in the study area. Most prescription medications in Denmark are eligible for full or partial tax-paid reimbursement. Only over-the-counter drugs and a few prescription medications, such as oral contraceptives and sedatives, are normally not eligible for reimbursement, although reimbursement can be granted on a case-by-case basis. All records in the AUPD include the date of dispensing, the patient's civil registration number, codes for the prescribing physician/clinic/hospital department, the Anatomical Therapeutic Chemical code, and the medication name, pack size, dose units, and manufacturer. Patients were classified as having received Cbl therapy if they had one or more relevant prescriptions recorded in the AUPD up to 2 years before measurement of their plasma Cbl level. These patients were excluded from the analysis. In Denmark, Cbl therapy in pharmacological doses is available only by prescription.

The DCR (22) includes all cancer diagnoses at the individual level in Denmark since 1943, coded according to the *International Classification of Diseases*, *10th revision* (ICD-10). The DCR was used to identify subsequent diagnoses of cancer from 1998 to 2010 among patients with a Cbl test result in the LABKA database. Patients were excluded if they had a cancer diagnosis before the date of the plasma Cbl measurement.

The DNRP (23), established in 1977, contains information on all inpatient hospitalizations and, since 1995, all hospital outpatient clinic visits. The DNRP includes the patient's civil registration number, hospital admission and discharge date, date of hospital outpatient visit, and up to 20 diagnoses coded by physicians, including a primary diagnosis representing the main reason for the inpatient or outpatient hospital contact. All diagnoses are coded according to the *International Classification of Diseases*, *8th revision* during the period from 1977 to 1993 and ICD-10 since 1994. To assess possible confounding from underlying diseases, we obtained data on all diagnoses before Cbl measurement from the DNRP and grouped them according to ICD-10 codes. Patients were classified as inpatients or outpatients if they were admitted and/or had a hospital outpatient clinic visit up to 30 days before or 7 days after Cbl measurement. If no hospital contact was recorded during the defined period, patients were classified as outpatients.

Statistical analyses

We calculated the expected number of cancers, based on national incidence rates by age, sex, and year of diagnosis in 1-year intervals (25). The number of cancers to be expected if the patients had the same risk as the general population was calculated by multiplying person-years of follow-up by the population-based cancer incidence rates. The risk of cancer in the study cohort was calculated as the standardized incidence ratio (SIR; ie, the ratio of observed cancers to expected cancers). The 95% confidence intervals (CIs) for the SIR estimates were calculated assuming a Poisson distribution of the observed number of specific cancers in the follow-up period. Byar's approximation was used unless the observed number was less than 10, in which case, exact 95% confidence intervals were calculated (26). All statistical tests were two-sided.

In the analyses, we first excluded patients receiving Cbl therapy. A priori, we grouped incident cancers through 2010 into four different categories: smoking and alcohol-related cancers, hematological cancers, immune-related cancers, and hormone-related cancers (see Table 1). We also obtained results for specific cancers. We divided patients into three groups according to plasma Cbl level: 200 to 600 pmol/L (regional reference interval (27) = 271–813 pg/mL); 601 to 800 pmol/L (regional reference interval = 814–1084 pg/mL), and >800 pmol/L (regional reference level = >1084 pg/mL). Patients

Table 1. Cancer groups

Smoking and alcohol-related cancers	Hematological cancers	Immune-related cancers	(Sex) hormone-related cancers
Lip	Non-Hodgkin lymphoma	Cervix uteri	Breast
Mouth	Hodgkin lymphoma	Malignant melanoma	Corpus uteri
Oro- and nasopharynx	Multiple myeloma	Nonmelanoma skin cancer including basal cell and squamous cell carcinoma	Ovary
Larynx	Leukemia	Anus	Prostate
Lung	Unspecified cancer of lymph	Penis	Testes
Esophagus	Unspecified cancer of blood		
Pancreas			
Liver			
Colon and rectum			
Kidney			
Urinary bladder			

were also stratified according to age (0–50 years, ≥51 years), sex, length of follow-up (≤1, >1 year, and >5 years), and receipt of inpatient or outpatient care. Standardized incidence ratios were assessed in 100 to 200 pmol/L Cbl level intervals to examine a possible Cbl cutoff level for high cancer risk. Because high plasma Cbl levels also have been associated with other conditions (19), we assessed cancer risk in relation to selected morbidities, categorized according to ICD-10 codes (see Supplementary Table 1, available online).

All statistical analyses were performed using SAS version 9.2 (SAS Institute Inc, Cary, NC). The study was approved by the Danish Data Protection Agency (record number: 2009-41-3866).

Results

Study Cohort

We identified 352 831 persons without prevalent cancer who had plasma Cbl levels ≥200 pmol/L and 19 164 patients who were receiving Cbl therapy at the time of plasma Cbl measurement and were therefore excluded.•• Of the patients included in the study, 19,665 (6%) had levels greater than the upper reference limit (≥601 pmol/L). The total number of person-years of follow-up was 1 421 512 and median follow-up time was 3.5 years (interquartile range [IQR]= 1.9–6.2 years). The median age was 55.1 years (IQR = 40.2–69.2 years). The distribution of outpatients and inpatients was 276 229 (83%) and 57 438 (17%), respectively. A total of 22 652 (7%) patients in the study cohort subsequently developed cancer during the period from 1998 to 2010.

Cancer Risks

The overall standardized incidence ratio was 1.26 (95% CI = 1.24 to 1.28; $P < .001$) (Table 2). The overall cancer risk increased with higher Cbl levels and with a peak during the first year of follow-up (Cbl 601–800 pmol/L: SIR = 3.44, 95% CI = 3.14 to 3.76, $P < 0.001$; Cbl >800 pmol/L: SIR = 6.27, 95% CI = 5.70 to 6.88, $P < 0.001$). Overall Cbl-associated cancer risk decreased with increasing age. The risk was higher for men than for women. For all cancer groups, the first year risk increased with higher Cbl levels, most substantially for Cbl levels greater than 800 pmol/L. After the first year, the risk remained elevated for patients with Cbl levels greater than 800 pmol/L for smoking and alcohol-related cancers and for hematological cancers. The standardized incidence ratios also remained elevated after the first year in patients with Cbl levels in the 601 to 800 pmol/L range for smoking- and alcohol-related cancers (Table 2). For hematological cancers and smoking- and alcohol-related cancers, the standardized incidence ratios remained elevated for more than 5 years of follow-up, although for hematological cancers, this was the case only for patients with Cbl levels greater than 800 pmol/L (Supplementary Table 2, available online). The numbers and percentages of patients diagnosed with cancer according to plasma Cbl levels and length of follow-up are shown in Table 3 and are as follows: overall: 200 to 600 pmol/L: 6.7%, 601 to 800 pmol/L: 7.8%, and greater than 800 pmol/L: 11.0%; first year: 200 to 600 pmol/L: 2.3%, 601 to 800 pmol/L: 3.7%, and greater than 800 pmol/L: 6.6%; after the first year: 200 to 600 pmol/L: 4.4%, 601 to 800 pmol/L: 4.1%, and greater than 800 pmol/L: 4.4%.

