REVIEWS

IS EVERYTHING CLEAR ABOUT TAKO-TSUBO SYNDROME?

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ABSTRACT

INTRODUCTION: Tako-tsubo syndrome is a novel cardio-vascular disease affecting predominantly postmenopausal women exposed to unexpected strong emotional or physical stress, in the absence of significant coronary heart disease. It is characterized by acute onset of severe chest pain and/or acute left ventricular failure, ECG-changes, typical left ventricular angiographic findings, good prognosis and positive resolution of the morphological and clinical manifestations. First described in 1990 in Japan by Sato, Tako-tsubo cardiomyopathy is characterized by transient contractile abnormalities of the left ventricle, causing typical left ventricular apical ballooning at end-systole with concomitant compensatory basal hyperkinesia. There are also atypical forms, presenting with left ventricular systolic dysfunction which affects the mid-portions of the left ventricle.

The etiology of the disease still remains unclear. Many theories have been put forward about the potential underlying pathophysiological mechanisms that may trigger this syndrome among which are the theory of catecholamine excess, the theory of multivessel coronary vasospasm, the ischemic theory, and the theory of microvascular dysfunction and dynamic left ventricular gradient induced by elevated circulating catecholamine levels. Adequate management of Tako-tsubo syndrome demands immediate preparation for coronary angiography. Once the diagnosis is made, treatment is primarily symptomatic and includes monitoring for complications. Patients with Tako-tsubo syndrome most frequently develop acute LV failure, pulmonary edema, rhythm and conductive disturbances and apical thrombosis. Treatment is symptomatic and includes administration of diuretics, vasodilators and mechanical support of circulation with intra-aortic balloon counterpulsation.

Key words: Tako-tsubo, apical ballooning, stress cardiomyopathy

INTRODUCTION

The increasingly more frequent use of interventional techniques for diagnosing and management of acute coronary syndromes has brought about a more frequent differentiation of a specific syndrome of reversible stress-induced cardiomyopathy. Terms used to describe this condition in published reports include transient left ventricle apical ballooning syndrome, stress-induced cardiomyopathy, the broken-heart-syndrome, the ampullar syndrome, neurogenic stunned myocardium and Tako-tsubo cardiomyopathy.1,2 The latter term has gained the widest currency in medical literature.

Tako-tsubo cardiomyopathy is a novel nosologic entity first described in 1990 by Sato3 in Japan. It is characterized by transient contractile abnormality of the left ventricle which causes typical left ventricular apical ballooning at end-systole with concomitant compensatory basal hyperkinesia.1 This morphological finding resembles an octopus fishing pot used in Japan in the past which was called takotsubo (tako – octopus, tsubo - pot). This medical condition is characterized by transient left-ventricular (LV) systolic dysfunction with complete restoration of the contractile function to the normal within an interval of several days to a few weeks.2

Clinically, Tako-tsubo syndrome is characterized by a sudden onset of chest pain, manifestations of acute left ventricle failure with or without pulmonary edema, low cardiac output syndrome, wide spectrum of ECG changes such as ST-elevation, ST-depressions or deep T-wave inversions usually in the precordial leads, transient Q-waves, which
mimic acute coronary syndrome, prolonged QT-interval and rhythm and conductive disturbances. There are also electrocardiographic data of expressed systolic LV dysfunction.\textsuperscript{1,2,4-7}

The serum levels of the cardiac biomarkers creatinine phosphokinase (CPK), CPK-MB fraction and troponin T and I are often slightly elevated. Serial examinations of these markers demonstrate levels which are about 10 times lower than in genuine acute coronary syndrome (ACS). No enzyme release is observed in isolated patients.\textsuperscript{2,4,5} Angiography does not visualize significant lesions of the coronary arteries and left ventriculography most frequently demonstrates typical apical ballooning with compensatory basal hyperkinesia (Figs 1, 2).

Tako-tsubo cardiomyopathy is a rare condition, often unrecognised, which affects predominantly postmenopausal women exposed to sudden strong emotional or physical stress. Imaging examinations by myocardial perfusion scintigraphy and isotope magnetic resonance tomography in search for images of delayed contrast enhancement of gadolinium, performed within the first five days after clinical manifestation of the disease do not yield any evidence of myocardial cicatrix, nor is there any evidence of increased intake of the pharmaceutical agent by the myocardium which is characteristic of myocarditis.\textsuperscript{1}

After conservative symptomatic treatment is administered, some of the clinical features like apical LV ballooning, ECG changes and enzyme cardiac markers usually show tendency of spontaneous positive resolution within an average of 19 days (9–54 days).\textsuperscript{1,2} The first reports about Tako-tsubo came from Japan which is interpreted as appearance of a new cardiovascular disease of specific geographic predilection.\textsuperscript{5} As Tako-tsubo syndrome draws increasingly greater and greater interest the number of reports of its global distribution increases. Based on statistical data of the USA National Heart, Lung and Blood Institute for 2007, Akashi et al. report that clinically manifested Tako-tsubo cardiomyopathy may account for about 1\% of all Americans with diagnosed acute myocardial infarctions (MI). Bybee et al.\textsuperscript{8} compare literature data to the data of different clinical studies and suggest that Tako-tsubo syndrome account for 1.5 to 2.2\% of all cases with ACS with/without ST elevation. Most patients with Tako-tsubo are postmenopausal women, about 90\% of all cases. The mean age of the patients is 68 years. Different studies demonstrate the presence of cardiovascular risk factors in most of them but these risk factors are generally present at a lesser degree in patients with Tako-tsubo cardiomyopathy than in patients with coronary artery disease.\textsuperscript{2}

The etiology of the disease still remains an enigma. A lot of theories have been suggested concerning the potential underlying pathophysiological mechanisms of this syndrome.

**Catecholamine excess**

The theory that has gained the widest recognition is

Figure 1. Normal anatomy of left anterior descending artery (LAD) in a patient with Tako-tsubo syndrome.

Figure 2. Typical findings of left-ventriculography in the same patient with Tako-tsubo syndrome.
that of catecholamine excess due to hyperactivation of the sympathetic tone. Patients with Tako-tsubo have higher serum levels of the circulating catecholamines. Clinical follow up of such patients on days one and two after hospitalization show elevated plasma levels of epinephrine, norepinephrine and dopamine 7 to 34 times that of normal reference value. By comparison in patients with classic acute myocardial infarction the same indicators are usually 2 to 3 times higher than the normal values. Endomyocardial biopsy is not routinely indicated and, moreover, it is not recommended in diagnosing the disease in its acute phase for quite obvious reasons. The few experimental studies in this field show a great number of vacuoles of different size associated with cell hypertrophy, increased accumulation of glycogen, interstitial infiltrates consisting primarily of mononuclear cells (lymphocytes and macrophages) and contraction band necroses. There have been also found disorganization of the structural and contractile proteins and increased extracellular matrix. Myocyte necrosis, however, has not been found. Histological studies of impaired left ventricle demonstrate changes that are consistent with clinical conditions with elevated catecholamine levels. After LV functional recovery the described morphological manifestations are almost completely reversible within 12 ± 3 days. Increased concentrations of serum catecholamines have a reversible toxic effect on the cardiomyocytes. Similar conditions may be observed in other diseases with manifested catecholamine excess such as pheochromocytoma. In Tako-tsubo the apex of the left ventricular seems to be selectively vulnerable to the effects of increased sympathetic tone. LV basal segments are not affected by the cardiotoxic effects of circulating catecholamines which raises the question of certain differences in adrenergic innervation and/or differences in the sensibility of adrenergic receptors. The precise mechanism of catecholamine-induced injury of the left ventricle still remains unclear, as well as the selective involvement of its apical segment. The results of several studies show that β-adrenergic stimulation is associated with changes in the gene expression of calcium regulatory proteins.

**Multivessel Coronary Artery Spasm**

One of the first theories propounded about what triggers Tako-tsubo cardiomyopathy suggests that multivessel coronary spasms can result in transient stunning of the myocardium. This coronary vasospasm can provoke ischemia in the absence of obstructive coronary artery disease. There has been extensive research seeking to find an explanation of that phenomenon but during coronary angiography arterial vasospasm was induced in only a few patients - 18%. Regardless of the potential role of vasospasm in the genesis of Tako-tsubo, this theory can not explain most of the cases with this syndrome. It is worth a note that even in multiple vasospasms of the coronary arteries they do not correlate with the non-contracting area of the myocardium.

**Ischemic Theory**

Another possible mechanism of Tako-tsubo seems to be the transient occlusion of the left anterior descending (LAD) coronary artery by atheromatous plaque. Although obstructive coronary artery disease (> 50% narrowing) has been ruled out by catheterization some researchers hypothesize that ruptured atheromatous plaque could be the underlying cause of Tako-tsubo cardiomyopathy. Spontaneous intermittent occlusion and recanalization of the coronary arteries as a result of a combination of thrombosis and vasoconstriction are common findings in the evolution of ACS. So a question arises as to whether it is possible for a ruptured atherosclerotic plaque or coronary occlusion to undergo spontaneous recanalization before the forthcoming coronary angiography. In classic MI the size of the infarct corresponds strictly to the amount of myocardium supplied with blood by the occluded coronary artery. In Tako-tsubo the amount of the affected myocardium is much larger than the area normally supplied by a single coronary artery. This involvement in the pathological process of more myocardial segments than normally supplied by a single coronary artery seems to agree well with the theory of transient obstructive coronary disease. Ibanez et al. use intravascular ultrasound (IVUS) to visualize LAD in 5 patients with Tako-tsubo cardiomyopathy. All 5 patients have less than 50% stenosis on the angiograms but nevertheless IVUS shows a single ulcerated atheromatous plaque in the mid section of LAD in each patient and no involvement of the other LAD regions was found. In three of the patients the morphological changes in the plaques are of “fibrous capsule rupture” type accompanied by the formation of an intra-plaque cavity; one case resembles fibrous capsule rupture without ulceration and one case presents with intimal dissection. Further evaluation of LAD in these patients demonstrate that the artery has greater size and it wraps around the apex of the heart and extends widely along the diaphragmatic
surface of the left ventricle. The total length of LAD in these patients is 133.6 mm (117.7–147.7 mm). The length of the LAD recurrent segment is 30.4 mm (25.7–34.9 mm), and it presents 22.7% of the total artery length. Such a type of LAD (wraparound LAD phenomenon) supplies with blood a sufficiently big section of the LV inferior wall. This special feature of LAD’s anatomy seems to explain the specific LV morphology during systole. The akinetic zone corresponds exactly to the LV region which is between the plaque and the end of LAD. Ibanez et al. suggest that in patients with a “well developed” LAD (like these included in their study) the widely spread apical akinesia may be caused by transient occlusion, and come to the conclusion that Tako-tsubo cardiomyopathy in these patients is essentially an aborted myocardial infarction. Other researchers believe that the wraparound LAD phenomenon is not common enough to explain all cases with Tako-tsubo cardiomyopathy. This theory could not explain also the clinical cases with mid-ventricular and basal akinesia, also known as atypical form of Tako-tsubo.

