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TITLE PAGE

Title: A protocol for prospective cohort and nested case-control studies of vitamin D and obesity in relation to cutaneous melanoma incidence and survival

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ABSTRACT

Introduction: In Norway, the incidence rate of cutaneous melanoma (CM) now ranks third in Europe, while CM mortality ranks first. Ultraviolet radiation (UVR) is the main carcinogen causing CM, and is also the main source of vitamin D, which has been associated with reduced risk and better prognosis of several cancers. However, the relation to CM is unclear as both low and high vitamin D levels have been associated with increased risk. Obesity as measured by body mass index (BMI) is associated with risk of several cancers, and have also been suggested as a risk factors for CM, which may be related to insufficient vitamin D and/or high leptin levels. Moreover, contracting a CM diagnosis have been associated with increased risk of developing second cancer. We aim to study whether low prediagnostic serum levels of vitamin D and high prediagnostic levels of BMI and serum leptin influence CM incidence and mortality, and risk of second cancer and survival after a CM diagnosis.

Methods and analysis: Cohort and nested case-control studies will be carried out using the population-based Janus Serum Bank Cohort (archival prediagnostic sera, BMI, smoking and physical activity), with follow-up 1972–2014. The cohort will be linked to the Cancer Registry of Norway, the national Cause of Death Registry, Statistics Norway (education and occupation), and exposure matrices of UVR. Time to event regression models will be used to analyze the cohort data, while the nested case-control studies will be analyzed by conditional logistic regression. A multilevel approach will be applied when incorporating group-level data.

Ethics and dissemination: The project is approved by the Regional Committee for Medical Research Ethics and is funded by the Norwegian Cancer Society. Project results will be

published in peer-reviewed journals, as abstracts at scientific conferences and in the news media.

Strength and limitations of this study

- Strengths:
  - Linkage of independent, national data sources by use of a unique personal identification number, enabling establishment of a comprehensive research file and complete control of loss to follow-up.
  - Over 3000 CM cases from a high-quality population-based cancer registry relying on mandatory reporting of incident cancers.
  - Prediagnostic serum samples assuring a clear prospective temporal relationship between exposure and cancer, limiting bias introduced by reverse causality.
  - Lifetime ambient UVR exposure data (UVA, UVB, and erythemally weighted UV) and group-level data on sunburns, sunbathing vacations, and solarium use capturing variations in age, time period and county of residence.
  - Clinically measured height and weight, limiting misclassification.
- Limitations:
  - Ambient UVR exposure and data on sunburns, sunbathing vacations and solarium use can only be linked to the Janus Cohort on a group-level.
  - Lack of data on pigmentary characteristics and nevi.

INTRODUCTION

Rationale and evidence gaps

Ultraviolet radiation (UVR) is a recognized human carcinogen and the principal environmental risk factor for cutaneous melanoma (CM)[1 2], while skin characteristics such as skin sensitivity and number of nevi determine CM susceptibility.[3-7] Currently, Norway ranks third and first in Europe with respect to CM incidence and CM mortality, respectively[8], and they both continue to rise.[9] Excess UVR is likely the major cause of this increase,[10] but also vitamin D deficiency and obesity have been suggested to play a role.[11 12]

UVR is the main source of vitamin D as exposure of the skin induces synthesis of calcidiol (25-OHD), which when synthesized to calcitriol (1,25-$(OH)D_3$) has been shown to modulate several anticancer mechanisms.[13-15] Epidemiological studies have found elevated risks[16] and poor prognosis[17 18] of several cancers associated with low levels of vitamin D. For CM, the relation to vitamin D is unclear,[12 19] and recent studies have reported both inverse and positive relationships between vitamin D serum levels and CM risk.[2 20-23] The apparently positive associations reported, have been suggested to be due to elevated ultraviolet-B (UVB) exposure, in turn being the underlying cause of the increased CM risk.[20] Studies with prediagnostic serum samples of vitamin D and information on UVB exposure are warranted, as the issue of reverse causality with diagnostic samples has been discussed.[24 25]

Obesity as measured by body mass index (BMI) above 30 kg/m^2 has been positively associated with CM risk in males, but results for women are ambiguous, probably confounded by personal habits as obese individuals may refrain from sunseeking behavior compared to their normal weight peers.[11] Further, obesity has been found to increase risk

of melanoma progression,[26 27] and vitamin D deficiency is suggested to cause obesity. [28-30] Low vitamin D levels may therefore be a common cause for increased BMI and CM risk. Moreover, the hormone leptin may be involved in CM development. Leptin is synthesized in adipose tissues, plays an important role in weight regulation,[31] and is a more valid measure of obesity than BMI, which is prone to misclassification.[32] Recent studies have demonstrated that leptin receptors are present in melanoma cells, and that leptin bound to its receptor stimulates melanoma growth.[33-35] High serum leptin (≥4.1 ng/mL) is associated with a 3-fold increased risk of colon cancer in men.[36 37] Results from laboratory studies, suggest that this might also be the situation for CM. [31 33 34]

An increased risk of second cancer has been observed after a CM diagnosis, [38 39] with the risk of a second CM being elevated, but also that of other cancers. The risk of lymphoma after CM, but also *vice versa*, has received focus,[6 40] implicating UVR as a possible shared etiologic factor[41]. The finding that lymphoma risk (particularly non-Hodgkin lymphoma, NHL) is inversely associated with UVR, suggests that vitamin D may play a role.[42] We need more information about the mechanisms that influence risk of second cancer and survival after CM, and a better understanding of the complex risk patterns requires studies with serum levels of vitamin D and data on exposures associated with cancer risk.

Aims and hypotheses

The interplay between vitamin D and obesity and their relation to CM is poorly described, and increased knowledge of these factors is warranted to improve CM prevention and prognosis. In the present study protocol, we propose a set of prospective cohort and nested case-control studies with the primary aim of examining BMI and serum levels of vitamin D

and leptin in relation to CM incidence and mortality, and risk of second cancer and survival after a CM diagnosis. As a secondary aim, we propose a nested case-control study of contracting CM after lymphoma and *vice versa*, in relation to serum levels of vitamin D and leptin.

We hypothesize that:

1. BMI ≥ 30 kg/m^2 is associated with
    1.1. Increased CM incidence and mortality
    1.2. Increased risk of second cancer and reduced survival after a CM diagnosis
2. High prediagnostic serum levels of leptin (>4 ng/mL or highest quintile) and low prediagnostic vitamin D levels (<30 nmol/L or lowest quintile) are associated with
    2.1. Increased CM incidence
    2.2. Reduced survival after a CM diagnosis
    2.3. Increased risk of second cancer after a CM diagnosis
    2.4. Increased CM incidence after a lymphoma diagnosis and *vice versa*

METHODS AND ANALYSIS

Study population and data sources

Janus Serum Bank Cohort

The study is based on the Janus Serum Bank Cohort, a population-based prospective cancer biobank containing blood serum samples and questionnaire data from 292,866 Norwegians participating in five health surveys 1972–2003. A detailed description of the Janus Serum Bank Cohort (hereafter Janus Cohort), its data and establishment, is published elsewhere.[43] The Janus Cohort includes participants from the following surveys:

1. The Oslo Study I (1972–73), invited men residing in Oslo aged 20–49 years.
2. The Norwegian Counties Study was carried out as a three-wave survey (1974–78, 1977–83, and 1985–88), inviting men and women aged 20–49 years residing in Finnmark, Oppland or Sogn- og Fjordane.
3. Oslo Age 40 Programme invited all 40-year old men and women residing in Oslo 1981–99.
4. The National Age 40 Programme triennially invited all men and women aged 40–42 years in all Norwegian counties during 1985–99.
5. The TROFINN Health Study invited all men and women aged 30–75 years residing in Troms and Finnmark in 2001–03.

Blood serum samples

The Janus Cohort has detailed sample information including date of sample collection and county of residence at sample collection. The samples have been stored at –25°C for up to 43 years.[43] Serum samples of vitamin D and leptin have been demonstrated to have high stability after long term storage,[44 45] and previous studies have shown that serum from the Janus Cohort is well suited for analyses of vitamin D[46 47] and leptin.[36 37]

Height and weight measurements and questionnaire data

Together with blood sample collection, standardized height and weight measurements were taken by trained personnel. Participants in the surveys were also asked to complete questionnaires on smoking habits, alcohol consumption, diet, physical activity, use of medications etc. Slightly different questionnaires (different wording and number of response-categories) were used in the five health surveys, and a set of variables has been

harmonized.[48] For the present project, the following variables are made available: height (cm), weight (kg), BMI (kg/m^2 and categorized as 12–18.49, 18.5–24.9, 25.0–29.9, ≥30),[49] 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), level of total physical activity (inactive, low, medium, high), and level of activity at work (sedentary, walking, walking and lifting, heavy physical work).

Linking the Janus Cohort to population-based registries

Every resident in Norway is assigned a unique 11-digit personal identification number (PIN), which ensures a correct linkage of the Janus Cohort to population-based registries and databases as described below and in Figure 1.

Population-based registries

The *Cancer Registry of Norway (CRN)* has registered all new cancer diagnoses in Norway since 1953. Reporting of incident cancers to the CRN is compulsory by law, and information from pathologists, general practitioners, Norwegian Patient Registry, and the Norwegian Cause of Death Registry ensures a high degree of completeness.[3] For the present project, incident cancers from 1972 through 2014 will be linked to the Janus Cohort. The following cancer information will be used: date of diagnosis (month and year), tumor localization (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) and Breslow thickness (mm).

Date and cause of death (death from cancer and death from other causes than cancer) will be obtained from *the Cause of Death Registry* and vital status (alive, emigrated or dead) with corresponding dates will be obtained from *the National Population Registry*.

Data on occupation at baseline (categorized as indoor/outdoor and high risk/low risk as markers of UVR exposure) and highest attained educational level at baseline (none, compulsory, upper secondary, college/university) will be obtained from *Statistics Norway*.

UVR exposure matrices

County-specific, yearly average doses of ultraviolet-A (UVA), UVB and erythemally weighted UVR (ERY) will be created and assigned to each participant, according to place of residence, at baseline and cumulated throughout follow-up (*i.e.* until cancer, emigration, death or 31st December 2014, whichever occurs first). The UVR exposure matrices will be based on measurement data from UV-network stations operated by the Norwegian Radiation Protection Authority and on modelled values as described by Medhaug et al.[50] Furthermore, data on sunburns, sunbathing vacations and solarium use will be linked to the Janus Cohort on a group-level basis (age, county, time period) as derived from questionnaire data collected in the Norwegian Women and Cancer study.[51 52]

Study designs

Study I: a prospective cohort study

In a prospective cohort study among all 292,866 individuals in the Janus Cohort (study sample I in Figure 2), we will explore baseline BMI in relation to CM incidence and mortality, and second cancer and survival after a CM diagnosis (hypotheses 1.1 and 1.2), adjusting for age, sex, UVR exposure, smoking, education, and Breslow thickness (hypothesis 1.2 only).

Studies II-IV: prospective nested case-control studies

Three prospective case-control studies will be nested within the Janus Cohort (study samples II-IV in Figure 2). For serum analyses, the nested case-control design is cost-efficient compared to the cohort design as only a limited number of CM cases and cancer-free controls are selected and matched according to an incidence-density sampling scheme.[53] Also, the nested case-control design takes advantage of the prospective nature of the cohort study by using data and serum samples collected before any cancer diagnosis, thereby reducing the potential for bias. Table 1 gives a complete description of the case, control and matching criteria.

