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A single dose of vitamin D enhances immunity to mycobacteria

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Impact of this research on clinical medicine and basic science:

Vitamin D was used to treat tuberculosis in the pre-antibiotic era, but studies to evaluate the effect of vitamin D supplementation on antimycobacterial immunity have not previously been performed. We report that a single oral dose of vitamin D enhanced TB contacts' immunity to mycobacteria in a randomized controlled trial. Our findings provide a rationale for vitamin D supplementation of tuberculosis contacts, and highlight the need for further trials to determine whether vitamin D supplementation prevents reactivation of latent tuberculosis infection.

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Abstract

Rationale

Vitamin D was used to treat tuberculosis in the pre-antibiotic era. Prospective studies to evaluate the effect of vitamin D supplementation on antimycobacterial immunity have not previously been performed.

Objectives

To determine the effect of vitamin D supplementation on antimycobacterial immunity and vitamin D status.

Methods

A double-blind randomized controlled trial was conducted in 192 healthy adult tuberculosis contacts in London, UK. Participants were randomized to receive a single oral dose of 2.5 mg vitamin D or placebo and followed up at 6 weeks.

Measurements and Main Results

The primary outcome measure was assessed with a functional whole blood assay (BCG-*lux* assay) that measures the ability of whole blood to restrict luminescence, and thus growth, of recombinant reporter mycobacteria *in vitro*; the read-out is expressed as a luminescence ratio (luminescence post-infection/baseline luminescence). Interferon-gamma responses to the *M. tuberculosis* antigens early secretory antigenic target-6 and culture filtrate protein 10 were determined with a second whole blood assay.

Vitamin D supplementation significantly enhanced the ability of participants' whole blood to restrict BCG-*lux* luminescence *in vitro* compared to placebo (mean luminescence ratio at follow-up 0.57 vs. 0.71 respectively, 95% CI for difference 0.01 to 0.25; P=0.03) but did not affect antigen-stimulated Interferon-gamma secretion.

Conclusions

A single oral dose of 2.5 mg vitamin D significantly enhanced the ability of participants' whole blood to restrict BCG-*lux* luminescence *in vitro* without affecting antigen-stimulated Interferon-gamma responses. Clinical trials should be performed to determine whether vitamin D supplementation prevents reactivation of latent tuberculosis infection.

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Introduction

Tuberculosis (TB) is a global emergency: in 2004 there were an estimated 8.9 million new cases and 1.7 million deaths due to the disease (1). One third of the global population has latent TB infection (LTBI) (2), providing a reservoir for future reactivation disease well into the present century. An understanding of the factors causing reactivation is therefore of considerable public health significance.

Clinical studies suggest that vitamin D enhances antimycobacterial immunity, and that deficiency is associated with susceptibility to active disease. High doses of vitamin D were widely used to treat active TB in the pre-antibiotic era (3); more recently, case control studies have demonstrated that vegetarian diet (low in vitamin D) is an independent risk factor for active TB in south Asians (4) and that TB patients of Gujarati Hindu ethnic origin have significantly higher rates of vitamin D deficiency than ethnically matched tuberculin positive TB contacts (5).

Vitamin D is synthesized in the skin during exposure to ultraviolet light and is also available in the diet, principally from oily fish. It is readily metabolized in the liver to form 25-hydroxy-vitamin D (25(OH)D), the accepted measure of vitamin D status (6). 25(OH)D is then further metabolized by the 1-alpha-hydroxylase enzyme Cyp27B1 to its biologically active metabolite, the steroid hormone 1-alpha, 25-hydroxyvitamin D ($1\alpha,25(OH)_2D$) (7).

