Chapter 4
Methods
4. Methods

4.1. Study design

4.1.1. Ethical clearance

The investigation and biological sample collections were approved by the Institutional ethics committees of Thrombosis research Institute and Narayana Hrudayalaya hospital and conformed to the Declaration of Helsinki and the Indian Council of Medical Research (ICMR, India) guidelines. Informed consent was taken prior to enrolment from all the study participants.

4.1.2. Study population

The study included patients and respective controls from the same ethnic group of the Indian population. The study included patients in the age group 18-60 years, admitted to the cardiology department of the hospital between October 2012 and December 2015 with no previous report of cardiac problem. Samples were collected from patients and controls (Figure 4.1.1. and 4.1.2.). Patients enrolled were categorized based on the severity of the disease. 1) Patients admitted for coronary angiography with chest pain and more than 50% block in the coronary arteries (Stable angina: SA). 2) Those admitted with acute myocardial infarction requiring emergency treatment with a positive test for creatine kinase MB and troponin I (ACS). The ACS was further categorized into UA, NSTEMI and STEMI. The UA group reported chest pain for a prolonged duration at rest or with minimal effort with documented transient ST-segment elevation or ST-segment depression of 0.1 mV in at least two contiguous electrocardiographs (Figure 4.2). Pain in the chest for more than 30 min within 24 h before enrolment and a positive test for
creatine kinase MB and troponin I levels were classified as MI. Based on the elevation of the ST segment in at least two contiguous electrocardiographs, the patients were further classified as STEMI or NSTEMI. In house cardiologist, who was part of the study, was involved in the proper classification of patients. 3) The sample was collected during the recurrent cardiac event who had diagnosed for a cardiac event earlier. 4) Healthy individuals from the same ethnic population with no previous medical record or symptoms of coronary artery disease, no chest pain on rest or under exertion and normal ECG report (Figure 4.2).

**Figure 4.1.1 Study population distribution**

![Study population distribution](image)

Total samples collected in the study
Figure 4.1.2 Study population distribution

Total sample size = 532

Healthy subjects N=192
CAD patients N=315
Previous history of CAD N=25

Stable Angina N=78
ACS N=237

UA N=23
NSTEMI N=34
STEMI N=180

Recurrent Event N=23
Responsive to medication
Improvement in condition N=19

Previous history of CAD N=25

Age and Gender matched

Healthy N=34
SA N=34
UA N=23
NSTEMI N=34
STEMI N=34
Recurrent Event N=23
Improvement in condition N=19
Previous history of CAD N=25

Total samples used for flowcytometry and sample group where the samples for cell based assays were selected.

Figure 4.2 Electrocardiographs
4.1.2.1. Clinical data recorded

In the study, the history of hypertension, diabetes mellitus, smoking, body mass index, waist-hip ratio, and medication was recorded using a standardized questionnaire. The fasting blood glucose levels, resting blood pressure, ECG, and angiogram records (patients) were obtained for all the participants. Diabetes at the time of recruitment was defined on the basis of reported physician diagnosis and use of anti-diabetes medications. Subjects with high fasting blood sugar levels >7 mm/litre (125 mg/dL) were categorized as hyperglycemic. Hypertension at the time of recruitment was defined based on doctor’s report, use of antihypertensive drugs. Subjects were considered as active smokers if they were current smokers or had stopped smoking within a month before entry into the study.

4.1.2.2. Exclusion criteria

Study participants having the following criteria will be excluded from the study

- Presence of any major illness defined according to WHO criteria,
- Known primary myocardial disease (hypertrophy or dilative Cardiomyopathy)
- Presence of congenital heart disease
- Presence of any contagious disease
- Participation of subjects in another clinical trial within 30 days of recruitment into the study
- Having a family history of CAD

4.1.3. Follow-up and re-sampling

Study participants were followed up after one year from the date of enrollment into the study. Any new events/ recurrent cardiac events were recorded. Resampling was
done from subjects who showed improvement in condition between 6-12 months after
treatment and volunteered to continue in the study.

4.1.4. Sample size calculation

Sample size calculation was done and the sample size required for the study was calculated as 385 for 5% error and 95% confidence level.

