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# The presence of some cytokines and *Chlamydia pneumoniae* in the atherosclerotic carotid plaque in patients with carotid artery stenosis

## Obecność *Chlamydia pneumoniae* a ekspresja wybranych cytokin w blaszce miażdżycowej krytycznie zwężonej tętnicy szyjnej

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## Summary

**Background:** Over the last few years the role of microorganisms in the pathogenesis of atherosclerosis has been widely discussed. *Chlamydia pneumoniae* activates immune cells to produce cytokines that are responsible for the formation of atheromatous carotid lesions.

**Material and methods:** The study was carried out at the Department of Vascular, General and Transplantation Surgery, Wroclaw Medical University, in 2002-2003, on 100 consecutive symptomatic patients with internal carotid stenosis, who underwent an endarterectomy procedure. Each patient had their carotid artery sampled in order to find *C. pneumoniae* DNA using the nested PCR method and some cytokines (TGF-β, VEGF, FGF, TNF-α) using immunohistochemical examination. The control group consisted of 20 young organ donors who had been diagnosed with brain death and who had their healthy carotid artery harvested. Analogous genetic and immunohistochemical tests were performed.

**Results:** We did not confirm the presence of either cytokines or *C. pneumoniae* in the healthy carotid arteries. The presence of FGF was probably due to intima fibroblast activity, which is responsible for elastin and collagen synthesis for the extracellular matrix. *C. pneumoniae* was discovered in 68% of patients with carotid plaques. Three cytokines (TGF-β, FGF, TNF-α) were detected in atherosclerotic internal carotid arteries as well.

**Conclusion:** Chronic infection by *C. pneumoniae* may exacerbate carotid plaque development and may lead to its destabilization.

**Key words:** cytokines • Chlamydia pneumoniae (CP) • carotid artery stenosis • carotid endarterectomy (CEA)

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**Abbreviations:** **CEA** – carotid endarterectomy, **ECM** – extracellular matrix, **FGF** – fibroblast growth factor, **ICA** – internal carotid artery, **IHC** – immunohistochemistry, **IRS** – immunoreactive score, **PAOD** – peripheral artery occlusive disease, **PCR** – polymerase chain reaction, **TGF-ß** – transforming growth factor, **TNF-α** – tumor necrosis factor, **VEGF** – vascular endothelial growth factor.

## Introduction

Nowadays, atherosclerosis is considered to be a chronic inflammatory disease, and the balance between inflammation and extracellular matrix deposition is thought to be important for the maintenance of plaque stability. Therefore, research on the pathogenesis of carotid artery atherosclerosis is of great value. Hyperlipidemia, arterial hypertension, tobacco use and diabetes are well-known conventional risk factors, but little information is available about the influence of any bacterial or viral infection on arterial wall changes [1,2]. There is accumulating evidence that certain infectious agents play a role in the pathogenesis of atherosclerosis [3,5]. It has been proved that active inflammatory cells produce cytokines [6]. However, it is not known which cytokines are responsible for the development and destabilization of the carotid plaque [8]. *C. pneumoniae* was first isolated in Taiwan in 1965. In 1986, it was first described as a cause of acute respiratory tract infection, and it was given the acronym TWAR (Taiwan acute respiratory agent). TWAR was renamed *C. pneumoniae* in 1989. The first suggestion that *C. pneumoniae* may be associated with atherosclerosis was proposed in 1988 by Saikku.