Study II

Study II will examine CM risk according to prediagnostic serum levels of vitamin D and leptin (hypothesis 2.1). We will study CM cases (II a, Figure 2) without a history of cancer and controls alive and without a cancer history at the time of the case diagnosis (II b). We will include 1 control per case, matched on sex, age at serum sampling, and season due to seasonal variation in vitamin D levels (Table 1). UVR exposure, smoking and education will be adjusted for.

Survival analysis (as in study I) will be undertaken on the subsample of CM cases (II a) with measured vitamin D and leptin, adjusted for age, sex, UVR exposure, smoking, education, and Breslow thickness (hypothesis 2.2).

Study III

In study III, we will examine the risk of second cancer after a CM diagnosis according to prediagnostic serum levels of vitamin D and leptin (hypothesis 2.3). CM cases with a second

cancer (III a, Figure 2) and controls alive and without a cancer history at the time of the second cancer diagnosis (III b) will be selected. We will include 1 control per case, matched on sex, age at serum sampling, and season of serum sampling (Table 1). Covariates included in studies I-II will be taken into account.

Study IV

A group including cases (IV a, Figure 2) with CM before lymphoma or *vice versa* and controls (IV b) with no cancer history at the time of the second cancer diagnosis will be examined according to prediagnostic serum levels of vitamin D and leptin (hypothesis 2.4). All case-control pairs will be matched on sex, age at serum sampling, and season of serum sampling (Table 1). Covariates included in studies I-III will be taken into account.

Power and sample size calculations

Study I: With the large study sample (n = 292,866), including more than 3000 CM cases by 31st December 2014, we have sufficient statistical power to reveal minor risk differences between the BMI categories normal weight (18.5-24.9 kg/m^2), overweight (25-29.9 kg/m^2) and obese (≥30 kg/m^2). Thus, further power calculation is not conducted.

Studies II-IV: Study II will include 700 CM cases out of the approximately 3000 available. Study III will include 345 cases with a second primary cancer after CM and study IV will include 60 cases of lymphoma after CM or *vice versa*, which were the total available number of cases in the Janus Cohort by 31st December 2014. Table 2 shows the smallest detectable odds ratio (OR) according to assumed proportion of controls exposed to low serum levels of vitamin D and high leptin levels when using a power of 0.80 and a significance level of 0.05.

The assumed proportions of exposed controls were based on previous studies conducted on serum samples from the Janus Cohort. For vitamin D, a study on prostate cancer reported that 4.4% and 30.6% of the controls had vitamin D levels below 30 nmol/L and 50 nmol/L, respectively.[46] For leptin, a study on colon cancer reported that 20% of the controls had a leptin level of 4.1 ng/mL or higher.[36]

Data management

Case-control selection

As indicated in Figure 2 there will be some overlap between cases and controls. CM cases (II a) will be sampled at random from all available CM cases in the Janus Cohort, independent of second cancer status. However, some of the CM cases (II a) may have developed a new cancer and then be eligible for use in study III as CMs with second cancer (III a). Controls (II b) will be sampled at random with replacement (incidence density sampling) from the Janus Cohort and matched to CM cases (II a). Also controls (II b) matched to the CM cases (II a) who developed a second cancer (III a), will be eligible for use in study III as controls (III b) if they are alive, resident, and cancer-free at the time of second cancer after CM (III a). Controls (II b) who die, emigrate or develop cancer before date of diagnosis of the case, cannot be reused in study III and a corresponding number of new controls must be sampled from the Janus Cohort together with the remaining case-control pairs to reach the total number of 345. Study IV will follow the same approach as studies II and III with respect to reuse. A picking list of unique serum samples for all studies will be prepared by a data manager for the Janus Serum Bank Cohort laboratory team.

Laboratory analyses

The Janus serum bank laboratory team will send 220 µl aliquots of serum to the Hormone laboratory at Oslo University Hospital for analyses of vitamin D and leptin. Serum concentrations of vitamin D and leptin will be determined using established methods at the Oslo University Hospital Hormone laboratory.[31 54]

Hormone laboratory staff will be blinded to case-control status. Two identical quality control (QC) samples with serum from a pool of several persons will be placed on each batch. These two QC-samples will change position for each new batch to avoid bias from weak spots in the machine/kit, and will thus take into account both inter-batch variability and intra-batch variability. Each case-control pair will be placed and analyzed on the same batch.

Statistical methods

In the cohort studies, we will use Poisson and Cox regression and estimate relative risks (RRs) with 95% confidence intervals (CIs). Flexible parametric models will also be explored if a non-linear relationship between exposure and outcome is assumed. In the nested case-control studies, conditional logistic regression will be applied to estimate RRs with 95% CIs. A multilevel approach will be applied for analyses containing group-level data. Interaction effects will be studied. All tests will be two-sided and $p<0.05$ will be considered statistically significant. All statistical analyses will be performed using Stata (StataCorp, College Station, TX, USA).

Analysis plan

We plan to conduct the following analyses to test our hypotheses:

- Hypothesis 1.1: A prospective cohort analysis of prediagnostic BMI and other anthroprometric measures (height, weight, and body surface area calculated from height and weight[55]) and CM incidence and mortality using the complete Janus Cohort (n = 292,866).
- Hypothesis 1.2: A prospective cohort analysis of prediagnostic BMI and the risk of second cancer and survival after a CM diagnosis (n ≈ 3000).
- Hypothesis 2.1: A nested case-control analysis of CM risk according to prediagnostic serum levels of vitamin D and leptin in 700 pairs.
- Hypothesis 2.2: A prospective analysis of survival after a CM diagnosis (n = 700) according to prediagnostic serum levels of vitamin D and leptin.
- Hypothesis 2.3: A nested case-control analysis of risk of second cancer after a CM-diagnosis according to prediagnostic serum levels of vitamin D and leptin in 345 pairs.
- Hypothesis 2.4: A nested case-control analysis investigating risk of lymphoma after CM or *vice versa* according to prediagnostic serum levels of vitamin D and leptin (n = 60 cases).

Project strengths and limitations

A major strength of the project is the linkage of multiple data sources by use of the PIN, thereby establishing a comprehensive research file with independently and prospectively collected data, and a complete control of loss to follow-up. An important strength is also the use of high-quality cancer data with over 3000 CM cases from a population-based registry

relying on compulsory reporting of incident cancers. Further, the prediagnostic serum samples assure a clear prospective temporal relationship between exposure and cancer, which limits the possibility of reverse causality *i.e*. that the cancer or its precursor affect the vitamin D or leptin serum levels.

An important limitation of the project is that we will only be able to obtain group level data on UVR exposure (ambient UVA, UVB and ERY; sunburns, sunbathing vacations, and solarium use) but our data capture variation in these variables by age, time period and between counties. However, the long and complete time-series, covering the whole observation period and early childhood for many of the participants, enables analysis with time-varying UVR exposure. Another limitation is the lack of data on pigmentary characteristics and number of nevi.

ETHICS AND DISSEMINATION

The project has a running approval from the Regional Committee for Medical and Health Research Ethics to link the different population-based registries to establish a de-identified research file. In addition, each registry and data source has approved that its data will be linked and used in a de-identified research file. A linkage-key consisting of the 11-digit PIN and a project-specific ID number will be stored and governed by a third party unavailable to the research team. Moreover, participation in each of the health surveys constituting the Janus Cohort was voluntary and based on informed consent.

All results will be published in relevant peer-reviewed international scientific journals and presented at conferences, nationally and internationally. Results will also be directly communicated to user groups such as the Norwegian Cancer Society, The Norwegian Melanoma Association, and to health authorities and clinicians. Both the annual Norwegian

conferences (“Oncologic Forum”, the Norwegian Melanoma Group Meeting) and international conferences will serve as platforms for knowledge distribution to clinicians and researchers. Important results will also be disseminated through press releases. Further, lectures, the CRN website, social media and other potential channels will also be used to reach patient organizations, patients and the general public.

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Authors' contributions

TER conceived the study. JSS, TKG, JRR, LV, RB, MBV, and TER contributed to the project design. TER and JSS are responsible for data acquisition. JSS and TER drafted the manuscript, and MBV, TKG, JRR, LV and RB reviewed and revised it critically for important intellectual content, and approved the final version for submission. JSS and TER are the guarantors.

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Conflict of interest

None declared.

Ethics Approval

The project has approval from the Regional Committee for Medical and Health Research Ethics (no. 2014/185), and approval from each of the listed data sources.

Data sharing

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

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TABLES

Table 1. Overview of case, control, and matching criteria for studies II-IV.			
	Study II	**Study III**	**Study IV**
CASE CRITERIA			
No. of cases	700	345	60
Verification	Histologically or cytologically verified CM in the Janus Cohort (ICD-10: C43).	Histologically or cytologically verified 2nd cancer after CM in the Janus Cohort (ICD-10: C43+any type).	Histologically or cytologically verified CM+lymphoma+CM in the Janus Cohort (ICD-10: C43+ ICD-O-3[a]).
Definition	CM cases without a cancer history (not tied on date with another diagnosis).	2nd cancers (any type) after 1st primary CM diagnosis.	Lymphoma after 1st primary CM diagnosis or *vice versa.*
Selection	Sampled at random from pool of available CM cases.	All available cases from study II + randomly sampled from CM pool.	All available cases from study III and IV-1 + randomly sampled from pool.
Age at diagnosis	<75 years		
Year of diagnosis	<2009		
Minimum time from blood draw to diagnosis	2 years		
Sex	Male or female		
CONTROL CRITERIA			
No. of controls	700	345	180
Definition	Alive and resident in Norway at date of diagnosis of case (for study III: diagnosis of 2nd cancer). No cancer history before case diagnosis (for study III: diagnosis of 2nd cancer), but allow common cancers (colon, breast, prostate, skin, and lung only) after date of diagnosis of case to conserve sera of rare cancers for later studies.		
Selection	Random sampling with replacement from pool of available controls		
MATCHING CRITERIA			
Sex	Same sex as case		
Age at blood draw	+/- 2 years from age of case at blood draw. Stepwise extension by +/-3 months up to +/-3 years if necessary.		
Time period of blood draw	The following 3-month intervals: a) Dec–Feb, b) Mar–May, c) Jun–Aug d) Sept–Nov.		
[a]9727, 9728, 9729, 9835, 9836, 9837, 9670, 9823, 9731, 9734, 9732, 9733, 9675, 9678, 9679, 9680, 9684, 9591, 9760, 9671, 9761, 9762, 9673, 9690, 9691, 9695, 9698, 9687, 9826, 9689, 9699, 9764, 9700, 9701, 9709, 9718, 9708, 9702, 9705, 9714, 9716, 9717, 9948, 9719, 9827, 9831, 9834 9833, 9940, 9820, 9832 9590, 9750			

Table 2. Smallest detectable OR (above the null) according to proportion of controls exposed to low vitamin D and high leptin levels, using a power of 0.80 and a significance level of 0.05

Proportion of exposed controls	**Study II** Cases = 700 Ratio = 1:1	**Study III** Cases = 345 Ratio = 1:1	**Study IV** Cases = 60 Ratio = 1:3
5%[a]	1.82	2.26	3.81
30%[b]	1.37	1.57	2.34
20%[c]	1.43	1.65	–

