ORIGINAL ARTICLE

# Human cytomegalovirus neutralising antibodies and increased risk of coronary artery disease in Indian population

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Accepted 24 April 2012

## ABSTRACT

**Background** Several studies have reported a conflicting association between cytomegalovirus (CMV) infection and coronary artery disease (CAD) based on the levels of total anti-CMV antibodies. However, none have estimated the levels of specific neutralising antibodies (NA) to CMV, which may be clinically more relevant.

**Objective** To determine whether CMV–NA titres show a better association with CAD compared with total anti-CMV antibody levels.

**Design** CMV–NA titres were measured by micro-neutralisation assay and anti-CMV IgG antibodies using ELISA in 391 consecutive CAD patients compared with the same number of controls (N=782), and 91 patients reporting recurrent cardiac events during a 4-year follow-up compared with those without a recurrent event (N=182). Levels of inflammatory markers, interleukin 6, high-sensitivity C reactive protein, fibrinogen and secretory phospholipase A2 (sPLA2), were measured by ELISA. Analysis of variance and logistic regression were used for statistical analyses.

**Results** High CMV–NA titres showed a positive association with CAD occurrence (OR 2.24, 95% CI 1.31 to 3.85, p=0.003) and recurrent cardiac events in CAD patients (OR 4.65, 95% CI 1.21 to 17.86, p=0.025) compared with total CMV antibodies (OR 1.67, 95% CI 1.04 to 2.69, p=0.034, and 2.70, 1.04 to 7.02, p=0.040, respectively). Patients with higher quartile of CMV–NA titres and sPLA2 levels had an adjusted OR of 7.82 (95% CI 1.87 to 32.65, 0.005) for recurrent cardiac events compared with those with the lowest quartiles for both markers.

**Conclusion** These findings suggest that high CMV–NA titres in combination with inflammatory markers improve prediction of cardiac events in the Asian Indian population.

## INTRODUCTION

Human cytomegalovirus (CMV) can persist lifelong as a subclinical latent infection in an immunocompetent host whereas it can cause significant morbidity and mortality in immunocompromised patients. Viral genes modulate the host immune system, mimic host chemokines and their receptors,[1 2] and play a role in the ensuing inflammatory response.[3]

CMV can infect smooth muscle cells, enhance cell proliferation, and induce early atherosclerotic lesion formation and its subsequent progression.[4 5] Viral glycoproteins, especially glycoprotein B and glycoprotein H, mediate viral entry into the host cell, and have been implicated as targets for virus-neutralising antibodies (NA).[6 7] NA titres differentiate primary CMV infections from reactivated ones, as these antibodies appear on average 13 weeks after the onset of primary infection, and are consistently detected early during the reactivated phase.[8]

Several epidemiological studies reported a positive association between CMV infection and coronary artery disease (CAD).[9 10] In animal models also, CMV infection has been shown to exacerbate atherosclerotic lesions.[4] Regardless of enormous epidemiological data, several questions still remain unanswered. Is CMV infection a causative agent for CAD or the inflammatory responses associated with atherosclerosis lead to viral reactivation which in turn activates inflammatory pathways related to acute coronary syndrome? So far, an association has been reported but no clear causative role has been established. Despite these reports, several studies have failed to show any association between CMV infection and CAD.[11 12] We therefore wanted to develop a more sensitive assay for studying the association between CMV infection and CAD. We hypothesised that estimating the clinically relevant CMV–NA may be more pertinent to study their association with CAD than total anti-CMV antibodies.

In this study, we investigated whether CMV–NA titres, either alone or in association with well-known inflammatory markers, would be better predictors of risk for CAD occurrence and recurrent cardiac events than total anti-CMV antibodies in an Asian Indian population.

## METHODS

### Study population

The Indian Atherosclerosis Research Study (IARS) is an ongoing nested case-control family based study to investigate the molecular basis of atherothrombosis in Asian Indian Population.[13] This prospective study of individuals with and without CAD at baseline was initiated in March 2004. In the phase I of IARS, 2318 individuals were enrolled, from 2004 to 2007, comprising of 774 CAD patients and 1544 asymptomatic controls. Patients with CAD had a positive angiogram, and were admitted for coronary artery bypass graft surgery or percutaneous coronary intervention to participating

hospitals in Bangalore and Mumbai, India. Family members over 18 years of age with normal ECG data who showed no CAD-related symptoms and were apparently healthy were enrolled as controls. The present analysis was carried out in an age- and gender-matched population of 782 subjects, consisting of 391 consecutive CAD patients and 391 controls (Group I).