4.1.5. Samples collected

Total sample collected was 604, comprising of control (203) and patients (401). Patients include 376 subjects collected during the first event and 25 subjects collected during the second event.

4.1.6. Method of samples collection and storage

Peripheral blood samples were collected using a K3 EDTA vacutainer tube (BD USA) within 24 hours of identification of subjects. Peripheral blood mononuclear cells (PBMCs) from the whole blood were isolated using Ficoll (Histopaque-Sigma Chemicals, USA) density gradient centrifugation and used for cell-based assays or frozen in liquid nitrogen for future use. Plasma samples were stored at -80°C until use.

4.2. Biochemical measurements in plasma

Total cholesterol, triglycerides and high-density lipoprotein cholesterol (Bayer Diagnostics, Randox Laboratories, Dade-Behring Ltd, United Kingdom), were determined using the Cobas Fara II Clinical Chemistry autoanalyzer, (F Hoffman La Roche Ltd, Switzerland), following manufacturer’s instructions. Low-density lipoprotein cholesterol level was calculated using Friedwald’s formula [221]. APO B 100 was calculated as described by Hwang et al. [222].
4.3. Assays

4.3.1. Flow Cytometry

PBMCs were activated for 4 hours with phorbol myristate acetate (10 ng/ml) and ionomycin (1 μg/ml) in the presence of 100 ng/ml Brefeldin (Sigma Chemicals, St. Louis, USA) and labeled with specific antibodies. Surface staining was performed by using anti-CD3 and anti-CD4 antibodies conjugated to Allophycocyanin H7 (APC H7) and Fluorescein isothiocyanate (FITC) respectively for 20 minutes at room temperature. Intracellular staining was carried out with Allophycocyanin (APC) conjugated anti-IL-17, Peridinin-chlorophyll Cy5.5 conjugated anti-IL-4, Phycoerythrin conjugated anti-IL-10 and PE CY7 conjugated anti-IFN-γ. CD3⁺CD4⁻ cells were considered as CD8 cells. For regulatory T cell staining, APC conjugated CD25 and Phycoerythrin (PE) conjugated anti-human FOX P3 were used in a separate tube along with CD3 and CD4 as mentioned earlier. Surface staining for monocytes was performed by using anti CD14, anti CD16, anti-HLA-DR and anti-CD192 antibodies conjugated to Phycoerythrin (PE), Fluorescein isothiocyanate (FITC), APC CY7 and APC respectively for 20 minutes at room temperature in a separate tube. For Apoptotic T cells staining anti-Annexin V -PE and anti-7AAD- PerCP Cy5.5 were labeled along with CD4, CD3, and CD25 as mentioned earlier in a separate tube. All antibodies were purchased from BD Biosciences, USA. Samples were acquired using FACS Canto II flow Cytometer using FACS Diva software (Becton Dickinson, NJ, USA). Fluorescence minus one and unstained control were used for setting the gates. FLOWJO Version 7.6.5 (Tree star Ltd, Oregon, USA) was used for data analysis and results were expressed as a percentage of CD4⁺ T cells and monocytes by sequential gating on lymphocytes and monocytes.
4.3.2. Cell proliferation assays

PBMCs were used for cell proliferation assays. CFSE dilution method was used. Cells were cultured with or without antigens Ox-LDL, HSP 60 and PHA. Ox-LDL and HSP 60 were self-antigens used for antigen-specific stimulation and PHA for non-specific stimulation as a positive control. Cell proliferation experiments were performed in X-Vivo medium (Bio Whittaker, Walkersville, MD, USA) supplemented with 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), sodium pyruvate, and antibiotics. T cell proliferation was monitored by the reduction in 5, 6-carboxyfluorescein diacetate succinimidyl ester (CFSE) fluorescence in the CD3 positive cells for 3 days. PBMCs (10^7/ml) were labeled with 10 μM CFSE (Sigma Chemicals, USA) and activated with antigens (10 μg/mL). Purified HSP 60 and human LDL were purchased from Sigma Chemicals, St. Louis, USA. LDL was oxidized overnight with CuSO4 (5μM) to get copper oxidized LDL (Ox-LDL). Phytohaemagglutinin (PHA) and unstimulated cells were used as positive and negative control respectively. Cells were restimulated with the same antigen in the presence of 100 ng/ml Brefeldin for the last 4 hours of culture. After 72 hours of incubation, cells were stained with anti-CD3-APC CY7 (BD Biosciences, clone, CA, USA), anti-IL-17-APC (BD Biosciences, CA, USA) and analyzed by flow cytometry as described earlier. Lymphocytes were gated using forward and side scatter plots. The proliferation of T cells was measured by CFSE dilution using FACS CANTO II (Becton Dickinson, New Jersey, USA) and analysed using FlowJO software. The proliferation index of T cells was calculated from the FlowJO software. Proliferation index of test wells was divided by that of control wells without antigen for analysis.
4.3.3. Treg and Teff functional assay

