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# Original papers

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# Circulating MMP9, vitamin D and variation in the TIMP-1 response with VDR genotype: mechanisms for inflammatory damage in chronic disorders?

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## Summary

**Background:** Vitamin-D deficiency and vitamin-D receptor genotype (VDR) are risk factors for several disorders with inflammatory components, including coronary heart disease (CHD) and diabetes, though the mechanisms involved are unclear.

**Aim:** To examine the hypothesis that vitamin D status modulates the matrix metalloproteinase (MMP) system in a population with a high prevalence of vitamin D deficiency, a situation affecting susceptibility to CHD and diabetes.

**Design:** Prospective cross-sectional, interventional and embedded studies.

**Methods:** Circulating MMP2,9, the inhibitor TIMP-1 and C-reactive protein (CRP) were measured during studies of vitamin-D deficiency as a risk factor for type 2 diabetes and CHD in 171 healthy British Bangladeshi adults, free of known diabetes or major illness. Vitamin D status, VDR genotype, body-build, blood pressure, lipid and insulin profiles, glucose tolerance, fibrinogen, PAI-1, folate and homocysteine were measured. Vitamin-D-deficient subjects were re-assessed after 1 years' supplementation. MMP, TIMP-1 and CRP levels were measured in 41 subjects halfway through 5-year follow-up. Independent determinants of circulating concentrations of MMP9, TIMP-1 and CRP were assessed by multiple regression analysis.

**Results:** Vitamin D status was the sole determinant of circulating MMP9 (inversely) and an independent determinant of CRP (inversely). Determinants of TIMP-1 were MMP9, systolic blood-pressure (directly) and VDR genotype (*Taq*I). Significant reductions in MMP9 (−68%), TIMP-1 (−38%) and CRP (−23%) concentrations followed vitamin-D supplementation.

**Discussion:** Vitamin-D insufficiency is associated with increased circulating MMP2,9 and CRP, correctable by supplementation. This finding provides a possible mechanism for tissue damage in chronic inflammatory conditions, including CHD and diabetes.

## Introduction

Coronary heart disease (CHD) risk increases with diabetes and across the continuum of normoglycaemia. It is generally accepted that increases in serum C-reactive protein (CRP) mark an increased risk of IHD. Although the atherosclerotic burden is similar in Whites and Indo-Asians, CHD events

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are increased in Indo-Asians;[2] this may relate to increased plaque vulnerability and enhanced thrombogenesis. Inflammatory responses are now known to be 'integral' to the 'atherosclerotic process', but it has been said recently that we still don't know why.[1–3]

Vitamin D deficiency is more common in Indo-Asians than Whites in 'Westernized' communities; furthermore it has been suggested that it is a risk factor for CHD and for diabetes. Whether the adverse effects of deficiency on insulin-resistance, insulin secretion, glycaemia, blood pressure and cholesterol account for the increased risk, or whether there are other pathogenic mechanisms, remains unclear.[4–6]

Tissue matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) control remodelling in vascular wall and myocardium as in other tissues. MMPs are modulated by variation in transcription rates, through activation of MMP-proenzymes and by anti-proteinase inhibitors (TIMPs). TIMP-1 inhibits MMPs, including MMP9,[7] and is increased in active arterial plaque, aggravating inflammatory damage and plaque instability. Plasma MMP9 and MMP2 levels increase in the circulation in unstable angina and acute infarction.[8–10] Calcitriol (activated hormonal vitamin D) modulates tissue MMP expression experimentally.[11] Furthermore, vitamin D receptors (VDR) are expressed in vascular wall and arterial plaque macrophages. The MMP/TIMP system could, therefore, be regulated not only by circulating calcitriol, but also by activated hormonal vitamin D produced within vascular tissues as well as by therapeutic agents.[12–14]

We therefore examined the hypothesis that vitamin D repletory status might modulate the metalloproteinase system in healthy British Indo-Asians whose risk factors for type 2 diabetes (T2DM) and CHD, including glucose tolerance, insulin secretory profile, serum folate, total homocysteine, vitamin D status and VDR genotype were known.[15,16] Serum C-reactive protein levels were measured for comparison, since raised levels of this acute phase reactant are regarded as prospective markers of IHD risk.[2]

# Methods

## Study design

This study, approved by the Local District Ethical Committee, was agreed with participating family doctors in East London.

