CLINICAL STUDY

Effect of smoking on the serum levels of 25-hydroxyvitamin D depends on the assay employed

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Abstract

Objective: Because we found higher serum 25-hydroxyvitamin D (25(OH)D) levels among smokers than among non-smokers with analyses using an electrochemiluminescence immunoassay (ECLIA) from Roche, the purpose of the present study was to examine whether this difference between smokers and non-smokers was maintained using other serum 25(OH)D assays.

Design: A cross-sectional population-based study on 6932 participants from the Tromsø study, 1994–1995, and one validation study comparing six different serum 25(OH)D assays in 53 non-smokers and 54 smokers were performed.

Methods: The association between smoking, season and serum 25(OH)D as measured by ECLIA (Roche) was assessed in the population-based study using general linear models with multivariate adjustments. In the validation study, serum levels of 25(OH)D were analysed with liquid chromatography coupled with mass spectrometry assay from two different laboratories, RIA (DiaSorin), HPLC, RIA (IDS) and ECLIA (Roche). *T*-tests and linear mixed model analyses were performed to compare the serum 25(OH)D levels in smokers and non-smokers within and between the methods.

Results: In the population-based study, the serum levels of 25(OH)D using the ECLIA method were 51.9, 53.2 and 72.0 nmol/l in never, former and current smokers ($P < 0.01$). In the validation study, the serum concentration of 25(OH)D was 10.3 nmol/l higher in smokers than in non-smokers ($P < 0.01$) using the ECLIA (Roche), while non-significantly lower serum levels of 25(OH)D were found in smokers using the other five methods.

Conclusions: These two studies indicate that the ECLIA (Roche) overestimates serum 25(OH)D levels in smokers by unknown mechanisms. If confirmed, this might have clinical consequences, and the issue needs further exploration.

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Introduction

Current research on vitamin D is extensive, as illustrated by a PubMed search using the term 'vitamin D' revealing nearly 10 000 articles that were published during the last 5 years. Growing evidence relates low serum levels of 25-hydroxyvitamin D (25(OH)D) to a worsening state of a number of diseases. Thus, valid methods for 25(OH)D measurements are of great importance in both research and clinical practice.

The circulating level of 25(OH)D is normally used as the marker of an individual's vitamin D status. This reflects the amount of vitamin D produced in the skin during ultraviolet B (UVB) exposure (cholecalciferol (25(OH)D₃)) and the amount of vitamin D ingested from

food (ergocalciferol (25(OH)D₂) or 25(OH)D₃). Food sources of vitamin D include fatty fish, cod liver oil and fortified food such as dairy products in addition to supplements. In Norway, cholecalciferol is almost exclusively used as fortification and supplementation. A number of different methods are used to determine serum levels of 25(OH)D, including RIAs or competitive protein binding assays, and direct physical detection methods such as HPLC and liquid chromatography coupled with mass spectrometry (LC–MS/MS). These methods differ in several aspects, as described in a recent review by Hollis (1), and also discussed by Carter (2).

Results regarding the vitamin D status in smokers are conflicting. Several studies report lower serum 25(OH)D levels in smokers (3–10), while others find no

significant differences in serum 25(OH)D levels between smokers and non-smokers (11–21). In the Tromsø study, 1994–1995 (called the Tromsø study in the text that follows), where a relatively new electrochemiluminescence immunoassay (ECLIA) from Roche (called ECLIA (Roche) in the text that follows) was used, smokers had significantly higher serum 25(OH)D levels than non-smokers. As this finding was unexpected, we hypothesised the presence of interfering substance(s) in smokers causing bias in the ECLIA (Roche) 25(OH)D method. Consequently, we performed a validation study comparing the ECLIA (Roche) assay with five other methods of 25(OH)D measurement in serum samples from 54 smokers and 53 non-smokers. In this report, we present the results obtained from the Tromsø study and the validation study.

Subjects and methods

The Tromsø study

Study population The Tromsø study is a repeated population-based study conducted in the municipality of Tromsø, Norway, situated at 69°N (current population 67 000). The study was initiated in 1974, focusing originally on cardiovascular diseases. However, the study has evolved through the years with increasing emphasis on other conditions and chronic diseases, for example osteoporosis, diabetes, chronic obstructive pulmonary disease, cancer and dementia. The Tromsø study has been performed six times at regular intervals by inviting the inhabitants of the municipality of Tromsø. The latest health survey was conducted in 2007–2008.