## Material and methods

The study was carried out on 100 consecutive symptomatic patients (71 men and 29 women), aged between 46 and 79 years (with a mean age of 65.5), who had internal carotid artery (ICA) stenosis. The research protocol was approved by the ethics committee of Wroclaw Medical University (approval no. 739/2003). The patients were informed about the study and gave their written consent to taking part in it. All the patients had their medical history taken, underwent routine biochemical laboratory tests (Table 1) and also had a Doppler ultrasound by which plaque, flow parameters and the stage of stenosis were recorded. All the patients had a brain CT or MRI examination. The patients were qualified for surgery according to NASCET and ECBT criteria (those who had internal carotid artery stenosis of 70% or more). Patients underwent elective carotid endarterectomy (CEA) with patch angioplasty or eversion CEA. 36% of patients had had at least one stroke episode, and 64% did not have any symptoms of a stroke. Patients with diabetes or cancer were excluded from the study. During the endarterectomy internal carotid arterial wall specimens were excised to examine the presence of some cytokines: transforming growth factor (TGF-ß), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and tumor necrosis factor (TNF-α). They were assessed by a semi-quantitative immunohistochemical method (antibody/cytokine) on a four-step scale (0, 1+, 2+, 3+) through an analysis of the grade of staining intensity. An immunoreactive score (IRS) was given, as described by Remmele and Stegner (1987). The IRS is the effect of staining intensity: 0 – no reaction (no positive cells), 1-2 weak reaction (<10% positive cells), 3-4 intermediate reaction (10-50% positive cells), 6-12 strong reaction (>50% positive cells).

Over the 3-year period, 7 patients died and 2 patients were not contactable for follow-up, so the study group consisted of 91 patients.

Group 1 consisted of patients with *C. pneumoniae* found in the carotid arterial plaque, and Group 2 consisted of patients without *C. pneumoniae*.

Comparisons between cases and controls were performed using the Friedman ANOVA test, the non-parametric Mann-Whitney U test, and the Pearson chi-squared ($\chi^2$) test as appropriate. Comparisons were made using Spearman's rank correlation method. $P<0.05$ was considered as statistically significant.

The control group consisted of 20 young organ donors (12 men and 8 women), aged between 20 and 28 (the mean age being 25), who were confirmed brain dead. During the organ-harvesting surgery they had their normal internal carotid artery sampled in order to perform a PCR test for *C. pneumoniae* and an immunohistochemical test for TGF-β, VEGF, FGF, and TNF-α.

| Parameter | | Study group | | Control group | P |
|---|---|---|---|---|---|
| | | group II (patch) | group I (eversion) | Donors | 1–2 |
| age (years) | | 65,5 | 66,4 | 24±4 | NS |
| sex | Men | 35 | 36 | 12 | NS |
| | Women | 15 | 14 | 8 | NS |
| History of stroke | | 21 | 17 | 0 | NS |
| TIA | | 51 | 49 | 0 | NS |
| History of heart disease | | 48 | 45 | 0 | NS |
| Arterial hypertension | | 47 | 45 | 0 | NS |
| Diabetes | | 0 | 0 | 0 | ---------- |
| Atherosclerosis | | 32 | 26 | 0 | NS |
| Smoking | | 41 | 38 | 6 | NS |
| Fibrinogen | | 370±93 | 375±105 | 0 | NS |
| LDL-cholesterol | | 146±48 | 147±42 | 0 | NS |
| HDL-cholesterol | | 48,5±10 | 47,4±9,37 | 0 | NS |
| Triglyceride (TG) | | 150,9±66 | 165±70,28 | 0 | NS |
| ASA (-) | | yes | yes | 0 | NS |

## Results

A total of 120 tests for the DNA of *C. pneumoniae* were performed using the nested-PCR method. No *C. pneumoniae* was found within the internal carotid artery of the 20 healthy donors. In the study group *C. pneumoniae* was discovered in 68% of patients (85% were men, 15% were women) (Figure 1).

The three cytokines TGF-β, VEGF, and TNF-α were not found in any of the 20 healthy donors in the control group. In 25% of the healthy donors (5 people) FGF was confirmed by a weak positive IHC reaction. FGF was not found in 75% of the healthy donors (15 people).

In the study group no cytokines were discovered in 10 (11%) of the patients, and all four cytokines were present in 6 (7%) of the patients. The only time that no cytokines were found was in patients who did not have the *C. pneumoniae* infection. In contrast, the presence of all four cytokines was observed only in patients with a *C. pneumoniae* plaque infection which had been confirmed by nested PCR.