### *Cross-sectional study*

Healthy British adults of Bangladeshi origin aged 35–65, free of known diabetes, CHD, hypertension or other on-going illness ($n = 631$) gave written informed consent (as random attenders at their GP surgeries with relatives or minor intercurrent illness). Subjects giving informed consent and with spot blood glucose values $>6.4$ mmol/l at $<2$ h after food, or $>4.4$ mmol/l $>2$ h after food, were assessed at the family doctors surgery by the bilingual research worker (NM). Anthropomorphic measurements were made by standard techniques and subjects completed a questionnaire covering paan usage (quids containing betel nut), aspects of diet relevant to vitamin D intake (eggs, fish, meat and yoghurt) and cigarette smoking before an OGTT (in a hospital-based clinical research facility) as previously described, between 1995–6.[15,16] Vitamin D status was defined by serum 25-hydroxyvitamin D (25(OH)D) levels.

### *Interventional study*

Classically 'deficient' subjects (defined at the time the study was planned as $<11$ ng/ml[17]) ($n = 54$) were allocated using a computer-based form of Minimization with a random element,[18] into two groups matched for age and sex (NM) for a trial of vitamin-D supplementation over 1995–6. The study was designed to compare three-monthly depot injections of a depot (oily) solution of Cholecalciferol i.m. at 'high' (50 000 IU) or 'low' (500 IU) dosage over one year. The increase in serum 25(OH)D expected, in the high dosage group, was from approximately 5.0(2.0) ng/ml (all subjects having levels $<11$ ng/ml by definition) to approximately 14(7.4) ng/ml, (at 80% power; $p<0.05$; $n = 6$ per group). Since, if subjects proved to be as deficient as in our earlier study, we expected to see a mean (SD) rise in 30-min serum insulin at OGTT of 40 mU/l from a baseline 30-min value of 36(20) mU/l (at 80% power; $p<0.05$), we planned to recruit $n = 150$ to include allowance for patients dropping out. Supplements were given to subjects by practice nurses, who were informed of the dosages required, as there was only one preparation available for use. The primary outcome was an increase in vitamin D repletion by $>100\%$ (as above). The secondary outcomes were planned as increases in serum insulin as above and reduction in circulating MMP2,9.

Overall, 47 of 48 subjects who completed the trial (six having withdrawn before supplementation and one before re-examination; see CONSORT portion of 'Flowchart' in the Appendix), were re-examined (with repeat assessments on fasting

blood and at OGGT) within 4 weeks of the anniversary of the first assessment.

### *Embedded study*

Subjects selected as having been vitamin-D-insufficient initially (serum 25(OH)D 12–20 ng/ml) ($n = 41$) were re-bled midway through the 5-year follow-up period for a study of VDR expression including reassessment of the vitamin D axis and of MMP2,9 and TIMP-1 and CRP levels.[19]

## Five-year follow-up

Subjects from the cross-sectional study who had developed clinical evidence of ischaemic heart disease were identified at 5-year record review during 2000–2001.

## Laboratory methods

Serum and plasma aliquots were frozen (−20 °C) before being blinded for assay. Plasma TIMP-1, MMP2 (MMP2) and MMP9 (MMP9) proenzymes were measured by ELISA (Biotrack UK; within run CV <3%, reference ranges 99–330 ng/ml, 365–649 ng/ml and <10 ng/ml respectively), on previously unthawed samples; numbers available per group are shown in Table 2. Pre- and post-supplementation samples were assayed blind within single runs. Insulin profiles at OGTT were available (insulin, pro-insulin, 32:33 split pro-insulin[16]) as previously reported, together with fasting serum lipid profiles (by standard automated techniques), apolipoprotein A1 and B and sensitive CRP (normal range <4 mg/l; CV 3–5%) levels (by immunoturbidimetry on previously unthawed serum samples; numbers of samples available, see Table 2). Plasminogen activator inhibitor-1, measured by ELISA; plasma fibrinogen, by a modified Clauss technique (Immuno A.G.) and serum 25(OH)D, by immunoassay (IncStar) were available,[16] together with serum intact parathormone, measured by immunoassay (ICS; within- and between-assay CVs <7% and <10%, respectively; normal range 48–119 nmol/l). Measurements were also made of bone-specific alkaline phosphatase (IRMA, Hybritech Europe: within- and between-assay CVs 3.7–6.7% and 7–8.1%, respectively, normal range 5–22 mcg/l). Biochemical profiles (serum albumin, calcium, corrected calcium and alkaline phosphatase) were measured on fasting samples, taken without venous constriction at OGTT, by standard automated techniques. All subjects had been typed for *Bsm*I, *Apa*I and *Taq*I vitamin D receptor polymorphisms using standard PCR-RFLP[16] and serum total homocysteine measured by HPLC.[20]

