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A protocol for prospective study of vitamin D obesity, and leptin in relation to bladder cancer, incidence and survival.

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Abstract

Introduction: Bladder cancer (BC) (including renal pelvis, ureter and urethra) is one of the most common urogenital cancers and the fourth most frequent cancer in men in the United States. In Norway, the incidence of BC has increased over the last decades. The age-standardized incidence rates per 100,000 for 2011-15 were 50.4 in men and 15.5 in women. Compared to the 5-year period 2006-2010, the percentage increase in incidence was 9.4% in men and 8.9% in women. The recurrence rate of BC is over 50%, the highest recurrence rate of any malignancy. Smoking and occupational exposure to aromatic amines are recognized as the major risk factors. Recently, low serum level of 25(OH)D and obesity have been suggested to increase the BC risk and leptin, which is important in weight regulation, may be involved in bladder carcinogenesis. More knowledge on potential risk factors for BC is necessary for planning and implementing primary prevention measures.

Methods and analyses: Cohort and nested case-control studies will be carried out using the population-based Janus Serum Bank Cohort consisting of pre-diagnostic sera, clinical measurement data (body height and weight, blood pressure, cholesterol and triglycerides) and self-reported information on lifestyle factors (smoking, physical activity). Participants were followed from cohort inclusion (1972-2003) through 2014. The cohort will be linked to the Cancer Registry of Norway (cancer data), the national Cause of Death Registry (date and cause of death), National Population Registry (vital status) and Statistic Norway (education and occupation). Serum samples will be analyzed for 25(OH)D, vitamin D binding protein, leptin, albumin, calcium and parathyroid hormone.

Cox regression and conditional logistic regression models will be used to estimate association between the exposures and BC.

Ethics and dissemination: The study has been approved by the Regional Committee for Medical Research Ethics and is funded by the Norwegian Cancer Society. Results will be published in peer-reviewed journals, at scientific conferences and through press releases.

Strengths and limitations of this study

- Use of a large sample set of more than 2300 incident BC cases
- Pre-diagnostic serum samples assure the temporality of the relationship between exposure and BC, limiting the possibility of reverse causality.
- Use of unique personal identification number for linkages between multiple data sources, to establish a virtually complete study file and complete ascertainment of follow-up.
- Reviewing and characterizing T-stage for all BC.
- Cases of the premalignant stages (papillary transitional carcinoma and carcinoma in situ) are not included in sub-study II.

Introduction

Rationale and evidence gaps

Urinary bladder cancer (BC) (including renal pelvis, ureter and urethra) is the most common urogenital cancer after prostate cancer and is the fourth most frequent cancer in men in the United States¹. BC has over a 50 % recurrence rate, the highest of any malignancy, and is one of the most expensive cancers to treat on a per-patient basis². In Norway, the incidence of cancer of the bladder has been increasing over the last decades. In 2015, the age-standardized incidence rates were 53.7 and 16.5 in Norwegian men and women, respectively. Compared to the 5-year period 2006-2010, the increase in incidence has been 6.1 % in men and 12.3 % in women. Up to 50 % of all BC cases have been ascribed to smoking³, and 5–25 % of the cases have been attributable to occupational exposures⁴; still, the etiology of up to 45 % of BC remains unexplained. Low serum level of 25(OH)D and obesity have been suggested to increase BC risk, and the hormone leptin, which is important in weight regulation, may be involved in its carcinogenic process^{5,6}.

25(OH)D is converted to its active hormonal form, 1-25-dihydroxyvitamin D (1,25(OH)₂D), by 1- α -hydroxylase, which is present in most tissues in the body⁷. PTH and calcium level are important factors as they affect the enzymatic conversion from 25(OH)D to active 1, 25-(OH)D₃ in the kidney and may be involved in non-classical synthesis. Measurement of circulating 25(OH)D is considered the gold standard measurement of vitamin D status as it integrates vitamin D exposure from oral intake from diet or supplements, as well as from

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3 exposure to ultraviolet radiation (UVR) ^{8,9}. Despite being the gold standard, total circulating
4 25(OH)D may not be the best measure of 25(OH)D exposure for all tumors. The “free
5 hormone hypothesis” suggests that only unbound, free hormones can have biologic effects
6 on target tissues ¹⁰. To date, few studies have examined the role of vitamin D binding protein
7 (DBP) also known as group-specific component or Gc-globulin, in the association between
8 25(OH)D and various cancer processes. DBP transports both 25(OH)D and 1,25(OH)₂D in
9 circulation. This protein carries 88% of 25(OH)D and 85% of 1,25(OH)₂D; an additional 12% of
10 25(OH)D and 15% of 1,25(OH)₂D circulate bound to albumin. Clinical laboratory assays of
11 circulating 25(OH)D that are currently in use measure total 25(OH)D without differentiating
12 between the bound and free forms. Thus, it remains unclear whether total or free 25(OH)D
13 is more biologically relevant with respect to risk of BC. Two previous studies have examined
14 free, in addition to total 25(OH)D, in relation to risk of BC. One found an inverse association
15 between total 25(OH)D and bladder cancer that appeared to be restricted to participants
16 with low DBP, suggesting that free 25(OH)D might be more strongly associated with risk of
17 bladder cancer than total 25(OH)D ¹¹. The other study found no association between
18 25(OH)D overall or at any level of DBP concentration ¹².

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33 Body mass index (weight/height², BMI) is a reliable indicator of body fatness and can be
34 categorized as underweight (<18.5), healthy weight (18.5-24.9), overweight (25-29.9) and
35 obese (> 30). The prevalence of obesity in Norway has risen steeply the last decades. In the
36 1970s, about 15% of men and women were overweight (www.fhi.no/) while in 2013 the
37 proportions were 58.4% and 47.3%, respectively ¹³. Two meta-analyses, including 15 ¹⁴ and
38 11 ¹⁵ cohort studies conclude that obesity significantly increases the risk of BC. Leptin, a
39 hormone involved in weight regulation ¹⁶, may be involved in this potential association. High
40 leptin levels have been shown to impact development of several forms of cancer ¹⁷. A study
41 of Yuan et al. ⁶ shows that leptin receptors are aberrantly expressed in BC tissue and a high
42 leptin level has been associated with BC carcinogenesis ^{5,6}.

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50 Low 25(OH)D levels are more frequent in obese persons, suggesting that 25(OH)D deficiency
51 is associated with obesity and vice versa. 25(OH)D deficiency is suggested to be associated
52 with obesity ¹⁸⁻²⁰, and both low 25(OH)D and obesity are suggested to contribute to
53 development of BC.
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Aims and hypotheses

Better-targeted BC primary and tertiary prevention (risk and survival) is warranted. The interplay between 25(OH)D and obesity and their associations with BC risk are poorly understood. We propose a study aiming to examine BMI, serum levels of total and free 25(OH)D, and leptin levels in relation to BC risk and survival by using samples from Janus Serum Bank and associated data from population-based registries and surveys.

We hypothesize that

- 1 a. Obesity is associated with increased BC risk;
- 1b. Obesity is associated with reduced BC survival;
- 2a. Low free and total 25(OH)D level and high serum leptin levels (>4.1 ng/mL are associated with increased risk of BC;
- 2b. Low free and total 25(OH)D level and high serum leptin levels (>4.1 ng/mL are associated with reduced BC survival.

METHODS AND ANALYSIS

Study population and data sources

The Janus Serum Bank Cohort

The study will be carried out using the Janus Serum Bank Cohort, a population-based biobank for prospective cancer studies, containing serum samples and questionnaire data from 292,851 Norwegians who participated in one or more of five Norwegian Regional Health Studies in the period 1972–2003. Detailed description of the samples and data included in the Janus Serum Bank Cohort has been published elsewhere^{21 22}. The quality aspects of long-term stored samples have been of high priority in the Janus Serum Bank and component stability for a large number of hormones, proteins, metabolites and electrolytes has been investigated²³⁻²⁵, including for both 25(OH)D, and leptin²⁶⁻²⁹. A unique 11-digit personal identification number (PIN), assigned to all Norwegian residents, will be used to link the Janus Cohort with population-based registries and surveys.

Population-based registries and surveys

The Cancer Registry of Norway (CRN) has collected notifications on cancer at a national level since 1953. Cancer reporting is mandatory by law, and reports from various sources ensures high quality and completeness (98.8%)³⁰. The reporting system, based on pathology and cytology reports, clinical records, and death certificates, provides information about site, histological type and stage of disease at the time of diagnosis. CRN has been involved in the Janus Serum Bank operation since establishment in the early 1970s and has been responsible for the data handling; in 2004 the serum bank was integrated into the CRN. The following information is available for cancer cases: month and year of diagnosis, tumor site (International Classification of Diseases 7th revision [ICD-7 codes] converted into ICD-10 codes), histology (codes from ICD-Oncology 2nd and 3rd revision), clinical stage (local = no metastases, regional = metastasis in regional lymph nodes or surrounding area, distant = distant metastasis). In addition, all BC diagnoses in the Janus cohort will be reviewed and assigned a pathological T-stage, according to the AJCC 8th ed.³¹.