Table 2. Cancer risk diagnosed after a plasma cobalamin (Cbl) measurement according to sex, follow-up interval, age, cancer group, and plasma Cbl levels*••

	Persons, No.	Incident cancers, No.	Overall SIR (95% CI)	Plasma Cbl levels, SIR (95% CI)		
				200–600 pmol/L	601–800 pmol/L	>800 pmol/L
All Cancers	333 667	22 652	1.26 (1.24 to 1.28)	1.23 (1.21 to 1.24)	1.61 (1.51 to 1.71)	2.38 (2.22 to 2.56)
<1 year SIR••		8103	2.17 (2.13 to 2.22)	2.04 (1.99 to 2.09)	3.44 (3.14 to 3.76)	6.27 (5.70 to 6.88)
>1 year SIR		14 549	1.02 (1.00 to 1.04)	1.01 (1.00 to 1.03)	1.09 (1.00 to 1.18)	1.24 (1.10 to 1.39)
Men	135 485	10 815	1.36 (1.34 to 1.39)	1.32 (1.29 to 1.35)	1.93 (1.75 to 2.12)	2.94 (2.64 to 3.27)
Women	198 182	11 837	1.18 (1.16 to 1.20)	1.15 (1.13 to 1.17)	1.43 (1.31 to 1.55)	2.05 (1.86 to 2.27)
Age						
0–50 years	142 000	2411	1.29 (1.24 to 1.34)	1.26 (1.20 to 1.31)	1.63 (1.32 to 2.00)	2.99 (2.36 to 3.72)
<1 year SIR		752	2.26 (2.10 to 2.43)	2.14 (1.98 to 2.30)	3.23 (2.24 to 4.52)	9.04 (6.46 to 12.31)
>1 year SIR		1659	1.08 (1.03 to 1.14)	1.07 (1.01 to 1.12)	1.28 (0.97 to 1.64)	1.77 (1.26 to 2.42)
≥51 years	191 667	20 241	1.26 (1.24 to 1.27)	1.22 (1.21 to 1.24)	1.61 (1.50 to 1.71)	2.33 (2.15 to 2.51)
<1 year SIR		7351	2.17 (2.12 to 2.22)	2.03 (1.98 to 2.08)	3.46 (3.14 to 3.79)	6.09 (5.51 to 6.71)
>1 year SIR		12 890	1.01 (1.00 to 1.03)	1.01 (0.99 to 1.03)	1.07 (0.97 to 1.17)	1.19 (1.05 to 1.34)
Cancer groups						
Smoking- and alcohol-related		9501	1.46 (1.43 to 1.49)	1.40 (1.37 to 1.43)	2.13 (1.95 to 2.33)	3.05 (2.74 to 3.39)
<1 year SIR		3799	2.75 (2.67 to 2.84)	2.56 (2.47 to 2.65)	4.89 (4.30 to 5.54)	8.37 (7.31 to 9.55)
>1 year SIR		5702	1.11 (1.08 to 1.14)	1.09 (1.07 to 1.12)	1.33 (1.17 to 1.52)	1.44 (1.20 to 1.71)
Hematological		1748	1.85 (1.76 to 1.94)	1.72 (1.63 to 1.81)	2.27 (1.79 to 2.85)	7.96 (6.66 to 9.44)
<1 year SIR		912	4.52 (4.23 to 4.82)	4.03 (3.75 to 4.32)	6.82 (5.08 to 8.97)	24.14 (19.51 to 29.54)
>1 year SIR		836	1.12 (1.05 to 1.20)	1.10 (1.02 to 1.18)	0.94 (0.60 to 1.40)	2.99 (2.12 to 4.11)
Immune-related		3565	0.93 (0.90 to 0.96)	0.92 (0.89 to 0.95)	1.17 (1.00 to 1.37)	0.97 (0.74 to 1.23)
<1 year SIR		834	1.08 (1.01 to 1.16)	1.04 (0.97 to 1.12)	1.89 (1.42 to .2.46)	1.37 (0.84 to 2.12)
>1 year SIR		2731	0.89 (0.85 to 0.92)	0.88 (0.85 to 0.92)	0.97 (0.80 to 1.18)	0.85 (0.62 to 1.14)
Hormone-related		5116	1.10 (1.07 to 1.13)	1.10 (1.06 to 1.13)	1.12 (0.96 to 1.29)	1.19 (0.96 to 1.45)
<1 year SIR		1555	1.62 (1.54 to 1.70)	1.59 (1.51 to 1.67)	1.96 (1.53 to 2.47)	2.61 (1.92 to 3.47)
>1 year SIR		3561	0.96 (0.93 to 0.99)	0.97 (0.94 to 1.00)	0.88 (0.72 to 1.05)	0.78 (0.57 to 1.03)

* All statistical tests were two-sided. CI = confidence interval; SIR = standardized incidence ratio.

Table 3. Percentages and numbers of patients diagnosed with cancer during the study period disaggregated according to plasma cobalamin levels*

	Plasma Cbl levels		
	200–600 pmol/L (n = 314 002)	601–800 pmol/L (n = 12 909)	>800 pmol/L (n = 6756)
Overall, % (No.)	6.7 (20899)	7.8 (1013)	11.0 (740)
<1 year, % (No.)	2.3 (7180)	3.7 (480)	6.6 (443)
>1 year, % (No.)	4.4 (13719)	4.1 (533)	4.4 (297)

* Percentages are the fraction of patients diagnosed with cancer in each cobalamin (Cbl) level group, presented as overall percentages and according to follow-up interval.

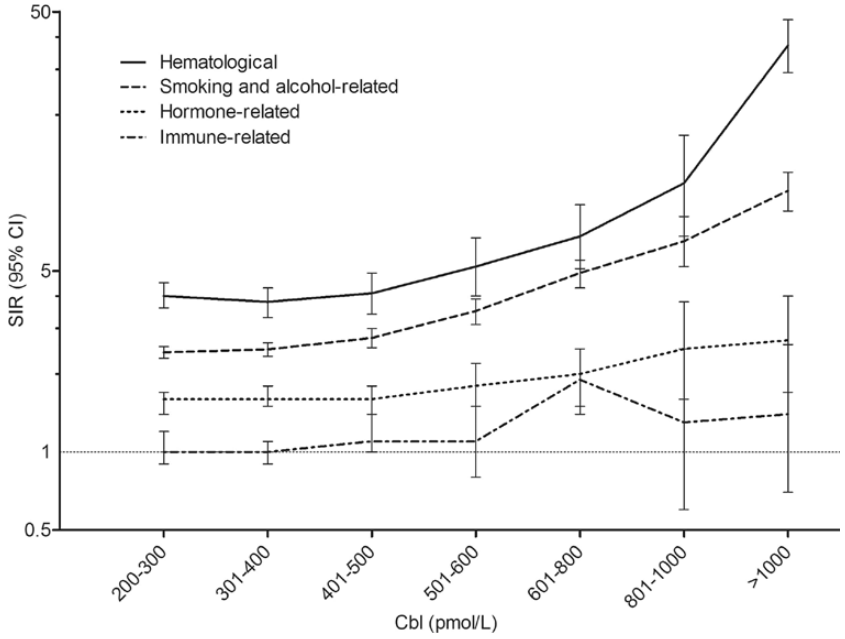

Figure 1. One-year risk of cancer in groups according to plasma cobalamin (Cbl) levels in 100 to 200 pmol/L intervals. The figure shows the 1-year standardized incidence ratios (SIRs) with corresponding 95% confidence intervals (CIs; **vertical bars**) disaggregated according to Cbl levels for hematological cancers (**solid line**), smoking- and alcohol-related cancers (**dashed line**), immune-related cancers (**dotted/dashed line**), and hormone-related cancers (**dotted line**). Note the logarithmic scale for standardized incidence ratio on the y-axis. The **horizontal gray line** indicates a standardized incidence ratio of 1. All statistical tests were two-sided.

Figure 1 presents the first year standardized incidence ratios in detailed Cbl-level intervals. The figure indicates that only risks of smoking- and alcohol-related cancers and hematological cancers substantially increased with higher Cbl levels. Also, it shows that a specific Cbl level cutoff for high cancer risk could not be established. The standardized incidence ratios were elevated fourfold to 37-fold for hematological cancers, twofold to 10-fold for smoking- and alcohol-related cancers, and twofold to threefold for hormone-related cancers; they were not elevated for immune-related cancers.