The regulatory and effector T cells were purified from PBMC by human CD4+CD25+ Regulatory T Cell Isolation Kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) following manufacturers instruction. CD4+ T cell purified were further sorted into CD4+CD25- effector T Cells (Teff) and CD4+CD25+ Regulatory T Cell (Treg). Teff cells were labeled with CFSE and plated as 5x10^4 cells/ wells with/without regulatory T cells in the ratio 1:1 and cultured in the presence of HSP 60, Ox-LDL and PHA for 72 hours. Cells after incubation were acquired on a flow cytometer and analysed for CFSE dilution.

4.3.4. Foam cell formation assay

Foam cell formation assay was carried out as reported by Larigauderie et al. [223,224]. The CD4+CD25+ regulatory T cells and CD4+CD25+(IL17+) effector T cells were isolated as mentioned earlier. THP1 cells (2x10^5) were cultured in the presence of Treg or Teff cells for 40 hours in RPMI medium. Nonadherent T cells were removed and 50µg/ml of Ox-LDL was added to the wells and incubated for 18 hours. THP1 cells were labeled for ORO and ORO uptake was estimated by absorbance at 492nm.

4.3.5. Monocyte to macrophage conversion assay

Monocytes were isolated using monocytes isolation kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The monocytes were cultured alone or co-cultured with effector T cells or regulatory T cells, which isolated as mentioned earlier. After 72 hours, the non-adherent T cells (effector and regulatory T cells) were removed from the supernatant and the adherent monocytes were continued in culture for another 4 days. On
day7, the adherent cells were harvested for total RNA isolation and gene expression analysis was performed.

4.3.6. Glucose assays

4.3.6.1. Cell proliferation assays on high glucose

PBMCs were cultured in normal (5 mM) and high (15 mM) glucose culture for 72 hours. PBMCs (10^7/ml) cultured were prior labeled with 10 μM 5, 6-carboxyfluorescein diacetate succinimidyld ester (CFSE) (Sigma Chemicals, USA) and activated with HSP 60, Ox-LDL and PHA (10 μg/mL). Cells were restimulated with the same antigen in the presence of 100ng/ml Brefeldin for the last 4 hours of culture and stained with anti-CD3-APC CY7 (BD Biosciences, California, USA), anti-IL-17-APC (BD Biosciences, California, USA) for flow cytometry. The proliferation of T cells was measured by CFSE dilution using FACS CANTO II (Becton Dickinson, New Jersey, USA) and analysed using FlowJO software. The proliferation index of T cells was calculated and divided by that of control wells without antigen for analysis.

4.3.6.2. Apoptotic Tregs assay

PBMCs were cultured in culture medium containing normal (5 mM) and high (15 mM) glucose for 18 hours. After incubation, the cells were washed and labeled for apoptotic markers. The cells were labeled for apoptotic Tregs with CD4+, CD3+, CD25+, Annexin V and 7AAD for determining early apoptotic, late apoptotic and necrotic Tregs.
4.3.6.3. Effect of high glucose and incubation period on monocytes

THP1 cells were cultured under normal (5mM) and high glucose (15mM) conditions for 24 hours, 48 hours and 72 hours. After the respective culture period, THP1 cells were harvested for total RNA was isolation and gene expression analysis was performed.

4.4. Cytokine detection

Plasma concentrations of cytokines interleukin (IL6) and interleukin 17 (IL17) were measured by multiplex assay by Milliplex kits using the Luminex platform (Merck –Millipore, USA).