[a]Exposure = vitamin D deficiency (<30 nmol /L);
[b]Exposure = vitamin D deficiency (<50 nmol /L);
[c]Exposure = high serum leptin levels (≥4.1 ng/mL);

FIGURES AND LEGENDS

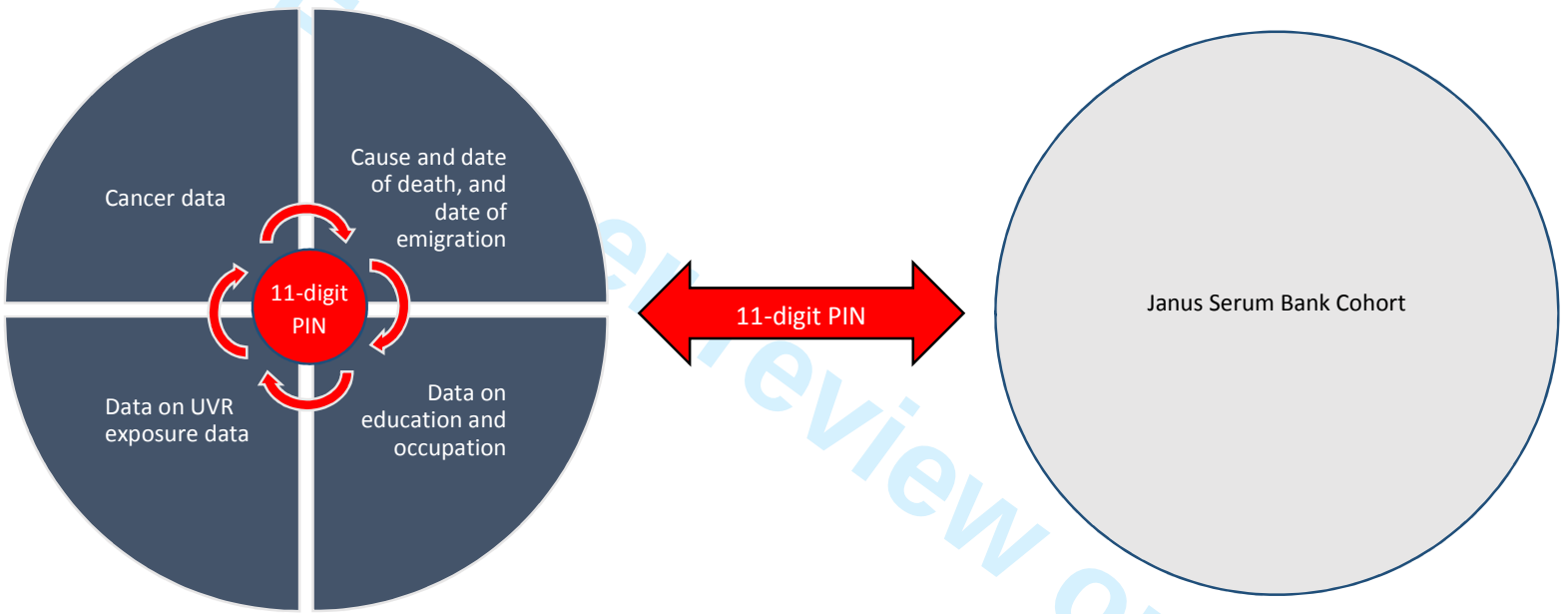

Figure 1. Overview of data and linkage using the 11-digit personal identification number (PIN)

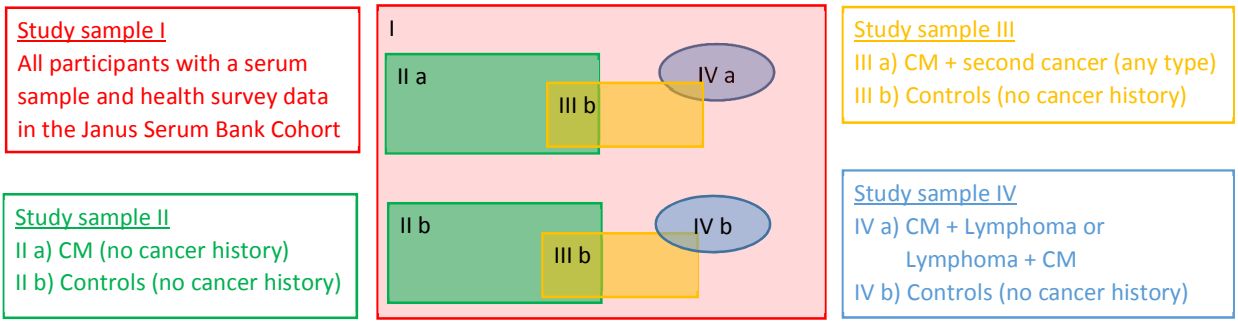

Figure 2. Overview of study samples and overlap between cases and controls between studies.

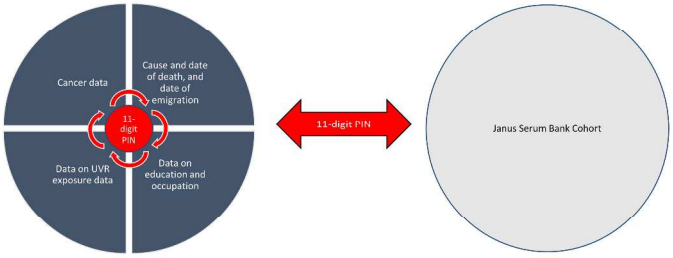

Figure 1. Overview of data and linkage using the 11-digit personal identification number (PIN)
Figure 1
338x190mm (300 x 300 DPI)

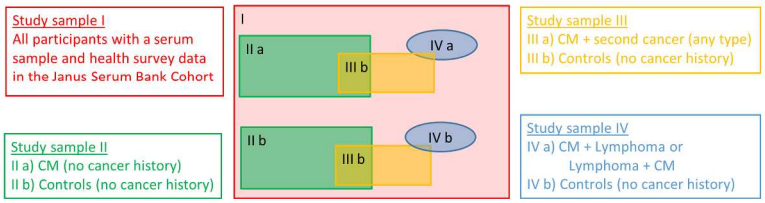

Figure 2. Overview of study samples and overlap between cases and controls between studies.
Figure 2
190x275mm (300 x 300 DPI)

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
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found
Introduction		
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported
Objectives	3	State specific objectives, including any prespecified hypotheses
Methods		
Study design	4	Present key elements of study design early in the paper
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed
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
Bias	9	Describe any efforts to address potential sources of bias
Study size	10	Explain how the study size was arrived at
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
		(*b*) Describe any methods used to examine subgroups and interactions
		(*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
		(b) Give reasons for non-participation at each stage
		(c) Consider use of a flow diagram
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders
		(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
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

Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses
Discussion		
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

*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.

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A protocol for prospective studies of 25-hydroxyvitamin D, leptin and body mass index in relation to cutaneous melanoma incidence and survival

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TITLE PAGE

Title: A protocol for prospective studies of 25-hydroxyvitamin D, leptin and body mass index in relation to cutaneous melanoma incidence and survival

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ABSTRACT

Introduction: The incidence and mortality rates of cutaneous melanoma (CM) are increasing among fair-skinned populations worldwide. Ultraviolet radiation (UVR) is the principal risk factor for CM, but is also the main source of 25-hydroxyvitamin D (25(OH)D), which has been associated with reduced risk and better prognosis of several cancers. However, both low and high 25(OH)D levels have been associated with increased risk of CM. Obesity as measured by body mass index (BMI) is associated with risk of several cancers, and has also been suggested as a risk factors for CM, and may also be related to insufficient 25(OH)D and/or high leptin levels. Moreover, contracting a CM diagnosis have been associated with increased risk of developing second cancer. We aim to study whether low prediagnostic serum levels of 25(OH)D, high prediagnostic levels of BMI and high serum leptin levels influence CM incidence, Breslow thickness and CM mortality, and risk of second cancer and survival after a CM diagnosis.

Methods and analysis: Cohort and nested case-control studies will be carried out using the population-based Janus Serum Bank Cohort (archival prediagnostic sera, BMI, smoking and physical activity), with follow-up 1972–2014. Additional data will be received from the Cancer Registry of Norway, the national Cause of Death Registry, Statistics Norway (education and occupation), and exposure matrices of UVR. Time to event regression models will be used to analyze the cohort data, while the nested case-control studies will be analyzed by conditional logistic regression. A multilevel approach will be applied when incorporating group-level data.

Ethics and dissemination: The project is 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 in the news media.

Strength and limitations of this study

- Strengths:
  - Linkage of independent, national data sources by use of a unique personal identification number for a comprehensive research file and complete control of loss to follow-up
  - Over 3000 CM cases from a high-quality population-based cancer registry relying on mandatory reporting of incident cancers.
  - Prediagnostic serum samples assuring a true prospective relationship between exposures and cancer, limiting bias introduced by reverse causality
  - Lifetime ambient UVR exposure data (UVA, UVB, and erythemally weighted UV) and group-level data on sunburns, sunbathing vacations, and solarium use capturing variations in age, time period and county of residence.
  - Clinically measured height and weight, limiting misclassification
- Limitations:
  - Ambient UVR exposure and data on sunburns, sunbathing vacations and solarium use can only be linked to the Janus Cohort on a group-level
  - Lack of data on pigmentary characteristics and nevi

INTRODUCTION

Rationale and evidence gaps

Ultraviolet radiation (UVR) is a recognized human carcinogen and the principal environmental risk factor for cutaneous melanoma (CM)[1 2], while skin characteristics such as skin sensitivity and number of nevi indicate CM susceptibility.[3-7] CM incidence and mortality rates have been increasing in fair-skinned populations worldwide the past decades, and CM is currently the third most common cancer in Europe after cancers of the colon/rectum and the lung.[8 9] In Norway, CM incidence has increased more than 3% annually between 1982 and 2011 and has been projected to continue to rise.[9] Excess UVR exposure is likely the major cause of this increase,[10] but also low vitamin D levels and obesity have been suggested to play a role.[11 12]

Vitamin D synthesis in the skin is initiated by UVR exposure to the skin surface at wavelengths of 290–320 nm, which converts 7-dehydrocholesterol in the keratinocytes to previtamin D3 (cholecalciferol). Together with previtamin D2 (ergocalciferol), previtamin D3 may also be obtained by diet. Both previtamin D2 and D3 are then hydroxylated in the liver to 25-hydroxyvitamin D (25(OH)D), which represents the circulating storage form of vitamin D. A second hydroxylation in the kidney converts vitamin D to its biologically active form 1,25-hydroxyvitamin D (1,25(OH)D),[13 14] which has been associated with anticancer mechanisms.[13 15-17] Based on four studies, a recent meta-analysis reported a summary relative risk of CM of 1.46 (95% CI: 0.60-3.53) for the highest compared to the lowest (reference) quantile of 25(OH)D.[12] In three of these studies, risks increased with increasing 25(OH)D serum levels, while the fourth study reported the opposite.[18-21] None of these studies individually showed any statistically significant associations, and the inconclusive results may be due to difference in statistical power, the covariate

adjustments, whether CM cases had a cancer history or not, and whether serum was sampled before or after the CM diagnosis. Several recent studies have reported an inverse association between Breslow thickness and 25(OH)D serum level at diagnosis.[20 22-25] As both tumor thickness and 25(OH)D level were measured at the same time in these studies, these associations may have been affected by reverse causality.[26 27] However, for prognosis after a CM diagnosis, higher diagnostic 25(OH)D levels have been shown to predict lower risk of relapse and increased survival, independent of Breslow thickness.[22 24] A recent study, ascribed the effect on CM survival to change in 25(OH)D during follow-up from CM diagnosis to death, and not the 25(OH)D level at diagnosis.[28]

