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24

Paraoxonase Polymorphisms and Platelet Activating Factor Acetylhydrolase Activity as a Genetic Risk Factors in Cerebral Atherosclerosis

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1. Introduction

Atherosclerosis is a progressive disease characterized by the accumulation of lipids and fibrous elements in the large arteries (Lusis, JA. 2000). Investigations into the genetics of atherosclerosis, along with biochemical approaches, have greatly advanced today knowledge of the mechanisms of this complex multifactorial disease (Lusis et al., 2004a, 2004b; Lusis &Weiss , 2010). According to the oxidation hypothesis, oxidative stress is a key mechanism through which atherosclerosis as a chronic inflammatory disease develops. It is mediated by reactive oxygen species that alter the fundamental properties of cholesterol, cholesterol esters, and phospholipids on lipoproteins, as well as other proteins, to make them dysfunctional, immunogenic, and pro-atherogenic (Tsimikas et. al., 2009). Oxidative stress can be enhanced by non-enzymatic pathways, such as by copper and iron cations, as well as by enzymatic pathways, such as by lipoxygenases, myeloperoxidase, and NADPH oxidase. These pro-oxidant pathways are balanced by anti-oxidant mechanisms, such as anti-oxidant vitamins (alpha-tocopherol and carotenoids) present within lipoproteins, and anti-oxidant enzymes, such as superoxide dismutase and glutathione peroxidase. Many of these enzymes and products of oxidation can be measured in the circulation, including oxidized low-density lipoprotein, oxidized phospholipids, isoprostanes, and myeloperoxidase, and have been shown to predict the presence of cardiovascular disease (CVD) and incident cardiovascular events (Tsimikas et al., 2007, 2009).

Human serum paraoxonase [(PON1); aryldialkylphosphatase (EC 3.1.8.1)] is associated with high density lipoprotein particles (HDL) responsible in part for the ability of HDL to prevent lipid peroxidation. The decreased serum paraoxonase (PON1) activity in patients with atherosclerosis disease may cause decreased HDL antioxidant capacity and therefore significantly influence the risk of the development of atherosclerosis (Aviram, M. 2004;

Nieminen et al., 2006; Shih DM. & Lusis AJ. 2009). The enormous between-individual biological variability in serum PON1 activity seems to be regulated mainly by genetic determinants. The paraoxonase gene family includes *pon1*, *pon2* and *pon3* genes which produce three enzyme paraoxonase 1 (PON1), paraoxonase 2 (PON2) and paraoxonase 3 (PON3). These genes are located on the long arm of chromosome 7 and they are structurally similar. They share about 70% of identity in nucleotide sequences and about 60% of identity in amino acid sequences. PON1 mRNA is expressed in the liver, and PON3 mRNA is expressed primarily in the liver but also in the kidneys. On the other hand PON2 mRNA is ubiquitously expressed in different kinds of tissues like kidneys, liver, lungs, small intestine, placenta, spleen, stomach and testicles and in the cells of the artery wall (including endothelial cell, smooth muscle cell and macrophages) (Draganov, DI. & La Du, BN. 2004; Ng et al., 2005).

PON1 is a 354 amino acid long glycosylated protein and has an apparent mass of 43-47 kDa. The enzyme is synthesized in the liver and is secreted into plasma. In the plasma, PON1 is mainly bounded to high density lipoproteins (HDL) but also small amount of this enzyme was detected in very low-density lipoprotein (VLDL), and postprandial chylomicrons. PON1 has hydrophobic signal sequence on the N-terminal region, from which only the initiator methionine residue is removed, and this region is for the association of PON1 with HDL (Draganov, DI. & La Du, BN. 2004; Fuhrman et al., 2005.) PON1 possesses organophosphatase, arylesterase and lactonase activities and hydrolyzes different kinds of substrates (like paraoxon, chlorpyrifos oxon, diazoxon, sarin, soman, phenylacetate, tiophenylacetate homogentisic acid lactone, dihydrocoumarin, γ-butyrolactone and homocysteine thiolactone) (Draganov, DI. & La Du, BN. 2004; Ng et al., 2005). PON1 is also well known to possess antioxidative and antiatherogenic activity, to protect HDL and low-density lipoprotein (LDL) from oxidation, and to destroy biologically active oxidized lipids on lipoproteins and in arterial cells (Draganov, DI. & La Du, BN. 2004; Aviram, M. 2004).

