

## Induction of the Chemokines CCL3 $\alpha$ , CCL3 $\beta$ and CCL5 in Atherosclerotic Patients

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**Abstract:** Chemokines recruit immune cells to inflammatory sites and promote the process of inflammation. The role of these mediators in the disease process in atherosclerosis is not fully studied. The spontaneous mRNA expression and intracellular protein production of the potential inflammatory chemokines CCL3 $\alpha$  and CCL3 $\beta$  (macrophage-inflammatory protein-1 $\alpha$  and  $\beta$ ; CCR3 $\alpha/\beta$  ligand) and CCL5 (regulated upon activation, normal T cell expressed and secreted (RANTES; CCR5 ligand) in atherosclerotic patients was examined together with the effects of the chlamydial antigen HSP60 and LPS on the gene expression and protein induction of these mediators. Detection of chemokine mRNA and protein levels was assessed by in situ hybridization and immunohistochemistry respectively. The examined chemokines were detected at significantly high levels on atherosclerotic patients compared to healthy controls at both mRNA and protein levels. Stimulation with HSP60 and LPS from *Chlamydia pneumoniae* (*C. pneumoniae*) and *E. coli* showed increased numbers of CCL3 $\alpha$ , CCL3 $\beta$  and CCL5 mRNA expressing cells in patients compared to health controls. Protein translation of these chemokines was depicted in correspondence to the mRNA gene expression for all examined chemokines spontaneously and after stimulation with chlamydial HSP60 and LPS and *E. coli* LPS. Thus, the herein data demonstrate the induction of potential inflammatory chemokines in atherosclerotic patients and that bacterial antigens play a role in the immunopathologic events in this disease by generating more inflammatory mediators.

**Key words:** Atheroma, inflammation, mRNA, *Chlamydia pneumoniae*, *E. coli*

### INTRODUCTION

Atherosclerosis is a pathological process that takes place in the major arteries and is the underlying cause of heart attacks, stroke and peripheral artery disease [1]. It is no longer considered a disorder of lipid accumulation, but a disease process characterized by the dynamic interaction between endothelial dysfunction, subendothelial inflammation and the 'wound healing response' of the vascular smooth muscle cells [2]. The principal cell types of the artery wall the endothelial cell, the smooth muscle cell and the monocyte/macrophage are major players in the events involved in initiation and evolution of the atherosclerotic plaque [3]. Other important participants are platelets and lymphocytes, which have modulating influences on smooth muscle cell, endothelial cell and macrophage behavior. The earliest detectable lesions, called fatty streaks, contain macrophage foam cells that

are derived from recruited monocytes. More-advanced atherosclerotic lesions, called fibro-fatty plaques, are the result of continued monocyte recruitment and smooth muscle cell migration and proliferation. Variable numbers of CD4<sup>+</sup> T cells are found in atherosclerotic lesions, and cytokines secreted by T helper 1 (Th1) - or Th2-type cells can have a profound influence on macrophage gene expression within atherosclerotic plaques [4].

Chemokines are small disulphide-linked polypeptides of typically 60-70 amino acids that act as potent chemoattractants for many cell types including monocytes, macrophages, neutrophils and T-cells [5]. They are important for constitutive trafficking and recruitment of leukocytes in response to inflammatory mediators [6, 7, 8, 9]. Some chemokines that can act as potent mediators of monocyte migration and macrophage differentiation are expressed in human atherosclerotic lesions [10]. Furthermore, adipose cell enlargement was found to lead to a pro-inflammatory

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state in the cells with reduced secretion of adiponectin and with increased secretion of several cytokines and chemokines including interleukin (IL)-6, IL-8, and MCP-1. The chemokine MCP-1 has been shown to play an important role for the associated recruitment of macrophages into the adipose tissue [11]. Also, recent study demonstrated that circulating chemokines may accurately identify individuals with clinically significant atherosclerotic heart disease [12].