CORONARY MICROVASCULAR DYSFUNCTION
This theory is based on the idea about the role of microvascular dysfunction in the genesis of Tako-tsubo. In such medical conditions the small-size arteries fail to provide adequate quantities of oxygen to the myocardium. It is likely that there are numerous factors leading to such dysfunction including vasospasm, insufficiency of the microcirculation and abnormal response to catecholamine stimulation. The strong association between Tako-tsubo cardiomyopathy and concomitant strong stressors (up to 80% according to some authors), brought to the concept of the involvement of catecholamines in the genesis of this syndrome. Acute stress is usually associated with elevated plasma levels of the pro-inflammatory cytokines and catecholamines which in combination result in vasoconstriction and higher arterial blood pressure and may cause instability of the atherosclerotic plaque. Case series of studies over large groups of patients report that some patients develop Tako-tsubo cardiomyopathy after an emotional stress while others have a preceding clinical stress stimulus (such as an asthma attack) or a sudden manifestation of a disease (such as subarachnoidal hemorrhage). Data from other investigations show that in one third of the patients strong correlation with a preceding stress cannot be found.

DYNAMIC CATECHOLAMINE-INDUCED LV GRADIENT
Another hypothesis which can explain the clinical findings in Tako-tsubo cardiomyopathy is the combination between induced high-degree dynamic gradient in the LV mid-cavity portion and the effects of catecholamines which cause subendocardial blood flow reduction. Such a dynamic LV mid-cavity obstruction would separate the left ventricle into two compartments – a proximal one which is at normal pressure and normal wall stress and an apical compartment which is at higher pressure and higher stress of the contiguous LV wall. This in itself would result in reduction of the sub-endocardial coronary blood flow in the apical compartment. Such a dynamic obstruction would be additionally accentuated by concomitant hypovolemia. Increased circulating catecholamines have a similar synergic effect. Such a combined mechanism would result in discrepancy between the coronary blood flow in the large epicardial vessels and the microcirculation. This results in worsening of the sub-endocardial blood flow in the apical LV region which is subjected to higher pressure and stress. So a logical question arises: why the condition of resulting myocardial stunning does not progress to a sub-endocardial myocardial infarction? There is no definitive answer to this question as yet. Intensified subendocardial ischemia in the distal LV compartment would result in decreased subendocardial contractile function and the myocardium of the segments with higher stress would fail to generate such a gradient. So ischemia of that region would be reduced. As the blood flow of the epicardial arteries is not compromised it will provide perfusion of the inner left ventricle wall. Theoretically, this process would continue until the levels of circulating catecholamines fall. Such a sequence of events would require certain primary abnormality of the ventricular myocardium. This could either be caused by localized septal hypertrophy or by abnormal distribution of LV muscle fibers. Other evidence supporting the upper mentioned hypothesis is the well known fact that elderly women develop abnormal thickening of the basal and/or mid-portion of the LV wall (mean tele-diastolic dimensions 13 mm) thus forming the so called “sigmoid septum”. By comparison, normally the mean tele-diastolic dimensions of the apical septal segment is 9 mm. This structural abnormality is associated with the ability to induce dynamic LV mid-cavity gradient during either dobutamine stress test or when patients are dehydrated. Female prevalence in the development of such localized septal hypertrophy could explain the gender predisposition to Tako-tsubo cardiomyo-
pathy. Of course, this finding does not lead to a general conclusion for all cases with Tako-tsubo; what is more, this factor alone is not sufficient for the development of the disease. Dawn et al.\textsuperscript{17} provoke LV mid-cavity dynamic obstruction during dobutamine stress test in 54 patients but do not find induced ischemia in any of the patients. At dynamic follow-up they find out that dynamic obstruction with generated pathological LV mid-cavity gradient cannot be an independent predictor of occurrence of chest pain or syncope. Co-participation of other factors is also necessary to display the full spectrum of stress-induced cardiomyopathy.\textsuperscript{1,11}

**Atypical forms of Tako-tsubo**

Atypical forms of Tako-tsubo are increasingly being described nowadays. It concerns medical conditions of LV systolic dysfunction affecting the mid portions of the left ventricle. Literature report show that the correlation between the two forms is 60 to 40\% respectively for the typical wall motion abnormality with apical LV ballooning and the atypical one.\textsuperscript{18} The two groups of patients with transient LV systolic dysfunction do not differ in their demographic, clinical, laboratory and angiographic parameters. One of the large studies of the disease reports that the global left ventricular ejection fraction as presented by angiography is 50 ± 13\%. While the epicardial blood flow in the LAD artery is normal or nearly normal, the microcirculation in the LAD artery as estimated by TIMI MBG (myocardial blush grade) and CTFC (corrected TIMI frame count) is compromised in 71\% of the patients. Furthermore, coronary microcirculation in the other coronary arteries - the RCA and/or the LCx - is also compromised (in 69\% of the patients), regardless of the localization of segmental abnormalities of the LV wall. Myocardial perfusion scintigraphy demonstrates a more pronounced decrease in myocardial perfusion in patients with typical apical ballooning of the left ventricle as compared to patients with atypical mid-ventricular pattern of Tako-tsubo. Investigations with fluoro-deoxy-glucose positron emission tomography (FDG-PET) reveal significant and disproportional decrease in glucose metabolism in the corresponding segments. Myocardial glucose metabolism seems to be affected to a greater extent than myocardial perfusion compared to the post-ischemic “stunned” myocardium. The disease presents with inverse perfusion-metabolic disproportion (mismatch). The most serious changes in glucose metabolism are usually observed between the 2nd and 6th days and later between the 6th and 12th days after the onset of symptoms.\textsuperscript{18} Kurisu et al.\textsuperscript{15} report compromised fatty acids metabolism in the myocardium. Studies with β-methyl-p-[\textsuperscript{123}I]-iodophenyl-pentadecanoic acid on the 5 ± 3, and subsequently on the 29 ± 6 days demonstrate serious abnormalities in the metabolism of fatty acids. It is well known that the heart muscle normally needs glucose and fatty acids as a source of energy for oxidative processes. Different disturbances in these directions may lead to alterations of the enzyme processes at cell level which play role in the oxidative cascade. Cyclic adenosine monophosphate (cAMP) modulates the enzyme systems which participate in the oxidative processes. Catecholamines, on their side, significantly modulate the effect of cAMP. Elevated cAMP plasma levels result in decreased activity of K\textsuperscript{+} channels with prolonged cell depolarization and prolonged QT-interval as frequently registered in such patients. The receptors which mediate the elevation of cAMP plasma levels are β\textsubscript{1}, β\textsubscript{2}, β\textsubscript{3} and adenosine A\textsubscript{2} receptors. The adrenergic α\textsubscript{1}, adenosine A\textsubscript{2} and muscarine M\textsubscript{2} and M\textsubscript{3} receptors mediate cAMP reduction. Different regional distribution of these receptors in the myocardium may be responsible for the local differences in the vulnerability of the heart muscle to injury. A recent scientific report describes a case of Tako-tsubo cardiomyopathy with repeated hospitalizations and involvement of different segments.\textsuperscript{18,19} The distribution of myocardial receptors could be a dynamical process so clinical follow up is necessary to determine the progress of the medical condition.

While in typical Tako-tsubo FDG-PET studies reveal severely compromised glucose utilization in the apical and mid segments, in the atypical pattern of the syndrome disturbed glucose consumption is found in the mid LV segment and the apical segment does not show any abnormalities.

Because of Tako-tsubo’s polymorphism increasing number of researchers suggest that this medical condition should no longer be regarded as an apical ballooning syndrome but rather as a transient left ventricular systolic dysfunction.\textsuperscript{18}

**Gender differences in Tako-tsubo**

All studies dealing with this problem report that Tako-tsubo occurs predominantly in women. Presumably, it is due to some biological susceptibility to the disease whose causes are still enigma. It is well known that sex hormones may influence the sympathetic nervous system tone and may also affect the activity of coronary vessels. Although male individuals have higher levels of basal sympathetic...
tone, they produce higher catecholamine levels as a response to emotional stress and are more sensitive to catecholamine-mediated vasoconstriction. However, as a counterpoint, it seems that women are more vulnerable to sympathomimetic myocardial stunning. It is quite possible that the reason lies in the different ways of sex hormones metabolism. Another hypothesis trying to explain the higher predisposition of women to Tako-tsubo (particularly after menopause) is endothelial dysfunction as a result of reduced basal estrogen levels.4

TAKO-TSUBO SYNDROME AND CANCEROGENESIS

In the recent years interesting reports appeared about the association between Tako-tsubo cardiomyopathy and cancerogenesis. The results of several different studies demonstrate quite heterogeneous data. Some researchers explain the association between various malignant disorders and stress-induced cardiomyopathy with the latter being a result of a paraneoplastic phenomenon. There are two general hypotheses about the association of cancerogenesis with transient LV systole dysfunction syndrome. The first one postulates that oncologic diseases could change the physiological threshold to stress stimuli and therefore weak stimuli should generate inadequate increase of sympathetic activity. The second hypothesis postulates that the carcinoma itself could aggravate the cardiac adrenoreceptor sensitivity. We should not forget that about 20% of all the malignant disorders are associated with some chronic inflammatory activity and increased expression of the inflammatory mediators (such as cytokines, prostaglandins, free radicals, growth factors and catecholamines) which promote adrenergic activity. It is not impossible that the mechanisms described by both hypotheses participate simultaneously in the association between Tako-tsubo cardiomyopathy and cancerogenesis.20

RIGHT VENTRICLE INVOLVEMENT IN TAKO-TSUBO CARDIOMYOPATHY

An increasing number of researchers report on the involvement of the right ventricle in Tako-tsubo syndrome. Elesber et al.21 is the first to investigate right ventricle (RV) function in Tako-tsubo. They report compromised RV function in 8 of 25 patients. MRI findings show that RV dysfunction in these patients is associated with heavier LV dysfunction, lower LV ejection fractions, longer hospitalization and more frequent and severe complications such as congestive heart failure, necessity of intra-aortic balloon counterpulsation and cardiopulmonary resuscitation. Other studies demonstrate similar results of RV involvement in about 25% of patients. According to other research data severity of LV abnormality is not the only criteria determining RV involvement, i.e. data of RV dysfunction is found in patients with mild LV myocardial abnormalities. Some hypotheses postulate on certain differences in the regional distribution and the sensitivity of RV adrenoreceptors, as well as in the RV blood supply. It should be also noted that common MRI investigation of the RV function is usually slightly delayed and that may probably result in underestimation of the frequency of RV involvement in patients with Tako-tsubo syndrome.21,22

MANAGEMENT OF TAKO-TSUBO SYNDROME

As Tako-tsubo cardiomyopathy is initially indistinguishable from classic acute coronary syndrome, immediate treatment should be started and it should include the whole spectrum of medicines which are routinely used for the management of ischemia and acute heart failure. Adequate treatment demands the most immediate preparation for coronary angiography. Once the diagnosis is set symptomatic treatment should be started as well as monitoring for complications. Most complications of Tako-tsubo cardiomyopathy occur during the acute phase of the condition. Literature data report on complications in about 19% of patients.22,23 Most frequent complications are acute left ventricle failure, pulmonary edema, rhythm and conductive disturbances and apical thrombus formation. A case of left ventricular free wall rupture has also been reported.24 The relation between Tako-tsubo and sudden cardiac death still remains unclear.