More than 160 polymorphisms of *pon1* gene are known, and some of them have been recognized to affect PON1 concentration and activity (Deakin, SP. & James, RW. 2004; Costa et al., 2005). Two polymorphisms in the coding region of *pon1* gene result in the substitution of amino acid glutamine with arginine at the position 192 (Q192R polymorphism, the exchange of codon CAA to CGA in exon 6) and in the substitution of amino acid leucine to methionine at the position 55 (L55M polymorphism, the exchange of codon TTG to ATG in exon 3) (Adkins et al., 1993). Q192 and R192 alloenzymes have a different affinity and catalytic activity towards numerous substrates, the R192 alloenzyme hydrolyzes paraoxon six times faster than Q192 alloenzyme while Q192 alloenzyme hydrolyzes sarin, soman and diazoxon faster than R192 alloenzyme (Deakin, SP. & James, RW. 2004). These two alloenzymes are also different in their ability to protect LDL from oxidation *in vitro*, Q192 alloenzyme is more efficient than R192 alloenzyme (Deakin, SP. & James, RW. 2004; Mackness et al., 1999). L55M polymorphism affects PON1 mRNA levels, concentration and enzyme activity. M55 alloenzyme is associated with a lower level of PON1 mRNA, concentration and activity (Deakin, SP. & James, RW. 2004). These two alloenzymes are also different in protection of LDL against oxidation, where M55 alloenzyme shows to be more protective (Mackness et al., 1999). In the promoter region of *pon1* gene at least five polymorphisms were detected and -108C>T polymorphism is one of them. This polymorphism affects *pon1* gene expression, and enzyme concentration and activity. It is believed that -108C>T polymorphism is the main contributor to serum PON1 variation (accounting for 23-24% of total variation), while other polymorphisms in *pon1* promoter region made little or no difference to serum PON1 levels (Deakin, SP. & James, RW. 2004; Leviev, I. & James, RW. 2000; Suehiro et al., 2000).

As it was mentioned earlier, PON2 is a ubiquitously expressed intracellular protein with a relative molecular mass of approximately 44 kDa (Ng et al., 2005; Li et al., 2003). PON2 has antioxidant properties, lowers the intracellular oxidative stress and prevents the cell-mediated oxidation of LDL (Ng at al., 2005; Li et al., 2003). In the *pon2* gene two common polymorphisms were identified. Alanine or glycine could be at the position 148 (A148G), and serine or cysteine could be at the position 311 (S311C). S311C polymorphism has been related with eg. coronary artery disease, ischemic stroke in patients with type 2 diabetes mellitus, Alzheimer's disease and reduced bone mass in postmenopausal women (Ng at al., 2005; Li et al., 2003). The mechanisms by which PON2 exerts its atheroprotective effects remain to be clarified. Large-scale epidemiologic studies are needed to further examine the relationship between PON2 genetic polymorphisms and risk for CVD (Shih, DM. & Lusius, AJ. 2009).

Human PON3 is a '40-kDa protein primarily synthesized in the liver with biological activity similar to PON1. PON3 is a secreted protein associated with HDL in the plasma and can participate in the prevention of LDL oxidation. The PON3 protein may play a role, distinct from that of PON1, in the lipoprotein metabolism of the kidney. These characteristics link PON3 with a group of enzymes, such as PON1, platelet-activating factor–acetylhydrolase, and lecithin-cholesterol acyltransferase, which together may contribute to the antiatherogenic properties of HDL, but the role of PON3 in atherosclerosis needs further investigation (Reddy et al., 2001; Getz, GS. & Resardon, CA. 2004).