The IARS participants were followed-up from 2005 to 2010 (median follow-up time of 2.5 years). Acute coronary events requiring hospitalisation and treatment, or fatal myocardial infarction, were categorised as recurring cardiac events. Out of 774 CAD patients in the phase I of IARS, 91 patients (11.7%) reported a recurrent cardiac event requiring hospitalisation and treatment while 206 patients were lost to follow-up. To understand the role of baseline CMV–NA titres in the recurrence of cardiac events, an additional analysis was carried out in 182 CAD patients, consisting of 91 subjects who reported a recurrent cardiac event matched to an additional 91 patients who did not report a recurrent event during the same time period of follow-up (Group 2) (figure 1). Blood samples (22 ml) were collected at the time of recruitment, and serum and plasma separated and stored at −80°C in aliquots until use.

Individuals with a chronic illness, as defined by WHO, or with any concomitant infections were excluded from the study. Information pertaining to demographics, lifestyle, anthropometrics, and medical history of diabetes, hypertension, and medication use were recorded for each participant. Subjects with a fasting blood sugar level >6.67 mMol/l were considered diabetic and those with systolic/diastolic blood pressures >140/90 mm Hg were considered hypertensive as per WHO guidelines.

The study was conducted following guidelines defined by the Indian Council of Medical Research and the Declaration of Helsinki for undertaking human clinical research. An institutional ethics committee approved the IARS study, and voluntary signed informed consent was obtained from all participants.

### NA assay

The CMV neutralisation assay was carried out as described previously.[14] Briefly, serial twofold dilutions of the inactivated (56°C, 30 min) test serum were added to 96-well plates in duplicates. Guinea pig complement was mixed with $1\times10^4$ plaque-forming units/well of CMV (strain Towne (ATCC), grown and titred in MRC-5 cells (European Collection of Cell Cultures)) and added to serum-containing wells. The mixture of test sera, complement and CMV in the plates was incubated for 60 min at 37°C in a humidified 5% $CO_2$ incubator. MRC-5 cells were added at a density of $3\times10^4$ cells/well following the incubation period. Virus-infected cells in the absence of serum were used as a control for complete cytopathic effects (virus control). A pooled, normal human serum sample with a known CMV neutralisation titre was used as a technical control in every plate. Uninfected cells were plated as a cell-control in every set of experiments. Plates were incubated for 5 days. The final neutralising titre of the sera was determined when the total cytopathic effect developed in the virus control wells. The reciprocal serum dilution demonstrating ~99% protection against the cytopathic effect of the virus was recorded as the NA titre of the serum. The inter- and intra-assay coefficients of variation were 6.4% and 7.7%, respectively.

### Assay for CMV antibodies by ELISA

Levels of immunoglobulin G (IgG) antibody to CMV antigens in the serum were estimated using ELISA as per the manufacturer's instructions (Adaltis, Rome, Italy). Optical density values were converted into international units per millilitre of antibodies based on standards provided in the kit.

### Assay for inflammatory markers

Levels of inflammatory markers in the serum were estimated using ELISA as per the manufacturer's instructions: interleukin (IL)-6 (R&D systems, Minneapolis, Minnesota, USA), high-sensitivity C reactive protein (hsCRP) (Immune Diagnostic, USA), soluble intercellular cell adhesion molecule (sICAM) (R&D systems) and secretory phospholipase A2 (sPLA2; (Cayman Chemicals Company, Ann Arbor, Michigan, USA). Fibrinogen was analysed on the ACL 300 automated coagulation analyser (IL Systems, Milan, Italy) using clotting assays.