The symptomatic treatment of Tako-tsubo includes diuretics and vasodilators. In cases of low cardiac output conditions it is preferable to secure additional mechanical support of circulation by intra-aortic balloon counterpulsation instead of administration of cardioionotropic agents as the latter may increase the catecholamine burden. LV systolic dysfunction is treated according to the common accepted standards – application of ACE-inhibitors and beta-blockers. In cases of apical thrombus formation administration of anticoagulants is reasonable.

The mortality rates in Tako-tsubo within 30 days are about 8.6% and they do not differ from mortality rates in patients with MI who have undergone successful percutaneous transluminal coronary angioplasty.18 All reported cases of deaths demonstrate that the direct cause is some underlying disease - severe pneumonia, urosepsis and septic shock.13
Recurrent episodes of Tako-tsubo cardiomyopathy are reported in 7% of the patients. According to Gianni et al., the frequency of repeated episodes of this syndrome is 3.5%.1,18

Without significant accompanying comorbidity, the prognosis for patients with Tako-tsubo cardiomyopathy is good after the initial acute phase has been overcome. Recommendations for follow-up include serial echocardiography at approximately 4-6 weeks and 12 months after dehospitalization to verify the normalization of LV systolic function. Serial investigations demonstrate that the initial LV EFs values of 50 ± 13% are usually elevated to 68 ± 6% which means that in persisting LV contractile abnormality within a few weeks the diagnosis should be revised.18

In future, researchers will have to specify additionally the underlying pathophysiological mechanisms of Tako-tsubo, its gender predispositions, to identify the underlying risk factors and risk modification. It is also necessary to develop an algorithm which will help to differentiate Tako-tsubo from acute coronary syndrome and to determine the criteria for the diagnosis, medical treatment and follow-up of this syndrome.

REFERENCES


ТАКО-ЦУБО СИНДРОМ – ПОНИМАЕМ ЛИ ВСЕ?

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РЕЗЮМЕ

Тако-цубо синдром представляет собой новое сердечно-сосудистое заболевание, затрагивающее преимущественно женщин в менопаузе, подвергнутых внезапному сильному эмоциональному или физическому стрессам, при отсутствии значимой коронарной болезни сердца. Синдром характеризуется внезапным появлением сильной грудной болью и/или проявлениями острым левосторонней сердечной недостаточностью, ЭКГ-изменениями, характерной находкой при левовентрикулографии, хорошим прогнозом и позитивной резолюцией морфологических и клинических признаков. Болезнь впервые описана в 1990 г. в Японии Sato. Тако-цубо кардиомиопатия характеризуется транзиторным контрактильным аномалией левого желудочка, вызывающим типичное баллонирование левожелудочковой верхушки в систоле и сопутствующую компенсаторную базальную гиперкинезию. Наблюдаются и атипичные варианты – левожелудочковая систолическая дисфункция, затрагивающая средние отделы левого желудочка.

Этиология заболевания все еще остается невыясненной. Имеются множество теорий относительно потенциальных патофизиологических механизмов, ответственных за отключение этого синдрома (теория катехоламинового экссеса, теория многососудистого коронарного спазма, ишемическая теория, теория микроваскулярной дисфункции, теория динамического градиента в левом желудочке под воздействием катехоламинов).

Правильное поведение требует возможно самой быстрой подготовки к проведению коронарной ангиографии. При наличии диагноза лечение симптоматично и включает мониторинг в случаях осложнений. Чаще всего пациенты развивают острую сердечную недостаточность, легочный отек, нарушения ритма, нарушения в проводимости, апикальный тромбоз. Лечение симптоматично с включением диуретиков и вазодилататоров, механической поддержки циркуляции путем интракоронарной баллонной контрапульсации.
Clinical Investigations

EFFECT OF MODERATE AND HIGH DOSE SIMVASTATIN ON ADHESION MOLECULES IN SEVERE HYPERCHOLESTEROLEMIA AFTER TARGETING THE LDL-CHOLESTEROL – A RANDOMISED, PLACEBO-CONTROLLED STUDY

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ABSTRACT

INTRODUCTION: The effect of statins on the levels of cell adhesion molecules (CAM) is discussed in the literature as one of the pleiotropic effects of the drugs. This effect is one of the ways that could be used to control the initial stage of atherogenesis. The research in this field is inadequate and controversial. Prevention guidelines recommend that target levels of LDL cholesterol in high-risk patients should be less than 2.6 mmol/l. If the primary target is LDL-cholesterol, it is doubtful if patients can have any significant changes in the levels of the cell adhesion molecules (CAM).

AIM: Study the effect of simvastatin administered in a moderate dose of 40 mg and in a high dose of 80 mg on endothelium activation in the context of the plasma levels of soluble cellular adhesion molecules (sICAM-1, sVCAM-1, sE-selectin, sP-selectin) in recently diagnosed untreated severe hypercholesterolemia after reaching target levels for the LDL-cholesterol below 2.6 mmol/l.

PATIENTS AND METHODS: One hundred patients (aged > 16 years) were included in the study. Hypercholesterolemia was defined as fasting total serum cholesterol level greater than 7.5 mmol/l and LDL-cholesterol > 4.9 mmol/l. The study was carried out in three phases, the main goal being titration of simvastatin dose from 40 to 80 mg with the purpose of achieving the target LDL level of < 2.6 mmol/l in a randomised placebo-controlled study.

RESULTS: There was a statistically significant reduction of sVCAM-1 following the 80-mg simvastatin therapy for one month after reaching target levels of LDL-cholesterol < 2.6 mmol/l in hypercholesterolemic patients in comparison with the moderate dose (40 mg) of simvastatin for one month (p < 0.001). The results of the study demonstrated that simvastatin in a dose of 80 mg exerted an effect on the levels of some CAM, and particularly on VCAM-1 in contrast to the same drug used in a dose of 40 mg.

CONCLUSION: As different statins most likely have a distinctly specific effect on different adhesion molecules, this study seeks to establish a suitable panel of such adhesion molecules that may be used in monitoring statin therapy.

Key words: hypercholesterolemia, cell adhesion molecules, simvastatin, pleiotropic effect, statin

INTRODUCTION

International recommendations for management of hypercholesterolemia underscore the importance of diagnosis and treatment of asymptomatic individuals with high absolute cardiovascular risk. Individuals with marked hypercholesterolemia fall into this group as well.¹-³ On the other hand, in the context of the inflammatory genesis of the atherosclerosis the inflammatory reaction begins with the trundle of mononuclear cells on the endothelium followed by their adhesion, activation and transmigration to the sub-endothelium. The interaction between the leucocytes, the leucocytes and the endothelium, and between the leucocytes and the extracellular matrix is mediated by so called cell adhesion molecules (CAM).⁴-⁸ These molecules play a highly signifi-
ciant role in the uptake of circulating monocytes and T-lymphocytes in the atherosclerosis lesion. The mononuclear cell engaged in the intima of the large and medium blood vessels is exactly the first step in the beginning of atherosclerosis. This makes the uptake of leucocytes from the capillaries to be more precise and multiple.

Still, there are many ambiguous questions regarding the place of CAM in blood vessel pathology. Data on circulating CAM supports the thesis that they are potential atherosclerosis biomarkers. There is ample evidence that soluble intracellular cell adhesion molecules (sICAM-1) are highly informative in myocardial infarctions. Patients diagnosed with chronic ischemic heart disease have significantly higher levels of sVCAM-1 and E-selectin than healthy subjects. In Bulgaria there are data about the levels of sVCAM-1 and sICAM-1 in patients with hypercholesterolemia, but it is sVCAM-1 only that have been found to be statistically different in comparison with controls.

Statins have been demonstrated to be effective as lipid lowering agents – they can slow down and/or stop atherosclerotic plaque formation. The latter is likely to be partly due to their pleiotropic effects. The effect of statins on CAM levels is discussed in the literature as one of the pleiotropic effects of the drugs. This effect is one possible mechanism that could be used to control the initial stage of atherogenesis. The research in this field is inadequate and controversial. Prevention guidelines recommend that target levels of LDL cholesterol in high-risk patients should be less than 2.6 mmol/l. If the primary target is LDL-cholesterol, it is doubtful if patients can have any significant changes in CAM levels. This is the primary issue discussed in this article.

**AIM**

Study the effect of simvastatin used in a moderate dose of 40 mg and in a high dose of 80 mg on the endothelium activation in the context of the plasma levels of soluble cellular adhesion molecules (sICAM-1, sVCAM-1, sE-selectin, sP-selectin) in recently diagnosed untreated severe hypercholesterolemia after reaching target levels of LDL-cholesterol below 2.6 mmol/l.

**PATIENTS**

Between June 2006 and September 2008 we examined 460 outpatients with primary hypercholesterolemia in the Preventive Cardiology Surgery of the Clinic of Cardiology with St George University Hospital in Plovdiv. One hundred of these patients were included in the study; the patients were over 16 years of age. Hypercholesterolemia was defined as fasting total serum cholesterol level greater than 8.5 mmol/l and LDL-cholesterol below 5.9 mmol/l. All patients had a family history of atherosclerotic disease. All known endothelial function-related conditions and drugs were included in the exclusion criteria. The use of antioxidants as exclusion criteria is in context of the fasting condition of the patients 12 hours before examination. None of the patients had been taking lipid-lowering medication prior to the study.

The exclusion criteria were:

1. Diabetes mellitus, impaired glucose tolerance or fasting blood glucose > 5.6 mmol/l.
2. Cigarette smoking.
3. Clinical and laboratory evidence of:
   3.1. Coronary artery disease in all forms;
   3.2. Cerebrovascular disease;
   3.3. Arterial hypertension;
   3.4. Chronic obstructive pulmonary disease as well as bronchial asthma;
   3.5. Chronic arterial insufficiency of the extremities (peripheral arteries): ankle-brachial index < 0.9;
   3.6. Chronic renal and hepatic dysfunction;
   3.7. Systemic disorders of the connective tissue;
   3.8. Neoplasms;
   3.9. Acute or chronic inflammatory process requiring active treatment;
4. Prolonged use of non-steroidal anti-inflammatory drugs, corticosteroids, hormonal and psychotropic drugs, lipid regulating medications, and antioxidants;
4.1. Alcohol and drug abuse.

Another hundred patients were studied without finding hypercholesterolemia.