In 1994, all individuals aged 25 years or older were invited to participate in the fourth survey of the Tromsø study. A total number of 27 158 persons participated in the first visit, providing an attendance rate of 77% among eligible inhabitants. All men aged 55–74 years, all women aged 50–74 years and a sample of 5–10% of the remaining age groups between 25 and 84 years were invited to undergo a more extensive clinical examination (second visit), and 7965 persons, or 78% of those invited, attended (22). The study was conducted by the University of Tromsø in cooperation with the National Health Screening Service, and was approved by the Regional Committee of Research Ethics and the Norwegian Data Inspectorate. Each participant gave written informed consent prior to the examinations.

Measurements The participants filled in self-administered questionnaires on socio-demographic factors, medical history, lifestyle factors, and dietary habits including vitamin D supplementation. All participants attended a physical examination where the questionnaire was checked for incomplete answers, and blood samples were taken. We defined vitamin D supplementation as use of cod liver oil (yes/no) and/or multivitamins containing vitamin D (yes/no).

Physical activity level was calculated by adding together hours of moderate and hard physical activity during leisure time per week. To identify current, former and never smokers, we used a combination of the following questions: 'Do you smoke cigarettes daily?' (yes/no); for previous or current smokers: 'How many cigarettes do you, or did you smoke daily?' (hand rolled + factory made), and 'If you currently smoke, or have smoked before, how many years in all have you smoked daily?'. We also used the smoking variable 'If you previously smoked daily, how long is it since you stopped (years)?'. Subjects answering yes to 'Do you smoke a pipe daily?' ($n = 52$) or 'Do you smoke cigars/cigarillos daily?' ($n = 43$) were coded as current smokers. Owing to inconsistent answers in the questionnaires, 100 participants were excluded from the analyses since their smoking status could not be classified with certainty. Height and weight were measured wearing light clothing and no shoes, and body mass index (BMI) was defined as weight (kg) divided by height squared (m^2).

Laboratory analyses Serum calcium was analysed within a week of sampling using an automated analyser (Hitachi 917) with reagents from Boehringer Mannheim. The reference range was 2.20–2.60 mmol/l. Serum parathyroid hormone (PTH) was analysed in 2001 in a subgroup of 3277 participants using an automated clinical chemical analyser (Immulite 2000, Siemens Healthcare Diagnostics, Los Angeles, CA, USA), with a reference range of 1.1–6.8 pmol/l (≤ 50 years) and 1.1–7.5 pmol/l (> 50 years).

Sera from the second visit of the study were stored at -70°C , and after a median storage time of 13 years, they were thawed in March 2008 and analysed for 25(OH)D₃ by ECLIA (Roche) using an automated clinical chemistry analyser (Modular E170, Roche Diagnostics). The total analytical coefficient of variation (CV) for the 25(OH)D₃ assay was 7.3% as recorded by measuring a donor control (65.0 nmol/l) consecutively during the analytical period using a quality management programme (QM, Tieto Enator, Helsinki, Finland). This was in accordance with the total analytical precision $\leq 7.8\%$ as reported by the producer. The cross-reactivity with 25(OH)D₂ was $<10\%$, and the lower detection limit was 10 nmol/l. Five subjects had 25(OH)D₃ below the detection limit, and their value was set to 5 nmol/l. All analyses were performed at the Department of Medical Biochemistry, University Hospital of North Norway, Tromsø, and were approved by the Norwegian Accreditation Authority.

The validation study

Study population The validation study was performed as an extension of the sixth survey of the Tromsø study, which included 12 984 participants during 2007–2008 (attendance rate 65.7%). Here, a

questionnaire covering lifestyle factors was filled in by the participants at home, and it was checked for incomplete answers when they came for the first visit. Based on the information obtained from the questionnaire, a random group of participants matching the following predefined criteria was invited by mail to participate in the validation study:

- i) 'Non-smokers' as defined by never smoked or quit smoking ≥ 5 years ago, or
- ii) 'smokers' as defined by smoking ≥ 5 cigarettes/day.

Subjects reporting use of snuff were not invited. We included 108 participants. The validation study was approved by the Regional Committee of Research Ethics, and each participant gave a written informed consent prior to the examination.

Measurements The participants in the validation study filled in a new questionnaire covering sex, age, smoking and snuffing habits, hours spent outdoors during the last week, use of solarium during the last 3 months and the last 14 days, sunny holidays during the last 3 months, intake of fatty fish as dinner or as a topping on sandwiches, intake of semi-skimmed milk (which is the only vitamin D-fortified milk in Norway), use of vitamin D supplementation, and self-reported height and weight. The questionnaire was checked for incomplete answers, and blood samples were taken by trained health personnel at the University Hospital of North Norway. Time since intake of last meal and smoking of last cigarette was noted, as well as the number of cigarettes smoked on the same day. The data collection was performed during a period of 3 weeks in April/May 2009.