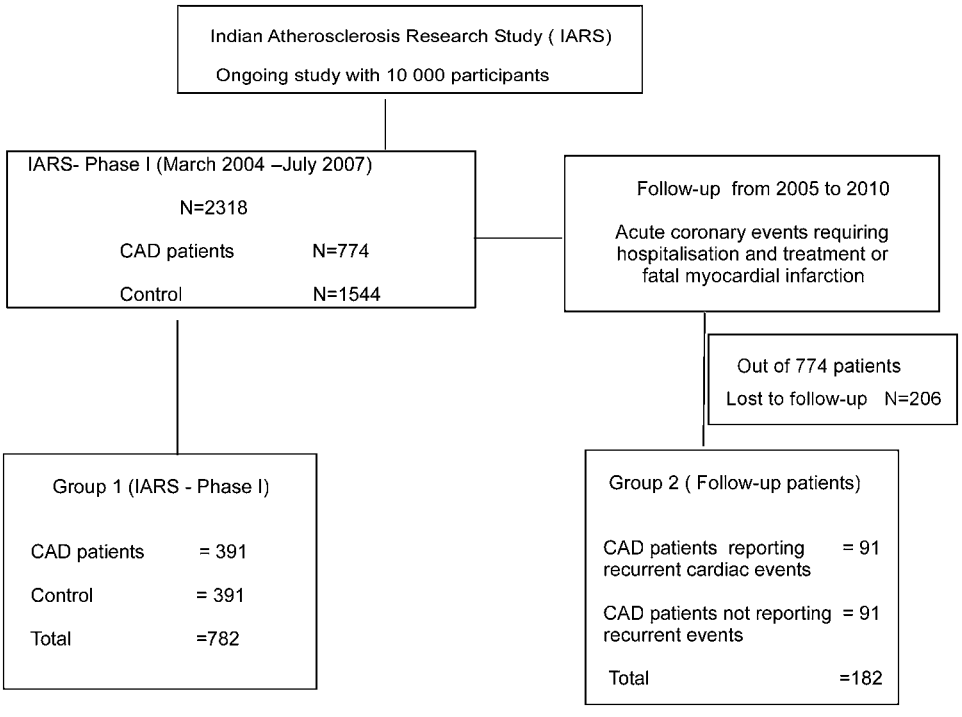


**Figure 1** Study design showing the details of patients and controls in the two groups of analysis. Group 1: The analysis was carried out in a population of 782 subjects, consisting of 391 consecutive coronary artery disease (CAD) patients compared with 391 controls with matched age and gender. Group 2: Analysis was carried out in 182 CAD patients, consisting of 91 subjects who reported a recurrent cardiac event matched to an additional 91 patients who did not report a recurrent event during the same time period of follow-up.

### Lipid assays

Total cholesterol, triglycerides and high-density lipoprotein cholesterol (Bayer Diagnostics, Randox Laboratories, Dade-Behring, UK) concentrations were determined on the Cobas Fara II Clinical Chemistry auto analyser (F. Hoffman La Roche, Basal, Switzerland), following the manufacturer's instructions. Serum low-density lipoprotein cholesterol was calculated using Friedwald's formula.[15]

### Statistical analysis

The quantitative data were tested for normality using Kolmogorov–Smirnov test and were transformed using natural logarithms wherever a deviation from normality was observed (lipids levels, inflammatory markers and antibody titres). The qualitative and categorical data were analysed using frequencies/proportions. Baseline characteristics were compared between the CAD patients and asymptomatic individuals (Group 1) and between patients reporting recurrent events and those not reporting an event (Group 2). To ensure the homogeneity in the sample, the individuals in the two groups were matched for age and gender. The epidemiological risk factors of hypertension, diabetes, hyperlipidaemia body mass index, waist to hip ratio, current smoking and statin use were used as covariates in the analysis of variance.

In the baseline information, the categorical variables were tested using $\chi^2$ tests and univariate analysis of variance and Student t test were used for testing the mean differences in various groups. Numerical results are expressed as mean (SEM). Multivariate analysis of variance was used to compare the mean levels of anti-CMV antibodies and CMV–NA titres and inflammatory markers between the different groups. To estimate the risk association of higher levels of anti-CMV antibodies and CMV–NA titres for CAD and its recurrence, the values are categorised into four quartiles. The risks of higher quartiles were assessed using logistic regression models using the first quartile as reference. In the logistic regression models with multiple biomarkers, risk associations were identified taking the first quartiles of both as the reference. ORs and their 95% CIs were obtained as estimates of associated risk for CAD. The receiver operating characteristic (area under the curve; AUC) analysis and C-Statistics are obtained to assess the contributing markers and as a criterion for model selection when multiple markers are used in the logistic regression. Statistical analyses were performed using SPSS V.17.0 for Windows and a p value $<0.05$ was considered statistically significant.