**METHODS**

Laboratory tests were performed by the Central Clinical Laboratory of St. George University Hospital in Plovdiv. The biochemical parameters of total serum cholesterol, triglycerides, HDL-cholesterol were investigated using biochemical analyzer Konelab 60i (Thermo Electron Co, Vantaa, Finland). Serum LDL-cholesterol levels were analysed using direct automated analysis and reagents of KonelabTM (Thermo Electron Co, Finland). Apolipoprotein-A1 (Apo-A1) and apolipoprotein-B (Apo-B) in serum were measured using reagents of KonelabTM (Thermo Electron Co, Vantaa, Finland) and biochemical
analyzer Konelab 60i (Thermo Electron Co, Vantaa, Finland). The intra-assay and inter assay coefficients of variation were calculated for Apo-A₁ – 2.7% and 4.8%, respectively and Apo-B – 1.6% and 3.1%, respectively. Reference values for Apo-A₁: reference range 1.09–1.84 g/l for males, 1.06–2.28 g/l for females. Reference values for Apo-B: reference range 0.63–1.88 g/l for males, 0.56–1.82 g/l for females. The serum soluble intracellular adhesion molecules (sICAM-1), soluble vascular adhesion molecules (sVCAM-1), P-selectin and E-selectin were determined by ELISA using kits from DLD Diagnostika GMBH (Hamburg, Germany) and BenderMed Systems (Vienna, Austria). The intra- and inter assay coefficients of variation were calculated (4.5–5.8% and 5.8–8.2%, respectively). Serum samples were obtained and stored as aliquots at -20°C until analysis was performed, up to two months. Grossly haemolysed or lipemic specimens were discarded. Prior to the assay frozen plasma was brought slowly to room temperature and mixed gently as required by the manufacture’s instructions. Repeated freeze-thaw cycles were avoided. Reference values and reference range for cell adhesion molecules were set at 128.9–374.5 ng/ml (sICAM-1), 170.4–478.4 (sVCAM-1), 9.15–65.19 (sE-selectin), and 101.7–209.7 (sP-selectin) for both males and females and for all age groups (older than 18 years).

Mediterranean diet was implemented for the entire duration of the study. Patients had not received statin therapy prior to the study. The study is a prospective follow-up trial conducted in three stages. The first stage includes determination of the group of patients reaching target levels with 40 mg simvastatin, compared to a placebo group. The reason we included a wash-out period was to create equal baseline conditions without therapy. After one-month wash-out the LDL-C levels were measured again – these levels are presented in

Effect of Moderate and High Dose Simvastatin on Adhesion Molecules in Severe Hypercholesterolemia after Targeting the LDL-Cholesterol – a Randomised, Placebo-Controlled Study

Figure 1. A flow chart of hypercholesterolemic patients who initially were started on 40 mg simvastatin, then the dose was titrated to 80 mg when they had to reach their LDL level target ≤ 2.6 mmol/l and monitoring until month 3.
According to literature there is a rebound of the lipid profile one week after therapy is discontinued. Lipid profile becomes stable within a month, approaching baseline values, which was the reason we had a one-month wash-out.

Following initial laboratory and instrumental work-up all patients received 40 mg simvastatin once daily, at 9.00 p.m. This dose is recommended as initial in patients with severe hypercholesterolemia. The laboratory parameters – lipid profile, apolipoproteins, soluble intracellular adhesion molecules – were measured at baseline and at the end of month 1. Patients received simvastatin in the evening before the study for one month. The phase lasts one month as, according to literature data, it takes 3-4 weeks to achieve optimal blocking of the enzyme (HMG-CoA reductase) of the speed-determining stage of the cholesterol synthesis, i.e. optimal lipid-regulation.

Study design: 460 dyslipidemic patients were screened; 100 of these had severe hypercholesterolemia with total cholesterol level ≥ 7.5 mmol/l and LDL-C level ≥ 4.9 mmol/l meeting Simon-Broom’s criteria for clinical diagnosis of family hypercholesterolemia. The study was carried out in three phases, the main goal being the titration of simvastatin dose from 40 to 80 mg with the purpose of achieving the target LDL level of ≤ 2.6 mmol/l in a randomised placebo-controlled study. During phase 1 all patients were randomized into two groups: one group of 50 patients received 40 mg simvastatin in one single dose at bedtime, 09.00 p.m. (it is recommended as an initial dose in all patients with severe hypercholesterolemia) and the other 50 patients received placebo. Of all patients on 40 mg simvastatin 15 patients reached target LDL level of less than 2.6 mmol/l, and 30 did not reach target. During phase 2 we discontinued the statin therapy for 1 month (at the end of month 1 to the end of month 2) in all patients who had not reached target LDL-C level of ≤ 2.6 mmol/l – washout period. During phase 3 all the patients (n = 30) who had not reached target LDL-C level of ≤ 2.6 mmol/l during phase 1 were randomized in two groups – one of them received 80 mg simvastatin, the other received placebo for a month (Fig. 1).

Table 1. Baseline characteristics of the examined hypercholesterolemic subjects and the placebo group

<table>
<thead>
<tr>
<th>Variables</th>
<th>Hypercholesterolemic patients (n = 50) mean ± SD</th>
<th>Placebo group (n = 50) mean ± SD</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>46 ± 4</td>
<td>46 ± 2</td>
<td>NS</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>30/32</td>
<td>30/28</td>
<td>NS</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>25 ± 3</td>
<td>25 ± 2</td>
<td>NS</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>83 ± 3</td>
<td>84 ± 1</td>
<td>NS</td>
</tr>
<tr>
<td>Blood pressure, systolic (mmHg)</td>
<td>119 ± 1</td>
<td>116 ± 1</td>
<td>NS</td>
</tr>
<tr>
<td>Blood pressure, diastolic (mmHg)</td>
<td>73 ± 1</td>
<td>69 ± 2</td>
<td>NS</td>
</tr>
<tr>
<td>Creatinine clearance (ml/min)</td>
<td>120 ± 7.3</td>
<td>118 ± 8.5</td>
<td>NS</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>4.5 ± 0.6</td>
<td>4.4 ± 0.3</td>
<td>NS</td>
</tr>
<tr>
<td>Alanin aminotransferase (U/L)</td>
<td>33 ± 6</td>
<td>32 ± 6</td>
<td>NS</td>
</tr>
<tr>
<td>Gamma-glutamyl transpeptidase</td>
<td>32 ± 6</td>
<td>33 ± 6</td>
<td>NS</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>8.47 ± 0.16</td>
<td>5.35 ± 0.24</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.85 ± 0.04</td>
<td>0.68 ± 0.05</td>
<td>NS</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>0.98 ± 0.04</td>
<td>1.31 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/l)</td>
<td>6.67 ± 0.14</td>
<td>3.51 ± 0.18</td>
<td>NS</td>
</tr>
<tr>
<td>Apo-A (g/l)</td>
<td>1.18 ± 0.02</td>
<td>1.22 ± 0.02</td>
<td>NS</td>
</tr>
<tr>
<td>Apo-B (g/l)</td>
<td>1.81 ± 0.03</td>
<td>1.34 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>Apo-B/Apo-A</td>
<td>1.53 ± 0.02</td>
<td>0.66 ± 0.02</td>
<td>NS</td>
</tr>
<tr>
<td>sICAM-1 (ng/ml)</td>
<td>235.68 ± 0.02</td>
<td>205.68 ± 0.02</td>
<td>NS</td>
</tr>
<tr>
<td>sVCAM-1 (ng/ml)</td>
<td>348.70 ± 0.02</td>
<td>333.60 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>sE-selectin (ng/ml)</td>
<td>30.02 ± 0.03</td>
<td>29.03 ± 0.02</td>
<td>NS</td>
</tr>
<tr>
<td>sP-selectin (ng/ml)</td>
<td>156.00 ± 0.02</td>
<td>148.00 ± 0.03</td>
<td>NS</td>
</tr>
</tbody>
</table>
SPSS v.14.0 was used in the statistical analysis of data. The distribution of all continuous data was analysed using the normality test on the distribution (one-sample Kolmogorov-Smirnov test). In other cases we applied nonparametric tests to compare two independent samples (Mann-Whitney U-test) and two related samples (Wilcoxon Signed Rank test). We used $p < 0.05$ as a level of significance for the null hypothesis. All $p$-values are two-tailed.

**RESULTS**

1. Therapeutic algorithm (phase 1 of the study). Baseline characteristics of the examined hypercholesterolemic subjects and placebo group are presented in Table 1. There was no statistically significant difference between the examined hypercholesterolemic subjects and the placebo group for

### Table 2. Effect of one-month simvastatin treatment (40 mg/d) on atherogenic biomarkers in hypercholesterolemic patients and placebo group

<table>
<thead>
<tr>
<th></th>
<th>Hypercholesterolemic patients (n = 50) (mean ± SD)</th>
<th>Placebo group (n = 50) (mean ± SD)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>at baseline</td>
<td>8.47 ± 0.16</td>
<td>9.05 ± 0.24</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>after 1 month</td>
<td>4.34 ± 0.04</td>
<td>4.39 ± 0.10</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>at baseline</td>
<td>0.85 ± 0.04</td>
<td>0.88 ± 0.05</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>after 1 month</td>
<td>0.67 ± 0.03</td>
<td>0.69 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>HDL-cholesterol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>at baseline</td>
<td>0.98 ± 0.04</td>
<td>0.91 ± 0.03</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>after 1 month</td>
<td>1.09 ± 0.20</td>
<td>1.10 ± 0.20</td>
<td>NS</td>
</tr>
<tr>
<td>LDL-cholesterol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>at baseline</td>
<td>6.67 ± 0.14</td>
<td>7.11 ± 0.18</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>after 1 month</td>
<td>3.55 ± 0.10</td>
<td>3.49 ± 0.10</td>
<td>NS</td>
</tr>
<tr>
<td>Apo-B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>at baseline</td>
<td>1.81 ± 0.03</td>
<td>2.34 ± 0.03</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>after 1 month</td>
<td>1.06 ± 0.30</td>
<td>1.07 ± 0.31</td>
<td>NS</td>
</tr>
<tr>
<td>Apo-A1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>at baseline</td>
<td>1.18 ± 0.02</td>
<td>1.22 ± 0.02</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>after 1 month</td>
<td>1.52 ± 0.21</td>
<td>1.50 ± 0.20</td>
<td>NS</td>
</tr>
<tr>
<td>Apo-B/Apo-A1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>at baseline</td>
<td>1.53 ± 0.02</td>
<td>1.91 ± 0.02</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>after 1 month</td>
<td>0.66 ± 0.19</td>
<td>1.50 ± 0.21</td>
<td>NS</td>
</tr>
<tr>
<td>sICAM-1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>at baseline</td>
<td>235.68 ± 0.02</td>
<td>230.64 ± 0.02</td>
<td>NS</td>
</tr>
<tr>
<td>after 1 month</td>
<td>215.28 ± 0.03</td>
<td>211.23 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>sVCAM-1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>at baseline</td>
<td>348.70 ± 0.02</td>
<td>348.70 ± 0.02</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>after 1 month</td>
<td>256.20 ± 0.03</td>
<td>296.20 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>sE-selectin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>at baseline</td>
<td>29.02 ± 0.01</td>
<td>32.02 ± 0.01</td>
<td>NS</td>
</tr>
<tr>
<td>after 1 month</td>
<td>28.02 ± 0.02</td>
<td>30.01 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>sP-selectin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>at baseline</td>
<td>156.00 ± 0.02</td>
<td>126.00 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>after 1 month</td>
<td>126.01 ± 0.01</td>
<td>126.01 ± 0.01</td>
<td>NS</td>
</tr>
</tbody>
</table>
There was statistically significant reduction of total cholesterol, triglyceride, LDL-C, Apo-B and Apo-B/Apo-A₁ levels, sICAM-1, sVCAM-1, sE-selectin, sP-selectin and an increase of HDL-C and Apo-A₁ levels following 40-mg-simvastatin therapy for one month in comparison with the placebo group (p < 0.001) (Table 2). Fifteen patients reached the target LDL-C level of ≤ 2.6 mmol/l with 40 mg of simvastatin, 3 patients were found to have a critical increase level of ALT ≥ 3 times, 2 patients resigned from the study, 30 patients did not attain the target LDL-C level of ≤ 2.6 mmol/l.