Another lipoprotein-associated enzyme, the platelet-activating factor acetylhydrolase (PAF-AH), also referred to as lipoprotein-associated phospholipase A_2 (Lp-PLA_2), is an enzyme (EC 3.1.1.47) recently described as a potentially useful plasma biomarker associated with cardiovascular disease (Srinivasan, B. & Bahson, BJ. 2010; Koenig et al., 2004; Yamada et al., 2000; Karasawa, K. 2006; Mallat et al., 2010). The biological role of Lp-PLA_2 (PAF-AH) has been controversial, with contradictory antiatherogenic and proatherogenic functions. The antiatherogenic properties of Lp-PLA2 were first suggested because plasma PAF-AH might play an anti-inflammatory role in human diseases by preventing the accumulation of PAF (1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) and PAF-like oxidized phospholipids (Karasawa, K. 2006; Mallat et al., 2010; Mitsios et al., 2006). PAF is a biologically active phospholipid involved in diverse pathologies such as inflammation and atherosclerosis. PAF can activate various cell types including platelets. In the presence of PAF, platelets aggregate and degranulate, releasing biologically potent agents. PAF is hydrolyzed and converted to lysoPAF by the catalytic reaction of PAF-AH (Mitsios et al., 2006). The atherogenic role of Lp-PLA_2 comes from the observation that this enzyme can also produce lysophosphatidylcholine and oxidatively modified nonesterified fatty acids which could promote the pathogenesis of atherosclerosis (Karasawa, K. 2006; Mallat et al., 2010). Lysophosphatidylcholine is an important chemoattractant for macrophages and T cells, it induces migration of vascular smooth muscle cells, affects endothelial function, and increases the expression of adhesion molecules and cytokines (Garza et al., 2007; Tsimikas et al., 2009).

Phospholipases A_2 (PLA_2s) comprise distinct sets of enzymes with different localizations: the intracellular (cytosolic) enzymes that are Ca^{2+} dependent (cPLA_2), Ca^{2+} independent (iPLA_2), or specific for PAF (intracellular PAF acetylhydrolase) and extracellular (plasma) enzymes, either associated with lipoproteins (Lp-PLA_2) or secreted PLA_2s (sPLA_2) (Mallat et al., 2010). Extracellular (plasma) PAF-AH shares 41% sequence identity with intracellular (cytosolic) Type II PAFAH, whereas both enzymes show less structural similarity to Type I PAF-AH (Karasawa, K. 2006; Mitsios et al., 2006). Secreted PLA_2s (sPLA_2) represent a

diverse family of structurally related, disulfide-rich calcium-dependent secreted enzymes that hydrolyze the sn-2 position of glycerophospholipids generating potent lipid mediators: lysophospholipids and free fatty acids, including the precursor of eicosanoids, arachidonic acid. Extracellular levels of secreted PLA_2s are increased in both plasma and inflammatory fluids in various inflammatory diseases (Karabina et al., 2010; Mallat et al., 2010).

The extracellular (plasma) enzyme Lp-PLA_2 is a single polypeptide that originates mostly from cells of the hematopoietic lineage, primarily from monocytes/macrophages (Karabina et al., 2010; Stafforini, DM. 2009). Lp-PLA_2 (PAF-AH) exhibits unique substrate specificity toward PAF and oxidized phospholipids. In human plasma, PAF-AH activity is associated mainly with the apolipoprotein B (apoB)-containing lipoproteins and primarily with low-density lipoprotein (LDL). A small proportion of the circulating enzyme activity is also associated with high density lipoprotein and lipoprotein(a), an atherogenic lipoprotein particle that appears to be a preferential carrier of oxidized phospholipids in human plasma (Mallat et al., 2010; Wolfert et al., 2004; Karasawa, K. 2006). In plasma, approximately 80% of Lp-PLA_2 is attached to low-density lipoproteins (LDLs), and the remaining 20% is linked to high-density lipoproteins (HDLs) and lipoprotein (a) (Garza et al., 2007). HDL protects LDL from oxidation and HDL-associated PAF-AH might be involved in this effect together with other HDL-associated enzymes, including PON1 and lecithin-cholesterol acyltransferase (LCAT) . Dyslipidemia-induced decrease in the ratio of HDL-associated PAF-AH to the plasma PAF-AH levels might thus lead to the promotion of atherosclerosis (Karasawa, K. 2006 ; Garza et al., 2007). Many studies appeared on the role of lipoprotein–associated PLA_2 and secreted PLA_2s in atherosclerosis at the level of biology and epidemiology. It is still unclear whether these PLA2s act as true biological effectors of cardiovascular diseases in humans and whether they have proven utility as biomarkers of disease severity (Mallat et al., 2010).

We explored relations between serum PON1 and PAF-AH activities as well as the distribution of polymorphisms of *pon1* and *pon2* genes and cerebral atherosclerosis in well-characterized groups of patients with angiografically assessed severe stenosis of cerebral arteries and matched control no-stenosis group.