## RESULTS

### Baseline characteristics

Baseline characteristics of the study participants are shown in table 1. Mean age of the study population was 55.0±0.7 years. The prevalence of hypertension, diabetes and smoking was higher in patients compared with controls while lipid levels were lower in patients, corresponding to a higher statin use.

During a follow-up period of 5 years (median 2.5 years), 91 patients reported an acute coronary event, 19 of which were fatal. Prevalence of hypertension and smoking were higher in CAD patients who reported a recurrent cardiac event compared with patients without a recurrent event. Patients with recurring cardiac events also had higher mean levels of hsCRP, sPLA2 and fibrinogen (table 1).

### Mean levels of NA and anti-CMV IgG antibodies in study population

The mean baseline NA titres were significantly higher in CAD patients compared with asymptomatic individuals for unadjusted model ($p<0.001$), and after adjustment for established risk factors (body mass index, waist to hip ratio, hypertension, diabetes, hyperlipidaemia, current smoking) and statin use.

**Table 1** Baseline characteristics

| | Total population (n=782) Group 1 | | Follow-up patients (n=182) Group 2 | |
|---|---|---|---|---|
| **Variable** | **Asymptomatic individuals (n=391)** | **CAD patients (n=391)** | **Without recurrent event (n=91)** | **With recurrent event (n=91)** |
| Demographic | | | | |
| Age, years | 53.06±0.50 | 53.57±0.50 | 57.16±0.98 | 57.08±0.98 |
| Gender (male/female) | 67.6%/32.4% | 66.5%/33.5% | 80.2%/19.8% | 80.2%/19.8% |
| Presenting characteristic | | | | |
| BMI, kg/m$^2$ | 25.93±0.21 | 26.10±0.21 | 25.67±0.41 | 26.05±0.42 |
| Waist to hip ratio | 0.93±0.004 | 0.93±0.004 | 0.94±0.01 | 0.94±0.01 |
| Systolic blood pressure, mm Hg | 127.91±0.90 | 127.95±0.89 | 132.06±1.94 | 127.07±1.94 |
| Diastolic blood pressure, mm Hg | 83.65±0.48* | 81.72±0.48* | 83.52±0.99 | 81.30±0.99 |
| Hypertension | 30.3%* | 51.0%* | 47.3% | 65.9%* |
| Diabetes | 25.0%* | 46.8%* | 46.2% | 57.1% |
| Current smoking | 7.2%* | 10.9%* | 4.4% | 6.6%* |
| Statin use | 5.3%* | 69.5%* | 62.2% | 68.9% |
| Serum cholesterol, mMol/l | | | | |
| Total cholesterol* | 4.72±0.51* | 3.94±0.05 | 3.88±0.11 | 4.18±0.11 |
| Triglycerides* | 1.62±0.04 | 1.71±0.03 | 1.66±0.07 | 1.58±0.07 |
| High-density lipoprotein* | 1.04±0.01* | 0.96±0.01 | 0.93±0.02 | 0.99±0.02* |
| Low-density lipoprotein* | 2.94±0.04* | 2.20±0.04 | 2.19±0.09 | 2.46±0.10 |
| Inflammatory markers | | | | |
| hsCRP,* ug/ml | 3.53±0.27 | 3.78±0.26 | 3.47±0.55 | 3.69±0.66 |
| sPLA2,* pg/ml | 3699.49±179.73 | 4143.13±178.58 | 3609.10±387.10 | 3821.57±394.56 |
| IL-6,* pg/ml | 4.16±0.61* | 6.34±0.61* | 5.01±0.66 | 4.97±0.68 |
| Fibrinogen,* mg/dl | 3.85±0.05* | 4.12±0.05* | 4.15±0.10 | 4.05±0.10 |

Values are per cent or mean±SE of mean (SEM).
*$p<0.05$.
BMI, body mass index; CAD, coronary artery disease; hsCRP, high-sensitivity C reactive protein; IL-6, interleukin 6; sPLA2, secretory phospholipase A2.