Blood samples were obtained in SST gel tubes and centrifuged within 1 h, and serum calcium, creatinine, phosphate and plasma PTH were analysed continuously. For analyses of serum 25(OH)D, sera were divided into portions of 0.5 ml, and frozen in 1-ml Cryotube vials at -70°C for later analyses. Transportation to different laboratories was done on ice packs, and all samples were confirmed to be frozen at arrival by the receiving laboratories, and thus there were no thawing-freezing cycles.

Laboratory analyses Serum calcium and PTH in plasma were analysed within 24 h using methods that were the same as those used in the Tromsø study. Serum creatinine was analysed by an enzymatic colorimetric method (CREA plus, Roche Diagnostics) using an automated clinical chemistry analyser (Modular P, Roche Diagnostics). Reference range was 50–90 $\mu\text{mol/l}$ for females and 60–100 $\mu\text{mol/l}$ for males. Serum phosphate was analysed by photometry using an automated clinical chemistry analyser (Modular P, Roche Diagnostics). Reference interval was 0.85–1.50 mmol/l for women aged ≥ 18 years, 0.75–1.65 mmol/l for men aged 18–49 years and 0.75–1.35 mmol/l for men aged ≥ 50 years. These analyses are approved by the Norwegian Accreditation Authority.

Serum 25(OH)D was analysed by the following six methods:

- i) LC–MS/MS (Tromsø): serum 25(OH)D₃ was analysed by mass spectrometry using an isotope dilution LC–MS/MS assay adapted from Maunsell *et al.* (23). Briefly, serum samples were denatured with methanol–propanol containing a stable isotope-labelled internal standard, and thereafter, they were extracted into hexane and dried under nitrogen. The reconstituted extract was chromatographed on an HPLC column, and the vitamin D metabolite and internal standard were detected by electrospray ionisation MS/MS (Quattro Premier, Waters, Manchester, UK). There was no cross-reactivity between vitamin 25(OH)D₂ and vitamin 25(OH)D₃ in the assay, and inter-assay CV was $\leq 6\%$. The analyses were performed at the Department of Medical Biochemistry, University Hospital of North Norway, Tromsø.
- ii) LC–MS/MS (Bergen): the isotope dilution tandem mass spectrometry method was developed at the Hormone Laboratory, Haukeland University Hospital, Bergen, Norway. In brief, serum was extracted with *n*-hexane:isopropanol, and injected into an isocratic HPLC system coupled with a API 3000 mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA, USA) equipped with an electrospray source. The within-day precision (CV) was $\leq 3.1\%$, and the between-day precision (CV) was $\leq 8.7\%$. There was no known interference from other substances, including no cross-reaction with vitamin 25(OH)D₂.
- iii) RIA (DiaSorin, Stillwater, MN, USA): serum levels of 25(OH)D were measured by RIA (DiaSorin) at the Hormone Laboratory, Department of Endocrinology, Oslo University Hospital, Norway. This assay measures both 25(OH)D₃ and 25(OH)D₂, and the total analytical CV was 14%.
- iv) HPLC: HPLC analyses were carried out using the manufacturer's reagent kit consisting of a mobile phase, calibrator, column, internal standard, and precipitation reagent and solid-phase extraction procedure (Chromsystems GmbH, 81243 München, Germany) at the Department of Clinical Chemistry, Akershus University Hospital, Lørenskog, Norway. Liquid chromatography was performed on a Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan). The CV for the assay was $\leq 9.5\%$.
- v) RIA (IDS): the RIA method from Immuno Diagnostic Systems Nordic AS (IDS, Herlev, Denmark) performed at the Hormone Laboratory, Haukeland University Hospital, Bergen, Norway, had an inter-assay precision (CV) $\leq 11.6\%$. 25(OH)D₂ had a 75% cross-reactivity with vitamin 25(OH)D₃.
- vi) ECLIA (Roche): see laboratory analyses under the Tromsø study.

Table 1 Baseline characteristics of the study population in the Tromsø study, 1994–1995. If not otherwise stated, values are shown as mean $\pm$ s.d. Differences in characteristics between categories were compared with χ^2 tests or ANOVA.