2. Patients and methods

2.1 Patients

The study comprised 119 patients, 35 women and 84 men with symptoms of cerebrovascular insufficiency and stenosis of carotid artery more than 50% of the lumen. Among them, 87 (73.1%) had transitory ischemic attacks, 19 (16.0%) had suffered a cerebrovascular insult with motor deficit 5-9 months previously, and 13 patients (10.9%) had headache and vertigo with carotid bruit. All patients were examined by neurologists and referred to Doppler examination. At the Doppler examination, all of them had stenosis of one or both carotid arteries more than 50% of the arterial lumen and were preceded to digital subtraction angiography (DSA) and possible endovascular carotid PTA/stent treatment. Based on the angiographic findings, for the purpose of present investigation they were divided in two groups. The first group consisted of 73 patients, 25 female, median age 67 years (range, 41-79 years) and 48 male, median age 65 years (range, 46-83 years) with a moderate degree of carotid extra cranial stenosis between 50% and 69% of the arterial lumen. In this group there was no intracranial stenosis of cerebral arteries. The second group consisted of 46 patients, 10 female, median age 67 years (range, 46-78 years) and 36 male, median age 68 years (range, 54-78 years) in whom stenosis between 70-99% or obliteration of the carotid artery

was angiographically determined. In this group, intracranial stenosis less than 50% of the lumen of carotid arteries in three patients were found.

The control no-stenosis group consisted of 90 patients, 46 female, median age 60 years (range, 44-76 years) and 44 male, median age 63 years (range, 46-82 years) with suspected cerebrovascular symptoms, but with normal Doppler examination of the carotid arteries. Vertigo, headache and transitory vision problems were indications for Doppler examination for 72 patients (80%). Twelve out of 90 (13.3%) patients had had nonischemic cerebrovascular insult a few months or years priorly with new symptoms like headache, suspected motor deficit or vertigo. The remaining six patients (6.7%) had the same symptoms combined with the carotid bruit. All of them had normal appearance and normal hemodynamic results at Doppler examination of carotid arteries. The third group of patients, with Doppler established carotid stenosis between 1-49% of cerebral arteries, was not included in the present investigation. They were proceeded to other non-invasive carotid investigations like MR angiography or multislice CT angiography.

All Doppler and DSA procedures were performed at the Institute for Diagnostic and Interventional Radiology of the Merkur University Hospital. Doppler examinations were performed at the center of excellence with more than 3,000 examinations per year. DSA was performed by the interventional radiologists skilled in neurovascular interventions.

Smokers were defined as those reporting daily smoking. Obesity was defined in terms of the patient's body mass index (BMI) calculated as weight in kg/height in m^2.

The patients with the BMI ≥ 25 were considered overweight. Written informed consent was obtained from all subjects according to the guidelines of our Ethics Committee.

This study was approved by the Ethics Committee of the Merkur University Hospital, Zagreb, Croatia.

2.2 Samples

Blood samples were collected by venopuncture after overnight fasting and under controlled pre-analytical conditions. Serum was prepared 30 min after blood collection into vacutainer tubes (Becton Dickinson) without additives by centrifugation at 3000 rpm for 15 minutes. Blood collected in EDTA-coated tubes was used for determination of *pon1 and pon2* genotypes while sera were analyzed for triacylglycerol, total cholesterol, LDL and HDL-cholesterol concentrations and PON1 and PAF-AH activities.

2.3 Methods

2.3.1 Serum triacylglycerol, total cholesterol, LDL and HDL cholesterol assays

Serum triacylglycerol and total cholesterol were measured by enzymatic PAP- method. HDL-cholesterol was measured with direct method based on selective inhibition of the non-HDL fractions by means of polyanions. A homogeneous assay for the selective measurement of LDL-cholesterol in serum was used. All measurements were performed on fresh sera on the day of blood collection using standard commercial kits (Olympus Diagnostic GmbH, Hamburg, Germany) on the Olympus AU 600 analyzer (Olympus Mishima Co., Ltd., Shizuoka, Japan).