Low 25(OH)D levels are more frequent in obese persons, suggesting that 25(OH)D deficiency is associated with obesity and *vice versa*.[29-33] Obesity as measured by body mass index (BMI) above 30 kg/m^2 has been positively associated with CM risk in males, but results for women are ambiguous, and possibly confounded by personal habits as obese women may refrain from sunseeking behavior compared to their normal weight peers.[11] Further, diet-induced obesity has been found to increase CM progression in mice models.[34] The biological mechanism underlying an obesity-induced increase in CM incidence is not well understood, although a hyperglycemia hypothesis has been suggested.[35] Another hypothesis suggests that adipocytes produce high levels of vascular endothelial growth factor (VEGF), associated with visceral fat, which contributes to angiogenesis and tumor growth.[36]

The metabolic hormone leptin may be a risk factor for both CM and CM progression. Leptin is released by adipose tissue and plays an important role in the regulation of insulin sensitivity and weight regulation.[37 38] Increased diagnostic serum levels of leptin have been associated with increased CM risk, possibly caused by a leptin-induced increase in

neoangiogenesis, reduction of melanogenesis and a decreased capacity of the melanocytes' DNA repair.[39 40] Recent studies have demonstrated that leptin receptors are present in melanoma cell-lines that proliferates in response to leptin, and that leptin bound to its receptor stimulates melanoma growth.[41-44]

After a CM diagnosis, there is an increased risk of diagnosis of additional CM, as well as other cancers.[45 46] For example, the risk of lymphoma before or after CM has received increased focus.[47] Immune perturbation has been suggested to contribute to the development of CM after non-Hodgkin lymphoma (NHL) subtypes such as chronic lymphocytic leukemia/small lymphocytic lymphoma.[48] As for CM, low 25(OH)D serum levels have also been associated with reduced survival and poor prognosis after NHL,[49 50] which raises the question of whether low 25(OH)D could alter the risk of lymphoma as a second cancer after CM or *vice versa*.

Aims and hypotheses

The interplay between 25(OH)D and obesity and their relation to CM is poorly described, and increased knowledge of these factors is warranted to improve CM prevention and prognosis. In the present study protocol, we propose a set of prospective cohort and nested case-control studies with the primary aim of examining BMI and serum levels of 25(OH)D and leptin in relation to CM risk, Breslow thickness and mortality, and risk of second cancer and survival after a CM diagnosis. As a secondary aim, we propose a nested case-control study of lymphoma risk after CM and *vice versa*, in relation to serum levels of 25(OH)D and leptin.

We hypothesize that:

1. High prediagostic BMI (≥30 kg/m^2, quantiles, continuous) is associated with
   1.1. Increased CM risk, Breslow thickness, and mortality
   1.2. Reduced survival after a CM diagnosis
   1.3. Increased risk of contracting CM followed by a second cancer (n = 292,866)
   1.4. Increased risk of second cancer among CM survivors (n ≈ 3000)
2. High prediagnostic serum levels of leptin (>4 ng/mL, highest quantile, continuous) and low prediagnostic 25(OH)D levels (<30 nmol/L, lowest quantile, continuous) are associated with
   2.1. Increased CM risk and Breslow thickness
   2.2. Reduced survival after a CM diagnosis
   2.3. Increased risk of contracting CM followed by a second cancer compared to no cancer history
   2.4. Increased risk of second cancer among CM survivors
   2.5. Increased lymphoma risk after a CM diagnosis and *vice versa* compared to no cancer history

METHODS AND ANALYSIS

Study population and data sources

Janus Serum Bank Cohort

This project is based on the Janus Serum Bank Cohort, a population-based biobank for prospective cancer studies containing serum samples and questionnaire data from 292,866 Norwegians who participated in five health surveys 1972–2003. A detailed description of the

Janus Serum Bank Cohort (hereafter Janus Cohort), its data and establishment, is published elsewhere.[51] The Janus Cohort includes participants from the following surveys:

1. The Oslo Study I (1972–73), invited men residing in Oslo aged 20–49 years.
2. The Norwegian Counties Study was carried out as a three-wave survey (1974–78, 1977–83, and 1985–88), inviting men and women aged 20–49 years residing in Finnmark, Oppland or Sogn- og Fjordane.
3. Oslo Age 40 Programme invited men and women aged 40 residing in Oslo 1981–99.
4. The National Age 40 Programme triennially invited all men and women aged 40–42 years in all Norwegian counties during 1985–99.
5. The TROFINN Health Study invited all men and women aged 30–75 years residing in Troms and Finnmark in 2001–03.

Blood serum samples

The Janus Cohort has detailed sample information including date of sample collection and county of residence at sample collection. The samples have been stored at –25°C for up to 43 years.[51] Serum samples of 25(OH)D and leptin have been demonstrated to have high stability after long term storage,[52 53] and previous studies have shown that serum from the Janus Cohort is well suited for analyses of 25(OH)D[54 55] and leptin.[56 57] Although the storage condition at -25°C is not ideal, a possible time-dependent degradation may be partly compensated for by matching cases and controls on time of blood draw.

Height and weight measurements and questionnaire data

Together with blood sample collection, standardized height and weight measurements were taken by trained personnel. Participants in the surveys were also asked to complete

questionnaires on smoking habits, alcohol consumption, diet, physical activity, use of medications etc. Slightly different questionnaires (different wording and number of response-categories) were used in the five health surveys, and a set of variables has been harmonized.[58] For the present project, the following variables are available: height (cm), weight (kg), BMI (kg/m^2 and categorized as 12–18.49, 18.5–24.9, 25.0–29.9, ≥30),[59] 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), level of total physical activity (inactive, low, medium, high), and level of physical activity at work (sedentary, walking, walking and lifting, heavy physical work).

Linking the Janus Cohort to population-based registries

Every resident in Norway is assigned a unique 11-digit personal identification number (PIN), which ensures a correct linkage of the Janus Cohort to population-based registries and databases as described below and in Figure 1.

Population-based registries

The *Cancer Registry of Norway (CRN)* has registered all new cancer diagnoses in Norway since 1953. Reporting of incident cancers to the CRN is compulsory by law, and information from pathologists, general practitioners, the Norwegian Patient Registry, and the Norwegian Cause of Death Registry ensures a high degree of completeness (overall 98.8%).[3] For the present project, incident cancers from 1972 through 2014 will be linked to the Janus Cohort. The following cancer information will be used: date of diagnosis (month and year), tumor localization (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) and Breslow thickness (mm).

Date and cause of death (death from cancer and death from causes other than cancer) will be obtained from *the Cause of Death Registry* and vital status (alive, emigrated or dead) with corresponding dates will be obtained from *the National Population Registry*.

Data on occupation at baseline (categorized as indoor/outdoor/mixed and high risk/medium risk/low risk for UVR exposure) and highest attained educational level at baseline (none, compulsory, upper secondary, college/university) will be obtained from *Statistics Norway*.

UVR exposure matrices

County-specific, yearly average doses of ultraviolet-A (UVA), ultraviolet-B (UVB) and erythemally weighted UVR (ERY) will be calculated and assigned to each participant, according to place of residence, at baseline and cumulated throughout follow-up (*i.e.* until cancer, emigration, death or 31st December 2014, whichever occurs first). The UVR exposure matrices will be based on measurement data from UV-network stations operated by the Norwegian Radiation Protection Authority and on modelled values as described by Medhaug et al.[60] Furthermore, age-, county-, time period-specific data on sunburns, sunbathing vacations and solarium (women only) use will be linked to the Janus Cohort on a group-level as derived from questionnaire data collected in the Norwegian Women and Cancer study.[61 62] Surveys conducted by the Norwegian Cancer Society show small gender-differences with respect to frequency of sunburns and sunbathing vacations among Norwegian women and men.[63] This is also supported by almost identical CM incidence rates between men and women in Norway the past 60 years.[64]

Study designs

Study I: a prospective cohort study

In a prospective cohort study among all 292,866 individuals in the Janus Cohort (study sample I in Figure 2), we will explore baseline BMI in relation to CM risk, Breslow thickness and mortality (hypothesis 1.1), survival after a CM diagnosis (hypothesis 1.2), and risk of second cancer after CM (hypotheses 1.3 and 1.4). Hypotheses 1.3 and 1.4 differ by use of study sample; hypothesis 1.3 includes all 292,866 individuals in the Janus Cohort, while hypothesis 1.4 includes only the 3000 CM cases. Sex-specific analyses exploring the potential confounding effects from age, UVR exposure, smoking and education will be conducted for all analyses in study 1.

Studies II-IV: prospective nested case-control studies

Three prospective case-control studies will be nested within the Janus Cohort (study samples II-IV in Figure 2). For serum analyses, the nested case-control design is cost-efficient compared to the cohort design as only a limited number of CM cases and cancer-free controls are selected and matched using an incidence-density sampling scheme.[65] Also, the nested case-control design takes advantage of the prospective nature of the cohort study by using data and serum samples collected before any cancer diagnosis, thereby reducing the potential for bias. Table 1 gives a complete description of the case, control and matching criteria.

Study II

Study II will examine CM risk and Breslow thickness according to prediagnostic serum levels of 25(OH)D and leptin (hypothesis 2.1). We will study CM cases (II a, Figure 2) without a

history of cancer and controls alive and without a cancer history at the time of the case diagnosis (II b). We will include 1 control per case, matched on sex, age at serum sampling, and season due to seasonal variation in 25(OH)D levels (Table 1). UVR exposure, smoking and education will be adjusted for. Survival analysis (as in study I) will be undertaken on the subsample of CM cases (II a) with measured 25(OH)D and leptin (hypothesis 2.2). Covariates included in study I will be taken into account.

Study III

In study III, we will examine the risk of second cancer after a CM diagnosis according to prediagnostic serum levels of 25(OH)D and leptin (hypotheses 2.3 and 2.4). CM cases with a second cancer (III a, Figure 2) and controls without a cancer history at the time of the second cancer diagnosis (III b) will be selected to address hypothesis 2.3. For hypothesis 2.4, controls with a CM diagnosis at the time of the second cancer diagnosis will be selected (III c). We will include 1 control per case, matched on sex, age at serum sampling, season of serum sampling (Table 1). In addition, control group III c will be matched on date of the CM diagnosis (Table I). Covariates included in studies I-II will be taken into account.

Study IV

A group including cases (IV a, Figure 2) with CM before lymphoma or *vice versa* and controls (IV b) with no cancer history at the time of the second cancer diagnosis will be examined according to prediagnostic serum levels of 25(OH)D and leptin (hypothesis 2.5). All case-control pairs will be matched on sex, age at serum sampling, and season of serum sampling (Table 1). Covariates included in studies I-III will be taken into account.