Patient and Cancer Subtypes

The standardized incidence ratios for inpatients and outpatients showed similar associations, both overall and by cancer group. The highest risks again were found for smoking- and alcohol-related cancers and for hematological cancers. The estimates were highest for inpatients in all cancer groups, although cancer risk was elevated also for outpatients with high Cbl levels (data not shown).

When we examined the association between high Cbl levels and specific cancer types within the first year of follow-up (Figure 2) (SIRs and 95% CIs for Cbl levels >800 pmol/L), we found associations with gastric (SIR = 13.24; 95% CI = 7.23 to 22.21; $P < .001$), colorectal (SIR = 5.48; 95% CI = 4.01 to 7.31; $P < .001$), liver (SIR = 40.70; 95% CI = 25.50 to 61.62; $P < .001$), pancreatic (SIR = 15.57; 95% CI = 10.34 to 22.50; $P < .001$), lung (SIR = 9.27; 95% CI = 7.24 to 11.69; $P < .001$), renal (SIR = 9.56; 95% CI = 4.58 to 17.58; $P < .001$), urinary bladder (SIR = 5.34; 95% CI = 3.11 to 8.55; $P < .001$), lymphatic leukemia (SIR = 8.88; 95% CI = 3.56 to 18.29; $P < .001$), myeloid malignancies (SIR = 105.73; 95% CI = 81.24 to 135.28; $P < .001$), non-Hodgkin lymphoma (SIR = 6.64; 95% CI = 3.31 to 11.89; $P < .001$), and multiple myeloma (SIR = 16.08; 95% CI = 7.70 to 29.58; $P < .001$). The associations increased with higher Cbl levels and were greatest among patients with the highest Cbl levels (>800 pmol/L). The cancer risk remained elevated after the first year of follow-up for liver, pancreatic, lung cancer, and myeloid malignancies, with highest standardized incidence ratios observed for patients with Cbl levels greater than 800 pmol/L (Supplementary Table 3, available online).

The associations between cancer risk and high Cbl levels remained robust in patients with selected morbidities, with no differences among specific morbidities (data not shown).

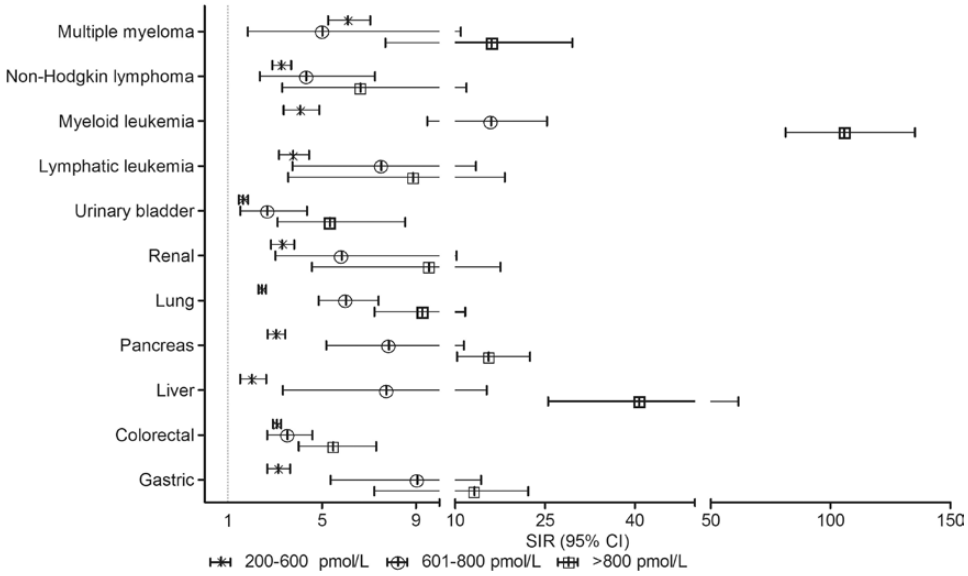

Figure 2. Risk of specific cancer types within the first year after plasma cobalamin (Cbl) measurement. The figure shows the 1-year standardized incidence ratios (SIRs) with corresponding 95% confidence intervals (CIs) disaggregated according to Cbl levels: x: 200 to 600 pmol/L; o: 601 to 800 pmol/L; and □: greater than 800 pmol/L. The **vertical gray line** indicates standardized incidence ratio of 1. All statistical tests were two-sided.

Discussion

Our study shows that elevated plasma Cbl levels are markers for various types of cancers, most notably hematological cancers within the first year after Cbl measurement. These findings remained robust in the stratified analyses.

Our results extend those of earlier research (3,5–11) by showing a strong association between elevated Cbl levels and cancer in a large study with a longitudinal design. Earlier studies have examined Cbl levels in patients with selected cancers. The majority of these previous studies have been negative (12–16), except for lung and prostate cancer (17,18). We focused on high Cbl levels and thereby avoided the limitations of these earlier studies. Our study was based on a large cohort of patients referred for plasma Cbl measurement rather than patients with a particular type of cancer or persons from the general population. Second, unlike the earlier studies, we assessed risk among patients with Cbl levels above a given reference range. Because we specifically focused on high Cbl levels, we were able to demonstrate associations with cancer, including types not previously associated with high Cbl levels, such as cancers of the pancreas and urinary bladder and multiple myeloma.

Our study also was able to examine clinical routine practice. We showed that approximately 15% of the population in Northern Denmark has had a plasma Cbl measurement during the study period and that these measurements show Cbl levels within or above the reference range. This suggests that plasma Cbl measurements are used routinely and frequently to screen for Cbl deficiency in the presence of symptoms or other risks. Although our study cannot directly assess use of high Cbl levels as a nonspecific marker of cancer, our findings may be relevant to the clinical interpretation of such high levels.

A number of different diseases have been suggested as causing high Cbl levels (19), but common to most of them is that the pathogenesis involving elevated Cbl levels is not fully understood. In principle, some of these diseases might be responsible for the long-term associations observed in our study, thereby possibly confounding the associations between high Cbl levels and cancer. For example, the association between liver cancer and high Cbl levels could, in theory, be caused by nonmalignant liver disease underlying the liver cancer. However, when we stratified the analyses according to prior diseases, the results remained robust. This indicates that high Cbl levels could be a marker for cancer both in the short- and long-term, exceeding 5 years for hematological cancers (Supplementary Table 2, available online). Such persistent long-term associations are more likely to be affected by unrecorded confounders, such as lifestyle factors. Although smoking is not associated with disruptions in Cbl status (28), alcoholism and alcohol-related liver disease have previously been linked to high Cbl levels (3,29). This could influence the long-term risk that we observed for some cancers, such as liver cancer, whereas the long-term association with hematological cancers is more peculiar.

Despite its large size and the use of registries to ensure complete follow-up, several possible limitations of our study require consideration. Misclassification of diagnosis is one concern. However, Danish medical registries have proven to be of high validity and completeness (20–23,30), and any potential misclassification of information is likely to be minor and nondifferential (ie, not associated with Cbl levels). Another concern is that we could not directly assess the clinical criteria for measuring plasma Cbl levels because we did not have access to medical files and there are currently no specific guidelines for requesting plasma Cbl measurement. Mainly speculative, unspecific symptoms (eg, anemia, fatigue, weight loss) could be the reason for requesting plasma Cbl measurement, and the same symptoms may also be related to the suspicion of cancer. This may explain why we found elevated standardized incidence ratios also for patients within normal Cbl levels. However, we find it unlikely that the finding of elevated Cbl levels in itself led to increased surveillance for cancer, although this statement cannot

be further assessed. Hence, we find it reasonable to believe that the risk of confounding by indication is minimal. Finally, we did not include concurrent blood test results on other biochemical parameters. Other abnormal test results could influence the probability of a subsequent cancer diagnosis. This may explain the decrease in cancer risk after the first year that we found for immune-related and hormone-related cancers, a compensatory deficit. Finally, we chose to classify cancers a priori to analysis according to type (hematological), life-style factors (smoking- and alcohol-related), and two groups based on potential pathogenesis (hormone related and immune related). To circumvent that some specific cancer types could fall in to more than one of the predefined groups, we also analyzed risk estimates for all specific cancers to obtain more detailed information within the predefined groups.