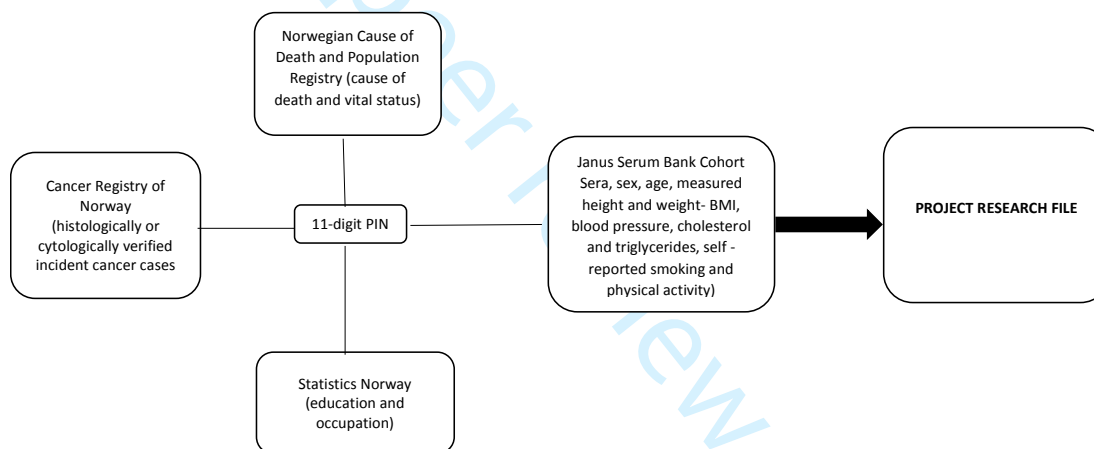
The Norwegian Institute of Public Health (NIPH) has been responsible for conducting the national health surveys, upon which the Janus Serum Bank is partly based. All participants have completed questionnaires for assessment of lifestyle factors (i.e. smoking habits, alcohol use), at the time of serum collection. A database has been established, including data from these questionnaires, as well as measured body height and weight, blood pressure, cholesterol and triglycerides^{32 33}. The Janus Cohort includes participants from five of the health studies: The Oslo Study I (1972–73), The Norwegian Counties Study (1974–78, 1977–83, and 1985–88), The Age 40 Program- Oslo (1981–99), The National Age 40 Program (1985–99) and The TROFINN Health Study (2001–03). A set of about 50 variables has been harmonized and standardized due to slightly different wording in the questionnaires²². Available variables include: height (cm), weight (kg), BMI (kg/m², categorized as 12–18.49, 18.5–24.9, 25.0–29.9, ≥30), smoking status (never, former, current), cigarettes per day (1–9, 10–14, ≥15), years of smoking (1–9, 10–29, ≥30), time since smoking cessation (<3mos, 3mos–1yr, 1–5yrs, >5yrs), and total physical activity (inactive, low, medium, high), based on leisure time activity.

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3 *The Cause of death Registry* has registered death certificates for all deaths in Norway since
4 1951. Cause of death registration is mandatory by law.
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7 *National Population Registry* contains information on vital status (alive, emigrated or dead)
8 of everyone that resides or has resided in Norway.
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11 *Statistics Norway* has the responsibility of covering the needs for official statistics on the
12 Norwegian population including individual data on settlements, migration, occupation and
13 level of education.
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16 Using the 11-digit PIN number we will link data from four different sources to set up the
17 research file, illustrated in Figure 1.
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38 Figure 1. Data collection from different sources using PIN
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Study design

Sub-study I: a prospective cohort study

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3 In a prospective cohort study among all individuals in the Janus Serum Bank Cohort (n =
4 292,851) (Figure 1), we will explore baseline BMI in relation to bladder cancer risk. Among
5 the included BC cases we will investigate baseline BMI and BC survival. By 2014, the cohort
6 included 2 347 BC cases (ICD-10: C67). BC cases of both muscle invasive and non-invasive
7 urothelial cell carcinoma, will be included in the study. Educational level, occupation, age,
8 sex, physical activity, smoking habits, cholesterol, triglycerides and blood pressure will be
9 included in the statistical analyses as confounders.
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16 Sub-study II: a nested case-control study

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18 The study will be nested within the prospective cohort described above (Study I), including a)
19 400 BC cases of muscle invasive urothelial cancer, and b) 400 controls alive and without a
20 cancer diagnosis at the time of the BC diagnosis of the cases, matched 1:1 on sex, age (+/-1
21 year) and date of serum sampling (+/-1 month). The serum samples will be analysed for total
22 25(OH)D, vitamin D binding protein and leptin. As parathyroid hormone (PTH) and albumin-
23 adjusted calcium level affect the enzymatic conversion from 25(OH)D to active 1,25-(OH)D₃
24 and might be involved in the non-classical synthesis as well, measurement of these
25 components will be taken into account.
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36 **Statistical methods**

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38 In the cohort study, Cox regression models will be used to estimate hazard ratios (HR) with
39 95% confidence intervals (CI) of BC and survival after BC, taking into account stage at
40 diagnosis, and BMI and including adjusting for season of blood collection for vitamin D. In
41 the nested case-control study, conditional logistic regression models will be used to estimate
42 the odds ratio (OR) with 95% CI.
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45 As we have a number of potential confounding variables, we will use directed acyclic graphs
46 to select variables to include in the statistical models. Confounding variables will be included
47 in the models and tests of interaction effects will be performed when relevant.
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52 All tests will be two-sided and $p < 0.05$ will be considered statistically significant. All statistical
53 analyses will be conducted using Stata version 14.1 (StataCorp, College Station, TX, USA).
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Laboratory analyses

The serum samples (aliquots of 400 μ L) will be analysed for total 25(OH) D, DBP, leptin, PTH, albumin, and calcium. The Hormone laboratory at Aker hospital, Oslo, Norway will analyze 25(OH)D, DBP, PTH and leptin. The Hormone Laboratory is accredited by the Norwegian Accreditation as a testing laboratory and complies with the requirements of the NS-EN ISO/IEC 17025 standards. Albumin and calcium will be analyzed at Department of Medical Biochemistry, Oslo University Hospital accredited by the Norwegian Accreditation reg. no TEST 103 that complies with the requirements of the NS-EN ISO 1518. The sample donors, identity and case control status will be blinded for the laboratory staff. Quality control (QC) samples from the biobank will be included in every batch to examine inter-batch and intra-batch variability.

Power and sample size

Sub-study I Within the prospective cohort ($n = 292,851$), there are more than 2,300 BC cases reported to the cancer registry until the end of follow up time. Assuming the risk of developing bladder cancer is 1% for the normal weight group and 5% for the obese group¹⁴, one would need a sample size of 586 to have 80% power. Since the sample size here is significantly larger, one can safely determine that the study has adequate statistical power.

Sub-study II In the case-control study nested into the prospective cohort, the statistical power will depend on: i) proportion of exposure in the population, ii) sample size (cases and controls) and iii) the minimum difference that is possible to detect.

Table 1 shows the smallest detectable OR according to proportion of controls exposed to low vitamin D (25(OH)D and high leptin levels, for different sample sizes. The power is 0.80 and a significance level of 0.05 (www.krothman.hostbyet2.com/Episheet.xls).

Table 1. Odds ratio based on proportion of exposed controls and sample size

		Study case:control= 1:1
Proportion		Number of cases

of exposed controls		n=500	n=400	n=300
55%*	OR	1.44	1.50	1.6
45%*	OR	1.43	1.49	1.58
30%**	OR	1.45	1.51	1.62

Exposure = 25(OH)D deficiency (25(OH)D < 50 nmol /L); **Exposure = high serum leptin levels (>4.1 ng/mL)

Based on results in the table above, we consider 400 matched case-control pairs as a sufficient sample size for the case-control study.

Data analysis plan

The following analyses will be conducted to test our hypotheses:

- Hypothesis 1.a: A prospective cohort analysis of pre-diagnostic BMI and other anthropometric measures in relation to BC risk using the complete Janus Cohort (n = 292,851)
- Hypothesis 1.b: A prospective cohort analysis of pre-diagnostic BMI and other anthropometric measures in relation to survival after a BC, using all BC cases in the Janus Cohort (n ≈ 2,650)
- Hypothesis 2.a: A nested case-control analysis of BC risk according to pre-diagnostic serum levels of total and free 25(OH)D, and leptin in 400 matched case-control pairs.
- Hypothesis 2.b: A prospective analysis of survival after a BC (n=400) according to pre-diagnostic serum levels of 25(OH)D and leptin.