A number of risk factors for atherogenesis, including infectious agents, have been shown to exert their influence via inflammatory means. There is a growing evidence that *Chlamydia pneumoniae* (*C. pneumoniae*) may be involved in the pathogenesis of atherosclerosis, as several studies have demonstrated the presence of the organism in atherosclerotic lesions [13]. *C. pneumoniae* can initiate and propagate inflammation in ways that could contribute to atherosclerosis. Infected leukocytes may serve to disseminate an infection from the lung to other susceptible tissues including arteries [14, 15]. *C. pneumoniae* also may influence atheroma biology by modulating macrophage-lipoprotein interactions. Infected macrophages ingest excess low-density lipoprotein to become cholesteryl ester-laden foam cells, the hallmark of early lesions in atherosclerosis [16, 17]. In addition, *C. pneumoniae* induces monocytes to oxidize lipoproteins, converting them to highly atherogenic forms [18]. *C. pneumoniae*-induced foam cell formation is mediated chiefly by lipopolysaccharide, whereas lipoprotein oxidation occurs mainly by CHSP60 [19].

In view of the depicted potential role for chemokines in inflammation and their suggested role in atherosclerosis in addition to the possibility that infection with *C. pneumoniae* may be an underlying contributing cause for the development of atherosclerosis, the present work studied the spontaneous induction of three potential chemokines (CCL3 $\alpha$ , CCL3 $\beta$  and CCL5) at gene and protein levels and, furthermore, the effect of chlamydial antigens in the expression of these chemokines was examined.

## MATERIALS AND METHODS

**Study Participants:** Patients presented to Bahrain Defense Force (BDF) Hospital with coronary artery disease undergoing angiograms (n = 15) of both sexes, age >35 year were included in the study, after the purpose of the study and its implications are fully explained to them. Control subjects comprised healthy controls (n = 15). Characteristics and clinical data of

patients and controls are included in table 1. All subjects (patients and controls) were asked to fill a consent form and sign it, indicating their acceptance to participate in the study. Venous blood samples (15 ml) were collected in EDTA tubes after angiogram was done and confirmed pathologic changes, and were stored at room temperature pending lymphocyte isolation.

**Lymphocytes isolation:** For lymphocyte isolation, peripheral blood cells were isolated by overlaying carefully the diluted blood on Ficoll and centrifugation at 3200 rpm for 20 min at room temperature, followed by discarding the plasma layer and collection of the buffy coat layer. Peripheral blood lymphocytes were then washed for 3 times with 1X (Phosphate Buffered Saline Ph7.2 (PBS).

**Cell cultures:** Lymphocytes were maintained in culture medium RPMI 1640 (ICN, Biomedicals, Inc.) supplemented with 5% fetal bovine serum, 1M HEPES buffer, 50 $\mu$ mole Mercapto ethanol, 2ml of 200 mM L-glutamine, and 5IU/5 $\mu$ g penicillin streptomycin (ICN, Biomedicals, Inc.). The cells were transferred to adhesion slides (BioRad Lab, Munich, Germany) and were stimulated by 5pg chlamydial and *E. coli* LPS or 5ng chlamydial HSP60 (Gift from Dr. M. Majeed, Division of Medical Microbiology, Linkping University, Sweden). The cells were then incubated for overnight at (37°C-in 5%CO<sub>2</sub>). Some negative control cells were left without stimulation. The positive control cells were stimulated by 5 $\mu$ g of phytohemagglutinin (PHA) (Sigma, Germany).

**Detection of cytokine mRNA expression by in situ hybridization:** *In situ* hybridization was performed as previously described [20]. Briefly, 200  $\mu$ l aliquots of suspensions containing 4x10<sup>5</sup> mononuclear cells (MNC) were plated into round-bottomed microtiter plates (Nunc) in triplicate. 10  $\mu$ l aliquots of 5pg chlamydial LPS or 5ng chlamydial HSP60 or *E. coli* LPS or PHA were added into appropriate wells. After culture for 24 h, the cells were washed, counted and applied onto restricted areas of electronically charged glass slides (ProbeOn slides; Fisher Scientific, Pittsburgh, PA). Synthetic oligonucleotide probes (Scandinavian Gene Synthesis AB, Köping, Sweden) were labeled using 35S deoxyadenosine-5'- $\alpha$ -(thio)-triphosphate with terminal deoxynucleotidyl transferase (Amersham). To increase the sensitivity of the method, a mixture of four different probes was employed for each cytokine. The oligonucleotide sequences were