The underlying pathogenesis leading to high Cbl levels is poorly elucidated, with a few exceptions (6,10,11). It is not thought to involve increased Cbl intake because intestinal absorption capacity is saturable (31) and high physiological consumption does not increase plasma Cbl levels substantially. Only Cbl therapy in the form of injections or extremely high oral doses can produce high circulating levels, and in this study, patients treated with Cbl were excluded. We therefore conclude that the mechanisms resulting in high Cbl levels may be related to malignant pathogenesis. Our recent study showed that levels of the circulating Cbl binding protein haptocorrin were high in patients with high plasma Cbl levels (3). Moreover, cancer was associated with high Cbl and high haptocorrin levels. This protein originates from a variety of tissues, but its physiological function remains unknown (32). It is elevated in patients with some cancer types (6,10,11) and has been suggested as a marker for disease progression (6,10). Thus, haptocorrin may be a candidate factor to include in future studies of the possible pathogenic mechanisms leading to high Cbl levels in cancer patients, in particular for the novel associations demonstrated in this study.

In conclusion, our study showed that high plasma Cbl levels increased the risk of subsequently diagnosed cancer, mostly within the first year of follow-up. However, this association was not present for all cancer types. Although our results may have clinical implications for interpreting high Cbl levels, further studies are warranted to examine the possible diagnostic value of high plasma Cbl levels.

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Notes

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

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Supplementary Table 1. ICD-10 diagnosis codes, NPU codes, local Danish analysis codes, procedure codes, and ATC codes.

Registry	**Codes**	
Danish Cancer Registry	ICD-10 codes for malignant disease	C00-D48
Danish National Registry of Patients	ICD-10 codes for non-malignant disease	A00-B99, D50-D89, E00-E90, F00-F99, G00-G99, H00-H95, I00-I99, J00-J99, L00-L99, M00-M99, N00-N99, O00-O99, P00-Q99, R00-R99, S00-Y09, Z12
	Procedure code for cobalamin therapy	BOHC2
Laboratory Information System Research Database	NPU/local Danish analysis codes for plasma cobalamin measurement	NPU01700, AAA00281, AAA00304
Aarhus University Prescription Database	ATC codes for cobalamin therapy	B03BA01, B03BA02, B03BA03, A11E

Supplementary Table 2. Cancer risk after a plasma Cbl measurement, overall and diagnosed after more than five years follow-up*

	Persons, n	Incident cancers, n	Overall SIR (95% CI)	Plasma Cbl levels, SIR (95% CI)		
				200-600 pmol/L	601-800 pmol/L	>800 pmol/L
All cancers	333,667	22,652	1.26 (1.24-1.28)	1.23 (1.21-1.24)	1.61 (1.51-1.71)	2.38 (2.22-2.56)
>5 year SIR (95% CI)		4,434	1.02 (0.99-1.05)	1.02 (0.99-1.05)	1.09 (0.93-1.27)	1.24 (0.99-1.53)
Cancer groups						
Smoking- and alcohol-related		9,501	1.46 (1.43-1.49)	1.40 (1.37-1.43)	2.13 (1.95-2.33)	3.05 (2.74-3.39)
>5 year SIR (95% CI)		1,727	1.13 (1.08-1.19)	1.11 (1.06-1.17)	1.48 (1.17-1.85)	1.56 (1.11-2.13)
Hematological		1,748	1.85 (1.76-1.94)	1.72 (1.63-1.81)	2.27 (1.79-2.85)	7.96 (6.66-9.44)
>5 year SIR (95% CI)		227	1.04 (0.91-1.19)	1.02 (0.88-1.16)	1.05 (0.45-2.08)	2.50 (1.14-4.74)
Immune-related		3,565	0.93 (0.90-0.96)	0.92 (0.89-0.95)	1.17 (1.00-1.37)	0.97 (0.74-1.23)
>5 year SIR (95% CI)		841	0.86 (0.80-0.92)	0.86 (0.80-0.92)	0.98 (0.68-1.38)	0.87 (0.48-1.46)
Hormone-related		5,116	1.10 (1.07-1.13)	1.10 (1.06-1.13)	1.12 (0.96-1.29)	1.19 (0.96-1.45)
>5 year SIR (95% CI)		1,127	0.99 (0.94-1.05)	1.01 (0.95-1.07)	0.66 (0.43-0.96)	0.65 (0.34-1.14)

*Expressed as SIRs with corresponding 95 % CIs. All statistical tests were two-sided. Abbreviations: Cbl: cobalamin, CI: confidence interval,

SIR: standardized incidence ratio.

Supplementary Table 3. Cancer risk for specific cancers after a plasma Cbl measurement, overall and diagnosed after more than one year follow-up*

Cancer types	Incident cancers, No.	Overall SIR (95% CI)	By plasma cobalamin levels, SIR (95% CI)		
			200-600 pmol/L	600-800 pmol/L	>800 pmol/L
Gastric cancer	431	1.67 (1.52-1.83)	1.59 (1.44-1.76)	2.64 (1.67-3.97)	4.06 (2.40-6.41)
>1 year SIR (95% CI)	235	1.16 (1.01-1.32)	1.17 (1.02-1.34)	0.74 (0.24-1.73)	1.18 (0.32-3.03)
Colorectal cancer	2,924	1.44 (1.39-1.50)	1.43 (1.38-1.49)	1.49 (1.22-1.80)	1.73 (1.33-2.22)
>1 year SIR (95% CI)	1,596	0.99 (0.95-1.04)	1.00 (0.95-1.06)	0.90 (0.67-1.19)	0.61 (0.35-0.97)
Liver cancer	281	2.09 (1.86-2.35)	1.62 (1.40-1.85)	7.99 (5.59-11.06)	17.16 (12.20-23.45)
>1 year SIR (95% CI)	196	1.86 (1.61-2.14)	1.51 (1.28-1.77)	8.06 (5.35-11.65)	9.81 (5.71-15.71)
Pancreas cancer	698	1.61 (1.49-1.73)	1.51 (1.39-1.63)	2.51 (1.79-3.44)	5.10 (3.64-6.95)
>1 year SIR (95% CI)	385	1.12 (1.01-1.24)	1.11 (1.00-1.23)	0.99 (0.51-1.74)	1.99 (1.03-3.47)
Lung cancer	2,878	1.50 (1.44-1.55)	1.43 (1.37-1.48)	2.52 (2.16-2.93)	3.37 (2.77-4.05)
>1 year SIR (95% CI)	1,777	1.17 (1.12-1.23)	1.15 (1.10-1.21)	1.51 (1.20-1.88)	1.60 (1.15-2.17)
Renal cancer	445	1.68 (1.53-1.85)	1.65 (1.50-1.82)	1.87 (1.09-3.00)	2.89 (1.54-4.94)
>1 year SIR (95% CI)	248	1.19 (1.05-1.35)	1.21 (1.06-1.38)	0.71 (0.23-1.66)	0.87 (0.18-2.54)
Urinary bladder cancer	916	1.16 (1.08-1.23)	1.13 (1.06-1.21)	1.29 (0.88-1.81)	2.22 (1.48-3.18)
>1 year SIR (95% CI)	612	0.99 (0.91-1.07)	0.99 (0.91-1.07)	0.86 (0.50-1.38)	1.21 (0.63-2.12)
Lymphatic leukemia	284	1.58 (1.40-1.77)	1.51 (1.33-1.70)	2.38 (1.33-3.92)	3.69 (1.90-6.44)
>1 year SIR (95% CI)	127	0.90 (0.75-1.07)	0.88 (0.73-1.06)	0.82 (0.22-2.11)	2.03 (0.66-4.73)
Myeloid malignancies	360	2.60 (2.33-2.88)	1.88 (1.65-2.13)	5.20 (3.36-7.67)	36.08 (28.94-44.46)
>1 year SIR (95% CI)	162	1.50 (1.28-1.75)	1.27 (1.06-1.50)	1.90 (0.76-3.91)	13.57 (8.78-20.03)
Non-Hodgkin lymphoma	628	1.51 (1.40-1.64)	1.50 (1.38-1.63)	1.58 (1.00-2.38)	2.07 (1.16-3.42)
>1 year SIR (95% CI)	334	1.02 (0.91-1.13)	1.03 (0.92-1.15)	0.80 (0.37-1.52)	0.72 (0.20-1.84)
Multiple myeloma	344	2.25 (2.02-2.50)	2.24 (2.00-2.49)	1.50 (0.65-2.95)	4.48 (2.31-7.82)
>1 year SIR (95% CI)	143	1.18 (1.00-1.39)	1.21 (1.02-1.43)	0.48 (0.06-1.74)	0.97 (0.12-3.51)