**Table 2** Mean levels of CMV–NA titres and CMV antibody levels for the study population

| Model | Control (n=391) | CAD patients (n=391) | p Value |
|---|---|---|---|
| A. Group 1: CAD patients versus controls | | | |
| CMV–NA titres | | | |
| Unadjusted | 4.716±0.051 | 4.966±0.050 | <0.001 |
| Adjusted for risk factors* | 4.754±0.064 | 4.943±0.106 | 0.018 |
| Anti-CMV antibodies, IU/ml | | | |
| Unadjusted | 2.114±0.058 | 2.302±0.057 | 0.022 |
| Adjusted* | 2.104±0.074 | 2.373±0.121 | 0.13 |
| | CAD patients | | |
| Model | Without recurrent event (n=91) | With recurrent event (n=91) | p Value |
| B. Group 2: CAD patients with versus without recurrent events during follow-up | | | |
| CMV–NA titres | | | |
| Unadjusted | 4.652±0.097 | 5.160±0.099 | <0.001 |
| Adjusted for risk factors* | 4.745±0.126 | 5.203±0.132 | 0.003 |
| Anti-CMV antibodies, IU/ml | | | |
| Unadjusted | 2.168±0.117 | 2.270±0.117 | 0.54 |
| Adjusted* | 2.395±0.150 | 2.377±0.157 | 0.54 |

Values are log-transformed mean±SEM.
*Body mass index, waist to hip ratio, hypertension, diabetes, hyperlipidaemia, current smoking and statin use.
CAD, coronary artery disease; CMV, cytomegalovirus; NA, neutralising antibodies.

Although concentrations of anti-CMV IgG antibodies were higher in CAD patients (p=0.022) compared with controls, the difference became non-significant following adjustment for risk factors in patients with recurrent events versus without recurrent events (p<0.001) (table 2).

Mean baseline titres of CMV–NA were also significantly higher in patients reporting recurrent events (p<0.001) compared with those without one, but the anti-CMV IgG antibody levels were comparable between these two groups (table 2).

### Higher odds for CAD occurrence associated with CMV–NA versus anti-CMV IgG antibodies

Logistic regression analysis showed a strong association between CMV–NA titres and CAD occurrence when the fourth and first quartiles of CMV–NA levels were compared (OR 1.89, 95% CI 1.18 to 2.99, p=0.007) (table 3). The ORs increased and remained significant (OR 2.25, 95% CI 1.31 to 3.85, p=0.003) after adjusting for risk factors.

**Table 3** Comparison of risk association of CMV–NA and anti-CMV IgG antibodies with CAD

| | Unadjusted model OR (95% CI), p value | Adjusted for risk factors* |
|---|---|---|
| A. OR for CAD | | |
| CMV–NA | | |
| Q2 | 1.27 (0.76 to 2.12), 0.350 | 1.43 (0.79 to 2.61), 0.240 |
| Q3 | 1.06 (0.66 to 1.70), 0.800 | 1.16 (0.67 to 2.01), 0.591 |
| Q4 | 1.89 (1.18 to 2.99), 0.007 | 2.24 (1.31 to 3.85), 0.003 |
| CMV IgG antibodies | | |
| Q2 | 1.38 (0.93 to 2.08), 0.110 | 1.14 (0.71 to 1.83), 0.571 |
| Q3 | 1.56 (1.05 to 2.34), 0.029 | 1.60 (1.03 to 2.55), 0.048 |
| Q4 | 1.57 (1.05 to 2.35), 0.028 | 1.67 (1.04 to 2.69), 0.034 |
| B. OR for recurrent cardiac events | | |
| CMV–NA | | |
| Q2 | 1.56 (0.42 to 5.80), 0.500 | 1.32 (0.35 to 5.02), 0.680 |
| Q3 | 3.56 (1.01 to 12.64), 0.050 | 2.98 (0.74 to 11.96), 0.740 |
| Q4 | 5.68 (1.66 to 19.42), 0.006 | 4.65 (1.21 to 17.86), 0.025 |
| CMV IgG antibodies | | |
| Q2 | 1.00 (0.42 to 2.36), 0.999 | 0.95 (0.37 to 2.42), 0.900 |
| Q3 | 0.89 (0.38 to 2.07), 0.801 | 0.90 (0.36 to 2.22), 0.820 |
| Q4 | 2.26 (0.94 to 5.41), 0.067 | 2.35 (0.95 to 5.83), 0.064 |

ORs were calculated by logistic regression analysis using the first quartile as reference.
*Age, gender, body mass index, waist to hip ratio, hypertension, diabetes, current smoking, hyperlipidaemia and statin use.
CAD, coronary artery disease; CMV, cytomegalovirus; IgG, immunoglobulin G; NA, neutralising antibodies; Q, quartile of CMV–NA titre.