2. Therapeutic algorithm – phase 2 of the study – washout period for all 30 patients not reaching the target LDL-C level of ≤ 2.6 mmol/l (Fig. 1).

3. Therapeutic algorithm – phase 3 of the study: There was a statistically significant reduction in total cholesterol, triglyceride, LDL-C, Apo-B and Apo-B/Apo-A₁ levels, sICAM-1, sVCAM-1, sE-selectin, sP-selectin and an increase in HDL-C, Apo-A₁ following 80-mg-simvastatin therapy for one month in comparison with the placebo group (p < 0.001) (Tables 3, 4). Eleven patients reached the target LDL-C level of ≤ 2.6 mmol/l with 80 mg of simvastatin, 2 patients had a critical increase of the ALT level ≥ 3 times, 2 patients did not attain the target LDL-C level of ≤ 2.6 mmol/l: the alternative is change of statin.

DISCUSSION

The results of the study suggest that 80 mg of simvastatin exerts an effect on the levels of some CAM, and particularly on VCAM-1 and sICAM in contrast to the moderate dose of 40 mg of simvastatin. In the literature the latter two are closely connected with the presence of stable atherosclerotic plaque which is the context of the examined patients with asymptotic severe untreated hypercholesterolemia.⁶ The effect of simvastatin on VCAM-1 is a form of expression of the drug’s pleiotropic effect. Most of the literature sources that report of the effect of various statins on CAM levels are similar and determine their activity.¹¹-¹³,¹⁶-²⁰ The positive effect in the present study obtained only with 80 mg of simvastatin on CAM in contrast to the effect achieved with 40 mg accounts for the fact that higher doses of statins can have a positive effect on cardiovascular events i.e. aggressive statin therapy versus therapy with moderate doses of the drugs.

Table 3. Characteristics of the examined hypercholesterolemic subjects and placebo group

<table>
<thead>
<tr>
<th>Variables</th>
<th>Hypercholesterolemic patients (n = 15) (mean ± SD)</th>
<th>Placebo group (n = 15) (mean ± SD)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>46 ± 4</td>
<td>46 ± 2</td>
<td>NS</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>7/8</td>
<td>8/7</td>
<td>NS</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>25 ± 3</td>
<td>25 ± 2</td>
<td>NS</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>8.47 ± 0.16</td>
<td>8.35 ± 0.24</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.85 ± 0.04</td>
<td>0.88 ± 0.05</td>
<td>NS</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>0.98 ± 0.04</td>
<td>0.91 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/l)</td>
<td>6.67 ± 0.14</td>
<td>6.51 ± 0.18</td>
<td>NS</td>
</tr>
<tr>
<td>Apo-A₁ (g/l)</td>
<td>1.18 ± 0.02</td>
<td>1.22 ± 0.02</td>
<td>NS</td>
</tr>
<tr>
<td>Apo-B (g/l)</td>
<td>1.81 ± 0.03</td>
<td>1.74 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>Apo-B/Apo-A₁</td>
<td>1.53 ± 0.02</td>
<td>1.61 ± 0.02</td>
<td>NS</td>
</tr>
<tr>
<td>sICAM-1 (ng/ml)</td>
<td>235.68 ± 0.02</td>
<td>215.68 ± 0.01</td>
<td>NS</td>
</tr>
<tr>
<td>sVCAM-1 (ng/ml)</td>
<td>328.70 ± 0.03</td>
<td>320.70 ± 0.02</td>
<td>NS</td>
</tr>
<tr>
<td>sE-selectin (ng/ml)</td>
<td>30.02 ± 0.03</td>
<td>30.17 ± 0.04</td>
<td>NS</td>
</tr>
<tr>
<td>sP-selectin (ng/ml)</td>
<td>125.23 ± 0.74</td>
<td>120.20 ± 0.70</td>
<td>NS</td>
</tr>
</tbody>
</table>
be done with caution. Close monitoring of patients is important in the course of therapy. As there is a risk of drug interactions at cytochrome P450 3A4 level in the liver and increased simvastatin levels, other drugs, influencing the same enzyme levels, should be taken into consideration. According to the Food and Drug Administration control examinations should be carried out every 3 months and every 6 months during therapy. Rezaie-Majd et al. studied the in vivo effects of simvastatin therapy in hypercholesterolemia patients on the expression of ICAM-1 in vitro. They had two groups of patients: one group received 20 mg and the other group was treated with 40 mg per day. After 6 weeks of treatment the surface expression of ICAM-1 and his ligand from the integrin group over the peripheral monocytes lowered significantly in both groups of patients.

Table 4. Effect of one-month-simvastatin treatment (80 mg/d) on atherogenic biomarkers in hypercholesterolemic patients and the placebo group

<table>
<thead>
<tr>
<th></th>
<th>Hypercholesterolemic patients (n = 15)</th>
<th>Placebo group (n = 15)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mean ± SD)</td>
<td>(mean ± SD)</td>
<td></td>
</tr>
<tr>
<td>Total cholesterol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>at baseline</td>
<td>8.47 ± 0.16</td>
<td>9.05 ± 0.24</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>after 1 month</td>
<td>4.34 ± 0.04</td>
<td>4.39 ± 0.10</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>at baseline</td>
<td>0.85 ± 0.04</td>
<td>0.88 ± 0.05</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>after 1 month</td>
<td>0.67 ± 0.03</td>
<td>0.69 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>HDL-cholesterol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>at baseline</td>
<td>0.98 ± 0.04</td>
<td>0.91 ± 0.03</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>after 1 month</td>
<td>1.09 ± 0.20</td>
<td>1.10 ± 0.20</td>
<td>NS</td>
</tr>
<tr>
<td>LDL-cholesterol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>at baseline</td>
<td>6.67 ± 0.14</td>
<td>7.11 ± 0.18</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>after 1 month</td>
<td>3.55 ± 0.10</td>
<td>3.49 ± 0.10</td>
<td>NS</td>
</tr>
<tr>
<td>Apo-B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>at baseline</td>
<td>1.81 ± 0.03</td>
<td>2.34 ± 0.03</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>after 1 month</td>
<td>1.06 ± 0.30</td>
<td>1.07 ± 0.31</td>
<td>NS</td>
</tr>
<tr>
<td>Apo-A(_1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>at baseline</td>
<td>1.18 ± 0.02</td>
<td>1.22 ± 0.02</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>after 1 month</td>
<td>1.52 ± 0.21</td>
<td>1.50 ± 0.20</td>
<td>NS</td>
</tr>
<tr>
<td>Apo-B/Apo-A(_1)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>at baseline</td>
<td>1.53 ± 0.02</td>
<td>1.91 ± 0.02</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>after 1 month</td>
<td>0.66 ± 0.19</td>
<td>1.50 ± 0.21</td>
<td>NS</td>
</tr>
<tr>
<td>sICAM-1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>at baseline</td>
<td>235.68 ± 0.02</td>
<td>235.68 ± 0.02</td>
<td>NS</td>
</tr>
<tr>
<td>after 1 month</td>
<td>215.28 ± 0.03</td>
<td>215.28 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>sVCAM-1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>at baseline</td>
<td>348.70 ± 0.02</td>
<td>348.70 ± 0.02</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>after 1 month</td>
<td>256.20 ± 0.03</td>
<td>299.20 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>sE-selectin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>at baseline</td>
<td>30.02 ± 0.03</td>
<td>31.01 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>after 1 month</td>
<td>29.02 ± 0.03</td>
<td>32.02 ± 0.01</td>
<td>NS</td>
</tr>
<tr>
<td>sP-selectin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>at baseline</td>
<td>125.23 ± 0.74</td>
<td>126.00 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>after 1 month</td>
<td>119.00 ± 0.03</td>
<td>116.02 ± 0.1</td>
<td>NS</td>
</tr>
</tbody>
</table>
which shows that simvastatin effectively lowers the expression of ICAM-1 in patients with hypercholesterolemia.

The positive effect of the therapy with simvastatin, atorvastatin and pravastatin on the serum level of different CAM in patients with hypercholesterolemia and clinical manifestation of atherosclerosis is confirmed by other authors too. 23

M. Sardo et al. 15, however, studied the effect of simvastatin on sICAM-1 and sE-selectin in patients with hypercholesterolemia without other risk factors for atherosclerosis and found no significant reduction of the levels of sICAM and sE-selectin after a 4-week therapy.

Jilma et al. 24 reported similar data in 2003 about the effect of 3-month treatment with atorvastatin, pravastatin and pravastatin on the levels of sICAM-1, sVCAM-1 and sE-selectin in 25 patients with moderate hypercholesterolemia. They found that the level of the examined CAM did not decrease even in patients with 50% reduction of the LDL-cholesterol. This result is in context of our LDL-target for all patients, i.e. optimal lipid regulation is basic for another statin’s effect.

CONCLUSIONS

When LDL-cholesterol ≤ 2.6 mmol/l is targeted in patients with newly diagnosed untreated severe hypercholesterolemia by receiving 80 mg of simvastatin, only the levels of VCAM-1 lower considerably and significantly. In contrast, patients reaching this target with 40 mg simvastatin do not affect CAM. With the accumulation of more experimental results by methodologically similar research and meta-analysis it could be possible to get a clear idea to what extent CAM can be used as an effective marker in therapeutic regulation of the disturbed endothelium function with statins in hypercholesterolemia.

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Effect of Moderate and High Dose Simvastatin on Adhesion Molecules in Severe Hypercholesterolemia after Targeting the LDL-Cholesterol – a Randomised, Placebo-Controlled Study

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SERUM LEVELS OF sICAM-1, sVCAM-1, sE-SELECTIN, sP-SELECTIN IN HEALTHY BULGARIAN PEOPLE

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ABSTRACT

BACKGROUND: Endothelial dysfunction is increasingly recognized as an important early feature of vascular disease. As the damage to endothelium is a key underlying factor in the development and progression of atherosclerotic processes, markers of endothelial abnormalities have been sought. Increased expression of cell adhesion molecules (CAMs) on the vascular endothelium has been postulated to play a significant role in atherogenesis. Both in vitro and in vivo studies have suggested that different risk factors of atherosclerosis may increase expression of CAMs. The elevated level of soluble forms of CAMs in circulation is associated with a higher risk to future cardiovascular events in subjects predisposed to atherosclerosis.

OBJECTIVE: To determine the reference range for serum concentration of soluble cell adhesion molecules - sICAM-1, sVCAM-1, sE-selectin, sP-selectin.

MATERIAL AND METHODS: We studied 110 healthy people of Bulgarian nationality aged 18-65. The selection criteria for the reference group were made in accordance with the requirements of the International Federation of Clinical Chemistry (IFCC). Serum concentrations of CAMs were analysed by means of ELISA assay.