*****Expressed as SIRs with corresponding 95 % CIs. All statistical tests were two-sided. Abbreviations: Cbl: cobalamin, CI: confidence interval, SIR: standardized incidence ratio

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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.

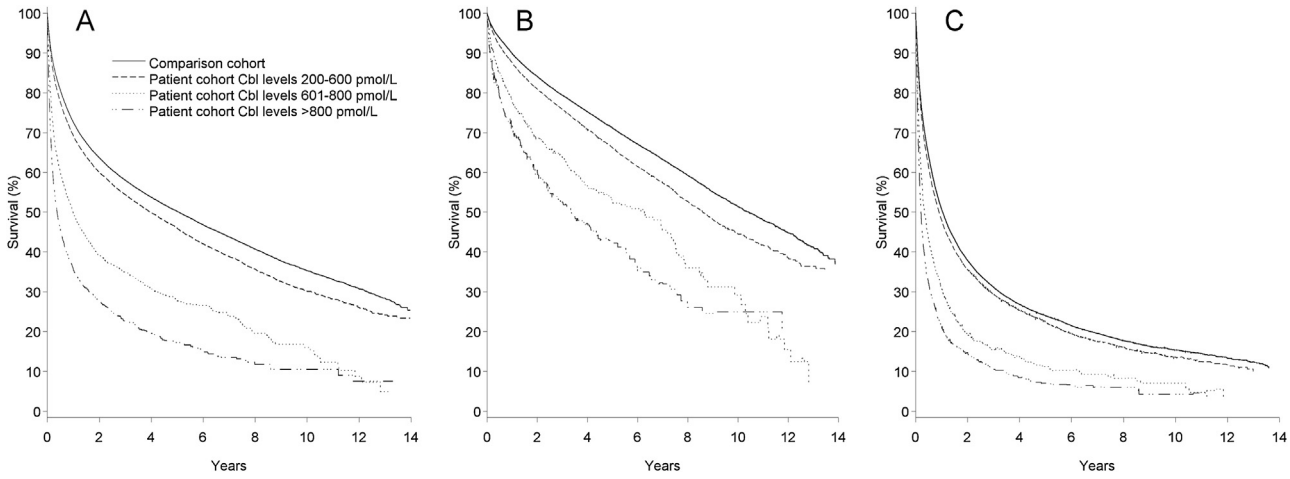

Fig. 1. Kaplan–Meier curves showing survival as a percentage in the comparison cohort (—) and in the patient cohort, disaggregated according to Cbl levels of 200–600 pmol/L (- - -), 601–800 pmol/L (···) and >800 pmol/L (·-·). These figures are based on multiple imputations to account for missing cancer 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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Supplementary Data

Methods. Description of registries and databases

The LABKA database contains the results of tests on blood and other bodily fluids routinely performed in hospital laboratories in Northern Denmark (total population = 2.2 million inhabitants). Results from more than 1,700 different types of analyses are included in the LABKA database, starting in 1998. Each record in the database contains the test result (or indicates a missing result), the patient's CPR number, the test date and the International Nomenclature, Properties and Units (NPU) code. Local analysis codes are used for some analyses (see Supplementary Table S1 for all codes used in this study).

The AUPD holds data on all prescription costs that are reimbursed for patients in the study area since 1998. Patients receive full or partial tax-paid reimbursement for most prescription medications in Denmark. Costs for over-the-counter drugs and prescribed oral contraceptives and sedatives are normally not reimbursed. AUPD records contain the date of dispensing, the patient's CPR number, codes identifying the prescribing physician/clinic/hospital department, the Anatomical Therapeutic Chemical (ATC) code and the medication name, pack size, dose units and manufacturer.

Since 1943, the DCR has collected individual-level data on all cancer diagnoses in Denmark, using the CPR number as identifier. Diagnoses are coded according to the International Classification of Diseases, 10^{th} revision (ICD-10). Registration of cancers in the DCR is mandatory for all physicians in Denmark.

The DNRP [26] was established in 1977 to collect information on all inpatient hospitalisations in Denmark. Information on all hospital outpatient clinic visits has also been recorded since 1995. The DNRP contains the patient's CPR number, date of hospital admission and discharge or date of hospital outpatient visit and up to 20 diagnoses coded by physicians. One diagnosis is coded as the primary diagnosis, *i.e.*, the main reason for the inpatient or outpatient hospital contact. Codes for different procedures and treatments are also held in the DNRP. All diagnoses have been coded according to the ICD-10 since 1994.

Supplementary Table S1

Registry	Codes	
Danish Cancer Registry	ICD-10 codes for malignant disease	C00-C97, D45, D46, D47.0, D47.1, D47.3, D47.4, D47.5, D48
Danish National Registry of Patients	ICD-10 codes for non-malignant disease used for CCI scoring	A00-B99, D50-D89, E00-E90, F00-F99, G00-G99, H00-H95, I00-I99, J00-J99, L00-L99, M00-M99, N00-N99, O00-O99, P00-Q99, R00-R99, S00-Y09, Z12
	Procedure code for Cbl therapy	BOHC2
Laboratory Information System Research Database	NPU/local Danish analysis codes for plasma Cbl measurement	NPU01700, AAA00281, AAA00304
Aarhus University Prescription Database	ATC codes for Cbl therapy	B03BA01, B03BA02, B03BA03, A11E

Supplementary Table S1. ICD-10 diagnosis codes, NPU codes, local Danish analysis codes, procedure codes and ATC codes used in the study.

Supplementary Table S2.