The ORs for the association between anti-CMV IgG and CAD were lower compared with CMV–NA for the unadjusted model (OR 1.57, 95% CI 1.05 to 2.35, p=0.028) as well as the adjusted model (OR 1.67, 95% CI 1.04 to 2.69, p=0.034) (table 3).

### Higher risk of recurrent cardiac events is associated with increased CMV–NA titres

The association between CMV–NA titres and recurrent CAD over the 4-year follow-up was assessed using age- and gender-matched patients who did not report a recurrent event. The risk of a recurrent event increased in the higher quartiles of CMV–NA titre. The OR was 1.32 (0.35 to 5.02), p=0.68 for the second quartile, 2.98 (0.74 to 11.96), p=0.743 for the third quartile and was the highest at 4.65 (1.21 to 17.86), p=0.025 when the CMV–NA titres were in the fourth quartile (table 3). CMV IgG antibodies did not show a significant association with recurrent cardiac events (OR of 2.35 (0.95 to 5.83), p=0.064 for the highest quartile).

### Risk association of CMV–NA titres combined with inflammatory markers

To further examine the discriminatory power of the CMV–NA titres in the presence of other inflammatory markers, risk association and c-statistics analysis were carried out. The ORs for CAD occurrence ranged between 2.24 and 2.68, with AUC values ranging between 0.783 to 0.785, when NA titres and biomarkers in the fourth quartile were compared with the first quartiles. Only marginal increases in the OR were observed in the presence of hsCRP (2.68 (1.52 to 4.73), p=0.001) and fibrinogen (2.32 (1.35 to 3.99), p=0.002) compared with that of CMV–NA alone (2.25 (1.31 to 3.85), p=0.003) (table 4).

The ORs for recurrent cardiac events increased from 4.65 (1.21 to 17.86), p=0.025 for CMV–NA alone to 7.82 (1.87 to 32.65), p=0.005 in the presence of sPLA2 and CMV–NA titres (table 4). Similar increases were observed in the presence of IL-6 (5.56 (1.50 to 20.65), p=0.010) and fibrinogen (5.05 (1.37 to 18.55), p=0.015). The addition of inflammatory markers did not

**Table 4** Association of CMV-NA and anti-CMV IgG antibodies with CAD and recurrent cardiac events in the presence of inflammatory markers

| | CMV−NA | | CMV IgG | |
|---|---|---|---|---|
| **Model** | **OR (95% CI), p Value** | **AUC, p Value** | **OR (95% CI), p Value** | **AUC, p Value** |
| A. Association of CMV−NA and anti-CMV IgG antibodies with CAD in the presence of other inflammatory markers | | | | |
| Antibody titres alone | 2.24 (1.31 to 3.85), 0.003 | 0.783, <0.001 | 1.67 (1.04 to 2.69), 0.034 | 0.777, <0.001 |
| + IL-6 | 2.15 (1.23 to 3.74), 0.007 | 0.784, <0.001 | 1.47 (0.91 to 2.38), 0.114 | 0.774, <0.001 |
| + sPLA2 | 2.17 (1.27 to 3.70), 0.017 | 0.782, <0.001 | 1.57 (0.98 to 2.50), 0.060 | 0.775, <0.001 |
| + hsCRP | 2.68 (1.52 to 4.73), 0.001 | 0.774, <0.001 | 1.48 (0.91 to 2.40), 0.116 | 0.765, <0.001 |
| + Fibrinogen | 2.32 (1.35 to 3.99), 0.002 | 0.785, <0.001 | 1.47 (0.92 to 2.34), 0.105 | 0.778, <0.001 |
| B. Association of CMV−NA and anti-CMV IgG antibodies with risk of recurrent cardiac events in the presence of other inflammatory markers | | | | |
| Antibody titres alone | 4.65 (1.21 to 17.86), 0.025 | 0.702, <0.001 | 2.35 (0.95 to 5.83), 0.064 | 0.674, <0.001 |
| + IL-6 | 5.56 (1.50 to 20.65), 0.010 | 0.729, <0.001 | 2.51 (0.98 to 6.38), 0.053 | 0.697, <0.001 |
| + sPLA2 | 7.82 (1.87 to 32.65), 0.005 | 0.712, <0.001 | 1.62 (0.57 to 4.58), 0.363 | 0.701, <0.001 |
| + hsCRP | 2.31 (1.39 to 3.85), 0.001 | 0.715, <0.001 | 1.77 (0.57 to 4.58), 0.779 | 0.684, <0.001 |
| + Fibrinogen | 5.05 (1.37 to 18.55), 0.015 | 0.703, <0.001 | 2.81 (1.07 to 7.36), 0.036 | 0.691, <0.001 |