## Statistical analyses

These were done using SPSS 10. Data distribution was normalized where required by logarithmic transformation before further analysis. Pre- and post-supplementation measurements were compared using paired t tests. Data analyses included $\chi^2$ tests, bivariate and partial correlation tests, stepwise multiple regression analyses to $p < 0.05$ and analysis of pre- and post-supplementation data by 'intention to treat'. Pairwise linkage disequilibrium between VDR gene polymorphisms was assessed by an 'estimate haplotype frequencies (EH)' programme.[21] The frequency distribution of VDR alleles for each genotype was examined ($\chi^2$ test) for compliance with Hardy-Weinberg equilibria in subjects with and without vitamin D deficiency (serum 25(OH)D $\leqslant 11$ and $>11$ ng/ml, respectively) and overall. These equilibria were compared (using $\chi^2$ tests) for evidence of ethnic admixture (the Wahlund effect).

# Results

## Cross-sectional study

Of 631 participants, 171 were recruited from the 230 screened as being eligible after exclusion of ineligible subjects (see flowchart) and completed this phase of the study; 31% were vitamin-D-deficient. The three VDR gene polymorphisms were in strong linkage disequilibrium with each other (*Taq*I to *Apa*I, $p < 0.001$; *Taq*I to *Bsm*I, $p < 0.001$; *Apa*I to *Bsm*I, $p < 0.001$). The allele distribution for these polymorphisms fitted Hardy-Weinburg equilibria in 'deficiency', 'insufficiency', in replete subjects (serum 25(OH)D >20 ng/ml)

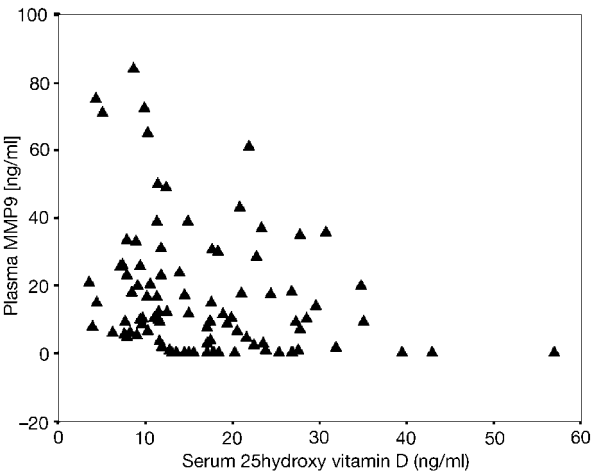


**Figure 1.** Plasma matrix metalloproteinase 9 (MMP9) levels with vitamin D status (serum 25-hydroxyvitamin D) in apparently healthy subjects of Bangladeshi origin living in London, UK.

and in this group as a whole ($p = 0.35$–$0.9$). Comparison of the findings in these groups did not show the Wahlund effect.

Plasma MMP9 related inversely to vitamin D status (whether calculated with or without the two MMP9 values >100 ng/l) on simple correlation analysis (Figure 1). Neither plasma TIMP-1, MMP9 nor MMP2 concentrations were related to age, body build, folate, homocysteine, PA1-1, fibrinogen, glycaemia, lipid or insulin profiles, diabetic status or blood pressure on simple correlation analysis. Multiple linear regression analysis of the normalized data, allowing for all of the factors that had been examined for correlation (as above), showed vitamin D status to be an independent determinant of MMP9 ($r = -0.41$; $p < 0.0001$). There were no other independent determinants of MMP9, nor were any independent determinants of MMP2 identified amongst these same factors by multiple regression analysis.