	Survival probability by follow-up, % (95% CI)		
	30 days	90 days	365 days
Comparison cohort			
Overall	95.4 (95.2 - 95.6)	88.9 (88.6 - 89.2)	74.8 (74.4 - 75.1)
Localised cancer	98.9 (98.7 - 99.0)	97.3 (97.1 - 97.5)	91.9 (91.6 - 92.2)
Non-localised cancer	90.7 (90.3 - 91.1)	77.5 (76.9 - 78.1)	51.5 (50.8 - 52.2)
Patient cohort			
Overall			
Plasma Cbl levels (pmol/L)			
200-600	95.0 (94.6 - 95.3)	87.0 (86.5 - 87.5)	72.1 (71.4 - 72.8)
601-800	88.7 (86.8 - 90.4)	74.2 (71.6 - 76.5)	52.9 (50.1 - 55.7)
>800	82.8 (80.3 - 85.1)	61.7 (58.5 - 64.7)	38.2 (35.1 - 41.3)
P for trend[a]	<0.0001	<0.0001	<0.0001
Localised cancer			
Plasma Cbl levels (pmol/L)			
200-600	98.8 (98.5-99.0)	96.5 (96.0 - 96.8)	90.1 (89.5 - 90.7)

601-800	97.6 (95.9 - 98.6)	93.5 (91.0 - 95.3)	80.8 (77.2 - 83.9)
>800	96.0 (93.1 - 97.7)	90.0 (86.1 - 92.9)	76.4 (71.2 - 80.8)
P for trend[a]	<0.0001	<0.0001	<0.0001
Non-localised cancer			
Plasma Cbl levels (pmol/L)			
200-600	90.1 (89.3 - 90.7)	74.6 (73.6 - 75.6)	48.4 (47.3 - 49.6)
601-800	81.9 (78.8 - 84.5)	59.3 (55.5 - 62.8)	31.4 (27.9 - 34.8)
>800	76.8 (73.4 - 79.8)	48.6 (44.8 - 52.4)	20.7 (17.6 - 23.8)
P for trend[a]	<0.0001	<0.0001	<0.0001

Supplementary Table S2. Survival probability (with 95% CIs) for cancer patients with complete data on cancer stage (patient cohort: n = 18,160; comparison cohort: n = 48,959). Lymphatic leukemia and malignant myeloid diseases were not included in the analyses. Disaggregated according to Cbl levels (in pmol/L) and follow-up time. [a]Log rank test for trend for survival across the three Cbl level groups. Abbreviations: Cbl: cobalamin; CI: confidence interval.

Supplementary table S3.

	366 days-2-years			3-4 years			≥5 years		
	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	1.6 (1.3 - 1.9)	1.6 (1.3 - 1.9)	0.9784	1.3 (1.1 - 1.6)	1.6 (1.3 - 2.0)	0.0961	1.2 (0.9 - 1.7)	1.2 (0.8 - 1.8)	0.8744
Localised cancer	1.4 (1.0 - 1.9)	2.1 (1.5 - 2.9)	0.0779	1.2 (1.0 - 1.6)	1.6 (1.2 - 2.2)	0.2089	1.3 (0.9 - 2.0)	1.4 (0.9 - 2.3)	0.9049
Non-localised cancer	1.6 (1.3 - 2.0)	1.3 (1.0 - 1.7)	0.2608	1.3 (0.9 - 1.7)	1.6 (1.2 - 2.2)	0.2564	1.0 (0.5 - 1.8)	0.8 (0.3 - 1.8)	0.6615

Supplementary table S3. Mortality risk ratios and 95% CIs for follow-up periods of 366 days-2-years, 3-4 years and ≥5 years 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 stage 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. [a]Wald chi-square test for equality in MRR estimates. Abbreviations: Cbl: cobalamin; CCI: Charlson Comorbidity Index; CI: confidence interval.

Supplementary table S4.

	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 (2.2 - 3.1)	0.0021	1.7 (1.5-2.0)	2.5 (2.1 - 2.9)	0.0006	1.6 (1.4-1.9)	2.1 (1.8-2.4)	0.0040
Sex									
Male	1.8 (1.4 - 2.3)	2.7 (2.1 - 3.5)	0.0100	2.0 (1.6 - 2.4)	2.1 (1.7 - 2.7)	0.6595	1.8 (1.5 - 2.2)	2.1 (1.7 - 2.6)	0.2400
Female	2.0 (1.5 - 2.5)	2.6 (2.1-3.3)	0.0705	1.5 (1.2-1.9)	2.8 (2.3-3.4)	<0.0001	1.5 (1.2-1.8)	2.1 (1.7 - 2.5)	0.0059
Age at diagnosis									
0-40	0.0 (.-.)	2.6 (0.3 - 21.8)	0.9974	0.0 (.-.)	6.8 (0.9 - 54.1)	0.9984	1.8 (0.5 - 6.5)	1.1 (0.1 - 8.8)	0.6821
41-60	3.2 (2.0 - 5.2)	2.7 (1.6 - 4.4)	0.5442	2.2 (1.5 - 3.3)	2.6 (1.8 - 3.8)	0.5261	1.3 (0.9 - 1.8)	3.1 (2.3 - 4.1)	<0.0001
61-80	1.6 (1.3 - 2.1)	2.9 (2.4 - 3.6)	0.0001	1.7 (1.4-2.1)	2.5 (2.0 - 3.0)	0.0045	1.7 (1.4-2.0)	1.9 (1.6-2.3)	0.3003
≥81	2.0 (1.4 - 2.7)	2.0 (1.4 - 2.8)	0.9406	1.6 (1.1 - 2.2)	2.1 (1.5-3.0)	0.1917	1.7 (1.3 - 2.3)	1.8 (1.3 - 2.6)	0.7593
Year of diagnosis									
2001-2005	1.8 (1.3 - 2.6)	2.2 (1.6 - 3.0)	0.4485	1.9 (1.4 - 2.6)	2.5 (1.9 - 3.3)	0.1677	2.0 (1.5 - 2.6)	1.9 (1.4 - 2.6)	0.8456

2006-2010	2.1 (1.6 - 2.7)	3.2 (2.5-4.0)	0.0080	1.8 (1.4 - 2.3)	2.3 (1.8-2.9)	0.1056	1.6 (1.3-2.0)	2.1 (1.7 - 2.5)	0.0972
2011-2013	1.5 (1.1 - 2.2)	2.4 (1.7 - 3.4)	0.0613	1.6 (1.2 - 2.1)	2.7 (2.1-3.6)	0.0028	1.5 (1.2-1.8)	2.4 (1.9 - 3.0)	0.0011
Cancer stage									
Localised	1.7 (1.0 - 3.1)	3.2 (1.8 - 5.6)	0.1225	1.7 (1.1 - 2.7)	2.4 (1.4 - 3.9)	0.3257	1.9 (1.5-2.5)	2.3 (1.6 - 3.1)	0.4498
Non-localised	1.9 (1.5 - 2.3)	2.6 (2.2-3.1)	0.0044	1.7 (1.5 - 2.0)	2.5 (2.1 - 2.9)	0.0010	1.5 (1.3-1.8)	2.1 (1.8 - 2.4)	0.0025

Supplementary table S4. 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 only include patients with complete data on cancer stage (patient cohort: n = 18,160). Lymphatic leukemia and malignant myeloid diseases were not included in the analyses. [a]Wald chi-square test for equality in MRR estimates. Abbreviations: Cbl: cobalamin; CCI: Charlson Comorbidity Index; CI: confidence interval.

Supplementary Table S5.