TGF-β cytokine: No cytokine was found in 57 (63%) patients, a weak reaction was present in 30 patients, an intermediate reaction in 2 patients and a strong reaction in 2 patients. In total, TGF-β cytokine was present in 37% of the patients.

VEGF cytokine: No cytokine was found in 70 (77%) patients, a weak reaction was present in 15 patients, an intermediate reaction in 6 patients, and no patient showed a strong reaction. In total, VEGF cytokine was present in 23% of the patients.

FGF cytokine: No cytokine was found in 12 (13%) patients, a weak reaction was present in 30 patients, an intermediate reaction in 27 patients, and a strong reaction in 22 patients. In total, FGF cytokine was present in 79 (87%) patients. In 49 (54%) patients there was increased FGF and an intermediate or strong reaction was observed. It should be remembered that the cytokine FGF was present in 25% of the donors of the control group as well.

TNF-α cytokine: No cytokine was found in 55 (60%) patients, a weak reaction was present in 27 patients, an intermediate reaction in 7 patients, and a strong reaction in 2 patients. In total, TNF-α cytokine was present in 36 (40%) patients.

A statistically significant correlation between the presence of all four cytokines and the positive DNA-PCR test for *C. pneumoniae* was observed. The strongest correlation was observed between FGF and *C. pneumoniae* ($R=0.729$, $p<0.05$). FGF was present in the atheromatous plaque of all the patients with *C. pneumoniae*. Cytokines TNF-α and TGF-β were present in 50% of the patients with *C. pneumoniae*. Statisti-