obtained from GenBank using MacVector software. Cells were hybridized with  $10^6$  cpm of labeled probe per 100  $\mu$ l of hybridization mixture. After emulsion autoradiography, development and fixation, the coded slides were examined by dark field microscopy for positive cells containing more than 15 grains per cell in a star-like distribution. The intracellular distribution of the grains was always checked by light microscopy and expressed as numbers per  $10^4$  MNC. In many positive cells, the grains were so heavily accumulated within and around the cells that it was not possible to count every single grain. In cells judged negative, the numbers of grains were mostly 0 - 2 per cell, and the grains were scattered randomly over the cells and not distributed in a star-like fashion. There were therefore no difficulties in differentiating between positive and negative cells. Variation between duplicates was < 10%. A control probe used in parallel with the cytokine probe on cells from each rat revealed no positive cells.

**Intracellular detection of chemokines by immunohistochemistry:** Cells fixation was performed in methanol for 5min. Slides were incubated with avidin-biotin blocking kit (Vector Laboratories, Burlingame, CA) to block endogenous biotin or biotin-binding proteins. Cells were permeabilized with 0.1% saponin dissolved in PBS to allow the intracellular access of the cytokine-specific antibody. A total of 30  $\mu$ l chemokine specific monoclonal antibody (mAb) (mouse anti-human CCL3 $\alpha$ , CCL3 $\beta$  and CCL5; R&D Systems, Oxon, UK) diluted in PBS-saponin to a final concentration of 5 $\mu$ l/ml was added and allowed to incubate for overnight at 4°C followed by several washes in PBS. Non-specific staining by the second-step biotinylated goat antibody caused by Fc-interactions was prevented by a subsequent incubation with 1% normal goat serum (Dako, patts, Glostrup, Denmark) dissolved in PBS-saponin for 30 min at room temperature. The biotin-conjugated secondary antibodies were then added. The cells were incubated with an avidin-biotin horse-radish peroxidase complex (vectasain, Vector Laboratories) for 30 min in the dark at room temperature. A color reaction was developed by 3- diminnobenzidine tetra hydrochloride (DAB) (Vector Laboratories) and stopped after 2-10 min by washes in PBS. The cells were counterstained with hematoxylin and the slides were left to dry before mounting in DPX (Fluka, Switzerland). The immunocytochemically stained cells were examined in a Leica RXM microscope (Leica, Wetzlar, Germany) equipped with a 3CDD color camera (Sony, Tokyo, Japan). Counting of cytokine producing cells was performed manually using X100 objective. The frequency of cytokine expressing cells was assessed by examination of at least 10.000 cells.

Table 1: Characteristics of Patients and controls

CHARACTERISTICS	PATIENTS (N=15)	CONTROLS (N=15)
Age (years, range )	54.3	49.9
Gender (n, %)	6 Female (35.71%) 9 Male (64.29%)	6 Female (35.71%) 9 Male (64.29%)
Hypertension (%)	84.4	0
Diabetes (%)	59.4	0
Hyperlipidemia (%)	65.9	0
Glucose (3.4-6.1mmol/l)	7.6	4.6
Triglyceride (0.11-2.15mmol/l)	1.8	0.9
Cholesterol (3.88-6.47mmol/l)	5.4	3.8
LDL-chol. (<3.4mmol/l)	3.8	1.8
HDL-chol. (F= 0.91-1.168 mmol/l) (M= 0.91-2.07mmol/l)	1.17	1.0
WBC (4.4-11 $\times 10^3/\mu$ l)	8.72	6.98
Platelets count (150-450 $\times 10^6/\mu$ l)	250.8	230.9

**Statistical analysis:** Student's unpaired *t*-test was used to measure statistical significance between two groups. In all tests  $p < 0.05$  was taken as the level of significance. (\*= $p < 0.05$ , \*\*= $p > 0.005$ , \*\*\*= $p > 0.0005$ ).

## RESULTS AND DISCUSSION

The measurement of inflammatory mediators was conducted because atherosclerosis has been considered as an unusual form of chronic inflammation occurring within the artery wall [21]. Significantly high levels of spontaneously expressed of CCL3 $\alpha$ , CCL3 $\beta$  and CCL5 at mRNA level in patients compared to healthy controls ( $p < 0.0005$ ) was detected. Cells stimulated with CHSP60 showed highly significant

differences ( $p < 0.0005$ ) between patients and healthy control, but lower significance for the cells which were stimulated with both chlamydial and *E. coli* LPS ( $p < 0.005$ ) was depicted. PHA was used as control, but there were no significant differences between of CCL3 $\alpha$ , CCL3 $\beta$  and CCL5 mRNA levels induced in the cells after stimulation with PHA between patients and healthy controls. However, PHA stimulation was much higher in both patients and healthy controls compared to non-stimulated cells (Fig 1 A-C; Fig 3 A-D).