4.5. ELISA

4.5.1. Seropositivity to pathogens

Seropositivity for the infection of H pylori (Genesis, UK), CMV (BioCheck, Inc, US) and CPN (Calbiotech, USA) was determined by kit method following manufactures instructions.

4.5.2. Autoantigen and autoantibody levels

4.5.2.1. Level of autoantigens in circulation

Human HSP 60 and Ox-LDL antigen estimation kits were bought from Wuhan fine biological Technology Co, LTD (Wuhan, China). The antigen concentration of HSP 60 and Ox-LDL in the serum was measured by ELISA as per the manufacturer’s instructions. Briefly, 100µl of standards controls or samples were added to the pre-coated plates incubated for 2hrs at 37°C. Contents of the plate were removed and 100µl of detection reagent A was added to wells without washing the plates which further
incubated for 1 hour at 37°C. The plate was washed three times with the wash buffer and 100µl detection reagent B was added followed by incubation for 60 minutes at 37°C. The plates were developed using TMB substrate and the absorbance read at 450nm. LDL and ApoB levels were analysed by clinical chemistry analyser as described earlier.

4.5.2.2. Measurement of autoantibodies to antigens

IgG and IgM autoantibodies to ApoB 100, HSP 60, Ox-LDL and LDL were determined by in-house ELISA determined by coating ApoB 100 (1 µg/ml), HSP 60 (1 µg/ml), Ox-LDL (10 µg/ml) and LDL (5 µg/ml) in ELISA plates. Antigens were coated in high absorbant immunoplate (Nunc) using bicarbonate buffer and incubated for 1 hour in 37°C and 4°C overnight. Plates were blocked with 2% milk proteins for 1 hour at 37°C. Plasma samples (1:100) were added to the plate and incubated at 37°C for 90 minutes. Antihuman IgG or IgM antibodies (1:2000) were added and incubated for 60 min at 37°C. ELISA plates were developed using TMB substrate, and absorbance was measured at 450nm with a reference wavelength of 570nm. Two peptides were taken for ELISA based on earlier data from the lab on immunoregulatory studies in mice [190,225]. These were the epitope derived from human ApoB 100 (peptide sequence: CI_{688}EIGLEGKGFPEPTLEALFGK_{707}, numbered including signal peptide) equivalent to p45 reported in the literature and an epitope from hHSP 60 (peptide sequence: CA_{153}ELKKQSKPVT_{163}) was used as HSP60 peptide. IgG antibodies to peptides were determined using ELISA. Maleimide-activated 96 well plates (Pierce, Thermo Scientific, USA) were coated with peptides individually. Peptides (1 µg/ml) were coated on Maleimide activated 96 well plates (Pierce, Thermo Scientific, USA). Cysteine (10 µg/ml) was used to block excess maleimide and were developed and measured as
mentioned earlier. The pooled plasma from normal human was used as a control in all the plates. About 10% of the samples were repeated at random and few samples were repeated at different positions in the same assay plate to increase the reliability of the antibody measurement. Inter and intraassay coefficients of variation were lower than 8% and 10% respectively. The pooled plasma from normal human was used as a control in all the plates.

4.5.3. Human HbA1c/Glycosylated hemoglobin A1c ELISA

The working solutions provided with the kit were brought to room temperature (37°C) before starting the assay. The pre-coated plate was washed two times before the addition of standards, sample and control. 100 µl of standard, control and samples (Diluted 1:100 in sample dilution buffer) was added to the wells and incubated at 37°C for 90 min. After incubation, the contents of the plate was discarded, followed by the addition of 100 µl of biotin detection antibody without washing the plate and incubated for 60 min at 37°C. After incubation, the content of the plate was discarded and the plate was washed three times. 100 µl/well of SABC working solution was added and incubated at 37°C for 30 min. The plate was washed five times which was followed by the addition of TMB substrate and incubated for 30 min dark at 37°C. After incubation 50 µl of stop solution was added and immediately read at 450 nm.

4.6. Gene Expression Analysis

The total RNA from PBMCs, monocytes, and THP1 cells after the assays were isolated using TRIzol® reagent and mRNA was reverse transcribed using cDNA synthesis kit (Thermo Scientific, USA). Polymerase chain reaction (RT-PCR) was performed using SYBR Green qPCR kit (Thermo Scientific, USA) using a 7900HT Fast
Real-Time PCR system from Applied Biosystems (Applied Biosystems, California, and USA). The primers used to study gene expression were shown in Table 4.1.