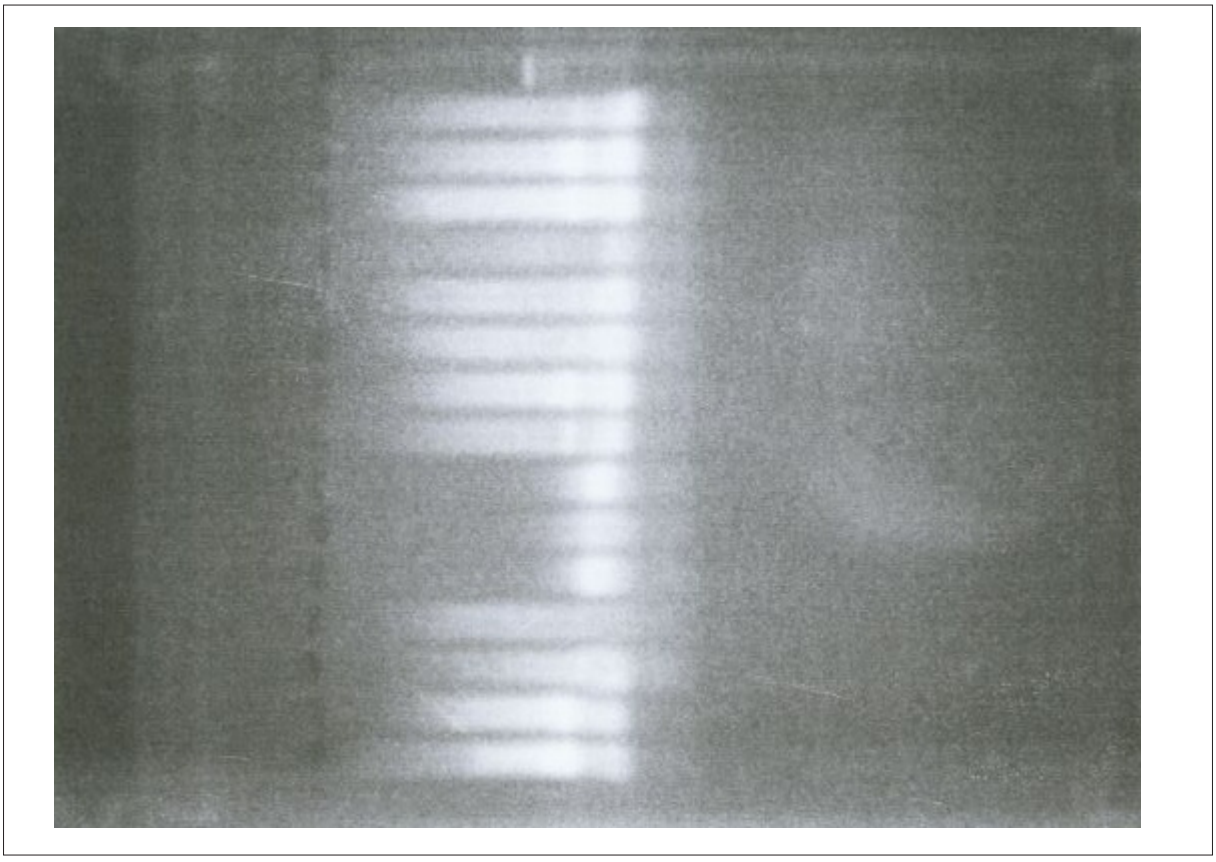

**Fig. 1.** Positive PCR test for DNA of *C. pneumoniae* in the carotid artery

cally significant correlations between *C. pneumoniae* and TNF-α (R=0.439, p=0.002), or TGF-β (R=0.368, p<0.05) were observed. The presence of VEGF did not depend on the result of the test for *C. pneumoniae* (no correlation was demonstrated).

FGF was present in 60% of the patients without *C. pneumoniae*, and the other cytokines were only found in 20% of these patients. The patients with *C. pneumoniae* infection manifested a twofold increase in the presence of cytokines TNF-α, TGF-β and FGF in comparison with patients without *C. pneumoniae* (Figure 2).

Our results show that a statistically significant correlation exists between the intensity of atherosclerosis in hematoxylin and eosin (H&E) staining and FGF (R=0.360) or *C. pneumoniae* infection (R=0.143).

A statistically significant correlation between TGF-β and FGF was proved (R=0.372).

## Discussion

*C. pneumoniae*, an obligate intracellular gram-negative bacterium, may play an important role in the pathogenesis of peripheral artery occlusive disease (PAOD) [9]. There is evidence supporting a causative role for *C. pneumoniae* in the initiation and progression of the disease [10]. The most usual mechanism postulated is that *C. pneumoniae* provokes an inflammatory immune response, triggering and possibly sustaining the inflammatory atherosclerotic lesion. *C. pneumoniae* activates inflammatory cells to produce cytokines, which migrate toward the infectious focus [11,12]. The increased concentration of cytokines leads to vascular epithelial dysfunction and to the progression of atherosclerosis [13]. They increase oxidative stress, initiate apoptosis and inhibit the synthesis of nitric oxide, an important protective molecule in the vasculature [14]. The question is which particular cytokine is responsible for the development of atherosclerosis. An absence of cytokines in the ICA wall of the control group may be a sign of a healthy intima with no need for regeneration (VEGF), and no necrosis (TNF-α) [15]. The presence of FGF in some healthy donors is due to the activity of intima fibroblasts, responsible for the synthesis of elastic fibers and collagen for the extracellular matrix (ECM) [16]. The continuous arterial blood pressure acting on the vessel wall causes the need for permanent intima elastic fibers and collagen regeneration [17]. In the study group, *C. pneumoniae* was discovered in the atheromatous plaque of 68% of patients, which may confirm its connection with the pathogenesis of atherosclerosis. However, its pathomechanism is not fully known [18]. These pathogens infect several different cell types (myocytes, macrophages, endothelial cells,