	Survival probability by follow-up, % (95% CI)		
Cancer type	30 days	90 days	365 days
Gastric			
200-600 pmol/L	89.8 (86.5-92.3)	72.6 (68.1-76.5)	41.2 (36.5-45.8)
601-800 pmol/L	79.3 (59.6-90.1)	65.5 (45.4-79.7)	27.6 (13.1-44.3)
>800 pmol/L	78.9 (62.3-88.9)	50.0 (33.4-64.5)	23.7 (11.8-37.9)
P for trend[a]	0.0086	0.0006	0.0002
Colorectal			
200-600 pmol/L	93.8 (92.9-94.6)	86.6 (85.3-87.7)	72.9 (71.3-74.5)
601-800 pmol/L	89.5 (84.2-93.1)	73.7 (66.8-79.4)	54.7 (47.4-61.5)
>800 pmol/L	82.8 (76.1-87.8)	65.6 (57.8-72.4)	41.7 (34.1-49.1)
P for trend[a]	<0.0001	<0.0001	<0.0001
Liver			
200-600 pmol/L	86.7 (80.9-90.9)	63.0 (55.5-69.6)	32.0 (25.4-38.9)
601-800 pmol/L	81.8 (70.2-89.2)	51.5 (38.9-62.7)	24.2 (14.7-35.0)
>800 pmol/L	67.5 (56.1-76.6)	43.7 (32.7-54.2)	21.3 (13.1-30.7)
P for trend[a]	0.0004	0.0006	0.0029
Pancreas			

200-600 pmol/L	82.2 (78.8-85.1)	54.3 (50.1-58.3)	20.3 (17.1-23.7)
601-800 pmol/L	76.9 (68.3-83.4)	45.5 (36.4-54.0)	14.0 (8.6-20.8)
>800 pmol/L	69.4 (61.2-76.2)	40.8 (32.8-48.6)	13.6 (8.7-19.7)
P for trend[a]	0.0005	0.0009	0.0009
Lung			
200-600 pmol/L	84.8 (83.4-86.1)	66.2 (64.4-68.0)	33.0 (31.3-34.8)
601-800 pmol/L	78.7 (73.6-82.9)	59.7 (53.9-65.0)	27.0 (22.1-32.1)
>800 pmol/L	74.2 (68.0-79.4)	46.5 (39.9-52.9)	19.7 (14.8-25.1)
P for trend[a]	<0.0001	<0.0001	<0.0001
Breast			
200-600 pmol/L	98.8 (98.1-99.3)	97.1 (96.1-97.8)	91.7 (90.2-93.0)
601-800 pmol/L	95.1 (88.6-97.9)	92.2 (84.9-96.0)	84.3 (75.7-90.1)
>800 pmol/L	94.9 (86.9-98.0)	83.3 (73.0-90.0)	78.2 (67.3-85.8)
P for trend[a]	0.0002	<0.0001	<0.0001
Prostate			
200-600 pmol/L	97.7 (97.1-98.3)	95.5 (94.6-96.3)	89.5 (88.2-90.7)
601-800 pmol/L	95.6 (88.7-98.3)	93.4 (85.9-97.0)	82.4 (72.9-88.8)
>800 pmol/L	86.7 (75.1-93.1)	81.7 (69.3-89.4)	65.0 (51.5-75.6)
P for trend[a]	<0.0001	<0.0001	<0.0001

Kidney			
200-600 pmol/L	94.5 (92.0-96.2)	83.0 (79.3-86.0)	65.9 (61.5-69.9)
601-800 pmol/L	84.8 (67.4-93.4)	75.8 (57.3-87.1)	60.6 (42.0-74.9)
>800 pmol/L	91.9 (76.9-97.3)	62.2 (44.6-75.6)	32.4 (18.2-47.5)
P for trend[a]	0.1479	0.0011	<0.0001
Urinary bladder			
200-600 pmol/L	93.6 (91.0-95.5)	82.1 (78.3-85.2)	60.3 (55.7-64.5)
601-800 pmol/L	94.3 (79.0-98.5)	85.7 (69.0-93.8)	48.6 (31.4-63.7)
>800 pmol/L	78.9 (62.3-88.9)	63.2 (45.9-76.3)	36.8 (22.0-51.8)
P for trend[a]	0.0029	0.0095	0.0003
Non-Hodgkin lymphoma			
200-600 pmol/L	95.0 (93.6-96.0)	88.0 (86.1-89.7)	74.3 (71.8-76.7)
601-800 pmol/L	82.3 (71.9-89.1)	73.4 (62.2-81.8)	63.3 (51.7-72.8)
>800 pmol/L	90.0 (80.2-95.1)	80.0 (68.6-87.6)	64.3 (51.9-74.3)
P for trend[a]	0.0003	0.0005	0.0041
Lymphatic leukemia[b]			
200-600 pmol/L	96.2 (93.8-97.7)	93.2 (90.2-95.3)	86.6 (82.9-89.6)
601-800 pmol/L	96.2 (75.7-99.4)	88.5 (68.4-96.1)	69.2 (47.8-83.3)
>800 pmol/L	91.7 (70.6-97.8)	79.2 (57.0-90.8)	70.8 (48.4-84.9)

P for trend[a]	0.3052	0.0094	0.0029
Malignant myeloid diseases[b]			
200-600 pmol/L	94.4 (92.4-95.9)	85.3 (82.4-87.7)	66.0 (62.3-69.4)
601-800 pmol/L	93.7 (88.3-96.7)	82.5 (75.2-87.8)	60.8 (52.3-68.3)
>800 pmol/L	88.7 (84.1-92.0)	79.7 (74.2-84.1)	57.4 (51.1-63.2)
P for trend[a]	0.0030	0.0262	0.0075
Brain and other CNS tumors			
200-600 pmol/L	93.8 (90.6-96.0)	76.0 (71.0-80.3)	40.3 (35.0-45.6)
601-800 pmol/L	90.0 (47.3-98.5)	60.0 (25.3-82.7)	40.0 (12.3-67.0)
>800 pmol/L	75.0 (12.8-96.1)	75.0 (12.8-96.1)	25.0 (0.9-66.5)
P for trend[a]	0.1013	0.3465	0.4279

Supplementary Table S5. Survival probability (with 95% CIs) for cancer patients with different cancer types. Disaggregated according to Cbl levels (in pmol/L) and follow-up time. These results are based on multiple imputations to account for missing cancer stage. [a]Log rank test for trend for survival across the three Cbl level groups. [b]Lymphatic leukemia and malignant myeloid diseases were not staged. Abbreviations: Cbl: cobalamin; CI: confidence interval.

Supplementary table S6.

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	2.0 (0.5-7.6)	2.1 (0.8-5.7)	0.9488	0.3 (0.0-2.6)	2.1 (1.0-4.6)	0.0944	1.5 (0.7-3.1)	2.1 (1.0-4.2)	0.5065
Colorectal	1.2 (0.6-2.3)	2.8 (1.7-4.6)	0.0302	2.2 (1.4-3.4)	3.0 (1.9-4.6)	0.3035	1.8 (1.2-2.5)	3.0 (2.1-4.3)	0.0273
Liver	1.9 (0.6-5.4)	5.4 (2.4-12.3)	0.0285	2.2 (1.0-4.6)	2.1 (1.0-4.5)	0.9671	1.4 (0.8-2.5)	1.4 (0.7-2.5)	0.9824
Pancreas	1.5 (0.9-2.4)	2.1 (1.3-3.2)	0.2306	1.3 (0.9-2.0)	1.4 (1.0-2.1)	0.7450	1.1 (0.7-1.7)	1.4 (0.9-2.1)	0.3731
Lung	1.5 (1.1-2.0)	1.9 (1.3-2.6)	0.3080	1.2 (0.9-1.6)	1.8 (1.4-2.4)	0.0291	1.2 (1.0-1.5)	1.4 (1.1-1.9)	0.3619
Breast	5.1 (1.0-26.5)	9.4 (2.2-39.7)	0.5046	1.6 (0.4-6.8)	10.3 (4.6-23.3)	0.0167	1.5 (0.6-3.4)	1.1 (0.3-3.4)	0.6420
Prostate	2.8 (0.6-12.2)	2.3 (0.5-10.0)	0.8688	1.9 (0.4-8.1)	0.9 (0.1-6.9)	0.5607	2.0 (0.9-4.7)	4.2 (2.1-8.4)	0.1805
Kidney	3.4 (1.1-10.2)	0.6 (0.1-4.2)	0.1074	1.1 (0.4-3.7)	1.9 (0.9-4.0)	0.4367	2.2 (0.9-5.7)	1.5 (0.6-3.5)	0.5083
Urinary bladder	0.7 (0.1-5.2)	0.8 (0.1-5.8)	0.9199	0.8 (0.3-2.4)	1.4 (0.5-3.5)	0.4803	1.5 (0.7-3.0)	1.5 (0.7-3.1)	0.9251
Non-Hodgkin lymphoma	8.8 (3.5 - 22.6)	2.6 (0.6 - 11.3)	0.1310	2.5 (0.9 - 7.5)	1.6 (0.6 - 4.6)	0.5462	0.3 (0.0 - 2.3)	1.2 (0.4 - 3.3)	0.2350
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	1.6 (0.0-.)	0.0 (.-.)	-	0.0 (.-.)	0.0 (.-.)	-	0.0 (.-.)	0.0 (.-.)	-

Supplementary table S6. 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 only include patients with complete data on cancer stage (patient cohort: n = 18,160). [a]Wald chi-square test for equality in MRR estimates. [b]Lymphatic leukemia and malignant myeloid diseases were not staged. Abbreviations: Cbl: cobalamin; CCI: Charlson Comorbidity Index; CI: confidence interval.