Power and sample size calculations

Study I: With the large study sample (n = 292,866), including more than 3000 CM cases by 31st December 2014, we have sufficient statistical power to reveal minor risk differences between the BMI categories, normal weight (18.5-24.9 kg/m^2), overweight (25-29.9 kg/m^2) and obese (≥30 kg/m^2). Thus, further power calculation is not conducted.

Studies II-IV: Study II will include 700 CM cases of the approximately 3000 available. Study III will include 345 cases with a second primary cancer after CM and study IV will include 60 cases of lymphoma after CM or *vice versa*, which were the total number of cases in the Janus Cohort by 31st December 2014. Table 2 shows the smallest detectable odds ratio (OR) according to assumed proportion of controls exposed to low serum levels of 25(OH)D and high leptin levels when using a power of 0.80 and a significance level of 0.05. The assumed proportions of exposed controls were based on previous studies conducted 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.[54] For leptin, a study on colon cancer reported that 20% of the controls had a leptin level of 4.1 ng/mL or higher.[56]

Data management

Case-control selection

As indicated in Figure 2 there will be some overlap between cases and controls between the studies. CM cases (II a) will be sampled at random from all available CM cases in the Janus Cohort, independent of second cancer status. However, some of the CM cases (II a) may have developed a new cancer and then be eligible for use in study III as CMs with a second

cancer (III a). Controls (II b) will be sampled at random with replacement (incidence density sampling) from the Janus Cohort and matched to CM cases (II a). Also controls (II b) matched to the CM cases (II a) who developed a second cancer (III a), will be eligible for use in study III (group III b) if they are alive, resident, and cancer-free at the time of the CM cases' second cancer (III a). Cases from study II (II a) may be reused as controls in study III (III c) if they fulfill the matching criteria (Table 1). The remaining case-control pairs for study III will be sampled from the Janus Cohort. Study IV will follow the same approach as studies II and III with respect to reuse. A picking list of unique serum samples for all studies will be prepared by a data manager for the Janus Serum Bank Cohort laboratory team.

Laboratory analyses

The Janus serum bank laboratory team will send 220 µl aliquots of serum to the Hormone laboratory at Oslo University Hospital for analyses of 25(OH)D and leptin. The laboratory participated in the Vitamin D External Quality Assessment Scheme (DEQAS) for total 25(OH)D. 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.

Serum concentrations of 25(OH)D will be determined by an in-house developed liquid chromatography – tandem mass spectrometry method. In brief, after protein precipitation, 25(OH)D will be extracted from samples using phospholipid depletion plates. Separation is achieved by reversed-phase chromatography and the isobaric C3 epimer 3-epi-25(OH)D3 will be separated from 25(OH)D3. Mass spectrometric detection will be performed by electrospray ionization and triple quadruple ion separation (multiple reaction monitoring).[66] Serum concentrations of leptin will be determined by using EMD Millipore Human Leptin Radioimmunoassay as described in Lee et al.[67]

Hormone laboratory staff will be blinded to case-control status. Two identical quality control (QC) samples with serum from a pool of several persons will be placed on each batch. These two QC-samples will change position for each new batch to avoid bias from weak spots in the machine/kit, and will thus take into account both inter-batch variability and intra-batch variability. Each case-control pair will be placed and analyzed on the same batch.

Statistical methods

In the cohort studies, we will use Poisson and Cox regression and estimate relative risks (RRs) with 95% confidence intervals (CIs). Flexible parametric models will also be explored if a non-linear relationship between exposure and outcome is assumed. In the nested case-control studies, conditional logistic regression will be applied to estimate ORs with 95% CIs. A multilevel approach will be applied for analyses containing group-level data. Directed acyclic graphs will be used in the process to select variables to include in the statistical models. Confounding variables will be included in the models and tests of interaction effects will be performed when relevant. In the case of interaction effects, stratified results will be presented. All tests will be two-sided and $p<0.05$ will be considered statistically significant. All statistical analyses will be performed using Stata (StataCorp, College Station, TX, USA).

Analysis plan

We plan to conduct the following analyses to test our hypotheses:

- Hypothesis 1.1: A prospective cohort analysis of prediagnostic BMI and other anthroprometric measures in relation to CM risk, Breslow thickness and mortality using the complete Janus Cohort (n = 292,866)

- Hypothesis 1.2: A prospective analysis of survival after a CM diagnosis, according to prediagnostic BMI (n ≈ 3000)
- Hypothesis 1.3: A prospective cohort analysis of prediagnostic BMI and the risk of second cancer after a CM diagnosis using the complete Janus Cohort (n = 292,866)
- Hypothesis 1.4: A prospective cohort analysis of prediagnostic BMI and the risk of second cancer among CM survivors (n ≈ 3000)
- Hypothesis 2.1: A nested case-control analysis of CM risk and Breslow thickness according to prediagnostic serum levels of 25(OH)D and leptin in 700 pairs
- Hypothesis 2.2: A prospective analysis of survival after a CM diagnosis (n = 700) according to prediagnostic serum levels of 25(OH)D and leptin
- Hypothesis 2.3: A nested case-control analysis of risk of second cancer after a CM-diagnosis according to prediagnostic serum levels of 25(OH)D and leptin. Using 345 pairs of cases with CM + a second cancer and controls without a cancer history
- Hypothesis 2.4: A nested case-control analysis of risk of second cancer among CM survivors according to prediagnostic serum levels of 25(OH)D and leptin. Using 345 pairs of cases with CM + a second cancer and controls with a CM diagnosis
- Hypothesis 2.5: A nested case-control analysis investigating risk of lymphoma after CM or *vice versa* according to prediagnostic serum levels of 25(OH)D (n = 60 cases) compared to controls without a cancer history

Project strengths and limitations

A major strength of the project is the linkage of multiple data sources by use of the PIN, thereby establishing a comprehensive research file with independently and prospectively collected data, and a complete control of loss to follow-up. An important strength is also the

use of high-quality cancer data with over 3000 CM cases from a population-based registry relying on compulsory reporting of incident cancers. Further, the prediagnostic serum samples assure a clear prospective temporal relationship between exposure and cancer, which limits the possibility of reverse causality *i.e*. that the cancer or its precursor affect the 25(OH)D or leptin serum levels.

An important limitation of the project is that we will only be able to obtain group level data on UVR exposure (ambient UVA, UVB and ERY; sunburns, sunbathing vacations, and solarium use) but our data capture variation in these variables by age, time period and between counties. However, the long and complete time-series, covering the whole observation period and early childhood for many of the participants, enables analysis with time-varying UVR exposure. Another limitation is the lack of data on pigmentary characteristics and number of nevi.

ETHICS AND DISSEMINATION

The project has a running approval from the Regional Committee for Medical and Health Research Ethics to link the different population-based registries to establish a de-identified research file. In addition, each registry and data source has approved that its data will be linked and used in a de-identified research file. A linkage-key consisting of the 11-digit PIN and a project-specific ID number will be stored and governed by a third party unavailable to the research team. Moreover, participation in each of the health surveys constituting the Janus Cohort was voluntary and based on informed consent.

All results will be published in relevant peer-reviewed international scientific journals and presented at conferences, nationally and internationally. Results will also be directly communicated to user groups such as the Norwegian Cancer Society, The Norwegian

Melanoma Association, and to health authorities and clinicians. Both the annual Norwegian conferences ("Oncologic Forum", the Norwegian Melanoma Group Meeting) and international conferences will serve as platforms for knowledge distribution to clinicians and researchers. Important results will also be disseminated through press releases. Further, lectures, the CRN website, social media and other potential channels will also be used to reach patient organizations, patients and the general public.

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Authors' contributions

TER conceived the study. JSS, TKG, JRR, LV, RB, MBV, and TER contributed to the project design. TER and JSS are responsible for data acquisition. JSS and TER drafted the manuscript, and MBV, TKG, JRR, LV and RB reviewed and revised it critically for important intellectual content, and approved the final version for submission. JSS and TER are the guarantors.

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Conflict of interest

None declared.

Ethics Approval

The project has approval from the Regional Committee for Medical and Health Research Ethics (no. 2014/185), and approval from each of the listed data sources.

Data sharing

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

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TABLES

Table 1. Overview of case, control, and matching criteria for studies II-IV.

	Study II	Study III		Study IV
CASE CRITERIA				
No. of cases	700	345		60
Verification	Histologically or cytologically verified CM in the Janus Cohort (ICD-10: C43).	Histologically or cytologically verified 2nd cancer after CM in the Janus Cohort (ICD-10: C43+any type).		Histologically or cytologically verified CM+lymphoma+CM in the Janus Cohort (ICD-10: C43+ICD-O-3[a] or ICD-O-3[a]+C43)
Definition	CM cases without a cancer history (not tied on date with another diagnosis).	2nd cancers (any type) after 1st primary CM diagnosis.		Lymphoma after 1st primary CM diagnosis or *vice versa.*
Selection	Sampled at random from pool of available CM cases.	All available cases from study II + randomly sampled from CM pool.		All available cases from study III and IV + randomly sampled from pool.
Age at diagnosis	<75 years			
Year of diagnosis	<2009			
Minimum time from blood draw to diagnosis	2 years			
Sex	Male or female			
CONTROL CRITERIA				
Control group	II b	III b	III c	IV b
No. of controls	700	345	345	180
Definition[b]	Alive, resident in Norway and no cancer history before case diagnosis	Alive, resident in Norway and no cancer history before diagnosis of 2nd cancer	Alive, resident in Norway, and a CM diagnosis but no 2nd cancer before diagnosis of 2nd cancer	Alive, resident in Norway and no cancer history before case diagnosis
Selection	Random sampling with replacement from pool of available controls			
MATCHING CRITERIA				
Sex	Same sex as case			
Age at blood draw	+/- 2 years from age of case at blood draw. Stepwise extension by +/-3 months up to +/-3 years if necessary.			
Time period of blood draw	The following 3-month intervals: a) Dec–Feb, b) Mar–May, c) Jun–Aug d) Sept–Nov.			
Date of CM diagnosis	Only applies to control group III c: +/- 6 months. Stepwise extension by +/-1 months up to +/-1 year if necessary.			

[a]9727, 9728, 9729, 9835, 9836, 9837, 9670, 9823, 9731, 9734, 9732, 9733, 9675, 9678, 9679, 9680, 9684, 9591, 9760, 9671, 9761, 9762, 9673, 9690, 9691, 9695, 9698, 9687, 9826, 9689, 9699, 9764, 9700, 9701, 9709, 9718, 9708, 9702, 9705, 9714, 9716, 9717, 9948, 9719, 9827, 9831, 9834 9833, 9940, 9820, 9832 9590, 9750

[b]Allow common cancers (colon, breast, prostate, skin, and lung only) after date of diagnosis of case to conserve sera of rare cancers for later studies

Table 2. Smallest detectable OR (above the null) according to proportion of controls exposed to low vitamin D (25(OH)D) and high leptin levels, using a power of 0.80 and a significance level of 0.05

Proportion of exposed controls	**Study II** Cases = 700 Ratio = 1:1	**Study III** Cases = 345 Ratio = 1:1	**Study IV** Cases = 60 Ratio = 1:3
5%[a]	1.82	2.26	3.81
30%[b]	1.37	1.57	2.34
20%[c]	1.43	1.65	–

[a]Exposure = 25(OH)D <30 nmol /L;
[b]Exposure = 25(OH)D <50 nmol /L;
[c]Exposure = high serum leptin levels ≥4.1 ng/mL)

FIGURES AND LEGENDS

Figure 1. Overview of linkage between different data sources. Abbreviations: BMI = body mass index; ERY = erythemally weighted UVR, PIN = personal identification number; UV = ultraviolet radiation.