$1\alpha,25(OH)_2D_3$ has no direct antimycobacterial action, but it does induce anti-tuberculous activity *in vitro* in both monocytes (8) and macrophages (9). Several mechanisms of action have been proposed. Exogenous $1\alpha,25(OH)_2D_3$ induces a superoxide burst (10) and enhances phagolysosome fusion (11) in *M. tuberculosis*-infected macrophages; both phenomena are mediated by phosphatidylinositol 3-kinase, suggesting that this response is initiated by binding membrane vitamin D receptor (VDR) (12). $1\alpha,25(OH)_2D_3$ also modulates immune responses by binding nuclear VDR, where it upregulates protective innate host responses including induction of nitric oxide synthase (13). Recently, 25(OH)D has also been shown to support messenger RNA induction of the antimicrobial peptide cathelicidin LL-37 which possesses antituberculous activity (14).

Randomized controlled trials evaluating the effect of vitamin D supplementation on antimycobacterial host response have yet to be performed. Placebo-controlled prospective studies in tuberculin positive individuals with the development of active TB as primary outcome measure would require very large sample sizes to detect clinically significant effects. We therefore adopted an alternative approach, conducting a double-blind randomized controlled trial of vitamin D supplementation in a large cohort of TB contacts in London, UK, using a surrogate primary outcome measure: the BCG-*lux* assay (15). In this assay, whole blood is cultured with a recombinant *M. bovis* Bacille Calmette Guérin (BCG) expressing luciferase, an enzyme that catalyzes the conversion of aldehyde substrate to produce light detectable in a luminometer. Because this reaction is ATP-dependent, light production relates to bacillary metabolic activity and colony forming units (CFU) (16). The ability of whole blood to suppress BCG-*lux* bioluminescence is determined at both 24 and 96 hours post-infection, in order to assay innate and acquired

components of host response, respectively. This model has yielded intuitive correlates of protection in several previous studies (15, 17, 18).

We combined BCG-*lux* analysis with a whole blood Interferon-gamma (IFN-γ) release assay (IFNGRA) that quantifies IFN-γ response to the *M. tuberculosis* antigens early secretory antigenic target-6 (ESAT-6) and culture filtrate protein 10 (CFP-10) (19). Antigen-stimulated IFN-γ production is an often-utilized correlate of protective immunity to *M. tuberculosis* infection (20), and this assay therefore served as a second indicator of the acquired host response. We also determined 25(OH)D concentrations in serum of all subjects at baseline and follow-up, and in BCG-*lux* assay supernatants of a randomly selected subgroup of participants.

Some of the results of this study have been previously reported in the form of an abstract (21).

Methods

Participants

Study participants were recruited from TB contact clinics at Newham and Northwick Park Hospitals, London, UK. All people over 17 years of age who had been exposed to a patient with active TB were assessed. Individuals were excluded if they had symptoms, clinical signs or radiographic evidence of active TB; HIV infection, renal failure, sarcoidosis or hyperparathyroidism; if they were taking corticosteroids, thiazide diuretics or

supplementary vitamin D (either alone or as part of a multivitamin preparation); or if they were breastfeeding or pregnant. Socio-demographic and dietary details were recorded on case report forms. Participants self-classified ethnicity into one of the following five categories (22): black African, south Asian, white, mixed or other. A blood sample was drawn for baseline assays, a tuberculin skin test was performed according to national guidelines (23) and a urine sample was collected from women of childbearing age for pregnancy test. TB contacts were reviewed at one week, and the grade of the tuberculin reaction was recorded. The study nurse then allocated a study ID number to eligible participants, and administered study medication from a container labeled with that number. These containers had previously been filled with either a single oral dose of 2.5 mg ergocalciferol or lactose placebo of identical appearance, according to a computer-generated sequence of random numbers in blocks of ten. Participants were reviewed at 6 weeks post-randomization, when a second blood sample was drawn for follow-up assays. The study research nurse and all participants and individuals performing laboratory assays were blinded to allocation. The study was approved by the Research Ethics Committees of North East London and Harrow (REC refs. P/02/146 and EC 2759 respectively), and written informed consent to participate was obtained from all participants.