Study strengths and limitations

A major strength of the large sample set of more than 2,300 incident BC cases. Also use of individual PIN for linkages between multiple data sources, to establish a virtually complete study file, with exception of data on histopathology, is a strength. The data sources are high

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3 quality population-based registries, with high degree of completeness. The bladder cancer
4 diagnoses are coded according to ICD-0. To get information on staging, and control the data
5 quality, all histopathological information will be reviewed and characterized by tumor-stage
6 (T-stage). Another strength of this study is that the public health data has been quality
7 assured, structured and harmonized²². The use of pre-diagnostic samples assure the proper
8 temporality of the relationship between exposure and BC, limiting the possibility of reverse
9 causality.
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15 It is a limitation that carcinoma in situ (Tis) regarded as a precursor lesion and the
16 premalignant stage of papillary translational cancer cases (Ta) and are not included in the
17 sub-study II. A high number (40-80 %) of patients with Tis stage will develop high grade
18 muscle invasive cancer if untreated, especially if associated with papillary tumors³⁴.
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25 **ETHICS AND DISSEMINATION**

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27 The Regional Committee for Medical and Health Research Ethics has approved the study.
28 The different data registries have approved that the use of a de-identified dataset. An ID-
29 key, consisting of the 11-digit PIN and a study-specific ID number, will be stored and
30 governed by a third party unavailable to the research team.
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35 All results will be published in relevant peer- reviewed international scientific journals and
36 presented at conferences, nationally and internationally. Results of importance will be
37 directly communicated to health authorities and to clinicians where the annual national
38 oncology conference “Onkologisk forum” can serve as a platform for knowledge distribution.
39 Results of importance will also be disseminated through press releases and to user groups
40 like the Norwegian Cancer Society. The CRN website is a potential channel to reach patients
41 organizations and the public.
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48 **Authors' contributions**

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50 REG prepared the study. TER, JSS, HL, BA, KA, EW and AM contributed to the study design
51 and reviewed and revised the protocol critically for important intellectual content, and
52 approved the final versions. REG is the guarantor.
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56 **Data sharing**

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3 Requests for data sharing/case pooling may be directed to the corresponding author. This
4 study uses third-party data derived from State government registries, which are ultimately
5 governed by their ethics committees and data custodians. Thus, any requests to share these
6 data will be subject to formal approval from each data source used in this study.
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11
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13 Society (no. 182308-2016) and the Cancer Registry of Norway Research Fund
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16 Conflicts of interest

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18 None declared
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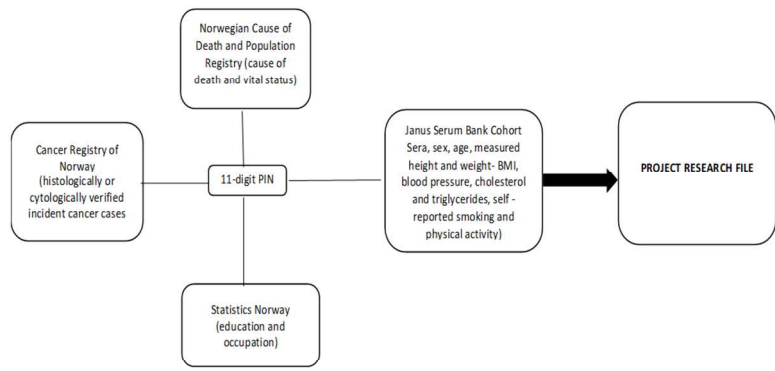


Figure 1. Data collection from different sources using PIN

338x190mm (96 x 96 DPI)

Review only

STROBE Statement—Checklist of items that should be included in reports of *cohort studies*

	Item No	Recommendation
Title and abstract	1	(a) Indicate the study's design with a commonly used term in the title or the abstract P 1 (b) Provide in the abstract an informative and balanced summary of what was done and what was found P 2
Introduction		
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported P 3-4
Objectives	3	State specific objectives, including any prespecified hypotheses P 5
Methods		
Study design	4	Present key elements of study design early in the paper P 7
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection P 5-7
Participants	6	(a) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up P 5-7 (b) For matched studies, give matching criteria and number of exposed and unexposed P 7-8
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group P 5-7, 9
Bias	9	Describe any efforts to address potential sources of bias P 8
Study size	10	Explain how the study size was arrived at P 9-10
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why
Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding P 8 (b) Describe any methods used to examine subgroups and interactions P 8 (c) Explain how missing data were addressed (d) If applicable, explain how loss to follow-up was addressed (e) Describe any sensitivity analyses
Results		
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed P 8 (b) Give reasons for non-participation at each stage (c) Consider use of a flow diagram P 7
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders P 7-8 (b) Indicate number of participants with missing data for each variable of interest (c) Summarise follow-up time (eg, average and total amount)
Outcome data	15*	Report numbers of outcome events or summary measures over time Not applicable
Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included (b) Report category boundaries when continuous variables were categorized (c) If relevant, consider translating estimates of relative risk into absolute risk for a

meaningful time period

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Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses
Discussion <i>Not applicable</i>		
Key results	18	Summarise key results with reference to study objectives
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence
Generalisability	21	Discuss the generalisability (external validity) of the study results
Other information		
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based P12

*Give information separately for exposed and unexposed groups.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at <http://www.plosmedicine.org/>, Annals of Internal Medicine at <http://www.annals.org/>, and Epidemiology at <http://www.epidem.com/>). Information on the STROBE Initiative is available at <http://www.strobe-statement.org>.

BMJ Open

A protocol for prospective study of vitamin D, obesity, and leptin in relation to bladder cancer, incidence and survival.

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Secondary Subject Heading:	Urology
Keywords:	Bladder cancer, Biobank, vitamin D, obesity

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Manuscripts

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3 A protocol for prospective study of vitamin D, obesity, and leptin in relation to bladder
4 cancer, incidence and survival
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Abstract

Introduction: Bladder cancer (BC) (including renal pelvis, ureter and urethra) is one of the most common urogenital cancers and the fourth most frequent cancer in men in the United States. In Norway, the incidence of BC has increased over the last decades. The age-standardized incidence rates per 100,000 for 2011-15 were 50.4 in men and 15.5 in women. Compared to the 5-year period 2006-2010, the percentage increase in incidence was 9.4% in men and 8.9% in women. The recurrence rate of BC is over 50%, the highest recurrence rate of any malignancy. Smoking and occupational exposure to aromatic amines are recognized as the major risk factors. Recently, low serum level of 25 hydroxy vitamin D (25(OH)D) and obesity have been suggested to increase the BC risk, and leptin, which is important in weight regulation, may be involved in bladder carcinogenesis. More knowledge on potential risk factors for BC is necessary for planning and implementing primary prevention measures.

Methods and analyses: Cohort and nested case-control studies will be carried out using the population-based Janus Serum Bank Cohort consisting of pre-diagnostic sera, clinical measurement data (body height and weight, blood pressure, cholesterol and triglycerides) and self-reported information on lifestyle factors (smoking, physical activity). Participants were followed from cohort inclusion (1972-2003) through 2014. The cohort will be linked to the Cancer Registry of Norway (cancer data), the national Cause of Death Registry (date and cause of death), National Population Registry (vital status) and Statistic Norway (education and occupation). Serum samples will be analyzed for 25(OH)D, vitamin D binding protein, leptin, albumin, calcium and parathyroid hormone.

Cox regression and conditional logistic regression models will be used to estimate association between the exposures and BC.

Ethics and dissemination: The study has been approved by the Regional Committee for Medical Research Ethics and is funded by the Norwegian Cancer Society. Results will be published in peer-reviewed journals, at scientific conferences and through press releases.

Strengths and limitations of this study

- Use of a large sample set of more than 2300 incident BC cases
- Pre-diagnostic serum samples assure the temporality of the relationship between exposure and BC, limiting the possibility of reverse causality.
- Use of unique personal identification number for linkages between multiple data sources, to establish a virtually complete study file and complete ascertainment of follow-up.
- Reviewing and characterizing T-stage for all BC.
- Lack of treatment data

Introduction

Rationale and evidence gaps

Urinary bladder cancer (BC) (including renal pelvis, ureter and urethra) is the most common urogenital cancer after prostate cancer and is the fourth most frequent cancer in men in the United States¹. BC has over a 50 % recurrence rate, the highest of any malignancy, and is one of the most expensive cancers to treat on a per-patient basis². In Norway, the incidence of cancer of the bladder has been increasing over the last decades. In 2015, the age-standardized incidence rates were 53.7 and 16.5 in Norwegian men and women, respectively. Compared to the 5-year period 2006-2010, the increase in incidence has been 6.1 % in men and 12.3 % in women. Up to 50 % of all BC cases have been ascribed to smoking³, and 5–25 % of the cases have been attributable to occupational exposures⁴; still, the etiology of up to 45 % of BC remains unexplained. Low serum level of 25 hydroxy vitamin D 25(OH)D and obesity have been suggested to increase BC risk, and the hormone leptin, which is important in weight regulation, may be involved in its carcinogenetic process^{5,6}.