RESULTS: The results are presented as central 95% interval and 0.90 confidence interval of the reference range. Reference ranges were determined for sICAM-1 (128.9 – 347.48 ng/ml), sVCAM-1 (170.42 – 478.36 ng/ml), sE-selectin (9.15 – 65.19 ng/ml) and sP-selectin (101.86 – 209.7 ng/ml). As we found no sex-related differences in the CAMs concentrations (p > 0.05) there needed to be no separate reference intervals for men and women. The single-factor dispersion analysis we used in analysing the effect of age found no age-related dependence (p > 0.05, F = 1.038) for the serum CAM concentrations in the 18–65 age range, which means that it is not necessary to establish reference intervals for smaller age ranges in this age group.

CONCLUSION: The reference ranges for sICAM-1, sVCAM-1, sE-selectin, sP-selectin computed in accordance with the results distribution can be used as baseline criteria in clinical laboratory studies.

Keywords: soluble cell adhesion molecules, reference range, endothelial dysfunction, atherosclerosis

INTRODUCTION

Knowledge of the role of endothelium in controlling vascular tone has expanded greatly in the past decade. Healthy endothelium usually provides an anticoagulant, vasodilatory and anti-inflammatory spectrum of function that is central in vascular homeostasis.1,2 In the recent years it has been established that an altered function of the overlying endothelium is an early important feature of atherogenesis. This endothelial activation or dysfunction is considered to be indicative of atherosclerosis and may even be a contributing factor to the progression of the disease.3,4

A large body of evidence now shows that endothelial dysfunction is not only associated with established atherosclerosis in humans but is also detectable when only risk factors for atherosclerosis are present.3,5 Our understanding of the process of atherogenesis has evolved from simple epidemiologic identification of cardiac risk factors to an increasing understanding of the molecular basis of vascular pathology. Evidence for the role of chronic in-
flammtation in atherogenesis has been accumulating over the past decade: it suggests that a generalized cellular and humoral inflammatory response promotes the formation of atherosclerotic plaques.\textsuperscript{6,7}

Adhesion of leucocytes to altered vascular endothelium and subsequent transmigration into the intima are key events in the initiation of atherogenesis. These processes mediate through the interactions of a group of specialized molecules, collectively referred to as cell adhesion molecules (CAMs). The cell-surface expression of these molecules in response to pathophysiological stimuli mediates the interaction between the endothelial and blood cells central to the development of atherosclerosis.\textsuperscript{3,8-10}

Several cell adhesion molecules have been identified and are believed to be important in the pathogenesis of atherosclerosis. These cell adhesion molecules (CAMs) include the selectins – P- and E-selectin and the members of the immunoglobulin superfamily, intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1). As they play a central role in the recruitment of inflammatory cells to the site of atheroma development, the CAMs are promising candidates to reflect underlying damage of endothelium. The increased expression of adhesion molecules has been indeed observed in animal models of human atherosclerosis, and in human atherosclerotic lesions.\textsuperscript{8-11}

Adhesion molecules from immune and endothelial cells are shed and released into the circulation from cell membranes; they are referred to as soluble cellular adhesion molecules (sCAMs). They can be detected by specific antibodies using enzyme-linked immunosorbent assay techniques (ELISA assay).\textsuperscript{12,13} Soluble shed forms of CAMs are found in plasma, serum and other body fluids, and their concentration may be regarded as a surrogate marker of cellular expression.\textsuperscript{13,14}

Both in vitro and in vivo studies have suggested that different risk factors of atherosclerosis may increase the expression of CAMs.\textsuperscript{13-16} In support of this concept, circulating levels of CAMs have been elevated in patients with diabetes, hypertension, hyperlipidemia, coronary heart disease, and high CAMs levels have been found associated with the increased risk of future coronary events in subjects with predisposed factors for atherosclerosis.\textsuperscript{17-24}

The aim of the present study was to determine the reference range for the serum concentration of soluble cell adhesion molecules - sICAM-1, sVCAM-1, sE-selectin and sP-selectin in contingent of Bulgarian people.

**MATERIAL AND METHODS**

To establish the reference range of sICAM-1, sVCAM-1, sE-selectin and sP-selectin in serum and study the impact of sex and age we recruited 110 healthy individuals of Bulgarian nationality. The selection criteria for the reference group were made in accordance with the requirements of the International Federation of Clinical Chemistry (IFCC).\textsuperscript{25} The study included 54 men and 56 women aged between 18 and 65: 94 were younger than 50 and 16 older than 50 (Fig. 1). With the purpose of assessing their health status, we performed a number of clinical and routine laboratory tests.

**Figure 1.** Distribution in the reference group by sex and age (n = 110).

Exclusion criteria included:
1. Diabetes mellitus, impaired glucose tolerance or fasting blood glucose > 6.1mmol/l;
2. Cigarette smoking;
3. LDL cholesterol > 4.1 mmol/l; hypertriglyceridemia - TG > 1.7 mmol/l; obesity – body mass index greater than 25 kg/m\textsuperscript{2};
4. Clinical and laboratory evidence of:
4.1. Coronary artery disease in all forms;
4.2. Cerebrovascular disease;
4.3. Arterial hypertension;
4.4. Chronic obstructive pulmonary disease, bronchial asthma;
4.5. Chronic arterial insufficiency of the extremities (peripheral arteries) – ankle-brachial index < 0.9;
4.6. Chronic renal and hepatic dysfunction;
4.7. Systemic disorders of connective tissues – collagenosis, rheumatoid arthritis, SLE;
4.8. Neoplasms;
4.9. Acute inflammation or chronic inflammatory
process requiring active treatment; 5. Prolonged use (over the last six months and during the period of investigation) of nonsteroid anti-inflammatory agents, corticosteroids, hormonal medications, psychotropic drugs, lipid regulating medications – fibrates, statins, antioxidants; 5.1. Chronic use of alcohol and drug abuse.

Serum concentrations of CAMs were analysed by means of ELISA assay (Bender MedSystems, Germany). The major characteristics of analytic reliability were tested: intra-assay imprecision – CV < 8.0%, inter-assay imprecision – CV < 10.0%; accuracy – d% < 5.0%; and recovery – 92.0-106.0%. The method shows high precision; the results are consistent with the recommended minimal non-reproducibility (intra-assay CV < 10%; inter-assay CV < 12%) for the method as given by the manufacturer.

The manners of withdrawal, processing and storage of blood samples and the choice of biologic materials were in compliance with the requirements and the recommendations given by the manufacturer to compensate for the factors of result variation and for standardisation of the preanalytical stage.

**STATISTICS**

Data were analysed with SPSS 11.0 statistical software package at a level of significance of null hypothesis of p < 0.05. The analysis of distribution of the results was assessed by Kolmogorov-Smirnov test. The effect of sex was determined using the independent sample t-test. The reference intervals were calculated using the IFCC algorithm developed for the software program REFVAL (v. 3.21). The results are presented as central 95% interval and 0.90 confidence interval of the reference range.

**RESULTS**

There were no statistically significant differences in the values of the studied indexes between males and females, for which reason we used a common group (Table 1).

The distribution of the results for sICAM-1, sVCAM-1 and sP-selectin according to Kolmogorov-Smirnov was Gaussian (p > 0.05, λ = 0.55, λ = 0.29, λ = 0.48) (Figs 2a, 2b, 2c), for E-selectin was non-normal (p < 0.05, λ = 1.21), (Fig. 3). The distribution of the results for E-selectin became normal after transformation of the data using exponential function to correct the asymmetry and excess.

The results of the statistical analysis of the data for CAMs are shown in Table 2. The reference limits with their 90% confidence interval were determined using parametric analysis. It is obvious that and Me are similar, which is due to the regular distribution of the majority of the results and the software program.

A reference range was defined for sICAM-1 serum levels with 128.9 (90% CI: 128.86-137.55) ng/ml to 347.48 (90% CI: 333.58-361.05) ng/ml, for sVCAM-1 serum levels with 170.42 (90% CI: 147.10-193.58) ng/ml to 478.36 (90% CI: 464.6-491.32) ng/ml, for sE-selectin serum levels with 9.15 (90% CI: 7.67-10.77) ng/ml to 65.19 (90% CI: 59.86-70.9) ng/ml and for sP-selectin serum levels with 101.86 (90% CI: 97.6-106.52) ng/ml to 209.7 (90% CI: 205.47-213.61) ng/ml. Single-factor dispersion analysis found no age dependency (p > 0.05), (F = 1.002; F = 1.156; F = 0.696; F = 1.433) of sICAM-1, sVCAM-1, sE-selectin and sP-selectin in the age range of 18-65 which makes unnecessary establishment of reference intervals for lower age range in this age group.

We used graphic presentation to achieve easier interpretation of the internal structure of the reference interval (Figs 4 a,b,c and Fig. 5).

**Table 1. Comparative characteristics of the results for CAMs by sex**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sex</th>
<th>N</th>
<th>$\bar{x}$</th>
<th>SD</th>
<th>SEM</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>sICAM-1</td>
<td>male</td>
<td>54</td>
<td>235.68</td>
<td>57.01</td>
<td>7.75</td>
<td>0.245</td>
</tr>
<tr>
<td></td>
<td>female</td>
<td>56</td>
<td>222.21</td>
<td>63.42</td>
<td>8.48</td>
<td></td>
</tr>
<tr>
<td>sVCAM-1</td>
<td>male</td>
<td>54</td>
<td>348.70</td>
<td>70.87</td>
<td>9.65</td>
<td>0.332</td>
</tr>
<tr>
<td></td>
<td>female</td>
<td>56</td>
<td>338.30</td>
<td>92.85</td>
<td>12.4</td>
<td></td>
</tr>
<tr>
<td>sE-selectin</td>
<td>male</td>
<td>54</td>
<td>30.02</td>
<td>14.93</td>
<td>2.03</td>
<td>0.423</td>
</tr>
<tr>
<td></td>
<td>female</td>
<td>56</td>
<td>32.45</td>
<td>16.65</td>
<td>2.22</td>
<td></td>
</tr>
<tr>
<td>sP-selectin</td>
<td>male</td>
<td>54</td>
<td>156.00</td>
<td>32.37</td>
<td>4.40</td>
<td>0.802</td>
</tr>
<tr>
<td></td>
<td>female</td>
<td>56</td>
<td>157.52</td>
<td>31.06</td>
<td>4.15</td>
<td></td>
</tr>
</tbody>
</table>

$\bar{x}$ - mean, SD – standard deviation, SEM – standard error of mean.
Serum Levels of sICAM-1, sVCAM-1, sE-selectin, sP-selectin in Healthy Bulgarian People

The reference range was plotted parallel to the Y-axis on which the units of measure of the parameter were marked. The horizontal lines mark the lower (0.025 fractile) and upper (0.975 fractile) reference ranges, the median (0.50 fractile) and the fractiles 0.25 and 0.75. The two middle square margins form the central 50% fraction, while the two terminal margins include 22.5% of all results. The vertical lines give the 90% confidence intervals of the upper and lower reference ranges.