Whole blood assays

The primary outcome measure was BCG-*lux* assay luminescence ratio. This assay has been described elsewhere (15). Briefly, *M. bovis*-BCG transformed with a replicating vector containing the luciferase (*lux*) gene of *Vibrio harveyi* was prepared as previously described (16). Frozen aliquots of BCG-*lux* bacilli were grown to mid-log phase in Middlebrook 7H9

broth supplemented with 10% Albumin Dextrose Catalase enrichment (BD, Franklin Lakes, NJ) and 15μg/ml hygromycin (Roche, Lewes, UK). Triplicate samples of 0.5 ml venous blood of each participant diluted with an equal volume of RPMI-1640/2 mM Glutamine/25 mM Hepes buffer (Sigma, Poole, UK) were infected with $3x10^5$ CFU of log-phase bacilli corresponding to a multiplicity of infection (mononuclear phagocyte:bacillus) of ~1:1 and incubated at 37ºC on a rocking platform. Mycobacterial luminescence was measured after harvesting of assay supernatants in triplicate samples at baseline, 24 and 96 hours, and a luminescence ratio calculated by division of the 24-hour or 96-hour luminescence value by the baseline value.

The IFNGRA used in this study has also been described elsewhere (19). Triplicate samples of venous blood diluted 1:10 with RPMI-1640 (Sigma, Poole, UK) were cultured with 2.5 μg/ml recombinant ESAT-6 (24), 5 μg/ml CFP-10 (Lionex, Braunschweig, Germany) or no stimulus at 37ºC in 5% CO_2. Supernatants were aspirated at 96 hours for determination of IFN-γ concentration by ELISA using an antibody pair from BD (assay sensitivity <10 pg/ml).

Determination of 25(OH)D concentration

Concentrations of $25(OH)D_2$ and $25(OH)D_3$ were determined by isotope-dilution liquid chromatography-tandem mass spectrometry (25) and summed to give values for total 25(OH)D. Assays were performed in a clinical biochemistry laboratory that participates in the International Vitamin D External Quality Assessment Program (http://www.deqas.org/). Positive standards of known concentration were run for both

$25(OH)D_2$ and $25(OH)D_3$. Total 25(OH)D was determined in serum of all participants and in BCG-*lux* assay supernatants of a randomly selected subgroup of 32 participants allocated to the intervention arm of the trial.

Statistical analysis

We calculated that a total of 198 participants would need to be recruited in order to detect a 20% difference in luminescence ratio between intervention and control groups with 80% power at the 5% significance level. Analysis of potential correlates of vitamin D deficiency was conducted using χ^2 tests for univariate analysis and binary logistic regression analysis for multivariate analysis. Effect of allocation on whole blood assay outcomes was evaluated using analysis of covariance. Mean concentrations of 25(OH)D pre- vs. post-supplementation were compared with paired t tests. We analyzed data using SPSS (version 12.0.1, 2003) and GraphPad Prism (version 4.03, 2005) software packages.

Results

We assessed 364 TB contacts for eligibility between December 16th 2002 and January 31st 2005; 39 were ineligible, and 133 declined randomization (Figure 1). The group of 133 TB contacts who declined randomization had similar sex ratio, age range and ethnic composition to the 192 participants were randomized to receive vitamin D or placebo. All received the intended treatment; 43 participants (22 in the placebo group and 21 in the intervention group) were lost to follow-up, and a further 18 (10 in the placebo group and 8

in the intervention group) were excluded from the analysis in the absence of a valid BCG-*lux* assay result at either baseline or follow-up. Analysis of primary outcome was conducted on the remaining 131 participants, of whom 64 received placebo and 67 received vitamin D.

Baseline characteristics of these 131 participants are compared in Table 1. Control and intervention groups did not differ significantly with respect to age, sex ratio, ethnic composition, BCG immunization rate, tuberculin reactivity, nature of TB exposure, vitamin D status, BCG-*lux* assay luminescence ratios or ESAT-6-stimulated IFN-γ concentration. The median CFP-10-stimulated IFN-γ concentration at baseline was higher in the placebo group than in the intervention group (median 40 pg/ml vs. 0 pg/ml, P=0.03).

