The impact of Vitamin D status on implantation and clinical pregnancy rates following *in vitro* fertilization

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Introduction

Infertility is a common problem affecting 15% of North American couples. Recent studies support a role for vitamin D in human reproduction, and have suggested that follicular fluid vitamin D levels predict reproductive success following *in vitro* fertilization (IVF) (1,2).

Vitamin D is a prohormone, acquired exogenously from the diet or produced endogenously in the skin. Vitamin D is primarily metabolized in the liver to 25 OH-D, serum concentrations of which are used as an indicator of vitamin D status. Vitamin D has a known role in calcium-phosphate homeostasis and bone mineralization (3). Recently, vitamin D has been linked to a variety of disease processes, such as autoimmunity, malignancy, and infertility (1,3,4). Classifications of vitamin D status vary in the literature (1,3,4). A recent Canadian guideline defined vitamin D deficiency (<25 nmol/L), insufficiency (25-74 nmol/L), and sufficiency (≥75 nmol/L) (5). The benefits of vitamin D for nontraditional roles, such as malignancies, impaired immune response, and fertility have been associated with 25 OH-D levels ≥75 nmol/L (1,3,4).

People living in countries at higher latitudes, such as the US and Canada, are more prone to vitamin D insufficiency, especially during winter months (6). Vitamin D insufficiency is highly prevalent in reproductive age women (1,6). A Canadian study by Veith *et al.*, reported 25.6% of non-white and 14.8% of white reproductive age (18-35 y) women were vitamin D insufficient (<40 nmol/L). The Ozkan study investigating a reproductive age infertility population, found a slightly higher prevalence of vitamin D insufficiency (50-74 nmol/L) of 36% and deficiency (<50 nmol/L) of 27% (1). Anifandis *et al.*, reported a higher prevalence of 79% vitamin D insufficiency/deficiency in their reproductive age infertility population (7).

The link between vitamin D and reproduction has been largely demonstrated in murine models (8,9,10,11). In an early study by Halloran and Deluca, female rats were fed vitamin D sufficient or vitamin D deficient diets and then mated. They found that vitamin D deficiency reduced overall fertility by 75% and diminished litter sizes by 30% (8). These results were supported by studies by Hickey *et al.,* in which female rats fed vitamin D deplete diets prior to mating had significantly smaller litter sizes (9).

Further evidence supporting the association between vitamin D and reproduction comes from studies on the vitamin D receptor (VDR) (11,12,13). VDRs have been found in various reproductive tissues including, the ovary and uterus (11,12,14). Studies by Yoshizawa *et al,* observed normal growth and development in VDR-deficient mice before weaning (analogous to human puberty); however, after weaning they failed to thrive and exhibited infertility (11). Vitamin D has also been shown to regulate HOX gene expression in the uterus (12,13). Vitamin D and HOX genes, specifically transcription factors HOXA10/11, are hypothesized to function as part of endocrine signal transduction pathway, regulating endometrial development in preparation for implantation (12,13). Various reproductive tissues, including the ovaries, fallopian tubes, endometrial stromal and decidual cells, express the essential components of the vitamin D-VDR pathway and therefore are capable of responding to vitamin D signaling (12,13).

There are four studies in the literature looking at the impact of vitamin D deficiency on reproductive success following IVF in human, with conflicting results (1, 2, 7,15). The original study by Ozkan *et al,* was a prospective cohort study that measured the follicular fluid (FF) 25 OH-D levels in 84 women. They found that women with higher vitamin D levels in their FF were significantly more likely to

achieve implantation and clinical pregnancy following IVF. (1) A subsequent retrospective analysis of vitamin D levels in donor-recipient cycles by Rudick *et al.* also demonstrated that vitamin D insufficiency in donor-oocyte recipients was associated with lower clinical pregnancy rates (2). Conversely, a small prospective study by Aleyasin *et al.* did not show a significant difference in biochemical or clinical pregnancy rates across tertiles of FF 25 OH-D (p=0.959, 0.995 and 0.604, respectively) (15). Lastly, a prospective cohort study by Anifandis *et al.* suggested that excess follicular fluid 25 OH-D levels in combination with decreased follicular fluid glucose levels may have a negative impact on IVF success. Specifically, the clinical pregnancy rates in the deficient, insufficient and sufficient groups were 32.3%, 32.7%, and 14.5%, respectively (p=0.047) (7).