Table 4.1 Primers used for studying the expression

<table>
<thead>
<tr>
<th>Primers</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL23</td>
<td>TGC AAA GGA TCC ACC AGG GTC TGA</td>
<td>TAG GTG CCA TCC TTG AGC TGC TGC</td>
</tr>
<tr>
<td>IL17A</td>
<td>AAT CTC CAC CGC AAT GAG GA</td>
<td>ACG TTC CCA TCA GCG TTG A</td>
</tr>
<tr>
<td>IL22</td>
<td>ACA ACA CAG ACG TTC GTC TCA TTG</td>
<td>GAA CAG CAC TTC TTC TAC AGG GGT GA</td>
</tr>
<tr>
<td>ROR Ɣ</td>
<td>CTC ACC GAG GCC ATT CAG TAC</td>
<td>CTG GTC ATT CTG GCA GAG CTC</td>
</tr>
<tr>
<td>IL6</td>
<td>ACT CAC CTC TTC AGA ACG AAT TG</td>
<td>CCA TCT TTG GAA GGT TCA GGT TG</td>
</tr>
<tr>
<td>Foxp3</td>
<td>GCA CCT TCC CAA ATC CCA GT</td>
<td>GGC CAC TTG CAG ACG ACA CCA T</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>GGA CAC CAA CTA TTG CTT CAG</td>
<td>TCC AGG CTC CAA ATG TAG G</td>
</tr>
<tr>
<td>β-actin</td>
<td>GGA TGC AGA AGG AGA TCA CTG</td>
<td>CGA TCC ACA CGG AGT ACT TG</td>
</tr>
<tr>
<td>IL10</td>
<td>CAT CGA TTT CTT CCC TGT GAA</td>
<td>TCT TGG AGC TTA TTA AAG GCA TAC</td>
</tr>
<tr>
<td>IL1B</td>
<td>AAG GGC TGC TTC CAA ACC TTT GAC</td>
<td>ATA CTG CCT GCC TGA AGC TCT TGT</td>
</tr>
<tr>
<td>IL8</td>
<td>ATG ACT TCC AAG CTG GCC GT</td>
<td>TCC TTG GCA AAA CTG CAC CT</td>
</tr>
<tr>
<td>NFkB</td>
<td>ATG GCT TCT ATG AGG CTG AG</td>
<td>GTT GTT GGT GGT CTG GAT GC</td>
</tr>
<tr>
<td>TNF</td>
<td>CCC AGG GAC CTC TCT CTA ATC</td>
<td>ATG GGC TAC AGG CTT GTC ACT</td>
</tr>
<tr>
<td>MCP1</td>
<td>TGC AGA GGC TCG CGA GCT A</td>
<td>CAG GTG GTC CAT GGA ATC CTG A</td>
</tr>
<tr>
<td>hsXBP1</td>
<td>CTGAGTCCGAAATCAGGTGCAG</td>
<td>ATCCATGGGGAGATGTTCTGG</td>
</tr>
<tr>
<td>hATF4</td>
<td>GTTCTCCAGCGACAAGGCTA</td>
<td>ATCCTGCTTGCTGTTGTTGG</td>
</tr>
<tr>
<td>hBiP</td>
<td>TGTCAACCAATTATCAGCAAACTC</td>
<td>TTCTGCTGATCCCTTCCTTACAGT</td>
</tr>
<tr>
<td>CHop</td>
<td>AGAACCAGGAAACGGAACAGA</td>
<td>TCTCCTTCATGCGCTGCTTT</td>
</tr>
</tbody>
</table>
The table contains a list of primers used to study gene expression in PBMCs of patients and control.

4.7. Markers used

To understand the immune response among the Indian CAD population different markers was used (Table 4.2). The markers included represents inflammatory and anti-inflammatory response, infection, and antigen specificity.