At follow-up, mean 24-hour luminescence ratio was 20.4% lower for individuals allocated to vitamin D compared with those allocated to placebo (0.57 vs. 0.71 respectively, 95% CI for difference 0.01 to 0.25; P=0.03; Figure 2). Mean 96-hour luminescence ratio at follow-up was not significantly different for individuals allocated to vitamin D compared with those allocated to placebo (1.34 vs. 1.30 respectively, 95% CI for difference -0.27 to 0.25; P=0.94). The median antigen-stimulated whole blood IFN-γ secretion at follow-up was not significantly different for individuals allocated to vitamin D compared with those allocated to placebo (for ESAT-6: 88 pg/ml vs. 54 pg/ml respectively, P=0.13; for CFP-10: 45 pg/ml vs. 26 pg/ml, P=0.23).

Baseline serum 25(OH)D concentrations were determined for all 192 randomized participants plus an additional ten TB contacts who gave a baseline blood sample but subsequently declined randomization (n=9) or were found to be ineligible for randomization (n=1) (Figure 1). Of these 202 TB contacts, 84 (41.6%) were vitamin D-deficient (serum 25-hydroxyvitamin D<20 nmol/l) (26) and 189 (93.6%) were vitamin D-insufficient (serum 25-hydroxyvitamin D<75 nmol/l) (27) at baseline. Vitamin D deficiency was associated with black African and south Asian ethnicity, lack of fish in the diet and enrolment from November to April on univariate analysis (Table 2). Multivariate analysis revealed each of these factors to be independent correlates of risk of vitamin D deficiency (for black African ethnic origin, exp(B)=0.16, 95% CI 0.04 to 0.67, P=0.01; for south Asian ethnic origin, exp(B)=0.20, 95% CI 0.06 to 0.73, P=0.02; for absence of fish in the diet, exp(B)=0.42, 95% CI 0.22 to 0.84, P=0.01; for enrolment from November to April, exp(B)=0.45, 95% CI 0.24 to 0.84, P=0.01).

Among randomized participants, administration of a single oral dose of 2.5 mg vitamin D induced a 91% increase in mean serum 25(OH)D (mean 25(OH)D pre- vs. post-supplementation 35.2 nmol/l vs. 67.4 nmol/l; 95% CI for difference 26.3 nmol/l to 38.7 nmol/l; P<0.0001; Figure 3A) and corrected deficiency in all 23 participants in the intervention arm with baseline serum 25(OH)D < 20 nmol/l. No study participant experienced hypercalcemia or any other adverse event.

Concentrations of 25(OH)D were also determined in BCG-*lux* assay supernatants performed at baseline and follow-up in a randomly selected subset of 32 participants allocated to the intervention arm of the study. Vitamin D supplementation induced a 95%

increase in mean 24-hour supernatant 25(OH)D concentration (pre- vs. post-supplementation: 7.3 vs. 14.2 nmol/l, 95% CI for difference 4.9 to 8.8 nmol/l; P<0.0001; Figure 3B) and a 123% increase in mean 96-hour supernatant 25(OH)D concentration (pre- vs. post-supplementation: 6.5 vs. 14.5 nmol/l, 95% CI for difference 5.4 to 11.8 nmol/l; P<0.0001; Figure 3C).

Discussion

We have shown that a single oral dose of 2.5 mg vitamin D enhanced the ability of TB contacts' whole blood to restrict BCG-*lux* luminescence *in vitro* without affecting antigen-stimulated IFN-γ responses. Profound vitamin D deficiency was found in more than 40% of this cohort, and was independently associated with absence of fish in the diet, south Asian and black African ethnic origin and sampling in winter or spring. Supplementation induced a 91% increase in mean serum 25(OH)D concentration and corrected deficiency in all individuals with baseline 25(OH)D < 20 nmol/l for at least 6 weeks without inducing hypercalcemia in any participant.