The goal of the proposed study is to determine whether serum 25 OH-D levels in infertile women are predictive of in IVF outcomes. The main objective was to investigate whether vitamin D deficiency in our infertile population is associated with lower implantation and lower clinical pregnancy rates after IVF.

MATERIALS AND METHODS

This study was conducted at an academic hospital-based fertility centre. Women undergoing IVF for any indication were eligible for the study. The inclusion criteria were; age ≥ 18, age ≤ 41, baseline day 3 FSH ≤ 12 and ability to provide informed consent. The exclusion criteria were 3rd party reproduction cycles, known uncorrected congenital or acquired uterine anomalies and inability to provide informed consent. One hundred and seventy-three patients meeting our inclusion criteria were recruited at Mount Sinai Hospital between April 2011 and November 2011. The study was approved by our institution's Research Ethics Board and informed written consent was obtained from all participants.

Recruited patients underwent IVF cycles per standard clinical care. Standard agonist and antagonist IVF protocols were employed. Agonist protocols used Buserelin Acetate (Suprefact; Sanofi Aventis, Canada) starting in midluteal phase at a dose of 0.5 mg/day SC for long protocols or at start of the cycle for microdose flare protocols. Antagonist cycles used Cetrolix acetate (Cetrotide; EMD Serono, Canada) or Ganirelix acetate (Merk, Canada), started as per flexible start protocol, when estradiol (E_2) levels were ≥2000 nmol/L or dominant follicle size was ≥14mm. Recombinant or urine purified FSH, with or without LH or HMG, was used for controlled ovarian hyperstimulation; starting doses were determined prior to study recruitment based on age, early follicular phase FSH, and number of antral follicles. Individualized step-down or step-up protocols were instituted when clinically indicated. Ovarian response was assessed by serial transvaginal ultrasound and serum LH and E_2 assays. Serum 25 OH-D samples were collected prior to oocyte retrieval. Nuclear maturation was triggered with 10,000 IU of hCG (Merk, Canada) when 3 or more dominant follicles ≥17 mm were achieved. Transvaginal ultrasound guided oocyte retrieval was performed 36-38 hours following hCG injection. Ultrasound-guided fresh embryo transfer was performed on day 3 or 5 post fertilization. Vaginal micronized progesterone suppositories (Abbott, Canada) were used for luteal phase support and were continued until documentation of fetal cardiac activity. Clinical pregnancy (CP) was defined as an intrauterine sac visible on ultrasound.

Patient's demographic data and IVF cycle data was obtained via chart review. Primary outcome was clinical pregnancy rate per cycle start in vitamin D deficient versus vitamin D sufficient women. Secondary outcomes included the prevalence of vitamin D deficiency in this infertility population and the effect of vitamin D on IVF cycle parameters including number of oocytes and implantation rate.

Statistical Analysis

Patients were divided into 2 groups based on 25 OH-D levels: Sufficient (≥75 nmol/L), insufficient (<75 nmol/L). Continuous variables were reported as mean +/- SD and categorical variables were reported as a percentage (%). Chi-square analysis and Students t test or Mann-Whitney U as appropriate were used to analyze categorical and continuous variables, respectively. Serum 25 OH-D tertiles were computed (Low 46.07 +/- 11.9, Middle 70.91 +/- 6.2, Highest 101.07 +/- 17.0). Multivariable logistic regression analysis was used to evaluate the relationship between serum 25 OH-D and implantation and clinical pregnancy after adjusting for parameters known to influence success of an IVF cycle (age, BMI, day 5 ET). Sample size (n=170) was calculated using a power of 0.8, a level of significance of 0.05, and a 95% confidence interval. Clinical pregnancy rates are expected to be 40% in vitamin D sufficient patients and 20% in vitamin D insufficient and deficient patients, as determined by our institutions clinical pregnancy rates and results from clinical pregnancy rates in vitamin D-IVF populations (1). All data analyses were performed using IBM SPSS Statistics version 19.0.