	All participants	Never smokers	Former smokers	Current smokers
Females/males (n)	4230/2702	1847/510* [‡]	1022/1258 [‡]	1361/934
Age as on December 31, 1994 (years)	59.0 $\pm$ 10.2	59.0 $\pm$ 10.9* [‡]	60.7 $\pm$ 9.0 [‡]	57.3 $\pm$ 10.2
BMI (kg/m ²)	26.0 $\pm$ 4.1	26.5 $\pm$ 4.3 [‡]	26.6 $\pm$ 3.7 [‡]	24.8 $\pm$ 3.8
Physical activity (h/week)	2.7 $\pm$ 1.6	2.7 $\pm$ 1.5 ^{‡,‡}	2.8 $\pm$ 1.6 [‡]	2.5 $\pm$ 1.6
Vitamin D supplements (%)	43	46 [‡]	45 [‡]	39
Serum 25(OH)D (nmol/l)	58.9 $\pm$ 20.2	50.9 $\pm$ 15.9* [‡]	53.5 $\pm$ 17.3 [‡]	72.4 $\pm$ 20.0
Serum 25(OH)D (nmol/l), adjusted ^a		51.2 $\pm$ 17.0* [‡]	53.6 $\pm$ 17.0 [‡]	72.0 $\pm$ 16.9
Serum calcium (mmol/l)	2.37 $\pm$ 0.11	2.37 $\pm$ 0.11	2.37 $\pm$ 0.11 [§]	2.38 $\pm$ 0.11
Serum PTH measured (n)	3180	1067	1073	1040
Serum PTH (pmol/l) (median (2.5, 97.5 percentile range))	2.6 (0.9, 6.2)	2.7 (1.0, 6.6) [‡]	2.8 (1.0, 6.3) [‡]	2.3 (0.8, 5.3)

BMI, body mass index; 25(OH)D, 25-hydroxyvitamin D; PTH, parathyroid hormone. * $P < 0.01$, [‡] $P < 0.05$ compared with former smokers; [‡] $P < 0.01$, [§] $P < 0.05$ compared with current smokers.

^aAdjusted for age, sex, month of blood sampling, vitamin D supplementation (yes/no), BMI and physical activity level.

Statistical analyses

The Tromsø study Distribution of the variables was evaluated by visual inspection of histograms for skewness and kurtosis, and after log-transforming PTH, the distributions of all dependent variables were considered normal. Differences between means of continuous variables of current, former and never smokers were assessed by ANOVA with the Bonferroni correction, while differences in the distribution of categorical variables were assessed by χ^2 tests. Since age, sex, BMI, vitamin D supplementation and physical activity differed between the groups and correlated with

serum 25(OH)D levels (data not shown), we estimated adjusted means of serum 25(OH)D for each month of blood sampling using a general linear model with age, sex, BMI, physical activity level and vitamin D supplementation as covariates. Owing to the general vacation in Norway in July, data from this month were not obtained, and a mean value for this month was estimated by adding the mean levels for June and August divided by 2 for use in the figures. The variables 'years of smoking', 'number of cigarettes' and 'years since smoking cessation' were grouped into categories, and linear trends in 25(OH)D levels were assessed using a general linear model including the same covariates as

Table 2 Serum 25(OH)D levels in relation to reported years of smoking and number of cigarettes smoked and years since smoking cessation in former and current smokers in the Tromsø study, 1994–1995.

	Former smokers			Current smokers		
	n ^a	Serum 25(OH)D (nmol/l) ^b	95% CI	n ^a	Serum 25(OH)D (nmol/l) ^b	95% CI
Years of smoking						
1–10	503	52.1	50.7–53.6	77	59.9	55.5–64.3
11–20	686	53.7	52.6–54.9	269	68.7	66.0–71.3
21–30	548	53.6	52.3–54.9	517	71.1	69.5–72.8
31–40	347	55.3	53.6–57.0	784	73.9	72.6–75.2
> 40	173	53.8	51.3–56.2	640	74.8	73.0–76.6
P for trend ^c		0.11			< 0.01	
Number of cigarettes/day						
1–5	431	53.9	52.4–55.5	296	67.5	65.4–69.7
6–10	863	53.1	52.0–54.1	942	72.3	71.0–73.5
11–20	678	54.0	52.8–55.2	860	74.2	72.9–75.5
> 20	177	53.4	51.0–55.8	103	78.5	74.8–82.3
P for trend ^c		0.64			< 0.01	
Years since smoking cessation						
1–2	246	55.5	53.5–57.5			
3–5	238	54.9	52.9–56.9			
> 5	1796	53.1	52.3–53.8			
P for trend ^c		0.03				

BMI, body mass index; CI, confidence interval; 25(OH)D, 25-hydroxyvitamin D.