Interestingly, vitamin D supplementation suppressed BCG-*lux* bioluminescence at the 24-hour timepoint, but did not affect BCG-*lux* bioluminescence or whole blood antigen-stimulated IFN-γ secretion at the 96-hour timepoint. Given that innate immune responses are mobilized more rapidly than acquired immune responses, 24-hour and 96-hour outcomes may be interpreted as indicators of innate and acquired responses, respectively. Our findings may therefore indicate that vitamin D supplementation primarily enhances

innate responses to mycobacterial infection. This interpretation is in keeping with a recent report that 25(OH)D supports induction of innate antimycobacterial immune responses (28). An alternative potential explanation, given the borderline statistical significance of the effect of vitamin D supplementation on 24-hour luminescence ratio (P=0.03) is that this result arose by chance. If, however, the effect is real, previous studies validating the assay (17, 18) suggest that suppression of BCG bioluminescence reflects a clinically meaningful enhancement of global antimycobacterial host response.

A large proportion of TB contacts assessed for eligibility declined randomization (Figure 1); however, the fact that this group did not differ significantly from participants in terms of median age, sex ratio or ethnic composition suggests that the external validity of this study is unlikely to have been severely compromised by its low recruitment rate. The significant numbers of participants who were lost to follow-up or excluded from the analysis also represent a weakness of the study, primarily by increasing the potential for type II error.

The very high rates of profound vitamin D deficiency that we report in this group of otherwise healthy adults are higher than those documented in the institutionalized elderly (29) and are a cause for grave public health concern, given emerging evidence implicating vitamin D deficiency in the pathogenesis of a wide range of chronic diseases (30). The observation that black African and south Asian ethnicity are risk factors for vitamin D deficiency independently of diet may be explained by the effect of increased skin pigmentation in reducing cutaneous vitamin D synthesis (31); however, this possibility cannot be evaluated in the absence of data on participants' skin pigmentation. Our finding

that a single oral dose of 2.5 mg vitamin D corrects profound vitamin D deficiency for at least 6 weeks without causing hypercalcemia underlines the potential use of this formulation as a safe, effective and cheap ($1.20) public health intervention.

In conclusion, we have demonstrated that a single oral dose of vitamin D enhances TB contacts' immunity to mycobacteria. Prospective trials to determine the effect of vitamin D supplementation on TB incidence rates should be performed in deficient populations with high rates of latent TB infection.

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Figure legends

Figure 1: *Study recruitment profile*

* Concentrations of 25(OH)D were determined in BCG-*lux* assay supernatants performed at baseline and follow-up in a randomly selected subset of 32 participants allocated to the intervention arm of the study.

Figure 2: *Influence of vitamin D supplementation on BCG*-lux *assay luminescence ratio*

Mean BCG-*lux* assay 24-hour luminescence ratio at follow-up was 20.4% lower for individuals allocated to vitamin D compared with those allocated to placebo (0.57 vs. 0.71 respectively, 95% CI for difference 0.01 to 0.25; P=0.03).

Figure 3: *Influence of vitamin D supplementation on serum and BCG*-lux *assay supernatant 25(OH)D concentrations*

A single oral dose of 2.5 mg vitamin D induced a 91% increase in mean serum 25(OH)D (mean 25(OH)D pre- vs. post-supplementation 35.2 nmol/l vs. 67.4 nmol/l; 95% CI for difference 26.3 nmol/l to 38.7 nmol/l; P<0.0001) and corrected deficiency in 23/23 participants with baseline 25(OH)D < 20 nmol/l allocated to the intervention arm of the trial (A). In a randomly selected subset of 32 participants allocated to the intervention arm of the trial, vitamin D supplementation induced a 95% increase in mean 24-hour BCG-*lux* assay supernatant 25(OH)D concentration (pre- vs. post-supplementation: 7.3 vs. 14.2 nmol/l, 95% CI for difference 4.9 to 8.8 nmol/l; P<0.0001; B) and a 123% increase in mean 96-hour BCG-*lux* assay supernatant 25(OH)D concentration (pre- vs. post-

supplementation: 6.5 vs. 14.5 nmol/l, 95% CI for difference 5.4 to 11.8 nmol/l; P<0.0001; C).