ORs were computed for antibody levels in the fourth quartile in the presence of quartiles of inflammatory markers using the first quartile as reference.
The models were adjusted for age, gender, body mass index, waist to hip ratio, hypertension, diabetes, current smoking, hyperlipidaemia and statin use.
AUC, area under the curve; CAD, coronary artery disease; CMV, cytomegalovirus; hsCRP, high-sensitivity C reactive protein; IgG, immunoglobulin G; IL-6, interleukin-6; NA, neutralising antibodies; sPLA2, secretory phospholipase A2.

influence the discriminatory power of CMV−NA, since the AUC values only increased from 0.702 to a maximum of 0.729 (in the presence of IL-6) (table 4). The addition of inflammatory markers to total anti-CMV antibodies did not result in a significant OR for CAD or recurrent cardiac events (table 4).

## DISCUSSION

In the present study, we report a significant association between CMV−NA titres and CAD, and more importantly, recurrent cardiac events, in an Indian population. We also demonstrate an increased risk of recurrent cardiac events in CAD patients in the presence of sPLA2 or IL-6 along with CMV−NA in the same model. The increase in ORs in the presence of inflammatory markers was highly significant with CMV−NA titres but not with anti-CMV IgG ELISA antibodies. These results establish the importance of estimating clinically relevant antibodies and are in line with our hypothesis that estimating specific antibodies gives a better understanding of the association between infection and CAD.

CAD is currently the most prevalent non-communicable disease in India and is a leading cause of morbidity and mortality.[16 17] Indians are also reported to have a higher incidence and prevalence of CAD at a younger age compared with other populations, leading to a greater social and economic impact.[17 18] Greater concern in recent years has been a steady increase in CAD related deaths in the rural Indian population who show a higher prevalence of cardiovascular risk factors including diabetes, hypertension and obesity.[16] Preventive medical care is less accessible for the rural Indian population and consequently these patients have a higher risk of acute coronary syndromes. Infection and related burden of pathogen are also reported to be higher in the Indian population.[19] We have previously noted that pathogen burden, especially CMV infection, plays an important role in CAD occurrence in the Asian Indian population (unpublished observation).

Viral glycoproteins mediate the entry of virus into the susceptible cells while antibody response against glycoproteins is an important defence mechanism, as these antibodies are capable of neutralising infectious viruses.[6 7] High titres of NA have been associated with improved survival after infection while a decline in their titre was associated with viral reactivation in immunosuppressed patients.[20] Since CMV can infect cardiac smooth muscle cells, reactivation of the virus may accelerate atherogenic process by activating the immune inflammatory responses. Although ELISA can measure antibodies for specific antigens from CMV, neutralisation of viral entry into cells can establish biological activity of CMV antigen specific antibodies. Therefore, estimation of NA against CMV should correlate better with CAD and recurrent cardiac events in patients.

In the present study, we found significantly higher mean levels of CMV−NA titres, but not total antibodies to CMV, in CAD patients compared with asymptomatic controls. The basal mean CMV−NA titres were also significantly higher in patients reporting a recurrent cardiac event compared with those without any recurrent events, suggesting that CMV−NA titres are more relevant for association studies. In substantiation, we observed 1.5-fold higher ORs for CAD occurrence and recurrent cardiac events with CMV−NA titres compared with total CMV antibodies.

Inflammation plays a major role in the manifestation of CAD. Systemic infections can promote inflammatory reactions in the vessel wall leading to progression of atherosclerosis.[21] Antigens from CMV and the viral DNA have been identified in the atherosclerotic lesions of human and mice, suggesting an inflammatory role of CMV in the progression of the disease.[4] Presence of the virus in the vessel wall induces smooth muscle-cell proliferation and migration, increased uptake of oxidised low-density lipoproteins, expression of inflammatory cytokines and an increased procoagulant activity leading to disease progression.[21] CMV can also cause molecular mimicry wherein an immune response to viral antigens can cross react with self-peptides expressed on uninfected host cells.[21 22] Thus, multiple mechanisms are likely to contribute to the risk association between CMV infection and CAD.