Figure 2. Overview of study samples and overlap between cases and controls between studies.

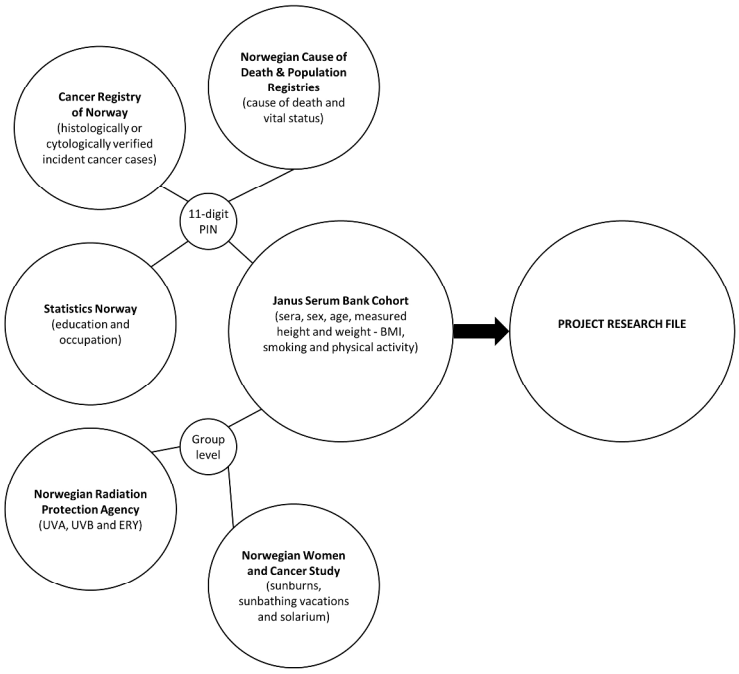

Figure 1. Overview of linkage between different data sources. Abbreviations: BMI = body mass index; ERY = erythemally weighted UVR, PIN = personal identification number; UV = ultraviolet radiation.
Figure 1
275x190mm (300 x 300 DPI)

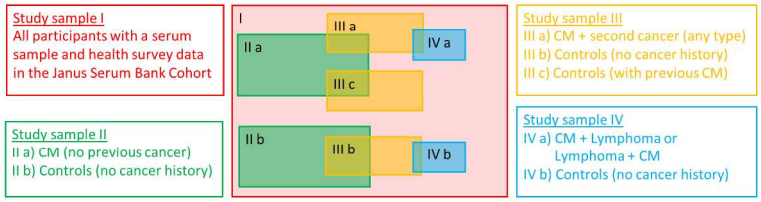

Figure 2. Overview of study samples and overlap between cases and controls between studies.
Figure 2
190x275mm (300 x 300 DPI)

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
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found
Introduction		
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported
Objectives	3	State specific objectives, including any prespecified hypotheses
Methods		
Study design	4	Present key elements of study design early in the paper
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed
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
Bias	9	Describe any efforts to address potential sources of bias
Study size	10	Explain how the study size was arrived at
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
		(*b*) Describe any methods used to examine subgroups and interactions
		(*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
		(b) Give reasons for non-participation at each stage
		(c) Consider use of a flow diagram
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders
		(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
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

Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses
Discussion		
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

*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.

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A protocol for prospective studies of 25-hydroxyvitamin D, leptin and body mass index in relation to cutaneous melanoma incidence and survival

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TITLE PAGE

Title: A protocol for prospective studies of 25-hydroxyvitamin D, leptin and body mass index in relation to cutaneous melanoma incidence and survival

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ABSTRACT

Introduction: The incidence and mortality rates of cutaneous melanoma (CM) are increasing among fair-skinned populations worldwide. Ultraviolet radiation (UVR) is the principal risk factor for CM, but is also the main source of 25-hydroxyvitamin D (25(OH)D), which has been associated with reduced risk and better prognosis of several cancers. However, both low and high 25(OH)D levels have been associated with increased risk of CM. Obesity as measured by body mass index (BMI) is associated with risk of several cancers, and has also been suggested as a risk factors for CM, and may also be related to insufficient 25(OH)D and/or high leptin levels. Moreover, contracting a CM diagnosis have been associated with increased risk of developing second cancer. We aim to study whether low prediagnostic serum levels of 25(OH)D, high prediagnostic levels of BMI and high serum leptin levels influence CM incidence, Breslow thickness and CM mortality, and risk of second cancer and survival after a CM diagnosis.

Methods and analysis: Cohort and nested case-control studies will be carried out using the population-based Janus Serum Bank Cohort (archival prediagnostic sera, BMI, smoking and physical activity), with follow-up 1972–2014. Additional data will be received from the Cancer Registry of Norway, the national Cause of Death Registry, Statistics Norway (education and occupation), and exposure matrices of UVR. Time to event regression models will be used to analyze the cohort data, while the nested case-control studies will be analyzed by conditional logistic regression. A multilevel approach will be applied when incorporating group-level data.

Ethics and dissemination: The project is 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 in the news media.

Strength and limitations of this study

- Strengths:
  - Linkage of independent, national data sources by use of a unique personal identification number for a comprehensive research file and complete control of loss to follow-up
  - Over 3000 CM cases from a high-quality population-based cancer registry relying on mandatory reporting of incident cancers.
  - Prediagnostic serum samples assuring a true prospective relationship between exposures and cancer, limiting bias introduced by reverse causality
  - Lifetime ambient UVR exposure data (UVA, UVB, and erythemally weighted UV) and group-level data on sunburns, sunbathing vacations, and solarium use capturing variations in age, time period and county of residence.
  - Clinically measured height and weight, limiting misclassification
- Limitations:
  - Ambient UVR exposure and data on sunburns, sunbathing vacations and solarium use can only be linked to the Janus Cohort on a group-level
  - Lack of data on pigmentary characteristics and nevi

INTRODUCTION

Rationale and evidence gaps

Ultraviolet radiation (UVR) is a recognized human carcinogen and the principal environmental risk factor for cutaneous melanoma (CM)[1 2], while skin characteristics such as skin sensitivity and number of nevi indicate CM susceptibility.[3-7] CM incidence and mortality rates have been increasing in fair-skinned populations worldwide the past decades, and CM is currently the third most common cancer in Europe after cancers of the colon/rectum and the lung.[8 9] In Norway, CM incidence has increased more than 3% annually between 1982 and 2011 and has been projected to continue to rise.[9] Excess UVR exposure is likely the major cause of this increase,[10] but also low vitamin D levels and obesity have been suggested to play a role.[11 12]

Vitamin D synthesis in the skin is initiated by UVR exposure to the skin surface at wavelengths of 290–320 nm, which converts 7-dehydrocholesterol in the keratinocytes to previtamin D3 (cholecalciferol). Together with previtamin D2 (ergocalciferol), previtamin D3 may also be obtained by diet. Both previtamin D2 and D3 are then hydroxylated in the liver to 25-hydroxyvitamin D (25(OH)D), which represents the circulating storage form of vitamin D. A second hydroxylation in the kidney converts vitamin D to its biologically active form 1,25-hydroxyvitamin D (1,25(OH)D),[13 14] which has been associated with anticancer mechanisms.[13 15-17] Based on four studies, a recent meta-analysis reported a summary relative risk of CM of 1.46 (95% CI: 0.60-3.53) for the highest compared to the lowest (reference) quantile of 25(OH)D.[12] In three of these studies, risks increased with increasing 25(OH)D serum levels, while the fourth study reported the opposite.[18-21] None of these studies individually showed any statistically significant associations, and the inconclusive results may be due to difference in statistical power, the covariate

adjustments, whether CM cases had a cancer history or not, and whether serum was sampled before or after the CM diagnosis. Several recent studies have reported an inverse association between Breslow thickness and 25(OH)D serum level at diagnosis.[20 22-25] As both tumor thickness and 25(OH)D level were measured at the same time in these studies, these associations may have been affected by reverse causality.[26 27] However, for prognosis after a CM diagnosis, higher diagnostic 25(OH)D levels have been shown to predict lower risk of relapse and increased survival, independent of Breslow thickness.[22 24] A recent study, ascribed the effect on CM survival to change in 25(OH)D during follow-up from CM diagnosis to death, and not the 25(OH)D level at diagnosis.[28]

Low 25(OH)D levels are more frequent in obese persons, suggesting that 25(OH)D deficiency is associated with obesity and *vice versa*.[29-33] Obesity as measured by body mass index (BMI) above 30 kg/m^2 has been positively associated with CM risk in males, but results for women are ambiguous, and possibly confounded by personal habits as obese women may refrain from sunseeking behavior compared to their normal weight peers.[11] Further, diet-induced obesity has been found to increase CM progression in mice models.[34] The biological mechanism underlying an obesity-induced increase in CM incidence is not well understood, although a hyperglycemia hypothesis has been suggested.[35] Another hypothesis suggests that adipocytes produce high levels of vascular endothelial growth factor (VEGF), associated with visceral fat, which contributes to angiogenesis and tumor growth.[36]

The metabolic hormone leptin may be a risk factor for both CM and CM progression. Leptin is released by adipose tissue and plays an important role in the regulation of insulin sensitivity and weight regulation.[37 38] Increased diagnostic serum levels of leptin have been associated with increased CM risk, possibly caused by a leptin-induced increase in

neoangiogenesis, reduction of melanogenesis and a decreased capacity of the melanocytes' DNA repair.[39 40] Recent studies have demonstrated that leptin receptors are present in melanoma cell-lines that proliferates in response to leptin, and that leptin bound to its receptor stimulates melanoma growth.[41-44]

After a CM diagnosis, there is an increased risk of diagnosis of additional CM, as well as other cancers.[45 46] For example, the risk of lymphoma before or after CM has received increased focus.[47] Immune perturbation has been suggested to contribute to the development of CM after non-Hodgkin lymphoma (NHL) subtypes such as chronic lymphocytic leukemia/small lymphocytic lymphoma.[48] As for CM, low 25(OH)D serum levels have also been associated with reduced survival and poor prognosis after NHL,[49 50] which raises the question of whether low 25(OH)D could alter the risk of lymphoma as a second cancer after CM or *vice versa*.

Aims and hypotheses

The interplay between 25(OH)D and obesity and their relation to CM is poorly described, and increased knowledge of these factors is warranted to improve CM prevention and prognosis. In the present study protocol, we propose a set of prospective cohort and nested case-control studies with the primary aim of examining BMI and serum levels of 25(OH)D and leptin in relation to CM risk, Breslow thickness and mortality, and risk of second cancer and survival after a CM diagnosis. As a secondary aim, we propose a nested case-control study of lymphoma risk after CM and *vice versa*, in relation to serum levels of 25(OH)D and leptin.