Table 1: Baseline characteristics of randomized participants included in analysis (n=131)

Variable	Placebo (n=64)	Vitamin D (n=67)
Median age, years (interquartile range)	37.5 (29.8 to 45.2)	30.1 (25.1 to 44.1)
Male sex, no. (%)	28 (43.8)	36 (53.7)
Ethnic group*		
- Black African, no. (%)	10 (15.6)	7 (10.4)
- South Asian, no. (%)	43 (67.2)	47 (70.1)
- White, no. (%)	9 (14.1)	9 (13.4)
BCG scar present, no. (%)	53 (82.8)	52 (77.6)
Tuberculin positive,† proportion (%)	14/53 (26.4)	11/56 (19.6)
Exposure site		
- Household, no. (%)	47 (73.4)	57 (85.1)
- Other, no. (%)	17 (26.6)	10 (14.9)
Index case		
- Smear positive pulmonary TB, no. (%)	30 (46.9)	38 (56.7)
- Smear negative pulmonary TB, no. (%)	11 (17.2)	8 (11.9)
- Extrapulmonary TB, no. (%)	17 (26.6)	16 (23.9)
Vitamin D-deficient,¶ no. (%)	30 (46.9)	23 (34.3)
24-hour luminescence ratio, mean (s.d.)	0.64 (0.24)	0.62 (0.27)
96-hour luminescence ratio, mean (s.d.)	1.21 (0.53)	1.28 (0.58)
ESAT-6-stimulated IFN-γ (pg/ml), median (IQ range)	88 (30 to 275)	49 (0 to 196)
CFP-10-stimulated IFN-γ (pg/ml), median (IQ range)	40 (0 to 204)	0 (0 to 67)

* Six not self-classified into categories above: two 'mixed ethnic group' (both in vitamin D arm) and four 'other ethnic group' (two in placebo arm, two in vitamin D arm).

†Defined as Heaf grade 3-4 in any participant, or Heaf grade 2 in non-BCG-vaccinated participants. Tuberculin skin test not performed in eight participants as per UK guidelines (23) (three in placebo arm, five in vitamin D arm); test performed but no result available in 14 participants (eight in placebo arm, six in vitamin D arm).

¶ Serum 25-hydroxyvitamin D < 20 nmol/l (26)