Initial serum CRP ($n = 116$) correlated positively with fibrinogen ($p = 0.009$), PAI-1 ($p < 0.001$), fasting pro-insulin ($p = 0.001$), blood pressure ($p = 0.001$) and BMI ($p = 0.01$). CRP correlated inversely with serum 25(OH)D when calculated allowing for each of the above factors by partial correlation ($r = -0.22$, $p = 0.031$) and directly with serum parathormone, as it did on simple direct correlation ($r = 0.21$, $p = 0.029$). Stepwise multiple regression analysis of normalized data showed initial CRP to increase independently with vitamin D deficiency (relation to serum 25(OH)D; $r = -0.22$, $p = 0.034$) and with triglycerides ($p = 0.003$), BMI ($p = 0.004$), PAI-1 ($p = 0.003$) and age ($p = 0.03$). Circulating TIMP-1 related directly to MMP9 overall (simple correlation, $r = 0.68$, $p < 0.0001$), in vitamin D deficiency ($r = 0.87$, $p < 0.0001$) and post-supplementation ($r = 0.63$, $p < 0.0001$). Multiple regression analysis (best-fit model, $p < 0.0001$) showed the independent determinants of TIMP-1 to be MMP9 ($p < 0.0001$), VDR *Taq*I genotype ($p < 0.001$) and systolic blood pressure ($p = 0.007$), while vitamin D status was a lesser determinant ($p = 0.045$). Sex, BMI, diastolic blood pressure, cigarette usage, other factors (as above) and VDR *Apa*I and *Bsm*I genotypes were not determinants. Mean (SD) TIMP-1 levels for *Taq*I VDR genotypes TT ($n = 69$), Tt ($n = 65$) and tt($n = 18$) were 257(117),

**Table 1a** Baseline age (years), sex ratio (women:men), Waist:hip ratio and body mass index (BMI) in subjects randomized to high ($n = 21$) or low ($n = 26$) dose supplementation in the interventional study

| Treatment group | Age (years) | Sex ratio (F:M) | BMI | Waist:hip ratio |
|---|---|---|---|---|
| High dose | 44.08 | 0.69 | 27.53 | 0.93 |
| Low dose | 41.76 | 0.62 | 27.41 | 0.94 |

**Table 1b** Vitamin D status and related variables (mean (SD)) in the initial study group and in deficient subjects before and after supplementation

| | Initial study group ($n = 171$) | Vitamin-D-deficient group: pre-supplementation | Post-supplementation |
|---|---|---|---|
| Serum 25(OH)D (ng/ml) | 17.61 (8.52) | 8.55 (2.47) ($n = 47$)<br>*(1) = 8.74 (2.27)*<br>*(2) = 8.26 (2.8)* | 14.05 (4.9)***<br>*(1) = 15.02 (4.19)****<br>*(2) = 13.16 (5.42)**** |
| Serum parathormone (mU/l) | 38.16 (18.18) | 44.32 (22.59) ($n = 46$)<br>*(1) = 45.6 (21.6)*<br>*(2) = 43.3 (25.6)* | 37.84 (16.9)*<br>*(1) = 37.4 (17.01)*<br>*(2) = 40.4 (17.85)* |
| Total ALP (IU/l) | 66.21 (20.56) | 64.32 (20.86) ($n = 44$)<br>*(1) = 64.8 (4.02)*<br>*(2) = 66.3(16.62)* | 66.16 (17.76)<br>*(1) = 64.3 (18.1)*<br>*(2) = 67.3 (22.5)* |
| Specific bone ALP (mcg/l) | N/A | 23.08 (6.70) ($n = 24$)<br>*(1) = 21.42 (4.02)*<br>*(2) = 25.1 (8.8)* | 23.65 (6.34)<br>*(1) = 21.1 (3.7)*<br>*(2) = 26.1 (7.8)* |
| Corrected serum calcium (mmol/l) | 2.19 (0.084) | 2.20 (0.062) ($n = 44$)<br>*(1) = 2.20 (0.057)*<br>*(2) = 2.19 (0.68)* | 2.24 (0.095)**<br>*(1) = 2.26 (0.088)*****<br>*(2) = 2.25 (0.01)***** |

The numbers of samples available in each group are shown in parentheses. Mean (SD) for the pooled subjects are shown, with data for subjects in the 'high' (1) and 'low' (2) treatment groups in italics. ALP, alkaline phosphatase; 25(OH)D, 25-hydroxyvitamin D. Comparisons of means between group 1 and 2 subjects non-significant both pre and post treatment. *$p = 0.02$; **$p = 0.01$; ***$p = 0.0001$; ****$p = 0.04$ for comparison of overall means before and after vitamin D supplementation (by paired t tests).

267(125), and 345(212) ng/ml, respectively, in the initial cross-sectional study group ($n = 152$).

## Interventional study

The numbers recruited were smaller than planned, due to a reduction in prevalence of vitamin D deficiency from ~80% in our earlier work to 31% during recruitment following an exceptionally good summer. Glucose tolerance in the subgroup selected as having vitamin D deficiency was normal in 75.5%, impaired in 16% and diabetic in 8% (WHO criteria, 1985), with similar findings post-supplementation (72.3%, 21.3% and 6.4%, respectively) ($\chi^2$, $p = 0.4$). The age- and sex-matched participants randomized to 'high' or 'low' treatment had comparable initial body build, vitamin D status (serum 25(OH)D and parathormone), glucose tolerance and insulin secretion index (see Table 1).