2.3.2 Paraoxonase activity measurement

PON1 paraoxonase activity was assessed by using paraoxon as the substrate in the presence of NaCl (NaCl stimulated activity) (Juretić et al., 2006). The assay was performed on Olympus AU 600 biochemical analyzer (Olympus Mishima Co., Ltd., Shizuoka, Japan) at

37°C, as previously described, with a minor modification (Grdić et al., 2008). Briefly, 15 μL of serum was added to 300 μL of reaction mixture containing 2.5 mmol/L paraoxon of ~90% purity, 2.2 mmol/L $CaCl_2$ and 1.0 mol/L NaCl in 0.1 mol/L Tris– HCl buffer, pH 8.0. The release of p-nitrophenol from paraoxon was measured at 410/480 nm (ε410/480=17900 L/ mol cm) and the enzyme activity is expressed in international units per 1 L of serum and standardized against concentration of HDL-cholesterol . Serum samples were kept frozen at -80°C until the day of analysis.

2.3.3 Paraoxonase polymorphisms determinations

Polymorphisms of *pon1* and *pon2* genes were determined by the polymerase chain reaction (PCR) followed by restriction fragment length polymorphism analysis (PCR-RFLP) (Table 1). The PCR reaction was performed in a Gene Amp PCR System 2720 (Applied Biosystems) PCR machine. *Pon1* gene polymorphisms (Q192R, L55M and -108C>T) were determined by the method described by Campo et al., (Campo et al., 2004). with some modifications concerning the sequence of 1CT primer, annealing temperature and restriction enzyme for -108C>T polymorphism (Grdić et al., 2008; Grdić Rajković et al., 2011).

Pon2 gene polymorphism (S311C) was determined by the method described by Sanghera et al. (Sangera et al., 1998) with a few modifications including the sequence of 2SC primer and annealing temperature (Grdić et al., 2011). Briefly, the amplification mixture (total volume 25 μL) for each *pon1* gene polymorphism and for *pon2* gene polymorphism contained 250 ng of genomic DNA, 0.4 μmol/L of each primer, 0.2 mmol/L of each dNTP, 2mmol/LMgCl2, 0.5 units of PlatinumTaqDNA Polymerase and 2.5 μL of reaction buffer (200mmol/L Tris–HCl, pH 8.4 and 500mmol/L KCl). PCR reaction was carried out using the following procedure: the first step of predenaturation at 95 °C for 12 min, 35 cycles of amplification (30 seconds at 94 °C followed by 30 seconds at specific primers annealing temperature and 60 seconds at 72 °C), and the last cycle of final extension at 72 °C for 7 min. PCR was attenuated by lowering the temperature to 4 °C for at least 6 min. The primers, annealing temperatures and lengths of PCR fragments are given in Table 1. Endonuclease mixture for each polymorphism explored in this study (total volume 15 μL) contained 9 μL of amplified fragment, an appropriate buffer for each restriction enzyme and 4 units of BspPI (for *pon1* Q192R), 5 units of Hin1II (for *pon1* L55M), 3 units of BsrBI (for *pon1* -108C>T) and 3 units of DdeI (for *pon2* S311C). For separation of restriction products electrophoresis on 4% agarose gel in TAE buffer (0.04 mol/L Tris–HCl, 5 mmol/L Na-acetate, 0.04 mmol/L EDTA, pH 7.9) and stained with ethidium bromide (final concentration was 0.5 μg/mL) were used. The length of RFLP fragments is given in Table 1.

Determination of *pon1* Q192R, *pon1* L55M and *pon1* -108C>T polymorphisms by the PCR-RFLP procedure using specific restriction enzymes were described in details previously (Grdić et al., 2008, 2011). Briefly, for *pon1* Q192R polymorphism undigested fragment (238 bp) was detected in genotype QQ, digested fragments (175 and 63 bp) were detected in genotype RR, and both digested and undigested fragments (238, 175 and 63 bp) were detected in genotype QR. For *pon1* L55M polymorphism undigested fragment (172 bp) was detected in genotype LL, digested fragments (103 and 69 bp) were detected in genotype MM, and digested and undigested fragments (172, 103 and 69 bp) were detected in genotype LM. For *pon1*-108C>T polymorphism undigested fragment (240 bp) was detected in genotype TT, digested fragment (212 bp) was detected in genotype CC, and both undigested and digested fragments (240 and 212 bp) were detected in genotype CT .