25(OH)D is converted to its active hormonal form, 1-25-dihydroxyvitamin D (1,25(OH)₂D), by 1- α -hydroxylase, which is present in most tissues in the body⁷. Parathyroid hormone (PTH) and calcium level are important factors as they affect the enzymatic conversion from 25(OH)D to active 1, 25-(OH)D₃ in the kidney and may be involved in non-classical synthesis. Measurement of circulating 25(OH)D is considered the gold standard measurement of

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3 vitamin D status as it integrates vitamin D exposure from oral intake from diet or
4 supplements, as well as from exposure to ultraviolet radiation (UVR)^{8,9}. Despite being the
5 gold standard, total circulating 25(OH)D may not be the best measure of 25(OH)D exposure
6 for all tumors. The “free hormone hypothesis” suggests that only unbound, free hormones
7 can have biologic effects on target tissues¹⁰. To date, few studies have examined the role of
8 vitamin D binding protein (DBP) also known as group-specific component or Gc-globulin, in
9 the association between 25(OH)D and various cancer processes. DBP transports both
10 25(OH)D and 1,25(OH)₂D in circulation. This protein carries 88% of 25(OH)D and 85% of
11 1,25(OH)₂D; an additional 12% of 25(OH)D and 15% of 1,25(OH)₂D circulate bound to
12 albumin. Clinical laboratory assays of circulating 25(OH)D that are currently in use measure
13 total 25(OH)D without differentiating between the bound and free forms. Thus, it remains
14 unclear whether total or free 25(OH)D is more biologically relevant with respect to risk of BC.
15 Two previous studies have examined free, in addition to total 25(OH)D, in relation to risk of
16 BC. One found an inverse association between total 25(OH)D and bladder cancer that
17 appeared to be restricted to participants with low DBP, suggesting that free 25(OH)D might
18 be more strongly associated with risk of bladder cancer than total 25(OH)D¹¹. The other
19 study found no association between 25(OH)D overall or at any level of DBP concentration¹².

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34 Body mass index (weight/height², BMI) is a reliable indicator of body fatness and can be
35 categorized as underweight (<18.5), healthy weight (18.5-24.9), overweight (25-29.9) and
36 obese (> 30). The prevalence of obesity in Norway has risen steeply the last decades. In the
37 1970s, about 15% of men and women were overweight (www.fhi.no/) while in 2013 the
38 proportions were 58.4% and 47.3%, respectively¹³. Two meta-analyses, including 15¹⁴ and
39 11¹⁵ cohort studies conclude that obesity significantly increases the risk of BC. Leptin, a
40 hormone involved in weight regulation¹⁶, may be involved in this potential association. High
41 leptin levels have been shown to impact development of several forms of cancer¹⁷. A study
42 of Yuan et al.⁶ shows that leptin receptors are aberrantly expressed in BC tissue and a high
43 leptin level has been associated with BC carcinogenesis^{5,6}.

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51 Low 25(OH)D levels are more frequent in obese persons, suggesting that 25(OH)D deficiency
52 is associated with obesity and vice versa. 25(OH)D deficiency is suggested to be associated
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3 with obesity¹⁸⁻²⁰, and both low 25(OH)D and obesity are suggested to contribute to
4 development of BC.
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8 **Aims and hypotheses**

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10 Better-targeted BC primary and tertiary prevention (risk and survival) is warranted. The
11 interplay between 25(OH)D and obesity and their associations with BC risk are poorly
12 understood. We propose a study aiming to examine anthropometric data (BMI, height,
13 weight, body surface area and weight change over time) and serum levels of leptin, total and
14 free 25(OH)D, in relation to BC risk and survival by using samples from Janus Serum Bank and
15 associated data from population-based registries and surveys.
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23 We hypothesize that

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25 1 a. Obesity is associated with increased BC risk;
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27 1b. Obesity is associated with reduced BC survival;
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29 2a. Low free and total 25(OH)D level and high serum leptin levels (>4.1 ng/mL are associated
30 with increased risk of BC;
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32 2b. Low free and total 25(OH)D level and high serum leptin levels (>4.1 ng/mL are associated
33 with reduced BC survival.
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40 **METHODS AND ANALYSIS**

41 **Study population and data sources**

42 The Janus Serum Bank Cohort

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44 The study will be carried out using the Janus Serum Bank Cohort, a population-based
45 biobank for prospective cancer studies, containing serum samples and questionnaire data
46 from 292,851 Norwegians who participated in one or more of five Norwegian Regional
47 Health Studies in the period 1972–2003. Detailed description of the samples and data
48 included in the Janus Serum Bank Cohort has been published elsewhere^{21,22}. The quality
49 aspects of long-term stored samples have been of high priority in the Janus Serum Bank and
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3 component stability for a large number of hormones, proteins, metabolites and electrolytes
4 has been investigated²³⁻²⁵, including for both 25(OH)D, and leptin²⁶⁻²⁹. A unique 11-digit
5 personal identification number (PIN), assigned to all Norwegian residents, will be used to link
6 the Janus Cohort with population-based registries and surveys.
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10 11 12 Population-based registries and surveys

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15 *The Cancer Registry of Norway (CRN)* has collected notifications on cancer at a national level
16 since 1953. Cancer reporting is mandatory by law, and reports from various sources ensures
17 high quality and completeness (98.8%)³⁰. The reporting system, based on pathology and
18 cytology reports, clinical records, and death certificates, provides information about site,
19 histological type and stage of disease at the time of diagnosis. CRN has been involved in the
20 Janus Serum Bank operation since establishment in the early 1970s and has been
21 responsible for the data handling; in 2004 the serum bank was integrated into the CRN. The
22 following information is available for cancer cases: month and year of diagnosis, tumor site
23 (International Classification of Diseases 7th revision [ICD-7 codes] converted into ICD-10
24 codes), histology (codes from ICD-Oncology 2nd and 3rd revision), clinical stage (local = no
25 metastases, regional = metastasis in regional lymph nodes or surrounding area, distant =
26 distant metastasis). In addition, all BC diagnoses in the Janus cohort will be reviewed and
27 assigned a pathological T-stage, according to the American Joint Committee on Cancer
28 (AJCC) staging manual 8th ed.³¹.
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41 *The Norwegian Institute of Public Health (NIPH)* has been responsible for conducting the
42 national health surveys, upon which the Janus Serum Bank is partly based. All participants
43 have completed questionnaires for assessment of lifestyle factors (i.e. smoking habits,
44 alcohol use), at the time of serum collection. A database has been established, including
45 data from these questionnaires, as well as measured body height and weight, blood
46 pressure, cholesterol and triglycerides^{32 33}. The Janus Cohort includes participants from five
47 of the health studies: The Oslo Study I (1972–73), The Norwegian Counties Study (1974–78,
48 1977–83, and 1985–88), The Age 40 Program- Oslo (1981–99), The National Age 40 Program
49 (1985–99) and The TROFINN Health Study (2001–03). A set of about 50 variables has been
50 harmonized and standardized due to slightly different wording in the questionnaires²².
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3 Available variables include: height (cm), weight (kg), BMI (kg/m², categorized as 12–18.49,
4 18.5–24.9, 25.0–29.9, ≥30), smoking status (never, former, current), cigarettes per day (1–9,
5 10–14, ≥15), years of smoking (1–9, 10–29, ≥30), time since smoking cessation (<3months,
6 3months–1yr, 1–5yrs, >5yrs), and total physical activity (inactive, low, medium, high), based
7 on leisure time activity.
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12 *The Cause of death Registry* has registered death certificates for all deaths in Norway since
13 1951. Cause of death registration is mandatory by law.
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16 *National Population Registry* contains information on vital status (alive, emigrated or dead)
17 of everyone that resides or has resided in Norway.
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20 *Statistics Norway* has the responsibility of covering the needs for official statistics on the
21 Norwegian population including individual data on settlements, migration, occupation and
22 level of education.
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26 Using the 11-digit PIN number we will link data from four different sources to set up the
27 research file, illustrated in Figure 1.
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37 **Study design**