### *Primary outcomes*

The increases in serum 25(OH)D and in serum corrected calcium did not differ between the 'high' and 'low' treatment groups, although reduction in parathormone was marginally greater after 'high' than 'low' supplementation ($p = 0.04$). Serum alkaline phosphatase and the marginally raised bone-specific alkaline phosphatase remained unchanged (see Tables 1a and 1b). These findings, on review with practice nurses, were thought to be due to the difficulty of delivering the smaller volume of the viscous depot preparation of vitamin D with accuracy, even with the fine 1 ml syringes used. Despite the failure to recruit adequate numbers of deficient subjects for the trial as planned, the data were examined by 'intention to treat'. There were no differences in any of the findings in the vitamin D axis, other than for serum parathormone as above, by 'intention to treat'. In view of these findings, the data were examined further after combination of the results of the two treatment groups. The pre- and post-supplementation findings were then compared for the combined group, as had been planned for each group individually.

### *Secondary outcomes*

Mean MMP9, MMP2, TIMP-1 and CRP levels fell post-supplementation (see Table 2). The reductions found were comparable for the 'high' and 'low' treatment groups for the MMP9, MMP2 and TIMP-1 ($p$ for comparison of the reductions found by 'intention to treat' = 0.34–0.88), while the reductions in CRP were greater in the 'high' than the 'low' treatment group (Table 2). The data for the two treatment groups were combined, as planned after review of the primary outcome data. Concentrations of MMP9 fell by −66.8%, TIMP-1 by −39.8% and CRP by −23.0%. Mean(SD) serum fibrinogen levels increased in the low and fell in the high treatment groups ($p > 0.1$ in both cases). While serum fibrinogen increased after supplementation in the interventional study as a whole, no subject in that study had a value above the normal range either before or after supplementation: mean(SD) (range); pre, 2.37(0.58) (1.18–3.45); post, 2.59(0.39) (1.7–3.46) g/l; $p = 0.009$.

**Table 2** Circulating mean (SD) MMP9, MMP2, TIMP-1 and CRP concentrations in deficient (serum 25-hydroxyvitamin D ⩽ 1 ng/ml) subjects before and after supplementation

| | Vitamin-D-deficient subjects pre-supplementation | Post-supplementation | Change (%) |
|---|---|---|---|
| MMP9 (ng/ml) ($n = 34$) | 27.75 (32.64) | 9.22 (12.14)** | −66.8*** |
| | *(1) = 20.21 (22.02)* | *(1) = 8.52 (11.53)* | *(1) = −57.84* |
| | *(2) = 28.03 (22.3)* | *(2) = 9.91 (13.5)* | *(2) = −64.68* |
| TIMP-1 (ng/ml) ($n = 38$) | 263.79 (173.54) | 160.68 (84.82)** | −39.8** |
| | *(1) = 243.13 (132.6)* | *(1) = 163.91 (82.45)* | *(1) = −32.6* |
| | *(2) = 291.78 (205.6)* | *(2) = 157.87 (92.88)* | *(2) = −45.89* |
| MMP2 (ng/l) ($n = 28$) | 322.14 (70.28) | 278.39 (78.87)* | −13.58* |
| | *(1) = 311.88 (67.0)* | *(1) = 265.33 (94.06)* | *(1) = −14.9* |
| | *(2) = 324.2 (89.06)* | *(2) = 291.25 (58.63)* | *(2) = −10.2* |
| CRP (mg/l) ($n = 24$) | 6.12 (5.87) | 4.71 (5.59) | −23.01* |
| | *(1) = 5.75 (6.98)* | *(1) = 3.47 (4.81)* | *(1) = −39.65* |
| | *(2) = 7.1 (4.38)* | *(2) = 6.76 (6.34)* | *(2) = −4.8* |

Numbers of samples available for examination in each group are shown in parentheses. Data for subjects in the 'high' (1) and 'low' (2) treatment groups are in italics. *$p = 0.01$–$0.04$, **$p = 0.002$, ***$p = 0.0001$ for comparison of means before and after supplementation in the interventional group as a whole (by paired t tests).