Poly-morphism	Primer	°C	Restriction enzyme	PCR fragment	RFLP fragments
pon1 Q192R	1_{QR}: 5′ TATTGTTGCTGTGGGACCTGAG 3′	60	BspPI	238 bp	Q allele: 238 bp
	2_{QR}: 5′ CCTGAGAATCTGAGTAAATCCACT 3′				R allele: 175+63 bp
pon1 L55M	1_{LM}: 5′ CCTGCAATAATATGAAACAACCTG 3′	63	Hin1II	172 bp	L allele: 172 bp
	2_{LM}: 5′ TGAAAGACTTAAACTGCCAGTC 3′				M allele: 103+69 bp
pon1 -108C>T	1_{CT}: 5′ AGCTAGCTGCCGACCCGGCGGGGAGGaG 3′	68	BsrBI	240 bp	C allele: 212+28 bp
	2_{CT}: 5′ GGCTGCAGCCCTCACCACAACCC 3′				T allele: 240 bp
*pon*2 S311C	1_{SC}: 5′ ACATGCATGTACGGTGGTCTTATA 3′	55	DdeI	265 bp	S allele: 123+75+67 bp
	2_{SC}: 5′ AGCAATTCATAGAAAATTAATTGTTA 3′				C allele: 142+123 bp

Table 1. Conditions for PCR-RFLP method. The lower case base "a" in *pon1* -108CNT 1CT primer indicates a mismatch, introducing a restriction site for restriction enzyme BsrBI.

Determination of *pon2* S311C polymorphism by PCR-RFLP procedure using DdeI restriction enzyme was carried out as follows. The exchange of the nucleotide C with G results in substitution of codon TCT to TGT (exon 9 of *pon2* gene), and with substitution of serine to cystein at position 311 (S311C, SNP ID rs7493). S and C alleles have a restriction site for DdeI restriction enzyme but the presence of codon TCT in S allele introduces an additional restriction site for this enzyme. The amplified fragment of 265 bp was digested in two fragments (142 and 123 bp) in both S and C allele. In the case of S allele 142 bp fragment is additionally digested in two fragments (75 and 67 bp). Fragments of 123, 75 and 67 bp were detected in genotype SS, fragments of 142 and 123 bp were detected in genotype CC, and fragments of 142, 123, 75 and 67 bp were detected in genotype SC.

2.3.4 PAF-AH activity assay

Platelet-activating factor acetylhydrolase (PAF-AH) activity was measured in plain serum with the new automated spectrophotometric assay (Azwell Inc., Auto PAF-AH, Osaka, Japan) at 37°C (Kosaka et al., 2000). In the first phase, 2μL of serum was added to 240 μL of 200 mmol/L HEPES (*N*-2-hydroxyethylpiperazine–*N*´-2-ethanesulfonic acid) buffer (Reagent 1), pH 7.6 and pre-incubated at 37°C for 5 min. The reaction was started by adding 80 μL of 20 mmol/L citric acid monohydrate buffer, pH 4.5 containing 90 mmol/L 1-myristoyl-2-(4-nitrophenylsuccinyl)phosphatidylcholine (Reagent 2). The liberation of *p*-nitrophenol was monitored at 405 and 505 nm at 1 and 3 min after the addition of Reagent 2 using the automatic biochemical analyzer OlympusAU600 (Olympus Mishima Co., Ltd., Shizuoka, Japan). Enzyme activities are expressed in international units per liter of serum and standardized against concentration of LDL-cholesterol. Serum samples were kept frozen at -80°C until the day of analysis.