Table 4.2: Markers used in the study

<table>
<thead>
<tr>
<th>Peripheral blood</th>
<th>Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Leukocytes</strong></td>
<td>Cytokines and Chemokines</td>
</tr>
<tr>
<td><strong>Monocyte subsets</strong></td>
<td>IFN-Ɣ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12(p70), IL-17, IP-10, MCP-1, MCP-3, CD40L and TNF-Α</td>
</tr>
<tr>
<td><strong>Mast cells</strong></td>
<td>CD23, CD203C, CD117 and FcεRIα</td>
</tr>
<tr>
<td><strong>T cell subtypes</strong></td>
<td><strong>Infections</strong></td>
</tr>
<tr>
<td>Th1 / Tc1</td>
<td>CD3⁺CD4⁺IFNG</td>
</tr>
<tr>
<td>Th2 / Tc4</td>
<td>CD3⁺CD4⁺IL4</td>
</tr>
<tr>
<td>Th17 / TC17</td>
<td>CD3⁺CD4⁺IL17</td>
</tr>
<tr>
<td>CD4⁺CD28⁻ cells</td>
<td>CD4⁺CD28⁻</td>
</tr>
<tr>
<td>Plasma</td>
<td>IgG</td>
</tr>
<tr>
<td>Native LDL</td>
<td>IgA</td>
</tr>
<tr>
<td>Antigen specificity</td>
<td>T regulatory/CD3⁺CD4⁺CD25⁺FOXP3 antibody response and antigen concentration</td>
</tr>
<tr>
<td>---------------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>Native LDL, Ox-LDL, Apo B 100 and Human HSP 60</td>
<td>IL6, IL17A, IL22, IL23, ROR-Ɣ, FOXP3, TGF-β, GRP 78, ATF4, CHOP, unspliced XBP1 and spliced XBP1</td>
</tr>
</tbody>
</table>

### 4.8. Statistical Analysis

Normality of quantitative data was tested using the Kolmogorov-Smirnov test and was log transformed for analysis (lipids levels, BMI, WHR, and cytokine levels). The percentage data were arcsine transformed for analysis. The qualitative and categorical data were analyzed using frequencies/proportions using Chi-square tests and quantitative data were compared by independent T-test. Inflammatory cell score was calculated to indicate low-grade systemic inflammation ranging 0 to 5, one point was given for a value greater than the median of the cohort sample for each of the cellular markers (Th17, Tc17, Th17/Tregs, Tc17/Tregs and classical monocytes). T cells, monocytes and inflammatory cell score were compared by multivariate analysis by taking age, gender as covariates. Numerical results of the markers are expressed as the mean ± standard error of the mean (SEM). Logistic regression analysis was performed by taking age, gender, current smoking, BMI, WHR, diabetes, and hypertension as covariates. For T cell, monocytes and inflammatory cell score risk association for CAD, the logistic regression analysis was performed by taking CAD as dependent variable and age, gender, BMI,
WHR, current smoking, hypertension and diabetes as covariates. For the comparison of 
subjects with and without hypertension, current smoking, BMI, WHR, diabetes were 
taken as covariates. Comparison of diabetic and nondiabetic individuals included 
hypertension as a covariate along with current smoking, BMI, WHR. Odds Ratios and 
their 95% CIs were obtained as estimates of associated risk. Correlation analysis was 
performed between T cells, monocytes subsets, inflammatory cell score and age, systolic 
blood pressure, diastolic blood pressure, fasting blood sugar level, IL6, IL17A, and 
infections for H pylori, CMV and CPN using Pearson's bivariate correlation analysis. 
Antibody levels in controls and cases were compared using multivariate analysis using 
epidemiological risk factors such as age, gender, current smoking, BMI, WHR, diabetes, 
and hypertension as covariates. Regression and correlation analysis was carried out only 
with the ACS subjects in comparison to control. To estimate the risk/protection 
association of lower levels of antibodies the values are categorized into tertiles. The 
protection associated with higher tertiles was assessed using logistic regression models 
using the first tertile as reference. In the logistic regression models with multiple 
biomarkers, risk associations were identified taking the first tertiles of both as the 
reference. ORs and their 95% CIs were obtained as estimates of associated risk for CAD. 
Correlation analysis of the antibody level to the classical risk factors, antigen level, and T 
cell subsets and cytokine level was performed using the Pearson bivariate correlation. All 
the statistical analyses were performed using SPSS version 17.0 for Windows and p-
value <0.05 was considered statistically significant.