^aNumbers may not sum to the same total in the corresponding columns due to missing data on smoke exposure.

^bValues are means adjusted for age, sex, month of blood sampling, vitamin D supplementation (yes/no), BMI and physical activity level.

^cTests for trends across categories were performed using a general linear model with adjustment for the same factors as mentioned above.

Figure 1 Serum 25(OH)D levels throughout the year in 2357 never smokers, 2280 former smokers and 2295 current smokers in the Tromsø study, 1994–1995. The values are shown as means and 95% confidence intervals adjusted for age, sex, vitamin D supplementation (yes/no), physical activity level and body mass index. *Values for the month of July are estimated based on the means of June and August.

mentioned above. All tests were done two-sided, and $P < 0.05$ was considered statistically significant. Statistical analyses were performed with SPSS version 14.0 (SPSS Inc., Chicago, IL, USA).

The validation study Background characteristics of smokers and non-smokers were compared by Student’s t -test or χ^2 tests. Correlation coefficients, simple scatter plots and Bland–Altman plots were used to compare the methods. A linear mixed model was used to assess the effect of smoking between laboratory methods for 25(OH)D. Smoking (yes/no) and indicator variables of each laboratory method and an interaction term between smoking and each indicator variable of

laboratory method comprised the fixed effects part of the model, whereas a random effect for each subject and a residual term for within-subject variation comprised the random part of the model. All tests were done two-sided, and $P < 0.05$ was considered statistically significant. Statistical analyses were performed with SPSS version 16.0 (SPSS Inc).

Results

The Tromsø study

Complete data sets were available in 4230 women and 2702 men. Characteristics of the whole group and those according to smoking status are given in Table 1. The main finding was the substantially higher serum 25(OH)D levels in current smokers than in never smokers, with former smokers exhibiting slightly higher levels than never smokers (Table 1). The differences in the serum 25(OH)D levels between never, former and current smokers remained almost unchanged after correction for the confounders age, sex, physical activity level, vitamin D supplementation, month of blood sampling and BMI (Table 1). The serum levels of 25(OH)D increased with reported years of smoking and number of cigarettes smoked per day in current smokers, but not in former smokers (Table 2). However, there was a minor decrease in serum 25(OH)D levels with increasing years since smoking cessation in former smokers (Table 2). Separate analyses of the small groups of exclusively pipe smokers ($n = 46$) and cigar/cigarillo ($n = 19$) smokers showed higher serum 25(OH)D levels compared with never smokers (70.7 and 85.2 vs 50.9 nmol/l, $P < 0.001$ for both comparisons). Current, former and never smokers showed similar seasonality in

Table 3 Baseline characteristics of the study population in the validation study, 2009. If not otherwise stated, values are shown as mean $\pm$ s.d. Differences in characteristics between smokers and non-smokers were compared with Student’s t -tests or χ^2 -tests.

	All	Non-smokers	Smokers
Females/males (n)	63/44	33/20	30/24
Age (years)	55.9 $\pm$ 12.4	56.9 $\pm$ 13.0	54.9 $\pm$ 11.8
BMI (kg/m ²) ^a	25.9 $\pm$ 3.8	25.8 $\pm$ 3.9	26.0 $\pm$ 3.7
Sunny holiday during the last 3 months (yes/no)	13/94	5/48	8/46
Use of solarium during the last 14 days (yes/no)	5/102	2/51	3/51
Use of solarium during the last 3 months (yes/no)	14/93	5/48	9/45
Hours spent outdoors in the last 7 days	13.7 $\pm$ 10.0	12.7 $\pm$ 8.6	14.6 $\pm$ 11.3
Fatty fish ≥ 1 /week (yes/no)	55/52	25/28	30/24
Semi-skimmed milk ^b ≥ 1 /day (yes/no) ^a	30/76	19/34	11/42
Daily vitamin D supplementation (yes/no)	39/68	23/30	16/38
Serum calcium (mmol/l)	2.35 $\pm$ 0.09	2.35 $\pm$ 0.12	2.35 $\pm$ 0.07
Serum phosphate (mmol/l)	1.17 $\pm$ 0.19	1.20 $\pm$ 0.18	1.15 $\pm$ 0.20
Serum creatinine (μ mol/l)	69.9 $\pm$ 12.8	71.3 $\pm$ 13.3	68.4 $\pm$ 12.2
Plasma PTH (pmol/l)	5.4 $\pm$ 1.3	5.6 $\pm$ 1.2	5.2 $\pm$ 1.4
Years of smoking			33.0 $\pm$ 13.5
Number of cigarettes/day			12.1 $\pm$ 4.7
Number of cigarettes/examination day			5.4 $\pm$ 3.0

BMI, body mass index; PTH, parathyroid hormone.
^aData available in 106 participants.
^bVitamin D-fortified milk.