2.3.5 Quality control of measurements

The Institute of Clinical Chemistry and Laboratory Medicine of the Merkur University Hospital has been accredited to ISO 15189, Medical laboratories - Particular requirements for quality and competence since 2007 (ISO 15189, 2008). Analytical methods for measurement of serum triacylglycerol, total cholesterol, LDL and HDL-cholesterol concentrations as well as for paraoxonase polymorphisms determinations used in this study have been accredited according to this norm (Flegar- Meštrić et al., 2010a). Traceability of analytical methods is achieved through a manufacturer's reference materials (calibrators) or reference methods for enzyme activities. Analyzer–based calibrations are routinely performed for compensation of systematic effects. Estimates of within-laboratory precision are provided by internal quality control data using commercial control sera (Olympus Diagnostic) for triacylglycerol, total cholesterol, LDL and HDL-cholesterol concentrations and pool serum samples for the paraoxonase and PAF-AH activities. Trueness estimates are based on the long-term results of external quality assessment (EQA) obtained by the participation of the Institute of Clinical Chemistry and Laboratory Medicine of the Merkur University Hospital in the National External Quality Assessment Scheme organized by the Croatian Society of Medical Biochemists and international EQA schemes for general and special medical biochemistry organized by Labquality - WHO Collaborating Centre for Education and Training in Laboratory Quality Assurance, FIN-00520 Helsinki, Finland (Flegar-Meštrić, Z. et al., 2010b). According to the requirements of the international standard ISO 15189, interlaboratory comparisons were performed for the paraoxonase polymorphisms determinations between the Institute of Clinical Chemistry and Laboratory Medicine of the Merkur University Hospital and Faculty of Pharmacy and Biochemistry, University of Zagreb, Croatia. Estimation of measurement uncertainties is done on the basis of the "Guide to the Expression of Uncertainty in Measurement" (GUM, 2005). The uncertainty components that we use are uncertainties related to calibrator, within-laboratory precision and trueness estimates based on the results of external quality assessment (EQA). The expanded measurement uncertainties (k=2) obtained for triacylglycerol, total cholesterol, LDL and HDL-cholesterol concentrations and pool serum samples for the paraoxonase and PAF-AH activities in the normal concentration range were 4.8, 4.0, 8.0, 11.1, 4.2 and 3.8%, respectively.

2.4 Statistical analysis

The Mann-Whitney U-test was applied to evaluate the differences between the groups, with $p < 0.05$ considered statistically significant. The correlations between serum PAF-AH activity and concentrations of total and LDL cholesterol were estimated using Pearson's correlation. Chi-square test was used for comparisons of allele and genotype proportions. MedCalc statistical program (MedCalc Software Version 8.1.0.0, 2005 Frank Schoonjans for Windows, available at the website:www.medcalc.be/) was used.

3. Results

3.1 Patients

The results of the Mann-Whitney U-test showed that, according to the demographic and lifestyle characteristics (age, body mass index), the control no-stenosis group matched the groups of patients with different degrees of cerebrovascular stenosis (Table 2). The chi-squared test showed no significant differences between sex and cerebrovascular stenosis

subgroups (Yates corrected χ^2 =0.003, p=0.338 in the group with <70% of stenosis; Yates corrected χ^2 =0.023, p=0.638 in the group with >70% of stenosis) or smoking habits and cerebrovascular stenosis subgroups (Yates corrected χ^2 =0.001, p=0.478 in the group with <70% of stenosis; Yates corrected χ^2 =0.012, p=0.962 in the group with >70% of stenosis). The proportion of daily smokers in the group of patients with <70% of stenosis was 33.3% and 32.6% in the group of patients with >70% of stenosis versus 25.8 % in control no-stenosis group. The mean values of the body mass index in all groups examined were more than 25 kg/m^2, indicating overweight.

3.2 Serum triacylglycerol, total cholesterol, LDL and HDL cholesterol concentrations

Comparing the results obtained for the traditional risk factors (triacylglycerol, total cholesterol, HDL-cholesterol, LDL-cholesterol) between the groups of patients with cerebrovascular stenosis and control no-stenosis group using the Mann-Whitney univariate statistic method, significant differences were found for all serum lipid parameters ($p<0.05$) (Table 2).

Parameter	Control no-stenosis group (N=90)	Patients with cerebrovascular stenosis			
		<70% of stenosis (N=73)	*P*	>70% of stenosis (N=46)	*P*
Age (years)	61 (44-82)	66 (41 - 83)	0.068	68 (46 - 83)	0.160
Body mass index (kg/m^2)	26.3 (20.2 – 35.7)	25.7 (19.1 – 34.1)	0.143	26.5 (19.0 – 35.1)	0.944
Total cholesterol (mmol/L)	6.3 (4.2 – 8.4)	5.4 (3.4 – 11.5)	0.001	5.7 (3.5 - 9.6)	0.000
Triacylglicerol (mmol/L)	1.39 (0.34 – 4.14)	1.75 (0.43 – 8.18)	0.003	1.66 (0.71 – 5.09)	0.026
HDL- cholesterol (mmol/L)	1.6 (1.0 – 3.1)	1.3 (0.7 – 2.3)	0.000	1.1 (0.8 – 1.8)	0.000
LDL- cholesterol (mmol/L)	3.9 (2.6 – 5.9)	3.6 (1.8 – 6.3)	0.021	3.5 (1.2 – 8.4)	0.001

Table 2. Demographic and biochemical parameters for control no-stenosis group and patients with <70% and >70% of cerebrovascular stenosis. Results are given as medians, with ranges in parentheses. p values: significance level for difference between the group of patients with cerebrovascular stenosis and the control no-stenosis group tested by Mann-Whitney test; $p<0.05$ was considered as statistically significant.