Decreases in plasma TIMP-1 correlated with initial TIMP-1 concentration and with the mean of the pre- and post-treatment values, ($r = 0.73$; $p < 0.0001$), as was also true for reductions in CRP ($r = 0.936$, $p < 0.0001$) making confounding due to regression to the mean unlikely. There was, therefore, an increase in circulating TIMP-1/MMP9 ratios after supplementation. Similar increases in this ratio were found with increasing vitamin D status in the initial cross-sectional study ($p < 0.0001$). These findings were unchanged by exclusion of individuals with newly detected diabetes (7.2–8.6% of the various study sub-groups in Tables 1 and 2). TIMP-1 levels in the deficient subjects entering the interventional study were similar to those found in the cross-sectional study, apart from a significant increase in TIMP-1 values in tt subjects (mean (SD) for TT, 232.4(129.9); for Tt, 284.6(158.3); for tt, 463.6(320); $p = 0.02$ on one-way ANOVA).

In addition, paan usage (betel-nut with or without tobacco) was an independent determinant of plasma TIMP-1 level in deficient subjects, while cigarette usage was an independent determinant of TIMP-1 in the initially replete subjects ($r = 0.29$, $p = 0.019$ and $r = 0.41$, $p = 0.009$, respectively, on multiple regression analysis). The small increases in serum insulin found were comparable in the 'high' and 'low' treatment groups and are not considered further in this report.

## Follow-up study

Five years after the cross-sectional study began, 20 subjects were identified as having developed overt IHD, hypertension, or both, among the 117 that could be adequately reviewed, 54 subjects having moved away, changed their doctor or failed to respond. Compared to those remaining free of overt IHD, the IHD/hypertension group had raised initial levels of fibrinogen (3.02(0.8) vs. 2.5(0.7); $p = 0.015$) but not of PAI-1, CRP or MMP2,9. Similar examination of the findings from the embedded study, midway through the 5-year follow-up, showed raised levels of MMP9 (mean (SD) = 179(103) vs. 103(85); $p = 0.034$), but not of CRP, to be associated with overt cardiovascular disease at 5 years.

# Discussion

Plasma MMP9 levels were higher in this group of Indo-Asian subjects than have previously been reported in healthy adults (reference range for assay used $< 10$ ng/ml). Both plasma MMP9 and serum CRP levels related inversely to vitamin D status in the cross-sectional study; furthermore, serum 25(OH)D was an independent determinant of CRP as well as of MMP9. The fact that both MMP9 and CRP levels were similarly related to vitamin D status supports the suggestion that the circulating MMP9 levels in this study are likely to reflect some contribution from atheromatous vulnerability, since sensitive CRP levels have been convincingly demonstrated to mark future risks of acute cardiovascular events. Interestingly, acute coronary events have recently been reported to occur in the presence of evidence of widespread coronary inflammation.[22] The fact that MMP9 was found to be raised in the embedded study, in those who did develop acute cardiovascular events, supports the suggestion that inflammatory vascular damage can contribute to circulating levels of MMP9 in the absence of acute events.

Supplementation by injection was used in the interventional trial to reduce the risk of non-compliance. However, we were unable to distinguish between the effects of 'high' and 'low' dose supplementation, both groups demonstrating comparable and significant increases in vitamin D status. This was thought to be due to difficulty measuring small doses of the viscous depot injection. Alternatively it may be that subjects took additional supplements after finding out that they were on 'low' dosages, since practice staff knew which dose they were having, our pharmacy being unable to provide us with a placebo preparation. The fact that the problem was in achieving low dosages was fortunate, since should both groups have received low dosages the effects of vitamin D supplementation on MMPs and CRP could well have been missed. Carrying out both cross-sectional and interventional studies has allowed us to compare the effects of variation in naturally achieved levels of vitamin D repletion with the effects of comparable levels achieved by supplementation; the interventional study showing supplementation to reduce both MMP9 and CRP to levels similar to, even rather lower than, those found with similar but naturally occurring levels of vitamin D repletion in the cross sectional study.

Though the cross-sectional findings suggest a 'dosage' effect of increasing repletory status, we are unable to say whether there may be dosage effects for supplementation. It is, however, unlikely that strong relationships between vitamin D status and MMP9 would have occurred in these separate studies by chance.