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39 Sub-study I: a prospective cohort study
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42 In a prospective cohort study among all individuals in the Janus Serum Bank Cohort (n =
43 292,851) (Figure 1), we will explore baseline BMI in relation to bladder cancer risk. Among
44 the included BC cases we will investigate baseline BMI and BC survival. By 2014, the cohort
45 included 2 347 BC cases (ICD-10: C67). BC cases of both muscle invasive and non-invasive
46 urothelial cell carcinoma, will be included in the study. Educational level, occupation, age,
47 sex, physical activity, smoking habits, cholesterol, triglycerides and blood pressure will be
48 included in the statistical analyses as confounders.
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55 Sub-study II: a nested case-control study
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3 The study will be nested within the prospective cohort described above (Study I), including a)
4 400 BC cases of high grade tumors, including muscle invasive (T2-T4) and non muscle
5 invasive (Ta, T1 and carcinoma in situ (Tis) cancer cases, and b) 400 controls alive and
6 without a cancer diagnosis at the time of the BC diagnosis of the cases, matched 1:1 on sex,
7 age (+/-1 year) and date of serum sampling (+/-1 month). Minimum time from blood draw
8 to diagnosis will be 5 years. The serum samples will be analysed for total 25(OH)D, vitamin D
9 binding protein and leptin. As PTH and albumin-adjusted calcium level affect the enzymatic
10 conversion from 25(OH)D to active 1,25-(OH)D₃ and might be involved in the non-classical
11 synthesis as well, measurement of these components will be taken into account.
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19 In sub-study I, we will investigate the association between BMI and BC, and for sub –study II
20 we will in addition include vitamin D levels. Overall we will focus on disentangle the
21 relationship of vitamin D, BMI and BC. This will be done in two ways:
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- 24 1. By implementing regression models including and interaction effect of vitamin D and
25 BMI on BC.
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- 28 2. By testing the hypothesis whether the effect of BMI on BC is mediated by vitamin D
29 using mediation analysis³⁴.
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34 **Statistical methods**

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36 In the cohort study, Cox regression models will be used to estimate hazard ratios (HR) with
37 95% confidence intervals (CI) of BC and survival after BC, taking into account stage at
38 diagnosis, and BMI and including adjusting for season of blood collection for vitamin D. In
39 the nested case-control study, conditional logistic regression models will be used to estimate
40 the odds ratio (OR) with 95% CI.
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44 In order to find out whether Vitamin D acts (totally or partly) as a mediator, modern causal
45 inference theory will be used to estimate different types of effects³⁴. The analysis will be
46 done by using the mediation R package³⁵
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50 As we have a number of potential confounding variables, we will use directed acyclic graphs
51 to select variables to include in the statistical models. Confounding variables will be included
52 in the models and tests of interaction effects will be performed when relevant.
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3 All tests will be two-sided and $p < 0.05$ will be considered statistically significant. All statistical
4 analyses will be conducted using Stata version 14.1 (StataCorp, College Station, TX, USA).
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9 **Laboratory analyses**

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11 The serum samples (aliquots of 400 μL) will be analysed for total 25(OH) D, DBP, leptin, PTH,
12 albumin, and calcium. The Hormone laboratory at Aker hospital, Oslo, Norway will analyze
13 25(OH)D, DBP, PTH and leptin. The Hormone Laboratory is accredited by the Norwegian
14 Accreditation as a testing laboratory and complies with the requirements of the NS-EN
15 ISO/IEC 17025 standards. Albumin and calcium will be analyzed at Department of Medical
16 Biochemistry, Oslo University Hospital accredited by the Norwegian Accreditation reg. no
17 TEST 103 that complies with the requirements of the NS-EN ISO 1518. The sample donors,
18 identity and case control status will be blinded for the laboratory staff. Quality control
19 samples from the biobank will be included in every batch to examine inter-batch and intra-
20 batch variability.
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38 **Power and sample size**

39 **Sub-study I** Within the prospective cohort ($n = 292,851$), there are more than 2,300 BC cases
40 reported to the cancer registry until the end of follow up time. Assuming the risk of
41 developing bladder cancer is 1% for the normal weight group and 5% for the obese group¹⁴,
42 one would need a sample size of 586 to have 80% power. Since the sample size here is
43 significantly larger, one can safely determine that the study has adequate statistical power.
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48 **Sub-study II** In the case-control study nested into the prospective cohort, the statistical
49 power will depend on: i) proportion of exposure in the population, ii) sample size (cases and
50 controls) and iii) the minimum difference that is possible to detect.
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54 Table 1 shows the smallest detectable OR according to proportion of controls exposed to
55 low vitamin D (25(OH)D and high leptin levels, for different sample sizes. The power is 0.80
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and a significance level of 0.05 (www.krothman.hostbyet2.com/Episheet.xls). The expected proportions of exposed controls were based on previous studies on serum samples from the Janus Cohort. For 25(OH)D, a study on prostate cancer reported that 4.4% and 30.6% of the controls had 25(OH)D levels below 30 nmol/L and 50 nmol/L, respectively.²⁹ For leptin, a study on colon cancer reported that 20% of the controls had a leptin level of 4.1 ng/mL or higher.²⁷

Table 1. Odds ratio (OR) based on proportion of exposed controls and sample size

Proportion of exposed controls		Study case:control= 1:1		
		Number of cases		
		n=500	n=400	n=300
55%*	OR	1.44	1.50	1.6
45%*	OR	1.43	1.49	1.58
30%**	OR	1.45	1.51	1.62

*Exposure = 25(OH)D deficiency (25(OH)D < 50 nmol /L);

**Exposure = high serum leptin levels (>4.1 ng/mL)

Based on results in the table above, we consider 400 matched case-control pairs as a sufficient sample size for the case-control study.

Data analysis plan

The following analyses will be conducted to test our hypotheses:

- Hypothesis 1.a: A prospective cohort analysis of pre-diagnostic BMI and other anthropometric measures in relation to BC risk using the complete Janus Cohort (n = 292,851)

- Hypothesis 1.b: A prospective cohort analysis of pre-diagnostic BMI and other anthropometric measures in relation to survival after a BC, using all BC cases in the Janus Cohort (n ≈ 2,650)
- Hypothesis 2.a: A nested case-control analysis of BC risk according to pre-diagnostic serum levels of total and free 25(OH)D, and leptin in 400 matched case-control pairs.
- Hypothesis 2.b: A prospective analysis of survival after a BC (n=400) according to pre-diagnostic serum levels of 25(OH)D and leptin.

Study strengths and limitations

A major strength of the large sample set of more than 2,300 incident BC cases. Also use of individual PIN for linkages between multiple data sources, to establish a virtually complete study file, with exception of data on histopathology, is a strength. The data sources are high quality population-based registries, with high degree of completeness. The bladder cancer diagnoses are coded according to ICD-0. To get information on staging, and control the data quality, all histopathological information will be reviewed and characterized by tumor-stage (T-stage). Another strength of this study is that the public health data has been quality assured, structured and harmonized²². The use of pre-diagnostic samples assure the proper temporality of the relationship between exposure and BC, limiting the possibility of reverse causality.

Treatment data is of importance when evaluating the survival analyses. These data are missing and will be a limitation of this study

ETHICS AND DISSEMINATION

The Regional Committee for Medical and Health Research Ethics has approved the study. The different data registries have approved that the use of a de-identified dataset. An ID-key, consisting of the 11-digit PIN and a study-specific ID number, will be stored and governed by a third party unavailable to the research team.

All results will be published in relevant peer-reviewed international scientific journals and presented at conferences, nationally and internationally. Results of importance will be

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3 directly communicated to health authorities and to clinicians where the annual national
4 oncology conference “Onkologisk forum” can serve as a platform for knowledge distribution.
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6 Results of importance will also be disseminated through press releases and to user groups
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8 like the Norwegian Cancer Society. The CRN website is a potential channel to reach patients
9
10 organizations and the public.

11 **Authors' contributions**

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14 REG prepared the study. TER, JSS, HHH, HL, BA, KA, EW and AM contributed to the study
15 design and reviewed and revised the protocol critically for important intellectual content,
16 and approved the final versions. REG is the guarantor.
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19 **Data sharing**

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22 Requests for data sharing/case pooling may be directed to the corresponding author. This
23 study uses third-party data derived from State government registries, which are ultimately
24 governed by their ethics committees and data custodians. Thus, any requests to share these
25 data will be subject to formal approval from each data source used in this study.
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29 **Funding**

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31
32 The research study has been reviewed and granted funding by the Norwegian Cancer
33 Society (no. 182308-2016) and the Cancer Registry of Norway Research Fund
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35

36 **Conflicts of interest**

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39 None declared
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Figure legends

Figure 1. Data collection from different sources using PIN

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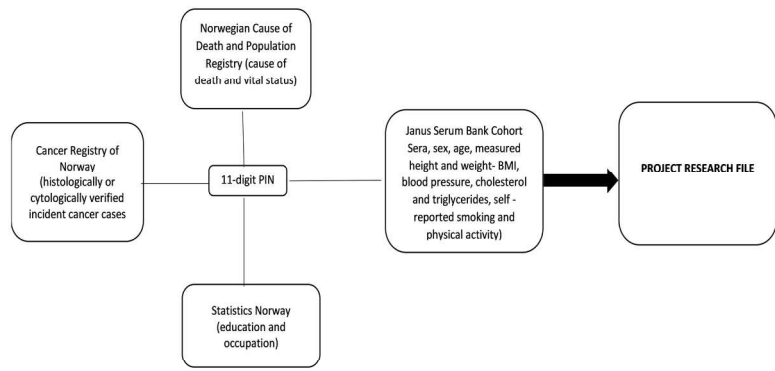