The graphic presentation, which actually shows the internal structure of the result distribution, clearly indicates that the central 50% fraction presents practically symmetry distribution for sICAM-1, sVCAM-1 and sP-selectin and relatively close confidence interval for upper reference interval. Results for E-selectin have a pronounced right asymmetry and relatively wider confidence interval for upper reference interval. This mode of presenting the reference intervals has the advantage of providing immediate information about the type of distribution, the width and reliability of the reference intervals and the uniqueness of a specific result.

DISCUSSION

The major findings of the present study are:
1. A reference value was defined for sICAM-1 serum levels with 128.9 ng/ml to 347.48 ng/ml, for sVCAM-1 serum levels with 170.42 ng/ml to 478.36 ng/ml, for sE-selectin serum levels with 9.15 ng/ml to 65.19 ng/ml and for sP-selectin serum levels with 101.86 ng/ml to 209.7 ng/ml.
2. We found no sex-related differences in CAMs concentration (p > 0.05), which obviates the need for separate reference intervals for men and women.
3. Single-factor dispersion analysis found no age dependency of CAMs (p > 0.05) in the age range 18-65 which makes unnecessary establishment of reference intervals for smaller age ranges in this age group.

In spite of the fact that establishing reference values is an expensive and time-consuming task, it is recommended that they be determined in case of
newly introduced parameters or parameters having genetic heterogeneity.\textsuperscript{25} This is a reason for building reference intervals for a specific population from a specific region and with a specific structure.

There is no information in related articles about reference limits for sICAM-1, sVCAM-1, sE-selectin, sP-selectin, and the results from the research are compared to the average values of the control group of healthy individuals calculated parametrically (±2SD), without being corrected for the type of distribution of the results.\textsuperscript{13,22,26-28}

It is accentuated that the increase (even a little higher than the average values) of the markers of endothelial dysfunction in healthy individuals is connected to an elevated risk of developing atherosclerosis.\textsuperscript{22,29-31} which requires the determination of reliable discriminant values (cut-off) to evaluate

---

### Table 2. Reference intervals for sICAM-1, sVCAM-1, sE-selectin, and sP-selectin

<table>
<thead>
<tr>
<th>Parameters</th>
<th>sICAM-1 ng/ml</th>
<th>sVCAM-1 ng/ml</th>
<th>sE-selectin ng/ml</th>
<th>sP-selectin ng/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>110</td>
<td>110</td>
<td>110</td>
<td>110</td>
</tr>
<tr>
<td>Range</td>
<td>113.00-353.00</td>
<td>145.00-530.00</td>
<td>10.00-96.50</td>
<td>99.00-218.00</td>
</tr>
<tr>
<td>Me</td>
<td>231.00</td>
<td>355.00</td>
<td>31.25</td>
<td>155.50</td>
</tr>
<tr>
<td>SD</td>
<td>60.50</td>
<td>82.78</td>
<td>15.80</td>
<td>31.57</td>
</tr>
<tr>
<td>0.025 fractile</td>
<td>128.90</td>
<td>170.42</td>
<td>9.15</td>
<td>101.86</td>
</tr>
<tr>
<td>90% CI</td>
<td>120.86-137.55</td>
<td>147.10-193.58</td>
<td>7.67-10.77</td>
<td>97.6-106.52</td>
</tr>
<tr>
<td>0.975 fractile</td>
<td>347.48</td>
<td>478.36</td>
<td>65.19</td>
<td>209.7</td>
</tr>
<tr>
<td>90% CI</td>
<td>333.58-361.05</td>
<td>464.60-491.32</td>
<td>59.86-70.9</td>
<td>205.47-213.61</td>
</tr>
</tbody>
</table>

\(\bar{x}\) - mean, SD – standard deviation, 90% CI – 90% confidence interval.

---

**Figure 4.** Graphic presentation of the reference intervals and their internal structure for sICAM-1, sVCAM-1 and sP-selectin.

**Figure 5.** Graphic presentation of the reference intervals and their internal structure for sE-selectin.
the risk of the appearance of vascular incidents.

CONCLUSIONS

The reference values of sICAM-1, sVCAM-1, sE- and sP-selectin serum levels are calculated according to the type of result distribution; they can be used as baseline criteria for clinical laboratory purposes. The changes in the concentration of analysed CAMs for the age range 18–65 are not statistically significant, which means that it is not necessary to establish reference intervals for smaller age ranges within this age group. The unavailability of sex-related differences in CAM concentration, does not require separate reference intervals for men and women. The graphic model of reference interval provides immediate information about the type of distribution, the width and reliability of the reference values and makes it possible to easily visualize and determine how typical the results in studies of risk populations are.

REFERENCES


ВЛИЯНИЕ СЫВОРОТЧИХ УРОВНЕЙ sICAM-1, sVCAM-1, sE-SELEXITIN, sP-SELEXITIN НА ЗДОРОВЬЕ БОЛГАРСКОЙ НАЦИОНАЛЬНОСТИ
Т. Денева-Койчева, Л. Владимирова-Китова, Е. Ангелова, Т. Цветкова

РЕЗЮМЕ
Повреждение эндотелия сосудов является ключевым фактором в развитии и прогрессии атеросклеротического процесса. Это определяет и поиски подходящих и достоверных маркеров для эндотелиальной дисфункции. Ряд экспериментальных и клинических исследований доказывает, что повышенная экспрессия клеточных адгезионных молекул (КАМ) играет значимую роль в атерогенезе. Увеличенные уровни циркулирующих адгезионных молекул связывают с повышенным риском сердечно-сосудистых осложнений среди лиц с факторами риска атеросклероза.

Цель: Исследование ставит себе целью определить референтные границы КАМ: – sICAM-1, sVCAM-1, sE-selectin и sP-selectin.

МАТЕРИАЛ И МЕТОДЫ: В целях определения границ референтной области КАМ в сыворотке и изучения влияния факторов “пол” и “возраст” обследовано 110 здоровых лиц болгарской национальности в возрасте от 18 до 65 лет. Критерии подбора референтной группы соображены с общепринятыми рекомендациями IFCC. Сывороточная концентрация КАМ определена иммунометрически с помощью ELISA-анализа.

РЕЗУЛЬТАТЫ: Референтные границы, выраженные как центральный 95-процентный интервал полученных стоимостей, следующие: 1128.9 - 347.48 ng/ml для sICAM-1; 170.42 - 478.36 ng/ml для sVCAM-1 ng/ml; для sE-selectin - 9.15 - 65.19 ng/ml; для sP-selectin - 101.86 - 209.7 ng/ml. Пол не оказывает влияние на концентрацию КАМ (р > 0.05), вот почему нет необходимости в использовании отдельных референтных границ для мужчин и женщин.

Прослеживание влияния возраста посредством однофакторного дисперсионного анализа не установливает возрастную зависимость: ρ > 0.05, (F = 1.038) для сывороточных концентраций КАМ при обследованной референтной группе в возрасте от 18 до 65 лет, что не требует формирования референтных границ для меньших возрастных интервалов.

ЗАКЛЮЧЕНИЕ: Референтные стоимости sICAM-1, sVCAM-1, sE-selectin и sP-selectin в сыворотке, вычисленные сообразно с видом распределения результатов, могут служить исходными критериями при проведении клинико-лабораторных исследований и позволят их пользование с клинической целью.
CLINICAL AND LABORATORY STUDY OF PRO-INFLAMMATORY AND ANTI-INFLAMMATORY CYTOKINES IN WOMEN WITH MULTIPLE SCLEROSIS

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ABSTRACT

Multiple sclerosis (MS) is an autoimmune demyelinating disorder of the central nervous system characterised with a complex system of interactions between proinflammatory and anti-inflammatory cytokines in its course.

AIM: The aim of the present study was to investigate the serum levels of cytokines TNF-α, IFN-γ, IL-4 and IL-10 in female patients with MS and healthy individuals, the changes occurring in the relapse and remission phases of the disease and their correlation with the severity of the neurological deficit.

PATIENTS AND METHODS: Thirty-five women with relapsing-remitting MS were examined. The patients’ age ranged between 18 and 50 years and MS was verified clinically and by magnetic resonance imaging according to the McDonald criteria. Thirteen of the patients were treated with interferon-β-1b. The serum concentrations of TNF-α, IFN-γ, IL-4 and IL-10 were determined twice – in relapse and in remission – using an enzyme-linked immunosorbent assay (ELISA). The control group consisted of 35 age-matched healthy females.

RESULTS: The comparison of cytokine serum concentrations during the two phases of the disease showed significant elevation of the TNF-α serum levels in the relapse phase and of IL-4 – in the remission phase. The comparison between the patients and the healthy control subjects demonstrated statistically significant lower concentrations of TNF-α in remission patients and higher concentrations of IL-10 in relapse patients. The patients with interferon-β-1b treatment showed different profile of cytokine secretion from the patients without interferon-β-1b treatment. Interferon-β-1b-treated patients showed significantly lower serum levels of TNF-α and IFN-γ during the relapse phase and higher TNF-α and IL-10 serum levels during the remission phase compared with the untreated patients.

CONCLUSIONS: Serum levels of TNF-α and IL-4 objectively reflect the immune response during relapse and remission of the disease. The severity of neurological deficit as estimated with the expanded disability status scale (EDSS) does not depend on the serum levels of TNF-α, IL-10 and IFN-γ in the two phases of MS.

Key words: multiple sclerosis, cytokines, interferon-β-1b

INTRODUCTION

Multiple sclerosis is an immunological organ-specific disease with persisting immunological dysbalance in the patient serum and cerebrospinal fluid. Experimental and clinical studies have revealed different profiles of cytokine secretion during relapse and remission of MS. It is thought that it is the Th1-dependent secretion of TNF-α, IFN-γ, IL-12 that triggers the autoimmune reaction, while the Th2 synthesis of IL-4, IL-6, and IL-10 has anti-inflammatory effects. There is a large body of evidence showing that some mediators of inflammation, which include cytokines, can protect the neurons from degeneration at some phases of the disease.

The studies of cytokine profiles in MS patients and the correlations with the clinical picture and progress of the disease have yielded contradictory results. Finding what roles play each of the immune components of the MS pathological process is of crucial importance if we want to determine the “targets” for the drugs and the intensity of their therapeutic effect determined very accurately.

The aim of the present study was to investigate...
the serum levels of cytokines TNF-α, IFN-γ, IL-4 and IL-10 in female patients with MS and healthy individuals, the changes which occur during the relapse and remission phases of the disease and their correlation with the severity of the neurological deficit.

PATIENTS AND METHODS

PATIENTS

We conducted an open, prospective and randomized study at the Department of Neurology, Medical University, Plovdiv between 2003 and 2007. The study included 35 female patients with MS and a control group of 35 healthy women aged between 18 and 50 years.

Inclusion criteria
1. Gender – females;
2. Age – between 18 and 50 years;
3. Relapsing-remitting MS diagnosed according to the McDonald criteria (2000) and verified by magnetic resonance tomography.

Exclusion criteria
1. Endocrinological, renal, hepatic and immunological disorders;
2. Acute and chronic infections;
3. Therapy with immunosuppressive drugs;
4. Therapy with corticosteroids three months before the first examination.

The serum levels of TNF-α, IFN-γ, IL-4 and IL-10 were measured twice:
• during the relapse phase defined as the appearance of new symptoms or deterioration of already existing ones, lasting more than 24 hours and at least a 30-day period of improvement or stabilization of the neurological status after the previous relapse.
• during the remission phase (at least 2 months after the previous exacerbation).