The failure to achieve the planned number of

recruits, or a meaningful difference in vitamin D repletion following 'high' and 'low' supplementation, meant that we could not examine insulin secretion post-supplementation. The only suggestion of dose dependency in the findings was that reductions in PTH were significantly greater in the 'high' than the 'low treatment groups. However, since other aspects of the vitamin D axis did not vary between the groups, this finding may be fortuitous. Further investigation is clearly required on the overall question of dosage effects, using a more reliable method of supplementation—for example, supervised administration of appropriate oral doses at intervals has been shown to be both effective and consistent.[23] The failure to recruit as many deficient subjects as had been expected, following an unusually good summer, did however provide an opportunity for the relationships of variables of interest to be examined across a wide range of levels of vitamin D repletion.

The Bangladeshi community in east London is close knit and derives from a single area, Sylhet. This meant that it was unlikely that any variations with VDR polymorphism, in variables of interest, would be missed because of confounding by ethnic admixture; the absence of evidence for such admixture (see results) provides further reassurance on this point. It is, however, possible that findings in this area may vary with ethnicity and this remains to be determined.

Members of the Bangladeshi community have tended to remain in Tower Hamlets over recent decades, making the 5-year follow-up more complete than had been expected. At the same time, travel to Bangladesh for long periods is common, leading to loss of subjects from the interventional study. These studies may be also be biased by the difficulty in recruiting subjects from among those in work, those with small children (large families being common), pregnant women or those planning pregnancy. There was, however, no bias towards English speakers since recruitment, and arrangements for investigations, were carried out by a bilingual scientist from within the local Bangladeshi community (NM).

Although there were strong positive independent contributions of increases in both blood pressure and circulating MMP9 to increases in TIMP-1 levels in these studies, the VDR *Taq*I polymorphism was also a major independent determinant of circulating TIMP-1. This finding may account for the modest independent effect of vitamin D status on TIMP-1 level and the absence of a simple direct correlation between them. The lowest TIMP-1 levels were associated with the T allele, present in 88% of study subjects.

The reduction in MMP9 levels in supplemented subjects to levels somewhat below those found in initially 'replete' subjects, despite only modest increases in measured vitamin D status, may reflect the benefits of continued supplementation compared to short-term seasonal increases relating to available sunlight. The implications of the inverse relationship of MMP9 to vitamin D status found in the cross-sectional study are supported by the reduction in MMP9 found with supplementation. Since this effect of supplementation was found in the vitamin-D-deficient subset of the original cross-sectional study group, it is possible that the severity of MMP9 mediated inflammatory damage could be reduced by long-term maintenance of adequate vitamin D repletion. The reduction in MMP9 found with supplementation also suggests that the tentative finding of normal plasma MMP9 once serum 25(OH)D concentration reaches 'non-deficient' levels ($>20$ ng/l) should be investigated further.

The reduction in MMP9 was proportionally greater than that of TIMP-1 after a year of vitamin D supplementation. The resultant increase in molar TIMP-1/MMP9 ratios, if present in tissues as well as the circulation, might provide additional benefits in terms of plaque stabilization and the reduction of chronic inflammatory damage in other tissues. This would apply especially to those subjects possessing the 'T' allele of the VDR *Taq*I polymorphism since they appear to mount poor TIMP-1 responses to increases in MMP9. Conversely, tt subjects could be at increased risk of disorders manifesting undue increases in fibrosis.

Variations in circulating MMP9, and in TIMP-1 responses, seem likely to reflect changes in a wide range of tissues. Whilst the changes found in circulating MMP9 (and 2) after vitamin D supplementation could reflect altered bone activity, corrected serum calcium increasing after supplementation, there was no change in total or specific bone alkaline phosphatase activity. In addition, circulating TIMP-1 increases in hypertensive heart disease and MMPs 9 and 2 appear in the circulation in unstable angina and during acute coronary events. It is, therefore, clear that MMPs generated in the vasculature can reach the blood stream.[10] That the relationships found for MMP9 with vitamin D are similar to those for the recognized risk factor CRP provides further support for the suggestion that plasma MMP9 can reflect vascular risk rather than solely reflecting acute coronary events. The finding that initial levels of fibrinogen were significantly increased in subjects developing IHD/hypertension or both within 5 years, while CRP, PAI-1 and MMP9 were not, suggests that fibrinogen

levels may be the best risk marker for IHD in this population group. The small increase in fibrinogen after vitamin D supplementation, is therefore a matter of concern requiring further investigation, although values remained entirely within normal limits. The finding of increases in MMP9 rather than of CRP in the mid-study samples of those later developing CHD suggests that MMP9 may be a more sensitive marker for *active* IHD than CRP, at least in population groups where vitamin D deficiency remains common.