Figure 1. Data collection from different sources using PIN

190x107mm (300 x 300 DPI)

review only

STROBE Statement—Checklist of items that should be included in reports of *cohort studies*

	Item No	Recommendation
Title and abstract	1	(a) Indicate the study's design with a commonly used term in the title or the abstract P 1 (b) Provide in the abstract an informative and balanced summary of what was done and what was found P 2
Introduction		
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported P 3-4
Objectives	3	State specific objectives, including any prespecified hypotheses P 5
Methods		
Study design	4	Present key elements of study design early in the paper P 7
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection P 5-7
Participants	6	(a) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up P 5-7 (b) For matched studies, give matching criteria and number of exposed and unexposed P 7-8
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group P 5-7, 9
Bias	9	Describe any efforts to address potential sources of bias P 8
Study size	10	Explain how the study size was arrived at P 9-10
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why
Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding P 8 (b) Describe any methods used to examine subgroups and interactions P 8 (c) Explain how missing data were addressed (d) If applicable, explain how loss to follow-up was addressed (e) Describe any sensitivity analyses
Results		
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed P 8 (b) Give reasons for non-participation at each stage (c) Consider use of a flow diagram P 7
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders P 7-8 (b) Indicate number of participants with missing data for each variable of interest (c) Summarise follow-up time (eg, average and total amount)
Outcome data	15*	Report numbers of outcome events or summary measures over time Not applicable
Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included (b) Report category boundaries when continuous variables were categorized (c) If relevant, consider translating estimates of relative risk into absolute risk for a

meaningful time period

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Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses
Discussion <i>Not applicable</i>		
Key results	18	Summarise key results with reference to study objectives
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence
Generalisability	21	Discuss the generalisability (external validity) of the study results
Other information		
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based P12

*Give information separately for exposed and unexposed groups.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at <http://www.plosmedicine.org/>, Annals of Internal Medicine at <http://www.annals.org/>, and Epidemiology at <http://www.epidem.com/>). Information on the STROBE Initiative is available at <http://www.strobe-statement.org>.

BMJ Open

A protocol for prospective study of vitamin D, obesity, and leptin in relation to bladder cancer, incidence and survival.

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Primary Subject Heading:	Oncology
Secondary Subject Heading:	Urology
Keywords:	Bladder cancer, Biobank, vitamin D, obesity

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3 A protocol for prospective study of vitamin D, obesity, and leptin in relation to bladder
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Abstract

Introduction: Bladder cancer (BC) (including renal pelvis, ureter and urethra) is one of the most common urogenital cancers and the fourth most frequent cancer in men in the United States. In Norway, the incidence of BC has increased over the last decades. The age-standardized incidence rates per 100,000 for 2011-15 were 50.4 in men and 15.5 in women. Compared to the 5-year period 2006-2010, the percentage increase in incidence was 9.4% in men and 8.9% in women. The recurrence rate of BC is over 50%, the highest recurrence rate of any malignancy. Smoking and occupational exposure to aromatic amines are recognized as the major risk factors. Recently, low serum level of 25 hydroxy vitamin D (25(OH)D) and obesity have been suggested to increase the BC risk, and leptin, which is important in weight regulation, may be involved in bladder carcinogenesis. More knowledge on potential risk factors for BC is necessary for planning and implementing primary prevention measures.

Methods and analyses: Cohort and nested case-control studies will be carried out using the population-based Janus Serum Bank Cohort consisting of pre-diagnostic sera, clinical measurement data (body height and weight, body surface area (BSA) and weight change over time, blood pressure, cholesterol and triglycerides) and self-reported information on lifestyle factors (smoking, physical activity). Participants were followed from cohort inclusion (1972-2003) through 2014. The cohort will be linked to the Cancer Registry of Norway (cancer data), the national Cause of Death Registry (date and cause of death), National Population Registry (vital status) and Statistic Norway (education and occupation). Serum samples will be analyzed for 25(OH)D, vitamin D binding protein, leptin, albumin, calcium and parathyroid hormone.

Cox regression and conditional logistic regression models, and mediation analysis will be used to estimate association between the exposures and BC.

Ethics and dissemination: The study has been approved by the Regional Committee for Medical Research Ethics and is funded by the Norwegian Cancer Society. Results will be published in peer-reviewed journals, at scientific conferences and through press releases.

Strengths and limitations of this study

- Use of a large sample set of more than 2300 incident BC cases
- Pre-diagnostic serum samples assure the temporality of the relationship between exposure and BC, limiting the possibility of reverse causality.
- Use of unique personal identification number for linkages between multiple data sources, to establish a virtually complete study file and complete ascertainment of follow-up.
- Reviewing and characterizing T-stage for all BC.
- Lack of treatment data

Introduction

Rationale and evidence gaps

Urinary bladder cancer (BC) (including renal pelvis, ureter and urethra) is the most common urogenital cancer after prostate cancer and is the fourth most frequent cancer in men in the United States¹. BC has over a 50 % recurrence rate, the highest of any malignancy, and is one of the most expensive cancers to treat on a per-patient basis². In Norway, the incidence of cancer of the bladder has been increasing over the last decades. In 2015, the age-standardized incidence rates were 53.7 and 16.5 in Norwegian men and women, respectively. Compared to the 5-year period 2006-2010, the increase in incidence has been 6.1 % in men and 12.3 % in women. Up to 50 % of all BC cases have been ascribed to smoking³, and 5–25 % of the cases have been attributable to occupational exposures⁴; still, the etiology of up to 45 % of BC remains unexplained. Low serum level of 25 hydroxy vitamin D 25(OH)D and obesity have been suggested to increase BC risk, and the hormone leptin, which is important in weight regulation, may be involved in its carcinogenetic process^{5,6}.

25(OH)D is converted to its active hormonal form, 1-25-dihydroxyvitamin D (1,25(OH)₂D), by 1- α -hydroxylase, which is present in most tissues in the body⁷. Parathyroid hormone (PTH) and calcium level are important factors as they affect the enzymatic conversion from 25(OH)D to active 1, 25-(OH)D₃ in the kidney and may be involved in non-classical synthesis. Measurement of circulating 25(OH)D is considered the gold standard measurement of

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3 vitamin D status as it integrates vitamin D exposure from oral intake from diet or
4 supplements, as well as from exposure to ultraviolet radiation (UVR)^{8,9}. Despite being the
5 gold standard, total circulating 25(OH)D may not be the best measure of 25(OH)D exposure
6 for all tumors. The “free hormone hypothesis” suggests that only unbound, free hormones
7 can have biologic effects on target tissues¹⁰. To date, few studies have examined the role of
8 vitamin D binding protein (DBP) also known as group-specific component or Gc-globulin, in
9 the association between 25(OH)D and various cancer processes. DBP transports both
10 25(OH)D and 1,25(OH)₂D in circulation. This protein carries 88% of 25(OH)D and 85% of
11 1,25(OH)₂D; an additional 12% of 25(OH)D and 15% of 1,25(OH)₂D circulate bound to
12 albumin. Clinical laboratory assays of circulating 25(OH)D that are currently in use measure
13 total 25(OH)D without differentiating between the bound and free forms. Thus, it remains
14 unclear whether total or free 25(OH)D is more biologically relevant with respect to risk of BC.
15 Two previous studies have examined free, in addition to total 25(OH)D, in relation to risk of
16 BC. One found an inverse association between total 25(OH)D and bladder cancer that
17 appeared to be restricted to participants with low DBP, suggesting that free 25(OH)D might
18 be more strongly associated with risk of bladder cancer than total 25(OH)D¹¹. The other
19 study found no association between 25(OH)D overall or at any level of DBP concentration¹².

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34 Body mass index (weight/height², BMI) is a reliable indicator of body fatness and can be
35 categorized as underweight (<18.5), healthy weight (18.5-24.9), overweight (25-29.9) and
36 obese (> 30). The prevalence of obesity in Norway has risen steeply the last decades. In the
37 1970s, about 15% of men and women were overweight (www.fhi.no/) while in 2013 the
38 proportions were 58.4% and 47.3%, respectively¹³. Two meta-analyses, including 15¹⁴ and
39 11¹⁵ cohort studies conclude that obesity significantly increases the risk of BC. Leptin, a
40 hormone involved in weight regulation¹⁶, may be involved in this potential association. High
41 leptin levels have been shown to impact development of several forms of cancer¹⁷. A study
42 of Yuan et al.⁶ shows that leptin receptors are aberrantly expressed in BC tissue and a high
43 leptin level has been associated with BC carcinogenesis^{5,6}.