Table 4 Serum 25-hydroxyvitamin D measurement with different methods in the validation study, 2009.

	Serum 25(OH)D (nmol/l) (mean $\pm$ s.d.)			Difference in serum 25(OH)D (nmol/l), mean (95% CI); smokers versus non-smokers
	All	Non-smokers	Smokers	
<i>n</i>	107	53	54	
LC-MS/MS (Tromsø)	55.6 $\pm$ 22.7	56.8 $\pm$ 23.0	54.5 $\pm$ 22.4	-2.3 (-11.0, 6.4) [†]
LC-MS/MS (Bergen)	65.0 $\pm$ 26.0	66.1 $\pm$ 26.3	64.0 $\pm$ 25.9	-2.2 (-12.2, 7.8) [†]
RIA (DiaSorin)	58.2 $\pm$ 21.1	59.6 $\pm$ 20.2	56.8 $\pm$ 22.0	-2.8 (-10.9, 5.3) [†]
HPLC	65.2 $\pm$ 25.5	67.2 $\pm$ 24.6	63.2 $\pm$ 26.4	-4.0 (-13.8, 5.7) [†]
RIA (IDS)	62.6 $\pm$ 20.2	64.0 $\pm$ 19.1	61.1 $\pm$ 21.2	-2.9 (-10.6, 4.9) [†]
ECLIA (Roche)	61.4 $\pm$ 16.5	56.2 $\pm$ 14.2	66.5 $\pm$ 17.2	10.3 (4.2, 16.3)*

Difference in serum 25-hydroxyvitamin D between smokers and non-smokers within each method was assessed by Student's *t*-test, while difference between methods was assessed using a linear mixed model with method and smoking status as factors. *Difference in smokers versus non-smokers, $P=0.001$. [†]Difference between methods, ECLIA (Roche) as reference, $P<0.001$.

25(OH)D levels, and the mean difference of about 3 nmol/l between former and never smokers and about 21 nmol/l between current and never smokers remained stable throughout the year (Fig. 1). The greatest peak was observed in September, and the nadir was observed in March for all groups. When males and females were analysed separately, the results were essentially the same (data not shown).

The validation study

After exclusion of one subject who had quit smoking 3 months before examination and therefore did not fulfil the inclusion criteria, we had available data in 107 subjects: 54 smokers and 53 non-smokers. As presented in Table 3, background characteristics did not differ between smokers and non-smokers. Although not statistically significant, five of the methods reported lower serum 25(OH)D levels in smokers than in non-smokers (Table 4). However, using the ECLIA (Roche) method, smokers had significantly higher serum 25(OH)D levels than non-smokers, and this method differed significantly from the other methods regarding the effects of smoking (Table 4). The correlations between the different methods were generally very high (Table 5), although the correlations between the ECLIA (Roche) and the other methods were somewhat lower (Table 5). When analysing

non-smokers separately, the correlation between the ECLIA (Roche) and the other methods improved (Table 6), without reaching the same level of agreement as that observed between other methods. The same was observed when analysing smokers separately (data not shown). To illustrate a typical relationship between two methods, a simple scatter plot and a Bland-Altman plot are shown in Figs 2 and 3, while similar plots including the ECLIA (Roche) method are shown in Figs 4 and 5. The other plots are available on request. In smokers, time since smoking of last cigarette varied from 4 to 370 min with a median of 45 min. The correlation between time since smoking of last cigarette in smokers and serum 25(OH)D concentration was non-significant (Pearson's correlation coefficient: -0.07 , $P=0.60$).

Discussion

We have performed one population-based study and one validation study, which together indicate an impact of smoking on the results of serum 25(OH)D analyses using the ECLIA (Roche) method, revealing relatively higher levels of serum 25(OH)D in smokers than what are found with other methods presently in use. This assay has previously been compared with both immunological and mass spectrometric methods of 25(OH)D measurement, and a good overall agreement

Table 5 Pearson's correlation coefficient between the different methods for serum 25-hydroxyvitamin D measurements in 107 subjects (53 non-smokers and 54 smokers) in the validation study, 2009.