The present findings help to explain how vitamin D deficiency might act as a risk factor for diseases where increased expression of MMP9 contributes to pathogenesis such as CHD, rheumatoid arthritis (RA) and tuberculosis (TB). Vitamin D reduces disease activity in RA, and can be predicted to be helpful in other conditions where MMP9 production is increased, such as periodontitis, already suspected of being linked to IHD.[24–26] We have not found the MMP/TIMP-1 system markers measured to relate to 'classic' risk markers for diabetes or CHD (lipids, fasting insulin profile, rheological factors, glycaemia or serum total homocysteine). Our findings do, however, suggest that chewing betel nut and cigarette smoking might have adverse effects on the MMP9 system; since these findings were independent of all other factors studied further work in this area is required.

This population has previously been shown to demonstrate variation in insulin secretion with VDR genotype[16] and we have now found variation in TIMP-1 response with VDR haplotype; this locus could, therefore, have a dual role in determination of CHD risk, firstly by influencing the risk of type 2 diabetes (by modulating insulin secretion) and secondly, by influencing risk of plaque instability (through reduction in the TIMP-1 response to increases in MMP9). Since reductions in CHD events found with ACE inhibition are known to be associated with reduction in vascular MMP9 expression, and therapeutic agents for suppression of MMP secretion (already used in periodontitis) are being assessed for reduction of plaque disruption and infarction,[13,14] these possibilities are being investigated further.

Mechanisms are known that explain how activation of the VDR by its ligand can modulate MMP9 expression in man. MMP9 and TIMP-1 gene expression is mediated by 'activating protein-1' (AP-1) whose response elements are present in promoters for these genes. For example, the increased AP-1 binding activity induced in human keratinocytes by 1$\alpha$,25-dihydroxyvitamin D3 (activated 'hormonal' vitamin D; calcitriol) *in vitro* depends on the binding of c-fos/c-jun elements to AP-1, and the regulatory region of c-jun contains a vitamin D response element (VDRE).[27] The arterial wall expresses the genes for MMP9, TIMP-1 and for the VDR and it can also activate 25(OH)D *in situ*.[13] Thus all the mechanisms necessary for regulation of MMP9 and TIMP-1 by vitamin D are clearly in place in the vasculature, and the VDR can be added to the list of nuclear hormone receptors that can inhibit MMP gene expression through various mechanisms.[28]

Increasing vitamin D repletory status is associated with increases in insulin secretion but with reductions in circulating MMP9; findings that appear to be contradictory but are consistent with the fact that vitamin-D-activated VDR complexes can induce repression as well as activation of different target genes.[29] This fact, together with our present findings, suggests that vitamin D deficiency is capable of being a significant factor in the pathogenesis of plaque instability and of other chronic disorders where MMP9 upregulation contributes to pathogenesis. While we have shown that folate repletion does not affect our findings, this does not exclude the possibility that other dietary factors may do so. Vitamin A, for example, is known to interact with vitamin D, being essential for normal vitamin D effector activity, though in excess it inhibits the effects of vitamin D.[6]

The suggestion that VDR genotype may contribute to disease severity is not new. Tuberculosis is one condition where *Taq*I polymorphism of the VDR has been reported to contribute independently to severity of disease.[30] Similar findings are reported for severity of myocardial damage in CHD and of diabetic retinopathy; increased risk being associated with TT in the former and tt in the latter.[31,32]

In conclusion, we have shown vitamin D deficiency to be associated with abnormal increases in circulating MMP9, and in the risk marker CRP, and that modest long-term supplementation of deficiency in these subjects corrects these abnormalities. TIMP-1 levels relate to circulating MMP9, as expected, but also vary with VDR genotype; the common 'T' allele of the *Taq*I polymorphism of the VDR marking poor TIMP-1 responders. This phenotype may, therefore, define people for whom vitamin D deficiency is a particular risk. If these findings are confirmed in other communities, many groups of people could be protected from undue inflammatory damage since vitamin D deficiency is both common and avoidable. The elderly, the very young, the sick, those working long hours indoors, people avoiding the sun or using sunscreens in both temperate and tropical countries, those living in northern countries with short summers or with dark skin living Western lifestyles are all well

known to be at especial risk of vitamin D deficiency. It has been said that 'it is high time that everyone was ensured an adequate long-term vitamin D repletory status', a view that our findings strongly support.[6,33]

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# Appendix

Flow chart for the cross-sectional, interventional and embedded studies [using CONSORT recommendations for the randomized trial of high versus low dose supplementation]