Results

One hundred and eighty two patients were recruited. One hundred and seventy three patients were included in the analysis. Nine patients did not meet our inclusion criteria and were excluded from the study. Four were > 41 years old and five did not start an IVF cycle and were excluded from the study. All 173 patients included in the study underwent oocyte retrieval and 162 had an embryo transfer (ET). Of the 11 patients that didn't have an ET, 4 patients did not have oocytes or embryo of sufficient quality and 7 had their embryos frozen secondary to ovarian hyperstimulation syndrome (OHSS) (n=3), fertility preservation (n=1), hydrosalpinx (n=1), no sperm available (n=1), and inappropriate endometrial lining (n=1). There was no trend observed in the distribution of these numbers between insufficient/deficient and sufficient groups.

The prevalence of vitamin D deficiency, insufficiency, and sufficiency was 1.2%, 53.8%, and 45.1% respectively. Patients were divided into two groups for analysis based on 25 OH-D levels: sufficient (≥75 nmol/L, ≥30 ng/L) and insufficient/deficient (<75 nmol/L, <30 ng/L). The Vitamin D deficient and insufficient groups were combined due to the small participant number in the deficient group (n=2).

Patient characteristics were compared between serum 25-OH D groups (Table 1). BMI (kg/m^2) was significantly higher in the vitamin D insufficient/deficient group (24.83 kg/m^2+/- 4.65) in comparison to the sufficient group (23.32 kg/m^2 +/- 3.80, p=0.023). The remaining patient characteristics did not differ significantly between groups. Table 2 shows the IVF cycle parameters for the two groups. The sufficient group had significantly more day 5 embryo transfers (71.8%) in comparison to the insufficient/deficient group (57.9%, p=0.04). There was no difference in number of oocytes retrieved, % of cycle with intracytoplasmic sperm injection (ICSI) and number of embryos implanted.

The main outcomes measures were implantation rate and clinical pregnancy rate (per cycle start and per ET) (Table 2). A significant increase in clinical pregnancy rate per cycle start was seen in the sufficient (52.5%) group in comparison to the insufficient/deficient group (34.7%, p < 0.0001). Similarly, a significant increase in clinical pregnancy rate per ET was seen in the sufficient group (54.7% vs. 37.9%, p < 0.0001). Although implantation rate was higher in the sufficient group in comparison to the insufficient/deficient group (34.5% vs. 25.6%), this was not statistically significant (p=0.62). Increasing clinical pregnancy rates were observed across serum 25-OH D tertiles (Low 46.07 +/- 11.9, Middle 70.91 +/- 6.2, Highest 101.07 +/- 17.0, p<0.0001) (Figure 1). Multivariable logistic regression analysis confirmed serum 25 OH-D levels as an independent predictor of success of an IVF cycle; adjusting for age, BMI, and day 5 ET (Table 3; p=0.05).

Interpretation

Vitamin D, as determined by serum 25 OH-D levels, was predictive of IVF outcomes in our cohort of infertile women. Infertile women with sufficient vitamin D levels had significantly higher clinical pregnancy rates following IVF. This finding is clinically significant and may hold potential therapeutic implications, as 54.9% of women in our study were vitamin D insufficient/deficient.