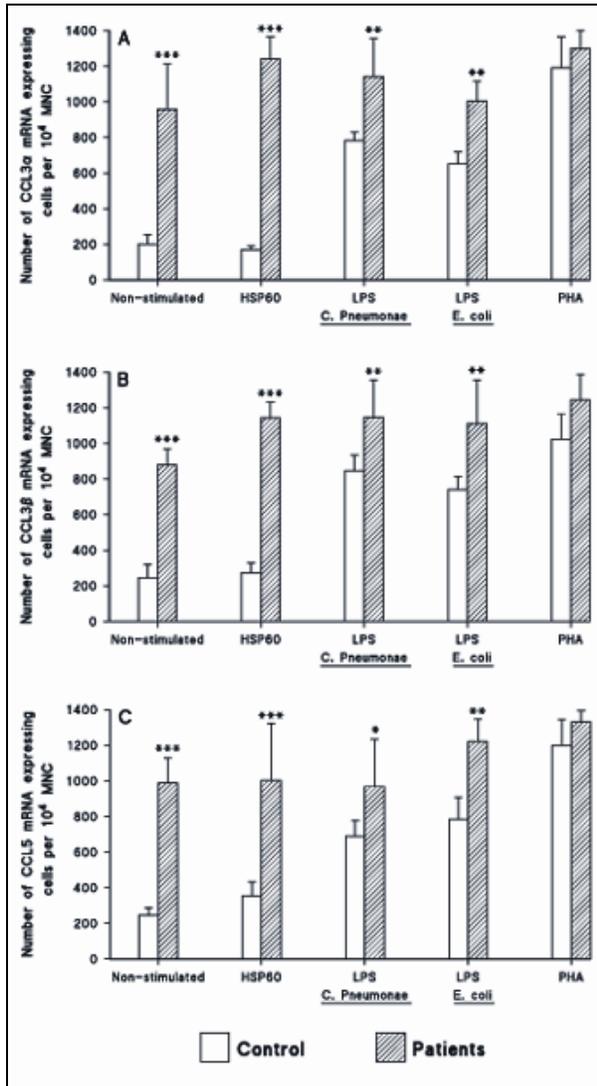


Fig. 1: Numbers of mRNA expressing cells for (A) CCL3 $\alpha$ , (B) CCL3 $\beta$  and (C) CCL5 among mononuclear cells from atherosclerotic patient challenged with chlamydial and *E. coli* LPS and HSP60. Means  $\pm$ SD are shown.