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51 Low 25(OH)D levels are more frequent in obese persons, suggesting that 25(OH)D deficiency
52 is associated with obesity and vice versa. 25(OH)D deficiency is suggested to be associated
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3 with obesity¹⁸⁻²⁰, and both low 25(OH)D and obesity are suggested to contribute to
4 development of BC.
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8 **Aims and hypotheses**

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10 Better-targeted BC primary and tertiary prevention (risk and survival) is warranted. The
11 interplay between 25(OH)D and obesity and their associations with BC risk are poorly
12 understood. We propose a study aiming to examine anthropometric data (BMI, height,
13 weight, body surface area (BSA) and weight change over time) and serum levels of leptin,
14 total and free 25(OH)D, in relation to BC risk and survival by using samples from Janus Serum
15 Bank and associated data from population-based registries and surveys.
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23 We hypothesize that

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25 1 a. Obesity, BSA and weight change over time are associated with increased BC risk;
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27 1b. Obesity, BSA and weight change over time are associated with reduced BC survival;
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29 2a. Low free and total 25(OH)D level and high serum leptin levels (>4.1 ng/mL are associated
30 with increased risk of BC;
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32 2b. Low free and total 25(OH)D level and high serum leptin levels (>4.1 ng/mL are associated
33 with reduced BC survival.
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40 **METHODS AND ANALYSIS**

41 **Study population and data sources**

42 The Janus Serum Bank Cohort

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46 The study will be carried out using the Janus Serum Bank Cohort, a population-based
47 biobank for prospective cancer studies, containing serum samples and questionnaire data
48 from 292,851 Norwegians who participated in one or more of five Norwegian Regional
49 Health Studies in the period 1972–2003. Detailed description of the samples and data
50 included in the Janus Serum Bank Cohort has been published elsewhere^{21,22}. The quality
51 aspects of long-term stored samples have been of high priority in the Janus Serum Bank and
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3 component stability for a large number of hormones, proteins, metabolites and electrolytes
4 has been investigated²³⁻²⁵, including for both 25(OH)D, and leptin²⁶⁻²⁹. A unique 11-digit
5 personal identification number (PIN), assigned to all Norwegian residents, will be used to link
6 the Janus Cohort with population-based registries and surveys.
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10 11 12 Population-based registries and surveys

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15 *The Cancer Registry of Norway (CRN)* has collected notifications on cancer at a national level
16 since 1953. Cancer reporting is mandatory by law, and reports from various sources ensures
17 high quality and completeness (98.8%)³⁰. The reporting system, based on pathology and
18 cytology reports, clinical records, and death certificates, provides information about site,
19 histological type and stage of disease at the time of diagnosis. CRN has been involved in the
20 Janus Serum Bank operation since establishment in the early 1970s and has been
21 responsible for the data handling; in 2004 the serum bank was integrated into the CRN. The
22 following information is available for cancer cases: month and year of diagnosis, tumor site
23 (International Classification of Diseases 7th revision [ICD-7 codes] converted into ICD-10
24 codes), histology (codes from ICD-Oncology 2nd and 3rd revision), clinical stage (local = no
25 metastases, regional = metastasis in regional lymph nodes or surrounding area, distant =
26 distant metastasis). In addition, all BC diagnoses in the Janus cohort will be reviewed and
27 assigned a pathological T-stage, according to the American Joint Committee on Cancer
28 (AJCC) staging manual 8th ed.³¹.
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41 *The Norwegian Institute of Public Health (NIPH)* has been responsible for conducting the
42 national health surveys, upon which the Janus Serum Bank is partly based. All participants
43 have completed questionnaires for assessment of lifestyle factors (i.e. smoking habits,
44 alcohol use), at the time of serum collection. A database has been established, including
45 data from these questionnaires, as well as measured body height and weight, blood
46 pressure, cholesterol and triglycerides^{32 33}. The Janus Cohort includes participants from five
47 of the health studies: The Oslo Study I (1972–73), The Norwegian Counties Study (1974–78,
48 1977–83, and 1985–88), The Age 40 Program- Oslo (1981–99), The National Age 40 Program
49 (1985–99) and The TROFINN Health Study (2001–03). A set of about 50 variables has been
50 harmonized and standardized due to slightly different wording in the questionnaires²².
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3 Available variables include: height (cm), weight (kg), BMI (kg/m^2 , categorized as 12–18.49,
4 18.5–24.9, 25.0–29.9, ≥ 30), smoking status (never, former, current), cigarettes per day (1–9,
5 10–14, ≥ 15), years of smoking (1–9, 10–29, ≥ 30), time since smoking cessation (<3months,
6 3months–1yr, 1–5yrs, >5yrs), and total physical activity (inactive, low, medium, high), based
7 on leisure time activity. Estimated variables include BSA (m^2) using the DuBois' equation
8 ($\text{weight}^{0.4253} \times \text{height}^{0.7253} \times 3.007184$)²⁰; and weight change calculated by subtracting the
9 1985–88 weight measure from the 1974–78 measure (median time between the weight
10 measurements of 10 years). Weight change will only be possible for a subgroup with
11 repeated measurement of weight.

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18 *The Cause of death Registry* has registered death certificates for all deaths in Norway since
19 1951. Cause of death registration is mandatory by law.

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National Population Registry contains information on vital status (alive, emigrated or dead)
of everyone that resides or has resided in Norway.

Statistics Norway has the responsibility of covering the needs for official statistics on the
Norwegian population including individual data on settlements, migration, occupation and
level of education.

Using the 11-digit PIN number we will link data from four different sources to set up the
research file, illustrated in Figure 1.

Study design

Sub-study I: a prospective cohort study

In a prospective cohort study among all individuals in the Janus Serum Bank Cohort ($n = 292,851$) (Figure 1), we will explore baseline BMI in relation to bladder cancer risk. Among the included BC cases we will investigate baseline BMI and BC survival. By 2014, the cohort included 2 347 BC cases (ICD-10: C67). BC cases of both muscle invasive and non-invasive urothelial cell carcinoma, will be included in the study. Educational level, occupation, age,

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3 sex, physical activity, smoking habits, cholesterol, triglycerides and blood pressure will be
4 included in the statistical analyses as confounders.
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8 Sub-study II: a nested case-control study 9

10 The study will be nested within the prospective cohort described above (Study I), including a)
11 400 BC cases of high grade tumors, including muscle invasive (T2-T4) and non muscle
12 invasive (Ta, T1 and carcinoma in situ (Tis) cancer cases, and b) 400 controls alive and
13 without a cancer diagnosis at the time of the BC diagnosis of the cases, matched 1:1 on sex,
14 age (+/-1 year) and date of serum sampling (+/-1 month). Minimum time from blood draw
15 to diagnosis will be 5 years. The serum samples will be analysed for total 25(OH)D, vitamin D
16 binding protein and leptin. As PTH and albumin-adjusted calcium level affect the enzymatic
17 conversion from 25(OH)D to active 1,25-(OH)D₃ and might be involved in the non-classical
18 synthesis as well, measurement of these components will be taken into account.
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26 In sub-study I, we will investigate the association between BMI and BC, and for sub –study II
27 we will in addition include vitamin D levels. Overall we will focus on disentangle the
28 relationship of vitamin D, BMI and BC. This will be done in two ways:
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- 32 1. By implementing regression models including and interaction effect of vitamin D and
33 BMI on BC.
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- 35 2. By testing the hypothesis whether the effect of BMI on BC is mediated by vitamin D
36 using mediation analysis³⁴.
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42 **Statistical methods**

43 In the cohort study, Cox regression models will be used to estimate hazard ratios (HR) with
44 95% confidence intervals (CI) of BC and survival after BC, taking into account stage at
45 diagnosis, and BMI and including adjusting for season of blood collection for vitamin D. In
46 the nested case-control study, conditional logistic regression models will be used to estimate
47 the odds ratio (OR) with 95% CI.
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52 In order to find out whether Vitamin D acts (totally or partly) as a mediator, modern causal
53 inference theory will be used to estimate different types of effects³⁴.
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3 As we have a number of potential confounding variables, we will use directed acyclic graphs
4 to select variables to include in the statistical models. Confounding variables will be included
5 in the models and tests of interaction effects will be performed when relevant.
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8 The tests will be two-sided and $p < 0.05$ will be considered statistically significant. Statistical
9 analyses will be conducted using R package and³⁵ Stata version 14.1 (StataCorp, College
10 Station, TX, USA).
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14 15 16 17 **Laboratory analyses**