We hypothesize that:

1. High prediagostic BMI (≥30 kg/m^2, quantiles, continuous) is associated with
   1.1. Increased CM risk, Breslow thickness, and mortality
   1.2. Reduced survival after a CM diagnosis
   1.3. Increased risk of contracting CM followed by a second cancer (n = 292,851)
   1.4. Increased risk of second cancer among CM survivors (n ≈ 3000)
2. High prediagnostic serum levels of leptin (>4 ng/mL, highest quantile, continuous) and low prediagnostic 25(OH)D levels (<30 nmol/L, lowest quantile, continuous) are associated with
   2.1. Increased CM risk and Breslow thickness
   2.2. Reduced survival after a CM diagnosis
   2.3. Increased risk of contracting CM followed by a second cancer compared to no cancer history
   2.4. Increased risk of second cancer among CM survivors
   2.5. Increased lymphoma risk after a CM diagnosis and *vice versa* compared to no cancer history

METHODS AND ANALYSIS

Study population and data sources

Janus Serum Bank Cohort

This project is based on 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 five health surveys 1972–2003. A detailed description of the

Janus Serum Bank Cohort (hereafter Janus Cohort), its data and establishment, is published elsewhere.[51] The Janus Cohort includes participants from the following surveys:

1. The Oslo Study I (1972–73), invited men residing in Oslo aged 20–49 years.
2. The Norwegian Counties Study was carried out as a three-wave survey (1974–78, 1977–83, and 1985–88), inviting men and women aged 20–49 years residing in Finnmark, Oppland or Sogn- og Fjordane.
3. Oslo Age 40 Programme invited men and women aged 40 residing in Oslo 1981–99.
4. The National Age 40 Programme triennially invited all men and women aged 40–42 years in all Norwegian counties during 1985–99.
5. The TROFINN Health Study invited all men and women aged 30–75 years residing in Troms and Finnmark in 2001–03.

Blood serum samples

The Janus Cohort has detailed sample information including date of sample collection and county of residence at sample collection. The samples have been stored at –25°C for up to 43 years.[51] Serum samples of 25(OH)D and leptin have been demonstrated to have high stability after long term storage,[52 53] and previous studies have shown that serum from the Janus Cohort is well suited for analyses of 25(OH)D[54 55] and leptin.[56 57] Although the storage condition at -25°C is not ideal, a possible time-dependent degradation may be partly compensated for by matching cases and controls on time of blood draw.

Height and weight measurements and questionnaire data

Together with blood sample collection, standardized height and weight measurements were taken by trained personnel. Participants in the surveys were also asked to complete

questionnaires on smoking habits, alcohol consumption, diet, physical activity, use of medications etc. Slightly different questionnaires (different wording and number of response-categories) were used in the five health surveys, and a set of variables has been harmonized.[58] For the present project, the following variables are available: height (cm), weight (kg), BMI (kg/m^2 and categorized as 12–18.49, 18.5–24.9, 25.0–29.9, ≥30),[59] 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), level of total physical activity (inactive, low, medium, high), and level of physical activity at work (sedentary, walking, walking and lifting, heavy physical work).

Linking the Janus Cohort to population-based registries

Every resident in Norway is assigned a unique 11-digit personal identification number (PIN), which ensures a correct linkage of the Janus Cohort to population-based registries and databases as described below and in Figure 1.

Population-based registries

The *Cancer Registry of Norway (CRN)* has registered all new cancer diagnoses in Norway since 1953. Reporting of incident cancers to the CRN is compulsory by law, and information from pathologists, general practitioners, the Norwegian Patient Registry, and the Norwegian Cause of Death Registry ensures a high degree of completeness (overall 98.8%).[3] For the present project, incident cancers from 1972 through 2014 will be linked to the Janus Cohort. The following cancer information will be used: date of diagnosis (month and year), tumor localization (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) and Breslow thickness (mm).

Date and cause of death (death from cancer and death from causes other than cancer) will be obtained from *the Cause of Death Registry* and vital status (alive, emigrated or dead) with corresponding dates will be obtained from *the National Population Registry*.

Data on occupation at baseline (categorized as indoor/outdoor/mixed and high risk/medium risk/low risk for UVR exposure) and highest attained educational level at baseline (none, compulsory, upper secondary, college/university) will be obtained from *Statistics Norway*.

UVR exposure matrices

County-specific, yearly average doses of ultraviolet-A (UVA), ultraviolet-B (UVB) and erythemally weighted UVR (ERY) will be calculated and assigned to each participant, according to place of residence, at baseline and cumulated throughout follow-up (*i.e.* until cancer, emigration, death or 31st December 2014, whichever occurs first). The UVR exposure matrices will be based on measurement data from UV-network stations operated by the Norwegian Radiation Protection Authority and on modelled values as described by Medhaug et al.[60] Furthermore, age-, county-, time period-specific data on sunburns, sunbathing vacations and solarium (women only) use will be linked to the Janus Cohort on a group-level as derived from questionnaire data collected in the Norwegian Women and Cancer study.[61 62] Surveys conducted by the Norwegian Cancer Society show small gender-differences with respect to frequency of sunburns and sunbathing vacations among Norwegian women and men.[63] This is also supported by almost identical CM incidence rates between men and women in Norway the past 60 years.[64]

Study designs

Study I: a prospective cohort study

In a prospective cohort study among all 292,851 individuals in the Janus Cohort (study sample I in Figure 2), we will explore baseline BMI in relation to CM risk, Breslow thickness and mortality (hypothesis 1.1), survival after a CM diagnosis (hypothesis 1.2), and risk of second cancer after CM (hypotheses 1.3 and 1.4). Hypotheses 1.3 and 1.4 differ by use of study sample; hypothesis 1.3 includes all 292,851 individuals in the Janus Cohort, while hypothesis 1.4 includes only the 3000 CM cases. Sex-specific analyses exploring the potential confounding effects from age, UVR exposure, smoking and education will be conducted for all analyses in study 1.

Studies II-IV: prospective nested case-control studies

Three prospective case-control studies will be nested within the Janus Cohort (study samples II-IV in Figure 2). For serum analyses, the nested case-control design is cost-efficient compared to the cohort design as only a limited number of CM cases and cancer-free controls are selected and matched using an incidence-density sampling scheme.[65] Also, the nested case-control design takes advantage of the prospective nature of the cohort study by using data and serum samples collected before any cancer diagnosis, thereby reducing the potential for bias. Table 1 gives a complete description of the case, control and matching criteria.

Study II

Study II will examine CM risk and Breslow thickness according to prediagnostic serum levels of 25(OH)D and leptin (hypothesis 2.1). We will study CM cases (II a, Figure 2) without a

history of cancer and controls alive and without a cancer history at the time of the case diagnosis (II b). We will include 1 control per case, matched on sex, age at serum sampling, and season due to seasonal variation in 25(OH)D levels (Table 1). UVR exposure, smoking and education will be adjusted for. Survival analysis (as in study I) will be undertaken on the subsample of CM cases (II a) with measured 25(OH)D and leptin (hypothesis 2.2). Covariates included in study I will be taken into account.

Study III

In study III, we will examine the risk of second cancer after a CM diagnosis according to prediagnostic serum levels of 25(OH)D and leptin (hypotheses 2.3 and 2.4). CM cases with a second cancer (III a, Figure 2) and controls without a cancer history at the time of the second cancer diagnosis (III b) will be selected to address hypothesis 2.3. For hypothesis 2.4, controls with a CM diagnosis at the time of the second cancer diagnosis will be selected (III c). We will include 1 control per case, matched on sex, age at serum sampling, season of serum sampling (Table 1). In addition, control group III c will be matched on date of the CM diagnosis (Table I). Covariates included in studies I-II will be taken into account.

Study IV

A group including cases (IV a, Figure 2) with CM before lymphoma or *vice versa* and controls (IV b) with no cancer history at the time of the second cancer diagnosis will be examined according to prediagnostic serum levels of 25(OH)D and leptin (hypothesis 2.5). All case-control pairs will be matched on sex, age at serum sampling, and season of serum sampling (Table 1). Covariates included in studies I-III will be taken into account.

Power and sample size calculations

Study I: With the large study sample (n = 292,851), including more than 3000 CM cases by 31st December 2014, we have sufficient statistical power to reveal minor risk differences between the BMI categories, normal weight (18.5-24.9 kg/m^2), overweight (25-29.9 kg/m^2) and obese (≥30 kg/m^2). Thus, further power calculation is not conducted.

Studies II-IV: Study II will include 700 CM cases of the approximately 3000 available. Study III will include 345 cases with a second primary cancer after CM and study IV will include 60 cases of lymphoma after CM or *vice versa*, which were the total number of cases in the Janus Cohort by 31st December 2014. Table 2 shows the smallest detectable odds ratio (OR) according to assumed proportion of controls exposed to low serum levels of 25(OH)D and high leptin levels when using a power of 0.80 and a significance level of 0.05. The assumed proportions of exposed controls were based on previous studies conducted 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.[54] For leptin, a study on colon cancer reported that 20% of the controls had a leptin level of 4.1 ng/mL or higher.[56]

Data management

Case-control selection

As indicated in Figure 2 there will be some overlap between cases and controls between the studies. CM cases (II a) will be sampled at random from all available CM cases in the Janus Cohort, independent of second cancer status. However, some of the CM cases (II a) may have developed a new cancer and then be eligible for use in study III as CMs with a second

cancer (III a). Controls (II b) will be sampled at random with replacement (incidence density sampling) from the Janus Cohort and matched to CM cases (II a). Also controls (II b) matched to the CM cases (II a) who developed a second cancer (III a), will be eligible for use in study III (group III b) if they are alive, resident, and cancer-free at the time of the CM cases' second cancer (III a). Cases from study II (II a) may be reused as controls in study III (III c) if they fulfill the matching criteria (Table 1). The remaining case-control pairs for study III will be sampled from the Janus Cohort. Study IV will follow the same approach as studies II and III with respect to reuse. A picking list of unique serum samples for all studies will be prepared by a data manager for the Janus Serum Bank Cohort laboratory team.

Laboratory analyses

The Janus serum bank laboratory team will send 220 µl aliquots of serum to the Hormone laboratory at Oslo University Hospital for analyses of 25(OH)D and leptin. The laboratory participated in the Vitamin D External Quality Assessment Scheme (DEQAS) for total 25(OH)D. 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.

Serum concentrations of 25(OH)D will be determined by an in-house developed liquid chromatography – tandem mass spectrometry method. In brief, after protein precipitation, 25(OH)D will be extracted from samples using phospholipid depletion plates. Separation is achieved by reversed-phase chromatography and the isobaric C3 epimer 3-epi-25(OH)D3 will be separated from 25(OH)D3. Mass spectrometric detection will be performed by electrospray ionization and triple quadruple ion separation (multiple reaction monitoring).[66] Serum concentrations of leptin will be determined by using EMD Millipore Human Leptin Radioimmunoassay as described in Lee et al.[67]

Hormone laboratory staff will be blinded to case-control status. Two identical quality control (QC) samples with serum from a pool of several persons will be placed on each batch. These two QC-samples will change position for each new batch to avoid bias from weak spots in the machine/kit, and will thus take into account both inter-batch variability and intra-batch variability. Each case-control pair will be placed and analyzed on the same batch.