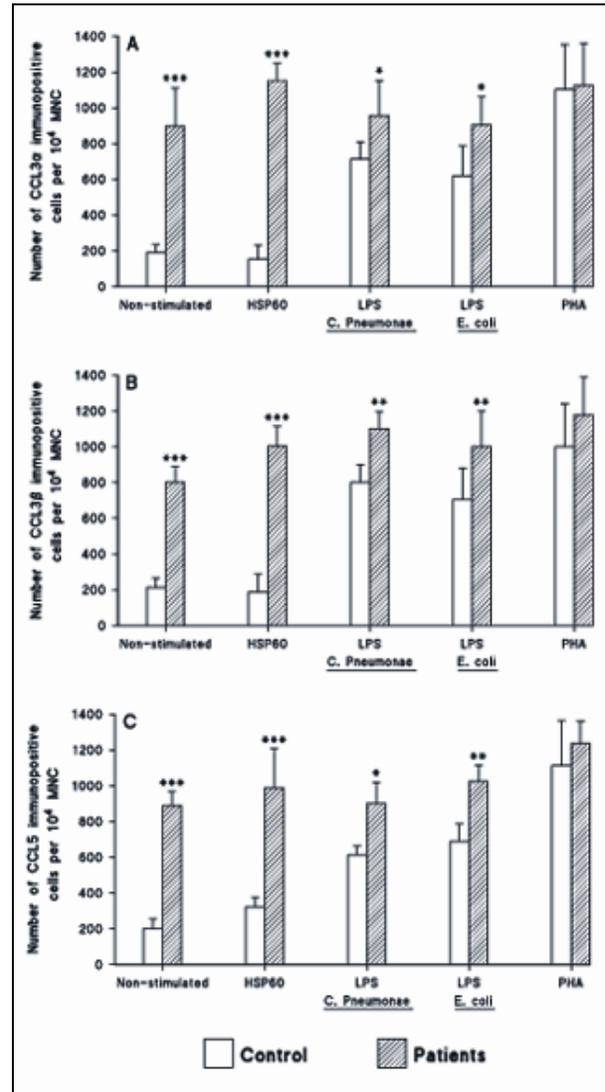


Fig. 2: Numbers of immunopositive cells for (A) CCL3 $\alpha$ , (B) CCL3 $\beta$  and (C) CCL5 among mononuclear cells from atherosclerotic patient challenged with chlamydial and *E. coli* LPS and HSP60. Means  $\pm$ SD are shown.

To ensure that the mRNA expression has resulted in actual protein production generated during the immunopathogenetic events of the disease, the levels of CCL3 $\alpha$ , CCL3 $\beta$  and CCL5 protein was measured by immunohistochemistry. As found in the measurement of mRNA for CCL3 $\alpha$ , CCL3 $\beta$  and CCL5, a significantly high levels of spontaneously expressed CCL3 $\alpha$ , CCL3 $\beta$  and CCL5 protein levels in patients compared

to healthy controls was demonstrated ( $p < 0.0005$ ). Also, cells stimulated with CHSP60 showed high significant differences ( $p < 0.0005$ ) compared to low significance for the cells stimulated with chlamydial and *E. coli* LPS ( $p < 0.05$ ) for CCL3 $\alpha$ , ( $p < 0.005$ ) for CCL3 $\beta$  and CCL5. Again, PHA was used as control and showed no significant differences in CCL3 $\alpha$ , CCL3 $\beta$  and CCL5 levels between the patients and control groups, but increased levels when compared to non-stimulated cells (Fig 2 A-C; Fig 3 E-H).

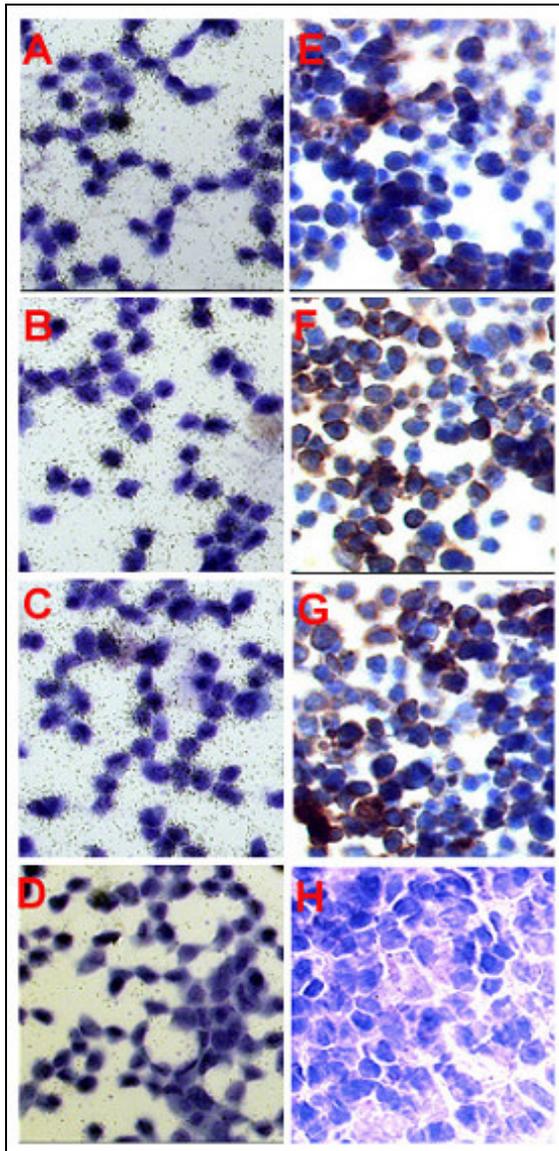


Fig. 3 A- Cells spontaneously expressing mRNA for CCL3 $\alpha$  produced by human mononuclear cells culture on slide.