18 The serum samples (aliquots of 400 μL) will be analysed for total 25(OH) D, DBP, leptin, PTH,
19 albumin, and calcium. The Hormone laboratory at Aker hospital, Oslo, Norway will analyze
20 25(OH)D, DBP, PTH and leptin. The Hormone Laboratory is accredited by the Norwegian
21 Accreditation as a testing laboratory and complies with the requirements of the NS-EN
22 ISO/IEC 17025 standards. Albumin and calcium will be analyzed at Department of Medical
23 Biochemistry, Oslo University Hospital accredited by the Norwegian Accreditation reg. no
24 TEST 103 that complies with the requirements of the NS-EN ISO 1518. The sample donors,
25 identity and case control status will be blinded for the laboratory staff. Quality control
26 samples from the biobank will be included in every batch to examine inter-batch and intra-
27 batch variability.
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44 **Power and sample size**

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46 **Sub-study I** Within the prospective cohort ($n = 292,851$), there are more than 2,300 BC cases
47 reported to the cancer registry until the end of follow up time. Assuming the risk of
48 developing bladder cancer is 1% for the normal weight group and 5% for the obese group¹⁴,
49 one would need a sample size of 586 to have 80% power. Since the sample size here is
50 significantly larger, one can safely determine that the study has adequate statistical power.
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Sub-study II In the case-control study nested into the prospective cohort, the statistical power will depend on: i) proportion of exposure in the population, ii) sample size (cases and controls) and iii) the minimum difference that is possible to detect.

Table 1 shows the smallest detectable OR according to proportion of controls exposed to low vitamin D (25(OH)D and high leptin levels, for different sample sizes. The power is 0.80 and a significance level of 0.05 (www.krothman.hostbyet2.com/Episheet.xls). The expected proportions of exposed controls were based on previous studies on serum samples from the Janus Cohort. For 25(OH)D, a study on prostate cancer reported that 4.4% and 30.6% of the controls had 25(OH)D levels below 30 nmol/L and 50 nmol/L, respectively.²⁹ For leptin, a study on colon cancer reported that 20% of the controls had a leptin level of 4.1 ng/mL or higher.²⁷

Table 1. Odds ratio (OR) based on proportion of exposed controls and sample size

		Study case:control= 1:1		
Proportion of exposed controls		Number of cases		
		n=500	n=400	n=300
55%*	OR	1.44	1.50	1.6
45%*	OR	1.43	1.49	1.58
30%**	OR	1.45	1.51	1.62

*Exposure = 25(OH)D deficiency (25(OH)D < 50 nmol /L);

**Exposure = high serum leptin levels (>4.1 ng/mL)

Based on results in the table above, we consider 400 matched case-control pairs as a sufficient sample size for the case-control study.

Data analysis plan

The following analyses will be conducted to test our hypotheses:

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3 • Hypothesis 1.a: A prospective cohort analysis of pre-diagnostic BMI and other
4 anthropometric measures in relation to BC risk using the complete Janus Cohort (n =
5 292,851)
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9 • Hypothesis 1.b: A prospective cohort analysis of pre-diagnostic BMI and other
10 anthropometric measures in relation to survival after a BC, using all BC cases in the Janus
11 Cohort (n ≈ 2,650)
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15 • Hypothesis 2.a: A nested case-control analysis of BC risk according to pre-diagnostic serum
16 levels of total and free 25(OH)D, and leptin in 400 matched case-control pairs.
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- 19 • Hypothesis 2.b: A prospective analysis of survival after a BC (n=400) according to pre-
20 diagnostic serum levels of 25(OH)D and leptin.
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25 **Study strengths and limitations**

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27 A major strength of the large sample set of more than 2,300 incident BC cases. Also use of
28 individual PIN for linkages between multiple data sources, to establish a virtually complete
29 study file, with exception of data on histopathology, is a strength. The data sources are high
30 quality population-based registries, with high degree of completeness. The bladder cancer
31 diagnoses are coded according to ICD-0. To get information on staging, and control the data
32 quality, all histopathological information will be reviewed and characterized by tumor-stage
33 (T-stage). Another strength of this study is that the public health data has been quality
34 assured, structured and harmonized²². The use of pre-diagnostic samples assure the proper
35 temporality of the relationship between exposure and BC, limiting the possibility of reverse
36 causality.
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40 Treatment data is of importance when evaluating the survival analyses. These data are
41 missing and will be a limitation of this study
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45 **ETHICS AND DISSEMINATION**

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47 The Regional Committee for Medical and Health Research Ethics has approved the study.
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49 The different data registries have approved that the use of a de-identified dataset. An ID-
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3 key, consisting of the 11-digit PIN and a study-specific ID number, will be stored and
4 governed by a third party unavailable to the research team.
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7 All results will be published in relevant peer- reviewed international scientific journals and
8 presented at conferences, nationally and internationally. Results of importance will be
9 directly communicated to health authorities and to clinicians where the annual national
10 oncology conference “Onkologisk forum” can serve as a platform for knowledge distribution.
11 Results of importance will also be disseminated through press releases and to user groups
12 like the Norwegian Cancer Society. The CRN website is a potential channel to reach patients
13 organizations and the public.
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19 **Authors' contributions**

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21 REG prepared the study. TER, JSS, HHH, HL, BA, KA, EW and AM contributed to the study
22 design and reviewed and revised the protocol critically for important intellectual content,
23 and approved the final versions. REG is the guarantor.
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27 **Data sharing**

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29 Requests for data sharing/case pooling may be directed to the corresponding author. This
30 study uses third-party data derived from State government registries, which are ultimately
31 governed by their ethics committees and data custodians. Thus, any requests to share these
32 data will be subject to formal approval from each data source used in this study.
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37 **Funding**

38
39 The research study has been reviewed and granted funding by the Norwegian Cancer
40 Society (no. 182308-2016) and the Cancer Registry of Norway Research Fund
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44 **Conflicts of interest**

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46 None declared
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Figure legends

Figure 1. Data collection from different sources using PIN

For peer review only

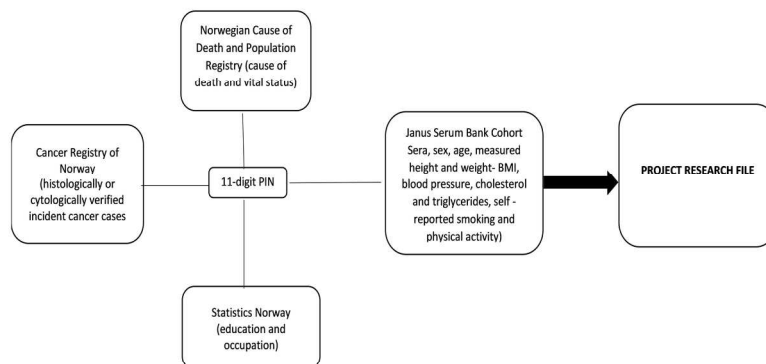


Figure 1. Data collection from different sources using PIN

190x107mm (300 x 300 DPI)

Review only

STROBE Statement—Checklist of items that should be included in reports of *cohort studies*

	Item No	Recommendation
Title and abstract	1	(a) Indicate the study's design with a commonly used term in the title or the abstract P 1 (b) Provide in the abstract an informative and balanced summary of what was done and what was found P 2
Introduction		
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported P 3-4
Objectives	3	State specific objectives, including any prespecified hypotheses P 5
Methods		
Study design	4	Present key elements of study design early in the paper P 7
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection P 5-7
Participants	6	(a) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up P 5-7 (b) For matched studies, give matching criteria and number of exposed and unexposed P 7-8
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group P 5-7, 9
Bias	9	Describe any efforts to address potential sources of bias P 8
Study size	10	Explain how the study size was arrived at P 9-10
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why
Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding P 8 (b) Describe any methods used to examine subgroups and interactions P 8 (c) Explain how missing data were addressed (d) If applicable, explain how loss to follow-up was addressed (e) Describe any sensitivity analyses
Results		
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed P 8 (b) Give reasons for non-participation at each stage (c) Consider use of a flow diagram P 7
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders P 7-8 (b) Indicate number of participants with missing data for each variable of interest (c) Summarise follow-up time (eg, average and total amount)
Outcome data	15*	Report numbers of outcome events or summary measures over time Not applicable
Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included (b) Report category boundaries when continuous variables were categorized (c) If relevant, consider translating estimates of relative risk into absolute risk for a

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Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses
Discussion Not applicable		
Key results	18	Summarise key results with reference to study objectives
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence
Generalisability	21	Discuss the generalisability (external validity) of the study results
Other information		
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based P12

*Give information separately for exposed and unexposed groups.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at <http://www.plosmedicine.org/>, Annals of Internal Medicine at <http://www.annals.org/>, and Epidemiology at <http://www.epidem.com/>). Information on the STROBE Initiative is available at <http://www.strobe-statement.org>.