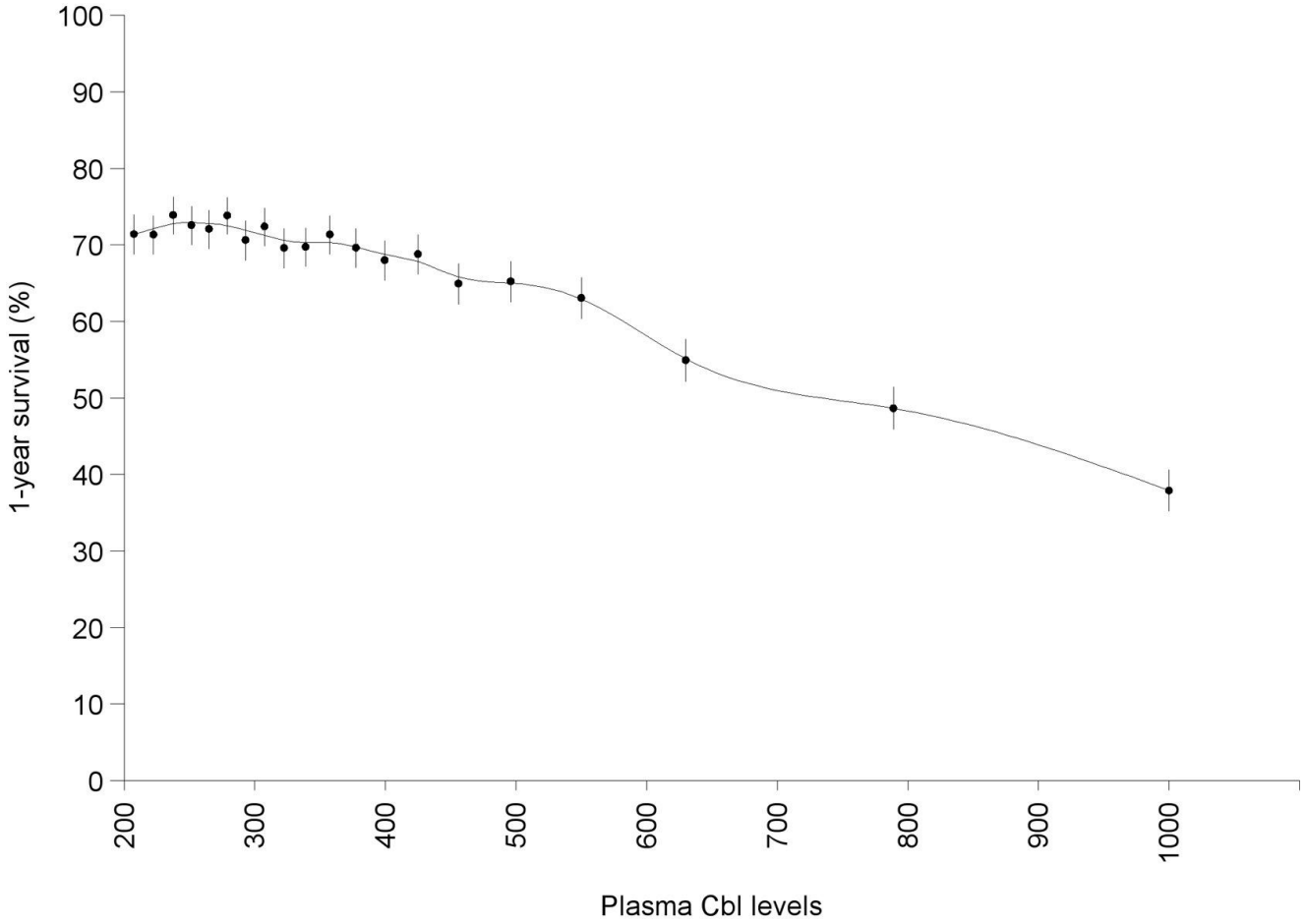

Figure S1. Spline regression analysis using plasma Cbl levels in 5%-percentiles to assess 1-year survival with increasing plasma Cbl levels in cancer patients. Abbreviations: Cbl: cobalamin.

Appendix 4

Table A4.1. Comparability across laboratory codes

	Plasma Cbl measurement							
NPU code	**Laboratory code**	Minimum	25th percentile	Median	75th percentile	Maximum	Mean	No. of measurements
NPU01700	-	2	250	331	453	2,376	393	1,835,456
	110212	41	377	471	628	4,000	609	7,075
	111212	40	252	332	460	1,764	420	67,792
	1316711	0	244	312	419	1,500	386	57,767
	1416711	10	237	307	417	1,500	382	145,776
	1516710	2	239	318	443	10,632	388	39,465
	1610117	38	242	316	442	1,400	410	55,997
	1711700	70	246	338	479	1,400	418	1,878
	1811700	22	244	318	444	1,400	407	116,439

Table A4.1. Plasma Cbl measurement in the LABKA database identified using different laboratory codes. Minimum, maximum, median, mean, 25th and 75th percentile and number of measurement per code are shown.

Table A4.2. Comparability across calendar years

	Plasma Cbl measurement						
Calendar year	Minimum	25th percentile	Median	75th percentile	Maximum	Mean	No. of measurements
1997	0	232	323	468	4,374	408	26,743
1998	0	238	330	475	10,632	421	33,293
1999	0	221	306	434	3,995	392	42,557
2000	0	244	323	452	1,500	413	61,548
2001	57	256	328	448	1,692	421	65,777
2002	15	241	314	439	1,500	407	84,741
2003	0	243	316	438	1,642	402	98,060
2004	10	240	313	431	1,500	394	110,637
2005	29	242	314	428	1,500	390	170,169
2006	22	246	323	440	1,500	397	195,252
2007	31	241	316	433	1,500	388	148,391
2008	8	246	321	434	1,500	383	185,919
2009	24	245	322	440	1,500	383	227,464
2010	22	245	327	450	1,500	387	237,373
2011	11	253	340	468	1,476	395	236,099
2012	22	252	338	466	1,488	393	263,199
2013	22	264	347	469	1,476	398	291,556

Table A4.2. Plasma Cbl measurement in the LABKA database identified per calendar year. Minimum, maximum, median, mean, 25th and 75th percentile and number of measurement per year are shown.

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1. Ane Marie Thulstrup: Mortality, infections and operative risk in patients with liver cirrhosis in Denmark. Clinical epidemiological studies. PhD thesis. *2000*.

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4. Charlotte Olesen: Use of the North Jutland Prescription Database in epidemiological studies of drug use and drug safety during pregnancy. PhD thesis. *2001*.

5. Yuan Wei: The impact of fetal growth on the subsequent risk of infectious disease and asthma in childhood. PhD thesis. *2001*.

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