**B-** Cells spontaneously expressing mRNA for CCL3 $\beta$  produced by human mononuclear cells culture on slide.

**C-** Cells spontaneously expressing mRNA for CCL5 produced by human mononuclear cells culture on slide.

**D-** Sense control.

**E-** Intercellular immunohistochemistry staining for CCL3 $\alpha$  produced by human mononuclear cells culture on slide.

**F-** Intercellular immunohistochemistry staining for CCL3 $\beta$  produced by human mononuclear cells culture on slide.

**F-** Intercellular immunohistochemistry staining for CCL5 produced by human mononuclear cells culture on slide.

**H-** Non-stimulated cells (negative control).

The photographs were taken in bright field microscopy (X200)

Evidence that inflammation is a risk factor for cardiovascular diseases is based on studies that show conditions caused by inflammatory states are at increased risk of cardiovascular disease. This has led to the search for inflammatory markers which could be of predictive value in terms of outcome, enabling early clinical intervention and thus, important mediators as the inflammatory chemokines CCL3 $\alpha$ , CCL3 $\beta$  and CCL5 were selected for this study. The data showed spontaneous upregulation of all examined chemokines at both gene and protein levels.

Chemokines exert their effects, in part, through mediating leukocytic infiltration into the vessel. Recent studies have determined that chemokines and their receptors are present, and function on other cellular components comprising the arterial wall, such as the endothelium and vascular smooth muscle [22]. The chemokines monocyte chemoattractant protein (MCP)-1 and macrophage colony-stimulating factor (M-CSF) are the only chemokines that demonstrated to contribute to coronary artery disease upon analysis of human coronary atherectomy specimens [23]. Thus, our herein data provide an evidence for the role of CCL5, CCL3 $\alpha$  and CCL3 $\beta$  in atherosclerosis as produced by mononuclear cells. Some chemokines that can act as potent mediators of monocyte migration and macrophage differentiation are expressed in human atherosclerotic lesions [24]. Considering such the roles for the above examined mediators and the recent findings indicating the potential role of infectious

agents in the pathogenesis and progression of atherosclerosis and the fact that among different microorganisms suspected, *C. pneumoniae* had represented as the most plausible pathogen to have a link in lesion development of atherosclerosis [25], we addressed the induction of the selected chemokines in response to the chlamydial antigen HSP60 and LPS. LPS from *E. coli* was used as control. The data illustrated the role of chlamydial antigens HSP60 and LPS in directing the immune response to inflammation via chemotactic activity by stimulating production of effective chemokines as CCL3 $\alpha$ , CCL3 $\beta$  and CCL5 in atherosclerotic patients.

Our chemokine data showed upregulation of CCL3 $\alpha$ , CCL3 $\beta$  and CCL5 indicating their activity in chlamydial infection. Their significant induction in patients with atherosclerosis compared to healthy individuals proposing that cells from atherosclerotic patients may specifically recognize these antigens suggesting the presence of chlamydia in patients with atherosclerosis as previously demonstrated [13]. This was confirmed by the absence of significant difference between patients and healthy subjects in all examined chemokines when using PHA as a positive control. Hence, another confirmation for the role of chlamydia in the pathogenesis of atherosclerosis is provided since chlamydial antigens stimulated essential inflammatory chemokines. It might not be only Chlamydia that might be involved in this activity, but other bacteria such as *E. coli* could contribute to the inflammatory process of atherosclerosis since LPS from *E. coli* did highly and significantly stimulate cells from patients compared to healthy subjects. Such effect has not been reported before, but on the other hand LPS from both *C. pneumoniae* and *E. coli* was recently reported to induce macrophage foam cell formation in a murine model, a mechanism that was mediated by Toll-like receptor 2 for *C. pneumoniae*, but not *E. coli* [26].

In conclusion, this work demonstrates the production of inflammatory chemokines spontaneously and after challenges with chlamydial and *E. coli* antigens. The recognition of these antigens by mononuclear cells from patients with atherosclerosis and the ensuing production of chemokines suggest important role for these bacterial infections in the immunopathogenetic events occurring in atherosclerosis.

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