CMV infection is known to increase plasma interferon-γ (IFN-γ) and tumour necrosis factor α (TNF-α) levels in rodents and induce IL-6 release, triggering the release of acute-phase proteins such as C reactive protein and exacerbating local inflammation.[23] These inflammatory cytokines can stimulate sPLA2 release from vascular smooth muscle cells, the proposed link between inflammation and lipid accumulation in atherosclerosis.[24] Serum levels of sPLA2 show an association with CAD risk and recurrent CAD events.[25] In humans, sPLA2 has been detected in both early and advanced atherosclerotic lesions. Interestingly, the presence of CMV DNA in the lesion is associated with sPLA2 expression and inflammation.[26] The protein has also been suggested to have a role in inflammatory damage to the heart ensuing in infarcted myocardium.[27] We chose IL-6, hsCRP and sPLA2 as markers of a subsequent inflammatory

response after CMV infection, as these are directly related to virus-induced pathogenesis. Although addition of inflammatory markers to CMV–NA titres resulted in only minor changes in the OR for CAD occurrence, CAD patients in the highest quartile of CMV–NA and sPLA2 had a 4.7-times increase in the OR for recurrent cardiac events. Addition of inflammatory markers to total CMV antibodies had minor or no change in the OR for CAD occurrence as well as recurrent events. These observations suggest that CMV infection as measured by NA levels in combination with inflammatory markers such as sPLA2 may improve risk prediction in the Asian Indian population.

Earlier studies have suggested that individuals with a subclinical inflammatory profile, as measured by CRP levels, have a higher susceptibility to CMV-induced atherogenic effects.[28] In our study, we did not observe an association between CRP levels and CMV infection, probably because of high statin use in the patient population, which has been reported to downregulate C reactive protein levels.[29]

The strengths of our study include the cohort of relatively young Asian Indian CAD patients, and age- and gender-matched asymptomatic controls, the assessment of covariates, and adjustment for confounding factors. The limitations comprise short follow-up and the collection of samples after the CAD event, which limit causal interpretation of the relationship between CMV–NA titres and CAD risk. During this time, we had only 21 asymptomatic individuals reporting a new event. Based on a follow-up of 8–10 years to predict cardiovascular risk, as described in major studies,[30] our future studies will include a larger cohort and longer follow-up to strengthen our hypothesis. Micro-neutralisation assay to estimate CMV–NA titres is time- and labour-intensive compared with ELISA. We are also developing methods to reduce the assay time without compromising its sensitivity.

In summary, our results reinforce the importance of CMV infection and the significance of estimating clinically relevant antibodies to pathogens in cardiovascular risk assessment. Further validation in different ethnic populations is required to confirm our hypothesis.

**Acknowledgements** We gratefully acknowledge the support of the trustees of Thrombosis Research Institute, London and Bangalore, the Tata Social Welfare Trust, India (TSWT/IG/SNB/JP/Sdm), and the Department of Biotechnology, Ministry of Science and Technology, Government of India (BT/01/CDE/08/07). The sponsors did not participate in the design, conduct, sample collection, analysis and interpretation of the data, or in the preparation, review or approval of the manuscript. We are indebted to Dr Sophie Rushton-Smith (Thrombosis Research Institute, London), for editorial assistance. We thank all investigators, staff, administrative teams and participants of the IARS from Narayana Hrudayalaya, Bangalore, and Asian Heart Centre, Mumbai, for their contribution. We are grateful to the patients, their family members and the unaffected subjects for participating in the study.

**Contributors** The study was conceptualised by VVK and EG, while LM designed and coordinated the same. VE and EG standardised the protocol and HS and MV carried out the antibody assays under the supervision of LM. Statistical analysis were designed and carried out by SH. Interpretation of the results were carried out by SH and LM. VSR designed and carried out the assays for inflammatory markers. LM wrote the first draft of the paper. VE and EG participated in the scientific discussions and improvement of the draft and it was finalised by VVK.

**Funding** Tata Social Welfare Trust, India (TSWT/IG/SNB/JP/Sdm), and the Department of Biotechnology, Ministry of Science and Technology, Government of India (BT/01/CDE/08/07).

**Competing interests** None.

**Patient consent** Obtained.

**Ethics approval** Ethics approval was provided by Indian Council of Medical Research.

**Provenance and peer review** Not commissioned; internally peer reviewed.

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*Heart* 2012 98: 982-987
doi: 10.1136/heartjnl-2012-301850