The prevalence of vitamin D deficiency (<25 nmol/L), insufficiency (25-74 nmol/L) and sufficiency (>75 nmol/L) in our population was 1.2%, 53.8%, and 45.1% respectively. These numbers are similar to previously aforementioned North American prevalence data in reproductive age women (6). The Ozkan study investigating a reproductive age infertility population, found a slightly higher prevalence of vitamin D insufficiency (50-74 nmol/L) of 36% and deficiency (<50 nmol/L) of 27% (1). Our study had a very low prevalence of vitamin D deficiency (<25nmol/L) of 1.2%. Explanations for this finding include: a difference in our deficiency definitions, the majority of our patients were taking prenatal vitamins (400 IU of vitamin D) and that our study did not extend over winter months. However, the percentage of insufficient/deficient vs. sufficient women in our study did not significantly differ in the spring, summer or fall months or with respect to race (Table 1).

BMI in the insufficient group (24.8 kg/m2 + 4.7) was significantly higher than in the sufficient group (23.3 kg/m2 +/- 3.8, p=0.02). This association is consistent with existing knowledge on vitamin D metabolism and has been reported in the literature (16). Specifically, Vitamin D is a fat soluble vitamin and adipose tissue is hypothesized to act as a reservoir for its storage, reducing its bioavailability (16). Lagunova *et al.* found a significant decrease in serum 25 OH-D levels in women with increasing BMI. The prevalence of vitamin D deficiency (≤50 nmol/L) was highest in individuals with BMI ≥40.

The mechanism by which vitamin D impacts fertility is unclear. Postulated mechanisms include its effect on ovarian steroidogenesis and implantation (1,2,7,12,13). No significant difference was seen between sufficient and insufficient/deficient groups with respect to IVF cycle parameters including: day of HCG, gonadotropin dose, peak E_2, endometrial thickness, oocytes number, or number of embryos transferred. Therefore, it is unlikely that the difference observed in clinical pregnancy rates was to due a difference in ovarian steroidogenesis between groups. The implantation rate was higher in the sufficient group, but not statistically significant ($p=0.62$), however, the clinical pregnancy rate per embryo transfer was ($p<0.0001$). A possible explanation may be that we lacked power to detect a difference in implantation rate. We did, however, observe that women with sufficient vitamin D status were significantly more likely to have a day 5 ET. At our institution, the decision between a day 3 and day 5 ET is made on the basis of embryo quality. Therefore, the difference in clinical pregnancy rates between insufficient/deficient groups may be related to improved embryo quality in the sufficient group. These results are in contrast to Anifandis *et al.,* which found higher clinical pregnancy rate in their deficient/insufficient group, suggesting that excess follicular fluid vitamin D may negatively impact IVF success. Their mean score of embryo quality (MSEQ), but not their Cumulative Embryo Score (CES) was significantly lower the vitamin D sufficient groups in comparison to the insufficient/deficient groups ($p<0.05$). (7) Our prospective study is larger than the previously cited studies on Vitamin D and IVF outcomes and focuses on serum (not follicular) vitamin D levels and IVF outcomes.

Although, BMI and number of day 5 ET was significantly different between vitamin D groups, only serum 25 OH-D levels proved to be an independent predictor of IVF success after multivariant logistic regression analysis. Therefore, future studies should focus on determining the mechanism by which vitamin D impacts clinical pregnancy, including an emphasis on measures of embryo quality,

implantation and uterine receptively. Studies should also be undertaken to investigate whether vitamin D supplementation can improve pregnancy rates following IVF.

Our findings suggest that women with sufficient vitamin D levels are significantly more likely to achieve clinical pregnancy following IVF. Therefore, vitamin D supplementation could provide an easy and cost effective means of improving pregnancy rates and merits further investigation. There was a high prevalence of vitamin D insufficiency/deficiency in our infertility population. Therefore, there may be a benefit assessment of vitamin D status as part of routine infertility assessment and prior to ART treatment, especially in women with higher BMI.