	LC-MS/MS (Tromsø)	LC-MS/MS (Bergen)	RIA (DiaSorin)	HPLC	RIA (IDS)	ECLIA (Roche)
LC-MS/MS (Tromsø)	1					
LC-MS/MS (Bergen)	0.98*	1				
RIA (DiaSorin)	0.94*	0.96*	1			
HPLC	0.94*	0.94*	0.96*	1		
RIA (IDS)	0.93*	0.94*	0.95*	0.92*	1	
ECLIA (Roche)	0.75*	0.77*	0.78*	0.75*	0.76*	1

* $P<0.01$.

Table 6 Pearson's correlation coefficient between the different methods for serum 25-hydroxyvitamin D measurements in 53 non-smokers in the validation study, 2009.

	LC-MS/MS (Tromsø)	LC-MS/MS (Bergen)	RIA (DiaSorin)	HPLC	RIA (IDS)	ECLIA (Roche)
LC-MS/MS (Tromsø)	1					
LC-MS/MS (Bergen)	0.99*	1				
RIA (DiaSorin)	0.93*	0.94*	1			
HPLC	0.95*	0.96*	0.98*	1		
RIA (IDS)	0.94*	0.95*	0.95*	0.95*	1	
ECLIA (Roche)	0.81*	0.80*	0.83*	0.81*	0.83*	1

* $P < 0.01$.

has been reported (24–26). However, the authors did not report data regarding smoking, and to our knowledge, this has not been studied previously using the ECLIA (Roche) method. If confirmed, this finding has clinically relevant consequences for many smoking patients who might have missed the diagnosis of easily treatable hypovitaminosis D. This is particularly unfortunate as smokers also have a higher risk of osteoporotic fractures (27), which might be prevented by the correction of hypovitaminosis D.

The data obtained from the Tromsø study were consistent in showing a higher serum 25(OH)D level throughout the year in current smokers than in never smokers using the ECLIA (Roche) method. The results also indicated a dose–response relationship as former smokers were close to, but still had higher serum 25(OH)D concentrations, than never smokers. In addition, the serum levels of 25(OH)D increased with increasing years of smoking and with increasing number of cigarettes smoked per day, while serum 25(OH)D decreased with increasing time since smoking cessation. Other studies registering data on smoking and serum levels of 25(OH)D report either lower levels (the difference ranging from 4 to 15 nmol/l) of 25(OH)D in smokers (3–10), or no statistically significant difference between smokers and non-smokers regarding serum 25(OH)D levels (11–20). The only exception we are aware of is an American study of 805 women aged 18–33 years, where smokers had higher serum 25(OH)D levels than non-smokers (58.43 nmol/l compared with 48.99 nmol/l, $P < 0.0001$) (21). However, following multiple adjustments, smoking did not affect the serum levels of 25(OH)D (21). All these studies used various forms of immunometric methods, although not the ECLIA (Roche) method (3–21).

The highest serum 25(OH)D levels were observed at the end of summer, and the difference between the peak value in September and the nadir value in March reached 14.2 nmol/l in current smokers, 17.0 nmol/l in former smokers and 15.3 nmol/l in never smokers. These differences between peak and nadir values are in accordance with studies in more sunny countries (11, 14, 28, 29); furthermore, it is consistent with the location of Tromsø at 69.7°N, where the period of the year with UVB radiation below the threshold for

vitamin D production in the skin is from the end of September till the middle of March (30).

In the validation study, we confirmed the finding that smokers had higher serum 25(OH)D levels than non-smokers when the levels were measured with the ECLIA (Roche) method, and that the ECLIA (Roche) method differed significantly from the other five methods in this aspect, as they all showed lower (although not significant) serum levels of 25(OH)D in smokers. Although the difference between smokers and non-smokers using the ECLIA (Roche) was not of the same magnitude in the validation study as in the Tromsø study, the difference still comprised more than 10 nmol/l. We do not know why these results differed, but the validation study was much smaller ($n = 107$ vs 6932), and the cohort was younger than that in the Tromsø study (55.9 vs 59.0 years). In addition, the current smokers in the Tromsø study had lower BMI than never smokers (24.1 vs 26.5 kg/m²), while the

Figure 2 Scatter plot between RIA (DiaSorin) assay for serum 25(OH)D and LC-MS/MS (Bergen) by smoking status in the validation study, 2009.

Figure 3 Bland–Altman plot where the x-axis indicates the mean of serum 25(OH)D measured with RIA (DiaSorin) and LC–MS/MS (Bergen) in 54 individual smokers and 53 individual non-smokers and the y-axis indicates the difference between the corresponding individual measurements in the validation study, 2009. Dotted line represents the line of identity. The solid line represents the mean of paired differences. Dashed lines represent mean $\pm$ 1.96 s.d.