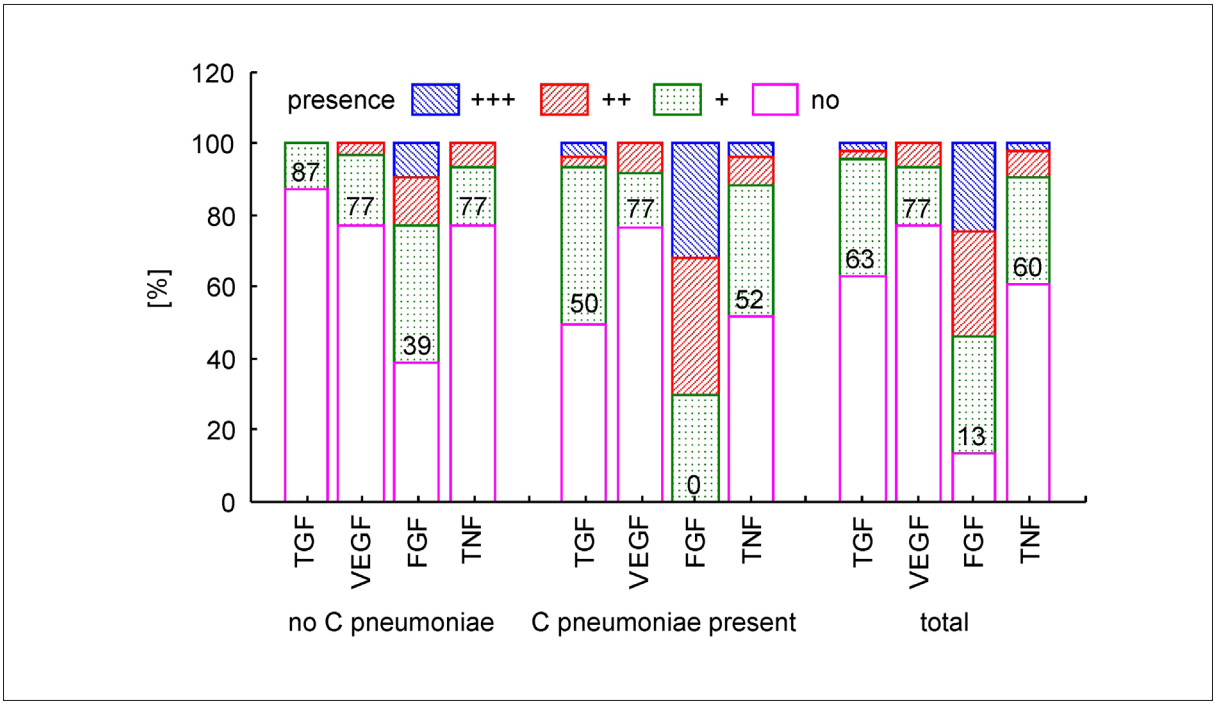


**Fig. 2.** Relationship between presence of the four cytokines and results of the PCR test for *C. pneumoniae*

etc.), not causing their apoptosis, but increasing their proliferation [19]. The activated leukocytes and endothelial cells stimulate the myofibroblasts to increased elastic fiber and collagen synthesis [20]. Eventually this leads to destabilization of the atheromatous plaque [21]. The multifactorial ANOVA analysis confirmed that the association between *C. pneumoniae* infection and the presence of FGF, TNF-α and TGF-β was statistically significant.

Atherosclerosis is a multifactorial disease involving several risk factors, but its etiopathogenesis is still largely unknown [7]. Therefore, both experimental and clinical research is still necessary to establish the exact role of *C. pneumoniae* infection and the presence of cytokines in this complex process [4].

## Conclusions

No *C. pneumoniae* was found in the internal carotid artery of healthy donors. The prevalence of *C. pneumoniae* DNA in the advanced atherosclerotic lesions examined in this study was 68%. There was a statistically significant correlation with the presence of three cytokines (TGF-β, FGF, TNF-α). No correlation between *C. pneumoniae* and VEGF was observed. The three cytokines TGF-β, FGF, and TNF-α were detected in atheromatous internal carotid arteries. Only FGF was present in the internal carotid artery of healthy donors.

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