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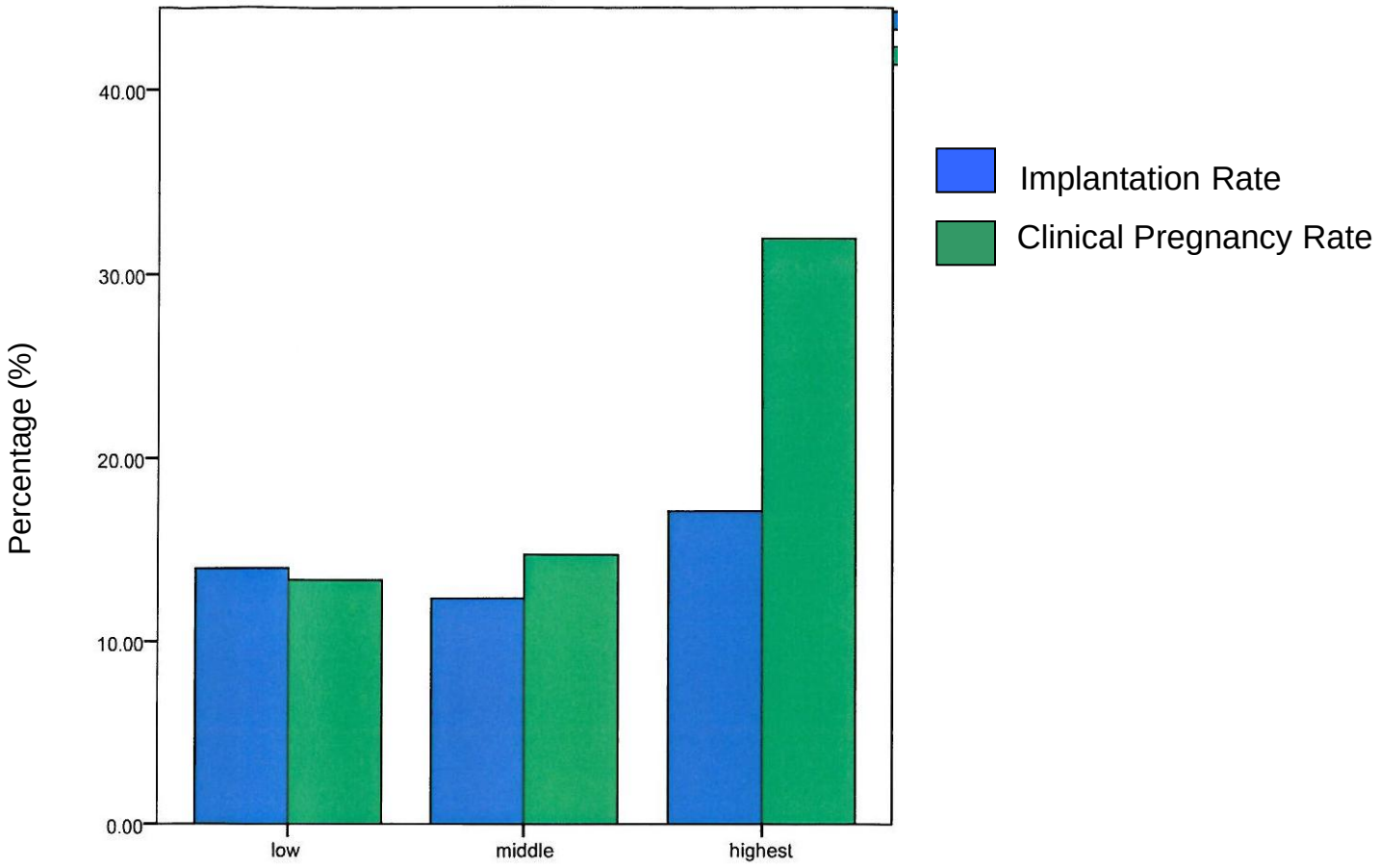
Figure Legend

Figure 1: Increasing clinical pregnancy rates in infertile women undergoing IVF across serum 25-OH D tertiles: Low 46.07 +/- 11.9, Middle 70.91 +/- 6.2, Highest 101.07 +/- 17.0 ($p<0.0001$).