BMI was similar among smokers and non-smokers in the validation study (26.0 vs 25.8 kg/m²).

The dose dependency between smoking and serum 25(OH)D levels found in the Tromsø study suggests the presence of one or more substances that are dose dependently associated with smoking, which interferes with the separation agent, antibodies or vitamin D-binding protein in the ECLIA (Roche) assay, and thereby modulates the measurement of serum 25(OH)D. Cigarette smoke consists of more than 4000 different substances (31). In addition, cigarette smoking affects concentrations of other compounds in the body. For instance, both PTH (6, 12, 32) and TSH (33) have been reported to be lower in smokers, while oestrone and oestradiol levels appear to be higher in male smokers (34). Accordingly, both direct effects from compounds in smoke and indirect effects through changes in the concentrations of other physiological substances may be responsible for the overestimation. This phenomenon may also be valid in immunoassays in use for other analytes. Accordingly, we suggest that special attention be paid to the possible effects of smoking when current and new immunoassays are validated. However, as the present studies do not give any molecular explanation for this observation, this issue needs further exploration.

On the other hand, it could be that the ECLIA (Roche) method displays the most accurate serum 25(OH)D values, and that the problem is that the other methods

underestimate serum 25(OH)D in smokers. If so, this could explain why plasma PTH levels are reported to be lower in smokers (6, 12, 32), and it would also be consistent with the fact that moderate smokers are found to have lower BMI than non-smokers (35), as BMI and serum 25(OH)D levels are inversely correlated in most studies (36). However, in the validation study, there was no difference in BMI between smokers and non-smokers, and neither were there any differences in reported intake of vitamin D or in sun-seeking behaviour that could explain higher serum 25(OH)D levels in smokers. It is also unlikely that the totally different assay principles, such as chromatography, immunometry and mass spectrometry, should express similar systematic errors. Accordingly, we find it very unlikely that the ECLIA (Roche) method reveals the most accurate values in the smokers.

The high correlations between the assays were in agreement with previous findings (24, 25, 37), but still, regardless of smoking, there were differences between the methods as illustrated by mean values and Bland–Altman plots. This is a well-known problem that might be attenuated by using internationally agreed standard calibrators to improve comparability between methods (37, 38).

The main strength of this study was the replication of the main finding in two different samples and study designs. Other strengths were the size and the year-round measurements in the Tromsø study and the thoroughness of the validation study where great efforts were made to avoid differences in sample handling

Figure 4 Scatter plot between ECLIA (Roche) assay for serum 25(OH)D and LC–MS/MS (Bergen) by smoking status in the validation study, 2009.

Figure 5 Bland–Altman plot where the x-axis indicates the mean of serum 25(OH)D measured with ECLIA (Roche) and LC–MS/MS (Bergen) in 54 individual smokers and 53 individual non-smokers and the y-axis indicates the difference between the corresponding individual measurements in the validation study, 2009. Dotted line represents the line of identity. The solid line represents the mean of paired differences. Dashed lines represent mean $\pm$ 1.96 s.d.

before laboratory analyses. The main weakness is that the ECLIA (Roche) was performed in the same laboratory in both studies. It is, however, unlikely that the problem should be laboratory specific, as the ECLIA (Roche) is a fully automated method which diminishes the risk of user errors. Although the ECLIA (Roche) assay does not recognise serum 25(OH)D₂, this is not a practical problem in these two studies, as all vitamin D fortification and over-the-counter supplementation in Norway consist of cholecalciferol. In addition, no 25(OH)D₂ was observed by LC–MS/MS. Thus, differences in method specificities could not explain our findings. Another limitation of the study is that we cannot differentiate between acute and chronic effects of cigarette smoking on the serum 25(OH)D measurements, as the smokers were allowed to smoke before blood sampling. However, there was no correlation between time since smoking of last cigarette and serum 25(OH)D concentration as measured by ECLIA (Roche) in smokers, and also the impact of smoking on serum 25(OH)D concentration in former smokers in the Tromsø study makes it more likely that the effect is chronic rather than acute.

In conclusion, the results from these two studies indicate an impact of smoking on the measured serum levels of 25(OH) using the ECLIA (Roche) method compared with other methods in use. If confirmed, this might have considerable clinical consequences.

The mechanisms behind the finding need to be explored, and possible effects of smoking should also be considered in other immunoassays in light of these findings.

Declaration of interest

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