Table 2: *Correlates of vitamin D status in London TB contacts (n=202)*

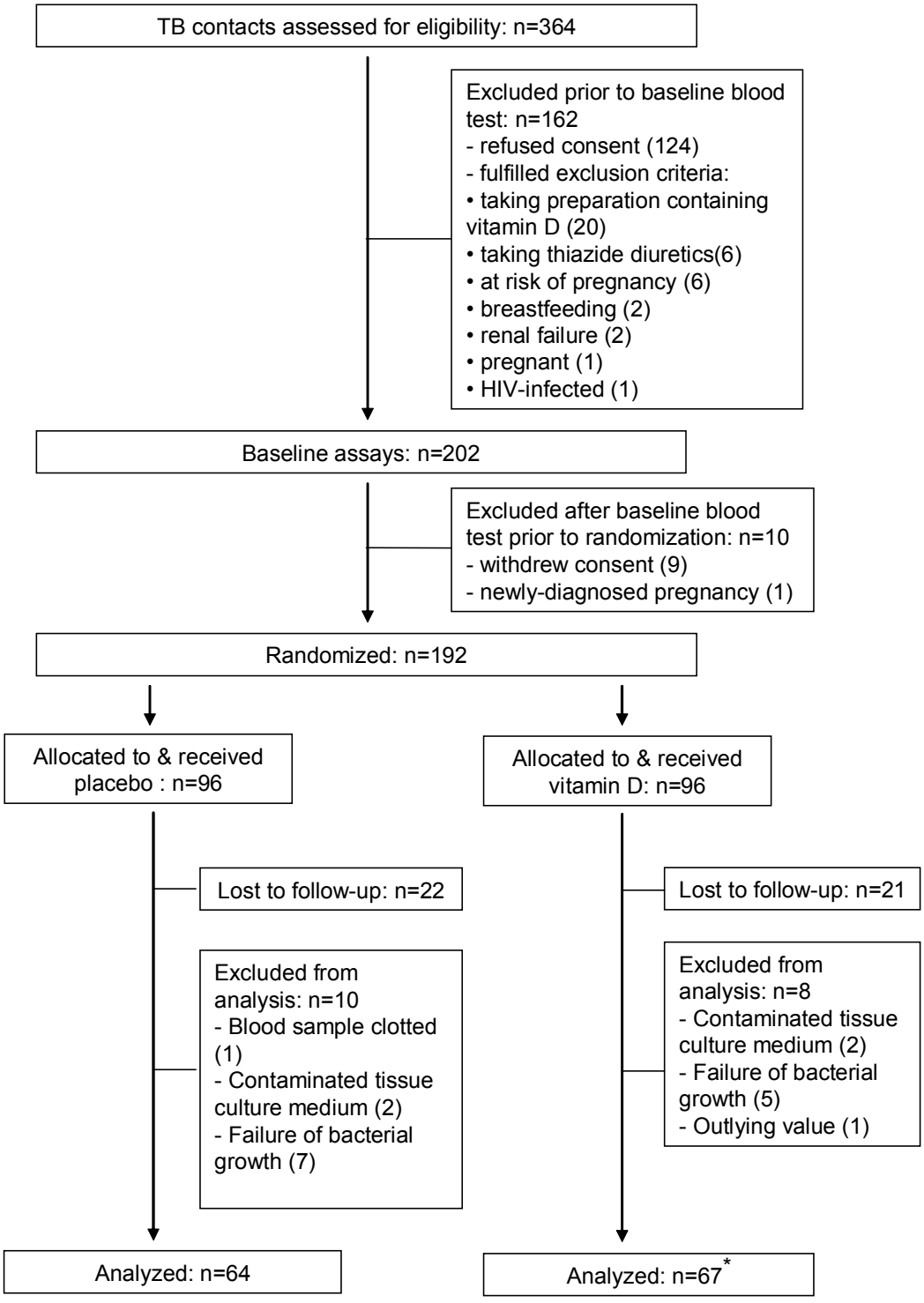
Variable		Proportion deficient (%)	P value
Sex	Male	39/107 (36.4%)	0.12
	Female	45/95 (47.3%)	
Ethnic group*	Black African	14/30 (46.7%)	0.008
	South Asian	66/139 (47.5%)	
	White	3/23 (13.0%)	
Diet†	Fish-eating	49/141 (34.8%)	0.005
	Non-fish-eating	33/59 (55.9%)	
Month of recruitment	November-April	57/114 (50.0%)	0.006
	May-October	27/88 (30.7%)	
Education¶	A	37/78 (47.4%)	0.18
	B	47/124 (37.9%)	
Age (Quartiles)	1	22/50 (44.0%)	0.65
	2	24/51 (47.1%)	
	3	20/51 (39.2%)	
	4	18/50 (36.0%)	

*9 not self-classified into categories above: 7 'other ethnic group', 2 'mixed'

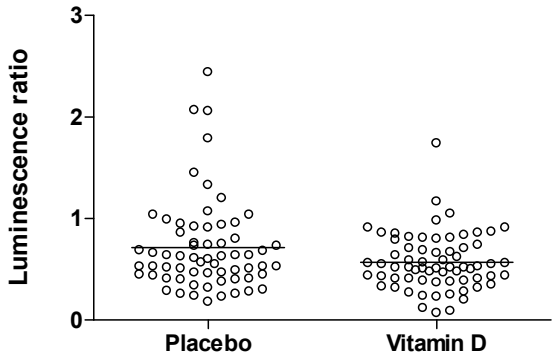
† Dietary data not available for 2 participants

¶ A: up to/including secondary education; B: tertiary or higher education

Martineau *et al.* Figure 1



Martineau *et al.* Figure 2



Martineau *et al.* Figure 3