Statistical methods

In the cohort studies, we will use Poisson and Cox regression and estimate relative risks (RRs) with 95% confidence intervals (CIs). Flexible parametric models will also be explored if a non-linear relationship between exposure and outcome is assumed. In the nested case-control studies, conditional logistic regression will be applied to estimate ORs with 95% CIs. A multilevel approach will be applied for analyses containing group-level data. Directed acyclic graphs will be used in the process to select variables to include in the statistical models. Confounding variables will be included in the models and tests of interaction effects will be performed when relevant. In the case of interaction effects, stratified results will be presented. All tests will be two-sided and $p<0.05$ will be considered statistically significant. All statistical analyses will be performed using Stata (StataCorp, College Station, TX, USA).

Analysis plan

We plan to conduct the following analyses to test our hypotheses:

- Hypothesis 1.1: A prospective cohort analysis of prediagnostic BMI and other anthroprometric measures in relation to CM risk, Breslow thickness and mortality using the complete Janus Cohort (n = 292,851)

- Hypothesis 1.2: A prospective analysis of survival after a CM diagnosis, according to prediagnostic BMI (n ≈ 3000)
- Hypothesis 1.3: A prospective cohort analysis of prediagnostic BMI and the risk of second cancer after a CM diagnosis using the complete Janus Cohort (n = 292,851)
- Hypothesis 1.4: A prospective cohort analysis of prediagnostic BMI and the risk of second cancer among CM survivors (n ≈ 3000)
- Hypothesis 2.1: A nested case-control analysis of CM risk and Breslow thickness according to prediagnostic serum levels of 25(OH)D and leptin in 700 pairs
- Hypothesis 2.2: A prospective analysis of survival after a CM diagnosis (n = 700) according to prediagnostic serum levels of 25(OH)D and leptin
- Hypothesis 2.3: A nested case-control analysis of risk of second cancer after a CM-diagnosis according to prediagnostic serum levels of 25(OH)D and leptin. Using 345 pairs of cases with CM + a second cancer and controls without a cancer history
- Hypothesis 2.4: A nested case-control analysis of risk of second cancer among CM survivors according to prediagnostic serum levels of 25(OH)D and leptin. Using 345 pairs of cases with CM + a second cancer and controls with a CM diagnosis
- Hypothesis 2.5: A nested case-control analysis investigating risk of lymphoma after CM or *vice versa* according to prediagnostic serum levels of 25(OH)D (n = 60 cases) compared to controls without a cancer history

Project strengths and limitations

A major strength of the project is the linkage of multiple data sources by use of the PIN, thereby establishing a comprehensive research file with independently and prospectively collected data, and a complete control of loss to follow-up. An important strength is also the

use of high-quality cancer data with over 3000 CM cases from a population-based registry relying on compulsory reporting of incident cancers. Further, the prediagnostic serum samples assure a clear prospective temporal relationship between exposure and cancer, which limits the possibility of reverse causality *i.e*. that the cancer or its precursor affect the 25(OH)D or leptin serum levels.

An important limitation of the project is that we will only be able to obtain group level data on UVR exposure (ambient UVA, UVB and ERY; sunburns, sunbathing vacations, and solarium use) but our data capture variation in these variables by age, time period and between counties. However, the long and complete time-series, covering the whole observation period and early childhood for many of the participants, enables analysis with time-varying UVR exposure. Another limitation is the lack of data on pigmentary characteristics and number of nevi. Also, differences in skin color between cases and controls could potentially bias our estimates. However, the average fraction of non-whites during 1970-1991 (when 97% of the Janus Cohort was established) was less than 1% of the total Norwegian population,[68] and hence we consider the risk of introducing bias by not taking individual information on skin color into account as negligible.

ETHICS AND DISSEMINATION

The project has a running approval from the Regional Committee for Medical and Health Research Ethics to link the different population-based registries to establish a de-identified research file. In addition, each registry and data source has approved that its data will be linked and used in a de-identified research file. A linkage-key consisting of the 11-digit PIN and a project-specific ID number will be stored and governed by a third party unavailable to

the research team. Moreover, participation in each of the health surveys constituting the Janus Cohort was voluntary and based on informed consent.

All results will be published in relevant peer-reviewed international scientific journals and presented at conferences, nationally and internationally. Results will also be directly communicated to user groups such as the Norwegian Cancer Society, The Norwegian Melanoma Association, and to health authorities and clinicians. Both the annual Norwegian conferences ("Oncologic Forum", the Norwegian Melanoma Group Meeting) and international conferences will serve as platforms for knowledge distribution to clinicians and researchers. Important results will also be disseminated through press releases. Further, lectures, the CRN website, social media and other potential channels will also be used to reach patient organizations, patients and the general public.

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Authors' contributions

TER conceived the study. JSS, TKG, JRR, LV, RB, MBV, and TER contributed to the project design. TER and JSS are responsible for data acquisition. JSS and TER drafted the manuscript, and MBV, TKG, JRR, LV and RB reviewed and revised it critically for important intellectual content, and approved the final version for submission. JSS and TER are the guarantors.

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Conflict of interest

None declared.

Ethics Approval

The project has approval from the Regional Committee for Medical and Health Research Ethics (no. 2014/185), and approval from each of the listed data sources.

Data sharing

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

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TABLES

Table 1. Overview of case, control, and matching criteria for studies II-IV.

	Study II	Study III		Study IV
CASE CRITERIA				
No. of cases	700	345		60
Verification	Histologically or cytologically verified CM in the Janus Cohort (ICD-10: C43).	Histologically or cytologically verified 2nd cancer after CM in the Janus Cohort (ICD-10: C43+any type).		Histologically or cytologically verified CM+lymphoma+CM in the Janus Cohort (ICD-10: C43+ICD-O-3[a] or ICD-O-3[a]+C43)
Definition	CM cases without a cancer history (not tied on date with another diagnosis).	2nd cancers (any type) after 1st primary CM diagnosis.		Lymphoma after 1st primary CM diagnosis or *vice versa.*
Selection	Sampled at random from pool of available CM cases.	All available cases from study II + randomly sampled from CM pool.		All available cases from study III and IV + randomly sampled from pool.
Age at diagnosis	<75 years			
Year of diagnosis	<2009			
Minimum time from blood draw to diagnosis	2 years			
Sex	Male or female			
CONTROL CRITERIA				
Control group	II b	III b	III c	IV b
No. of controls	700	345	345	180
Definition[b]	Alive, resident in Norway and no cancer history before case diagnosis	Alive, resident in Norway and no cancer history before diagnosis of 2nd cancer	Alive, resident in Norway, and a CM diagnosis but no 2nd cancer before diagnosis of 2nd cancer	Alive, resident in Norway and no cancer history before case diagnosis
Selection	Random sampling with replacement from pool of available controls			
MATCHING CRITERIA				
Sex	Same sex as case			
Age at blood draw	+/- 2 years from age of case at blood draw. Stepwise extension by +/-3 months up to +/-3 years if necessary.			
Time period of blood draw	The following 3-month intervals: a) Dec–Feb, b) Mar–May, c) Jun–Aug d) Sept–Nov.			
Date of CM diagnosis	Only applies to control group III c: +/- 6 months. Stepwise extension by +/-1 months up to +/-1 year if necessary.			

[a]9727, 9728, 9729, 9835, 9836, 9837, 9670, 9823, 9731, 9734, 9732, 9733, 9675, 9678, 9679, 9680, 9684, 9591, 9760, 9671, 9761, 9762, 9673, 9690, 9691, 9695, 9698, 9687, 9826, 9689, 9699, 9764, 9700, 9701, 9709, 9718, 9708, 9702, 9705, 9714, 9716, 9717, 9948, 9719, 9827, 9831, 9834 9833, 9940, 9820, 9832 9590, 9750

[b]Allow common cancers (colon, breast, prostate, skin, and lung only) after date of diagnosis of case to conserve sera of rare cancers for later studies

Table 2. Smallest detectable OR (above the null) according to proportion of controls exposed to low vitamin D (25(OH)D) and high leptin levels, using a power of 0.80 and a significance level of 0.05

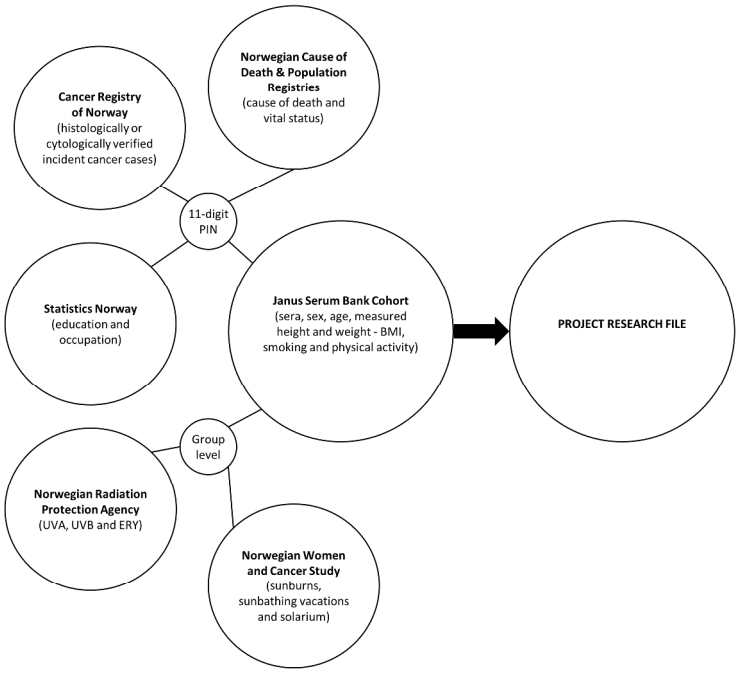
Proportion of exposed controls	**Study II** Cases = 700 Ratio = 1:1	**Study III** Cases = 345 Ratio = 1:1	**Study IV** Cases = 60 Ratio = 1:3
5%[a]	1.82	2.26	3.81
30%[b]	1.37	1.57	2.34
20%[c]	1.43	1.65	–

[a]Exposure = 25(OH)D <30 nmol /L;
[b]Exposure = 25(OH)D <50 nmol /L;
[c]Exposure = high serum leptin levels ≥4.1 ng/mL)

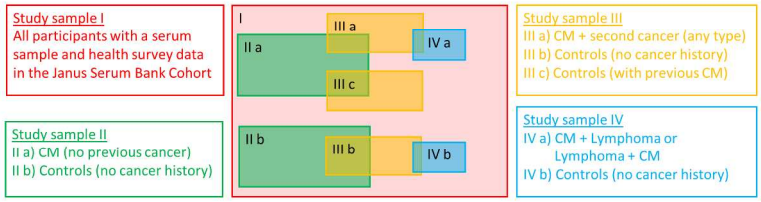
FIGURES AND LEGENDS

Figure 1. Overview of linkage between different data sources. Abbreviations: BMI = body mass index; ERY = erythemally weighted UVR, PIN = personal identification number; UV = ultraviolet radiation.

Figure 2. Overview of study samples and overlap between cases and controls between studies.



275x190mm (300 x 300 DPI)



190x275mm (300 x 300 DPI)

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
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found
Introduction		
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported
Objectives	3	State specific objectives, including any prespecified hypotheses
Methods		
Study design	4	Present key elements of study design early in the paper
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed
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
Bias	9	Describe any efforts to address potential sources of bias
Study size	10	Explain how the study size was arrived at
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
		(*b*) Describe any methods used to examine subgroups and interactions
		(*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
		(b) Give reasons for non-participation at each stage
		(c) Consider use of a flow diagram
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders
		(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
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

Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses
Discussion		
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

*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.