Patient Characteristics	Insufficient/Deficient n=95 (54.9%)	Sufficient n=78 (45.1%)	P value
25 OH-D (nmol/L)	54.5 +/- 14.0	95.5 +/- 17.8	<0.0001*
Age (y)	34.6 +/- 4.0	34.4 +/- 3.9	0.75
Race			0.31
White	56 (58.9)	58 (74.4)	
Black	3 (3.2)	1 (1.3)	
Other	36 (37.9)	19 (24.4)	
BMI (kg/m^2)	24.8 +/- 4.7	23.3 +/- 3.8	0.02*
Gravidy (n)	0.8 +/- 1.1	0.9 +/- 1.3	0.60
Parity (n)	0.2 +/- 0.5	0.3 +/- 0.6	0.52
Duration of Infertility (mo)	20.7 +/- 15.1	17.5 +/- 12.3	0.14
Baseline FSH (mIU/ml)	6.4 +/- 1.9	6.8 +/- 2.3	0.93
Baseline AFC (n)	15.9 +/- 8.4	17.7 +/- 10.3	0.21
Previous IVF cycles (n)	0.7 +/- 1.0	0.6 +/- 1.0	0.41
IVF cycle season (%)			0.79
Spring	50 (52.6)	41 (52.6)	
Summer	31 (32.6)	28 (35.9)	
Fall	14 (14.7)	9 (11.5)	

Continuous data are presented as mean +/- standard deviation

* Statistically significant, P ≤0.05

IVF Cycle Characteristics	Insufficient/Deficient n=95 (54.9%)	Sufficient n=78 (45.1%)	P value
IVF Protocol (%)			0.08
Long	29 (30.5)	34 (43.6)	
Antagonist	64 (67.4)	40 (51.3)	
Flare	2 (2.1)	4 (5.1)	
Day of hCG	12.1 +/- 1.3	11.9 +/- 1.4	0.39
Gonadotropin dose (IU)	2602.8 +/- 1287.3	2547.1 +/- 1330.3	0.78
Follicles >1.4 cm (n)	11.9 +/- 6.5	11.7 +/- 5.8	0.84
E_2 day of hCG (nmol/L)	8556.7 +/- 6187.3	9776.15 +/- 5902.6	0.19
Endometrium day of hCG (cm)	1.0 +/- 1.6	1.0 +/- 0.2	0.54
Oocytes retrieved (n)	12.6 +/- 7.4	12.7 +/- 6.6	0.98
ICSI (%)	74 (77.9)	63 (80.8)	0.39
Embryos transferred (n)	1.8 +/- 1.0	1.8 +/- 0.9	0.93
Day of Transfer			
Day 3	31 (32.6)	19 (24.4)	0.15
Day 5	55 (57.9)	56 (71.8)	0.04*
Implantation rate (%)	25.6	34.5	0.62
Clinical pregnancy rate/cycle start (%)	34.7	52.5	p <0.0001*
Clinical pregnancy rate/ET (%)	37.9	54.7	p <0.0001*

Note continuous data are presented as mean +/- standard deviation

* Statistically significant, P ≤0.05

Clinical Pregnancy	OR (95% CI)	P value	Adjusted OR (95% CI)	P value
Age (y)	0.96 (0.89-1.04)	0.34	0.98 (0.90 -1.07)	0.71
BMI (kg/m^2)	0.92 (0.86-1.00)	0.05	0.94 (0.87-1.01)	0.09
Day 5 ET (n)	1.60 (0.85-3.04)	0.15	1.34 (0.64-2.81)	0.44
25 OH-D nmol/L	1.02 (1.00-1.03)	0.17	1.01 (1.00-1.03)	0.05*

* Statistically significant, P ≤0.05

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.