METHODS

Clinical tests
• McDonald criteria (2000) for diagnosis of MS;
• Expanded Disability Status Scale (EDSS) to determine the severity of the neurological deficit.

Laboratory tests
Serum TNF-α, IFN-γ, IL-4 and IL-10 levels of the MS patients and the control subjects were measured twice; venous blood for examination was taken using ordinary procedures. The obtained serum was stored at -70°C until the analysis was carried out. The laboratory analyses of MS patients were performed during relapse and remission. For the control group the cytokine serum levels were estimated as the mean of two measurements. The TNF-α, IFN-γ, IL-4 and IL-10 serum levels were determined by ELISA using kits of BIOSOURCE Europe S.A. The absorption, which is proportional to the cytokine concentration, was measured colorimetrically using TECAN ELISA reader at 450 and 630 nm. The concentration of each of the cytokines was calculated with the help of a standard curve.

STATISTICS

Statistical analysis were performed using SPSS 13.0; the level of significance was accepted at p < 0.05. We used the Independent Samples t-test, the paired-samples t-test, the Wilcoxon signed-rank test and correlation analysis.

The study was approved by the Scientific Ethics Commission of the Medical University, Plovdiv, Proceedings №3/28.03.2005.

RESULTS

The clinical characteristics of the study contingent are presented in Table 1.

Thirteen of the study patients (37.14%) were treated with interferon-β-1b – group A, unlike the rest 22 patients (62.86%) included in group B.

The mean level of neurological deficit as estimated with the EDSS was significantly lower in remission compared with the one in relapse p < 0.001 (t = 9.62).

The results of the investigated immune indicators (serum cytokines) are presented in Fig. 1.

The changes of the cytokine serum levels were analyzed using the Independent Samples t-test. The comparison between the results of the patients and the healthy controls showed statistically significant lower concentrations of TNF-α in the patients during remission (p < 0.05, t = 1.99) and higher concentrations of IL-10 during relapse (p < 0.01, t = 3.18). We noticed the following tendencies: higher mean values of IFN-γ and IL-10 in the patients in remission compared with the controls. During the two phases of clinical manifestations the changes of the cytokine levels were analyzed using the Wilcoxon signed-rank test. The following statistically significant correlations were found: significant elevation of IL-4 levels (p < 0.05) during remission compared to the levels during relapse and of TNF-α levels (p < 0.01) during exacerbation compared to the levels during remission (Fig. 1).

Tables 2 and 3 present the results from the investigation of the cytokine levels in group A.
The IFN-γ levels were significantly elevated in group A during remission compared with the relapse levels (p < 0.05, t = 2.19). The changes in TNF-α, IL-4 and IL-10 levels during the two periods of clinical manifestations could not reach statistical significance.

In group B statistically significant elevation of TNF-α (p < 0.05, t = 2.56) and of IL-10 (p < 0.01, t = 4.07) during remission compared to the remission levels.

The correlation analysis revealed moderate positive correlation between the degree of disability as estimated with the EDSS in remission and the concentration of IL-4 during the same period (p < 0.05, r = 0.38).

**DISCUSSION**

The immune response to the myelin antigens in MS is still not entirely clear. TNF-α is given as one of the major pro-inflammatory cytokines in demyelinating disorders; it is considered to possess myelinotoxic effects.\(^8\) The present study revealed statistically significantly lower levels of TNF-α in remission and elevated levels of IL-10 in relapse in the MS patients compared with healthy individuals.
The significant decrease of TNF-α levels in remission which become lower than those of controls is probably an expression of abating autoimmune demyelination during clinical improvement. It is supposed that the elevated IL-10 serum levels during the relapse phase result from the activation of the regulatory mechanisms of the body which suppress autoimmune reactions. On the other hand several experimental studies reported exacerbation of the disease clinical manifestations after administration of IL-10 in doses higher than the physiological ones. The significant elevation of IL-4 plasma levels in all patients in remission compared with that in exacerbation is probably an indication of a shift from a Th1 to a Th2 immune response. The results of our study are consistent with the conclusion F Weber makes that a shift of the immune reaction from type Th1 to type Th2 should result in suppression of the inflammatory process in the central nervous system.²

The hypothesis of pro-inflammatory pathogenic effect of TNF-α and IFN-γ is based on clinical evidence: statistically significant elevation of TNF-α during relapse and increased frequency of disease exacerbations after intravenous treatment with IFN-γ. The significant elevation of TNF-α serum levels during exacerbation we found in this study in comparison with the levels in remission

Table 2. Serum cytokine concentrations in interferon-β-1b-treated MS patients

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>MS phase</th>
<th>n</th>
<th>Mean ± SE pg/ml</th>
<th>SD</th>
<th>t</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>relapse</td>
<td>13</td>
<td>21.72 ± 3.18</td>
<td>11.48</td>
<td>1.53</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td></td>
<td>remission</td>
<td>13</td>
<td>19.63 ± 3.68</td>
<td>13.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>relapse</td>
<td>13</td>
<td>2.86 ± 0.81</td>
<td>2.92</td>
<td>2.19</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>remission</td>
<td>13</td>
<td>4.35 ± 0.89</td>
<td>3.21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>relapse</td>
<td>13</td>
<td>6.70 ± 1.85</td>
<td>6.68</td>
<td>0.13</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td></td>
<td>remission</td>
<td>13</td>
<td>7.03 ± 1.69</td>
<td>6.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-4</td>
<td>relapse</td>
<td>13</td>
<td>2.36 ± 0.60</td>
<td>2.17</td>
<td>0.72</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td></td>
<td>remission</td>
<td>13</td>
<td>3.14 ± 0.86</td>
<td>3.11</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Serum cytokine concentrations in interferon-β-1b-non-treated MS patients

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>MS phase</th>
<th>n</th>
<th>Mean ± SE pg/ml</th>
<th>SD</th>
<th>t</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>relapse</td>
<td>22</td>
<td>27.81 ± 4.34</td>
<td>20.40</td>
<td>2.56</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>remission</td>
<td>22</td>
<td>14.06 ± 2.72</td>
<td>12.74</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>relapse</td>
<td>22</td>
<td>5.37 ± 0.59</td>
<td>2.74</td>
<td>0.75</td>
<td>&gt; 0.05</td>
</tr>
<tr>
<td></td>
<td>remission</td>
<td>22</td>
<td>6.07 ± 0.94</td>
<td>4.39</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>relapse</td>
<td>22</td>
<td>7.00 ± 0.98</td>
<td>4.59</td>
<td>3.28</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td></td>
<td>remission</td>
<td>22</td>
<td>3.70 ± 0.78</td>
<td>3.69</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-4</td>
<td>relapse</td>
<td>22</td>
<td>2.13 ± 0.43</td>
<td>2.03</td>
<td>2.31</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>remission</td>
<td>22</td>
<td>3.70 ± 0.66</td>
<td>3.10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
confirms the role of this cytokine as a factor triggering the immune inflammatory process. There are some difficulties in the clinical interpretation of the changes we found in the IFN-γ concentrations during the two phases of the disease. We established significant differences in all patients individually in the changes of IFN-γ and a tendency towards elevation of its plasma levels during remission compared with the relapse phase. Y. Miyazaki et al. reported that interferon-β-1b exerted a significant suppressive effect on the IFN-γ synthesis by the peripheral myelin-reactive CD4+ CD28+ T cell subpopulations, but had an insignificant effect on the production of IFN-γ by the CD4+ CD28- T lymphocyte branches. We suggest that the results of the present study reflect such an aspect of dysbalance of the cytokine-regulatory system. During remission interferon-β-1b-treated patients demonstrated statistically significantly elevated IL-10 plasma levels compared with this indicator in non-treated patients for the same clinical period. We accept that these results are associated with the increased synthesis of IL-10 by the auto-reactive T lymphocytes under the influence of interferon-β-1b which was experimentally proved by Zang YC, et al. and S Chabot and VW Yong in their studies.

We found no causal relationship between the
severity of disability (EDSS) and the level of TNF-α, IL-10 and IFN-γ during the two phases of clinical manifestations. That is why we suppose that the regulatory cytokine dysbalance and EDSS reflect the degree of immunological inflammation with different specificity. Literature data show direct correlation between the severity of neurological deficit and the loss of axons, but the axonal lesions are of different mechanisms: immunological-inflammatory, degenerative (Wallerian degeneration) and ischemic.4

CONCLUSIONS

The serum levels of TNF-α and IL-4 objectively reflect the activity of the immune response during relapse and remission of MS.

The severity of neurological deficit as estimated by EDSS does not depend on the serum levels of TNF-α, IFN-γ and IL-10 during the two periods of MS.

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КЛИНИКО-ЛАБОРАТОРНОЕ ИССЛЕДОВАНИЕ ПРОВОСПАЛИТЕЛЬНЫХ И АНТИВОСПАЛИТЕЛЬНЫХ ЦИТОКИНОВ У ЖЕНЩИН С МНОЖЕСТВЕННЫМ СКЛЕРОЗОМ

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РЕЗЮМЕ

Введение: Множественный склероз (МС) представляет собой аутоиммунное, демиелинизирующее заболевание центральной нервной системы в ходе которого развивается сложная сеть взаимодействий между проинфляматорными и антиинфляматорными цитокинами.

Цель: Исследовать сывороточные уровни TNF-α, IFN-γ, IL-4 и IL-10 у женщин с МС и у здоровых лиц, а также и изменения, наступающие во время приступа и во время ремиссии заболевания и их связь с тяжестью неврологического дефицита.

Материал и методы: Обследовано 35 женщин в возрасте от 18 до 50 лет с приступно-ремитентным, клиническим и резонансно подтвержденным МС по критериям Mc Donald. 13 женщин лечены интерфероном β-1b. Сывороточные концентрации TNF-α, IFN-γ, IL-4 и IL-10 определены двукратно — во время приступа и во время ремиссии с помощью энзимосвязанного иммуносорбентного теста (ELISA). Контрольная группа включает 35 здоровых женщин того же возрастного интервала.

Результаты: При сравнении сывороточных концентраций цитокинов во время обеих фаз заболевания устанавливаются значимо более высокие уровни TNF-α во время приступа и IL-4 — во время ремиссии. При сопоставлении результатов пациенток и здоровых женщин зарегистрированы статистически значимо более низкая концентрация TNF-α у больных во время ремиссии и более высокая концентрация IL-10 во время приступа. У пациенток с и без интерферона β-1b наблюдается различный профиль секреции цитокинов. Пациентки, леченные препаратом интерферон β-1b, имеют значимо более низкие TNF-α и IFN-γ во время приступа и более высокие TNF-α и IL-10 в состоянии ремиссии по сравнению с нелеченными.

Выводы: Сывороточные уровни TNF-α и IL-4 объективно отражают активность иммунной реакции во время двух периодов клинических проявлений МС (приступ и ремиссия). Тяжесть неврологического дефицита, оцененная с помощью EDSS, не зависит от сывороточных уровней TNF-α, IL-10 и IFN-γ во время двух периодов клинических проявлений.