3.3 Paraoxonase activity measurement

Basal and stimulated PON1 activities differ significantly between patients group with stenosis and the control no-stenosis group, and HDL standardized basal and stimulated PON1 activity did not show statistical difference. Kolmogorov –Smirnov test for normal distribution reject normality for all examined data (Table 3) . There were no statistically significant relationships between basal and stimulated PON1 activity and examined lipid and lipoprotein parameters (tryacylglicerol, total cholesterol, HDL cholesterol, LDL cholesterol) (Table 4).

Paraoxonase (unit)	Control no-stenosis group (N=90)	Patients with cerebrovascular stenosis (N=119)	p
	Median (IQR)	Median (IQR)	
Basal PON1 activity (U/L)	187 (137)	103 (180)	0.0056
NaCl –stimulated PON1 activity (U/L)	379 (326)	213 (339)	0.0079
HDL standardized basal PON1 activity (U/mmol)	110 (125)	93 (142)	0.9390
HDL standardized NaCl –stimulated PON1 activity (U/mmol)	228 (238)	189 (310)	0.9605

Table 3. Serum paraoxonase (PON1) activity and HDL standardized paraoxonase activity in control no-stenosis group and patients with cerebrovascular stenosis. Abbreviation: IQR, Interquartile range; p values: significance level for difference between the group of patients with cerebrovascular stenosis and the control no-stenosis group tested by Mann-Whitney test; $p < 0.05$ was considered as statistically significant.

	Correlation coefficient			
	Control no-stenosis group (N=90)		Patients with cerebrovascular stenosis (N=119)	
	Basal PON1 activity			
	r	p	r	p
Tryacylglicerol	0.1638	0.1229	0.0754	0.4211
Total cholesterol	0.0105	0.9219	0.1534	0.1003
HDL cholesterol	0.1278	0.2301	0.1146	0.2205
LDL cholesterol	-0.3067	0.3182	0.1201	0.1992
	NaCl stimulated PON1 activity			
Tryacylglicerol	0.1587	0.1358	0.0691	0.4606
Total cholesterol	-0.0140	0.8956	0.1589	0.0844
HDL cholesterol	0.1283	0.2281	0.1154	0.2173
LDL cholesterol	-0.1262	0.2359	0.1306	0.1623

Table 4. Relationships between paraoxonase activity and serum lipids and lipoproteins levels. $p < 0.05$ was considered as statistically significant.

3.4 Paraoxonase polymorphisms determinations

Genotype frequencies of *pon1* and *pon2* polymorphisms found in the group of patients with angiografically assessed stenosis of cerebral arteries vs. control no-stenosis group are presented in Table 5 and Figures 1-4. Observed and expected genotype frequencies of all examined *pon1* and *pon2* genes polymorphisms were in Hardy-Weinberg equilibrium. There were no statistically significant differences between genotype frequencies of *pon1* and *pon2* (Table 5) as well as for the alleles frequencies in patients group vs. control no-stenosis group ($p>0,05$) (Table 6).

Genotype	Control no-stenosis group (N=81)		Patients with cerebrovascular stenosis (N=71)		p
	n	%	n	%	
pon1 L55M					
LL	33	40	32	45	*P = 0,910*
LM	41	51	25	35	
MM	7	9	14	20	
pon1 Q192R					
QQ	38	47	33	47	*P = 0,995*
QR	39	48	32	45	
RR	4	5	6	8	
pon1 -108C>T					
CC	22	27	18	25	*P = 0,912*
CT	47	58	32	45	
TT	12	15	21	30	
pon2 S311C					
SS	44	54	45	63	*P = 0,981*
CS	37	46	24	34	
CC	0	0	2	3	

Table 5. Genotype frequencies of *pon1* and *pon2* polymorphisms in control no-stenosis group and patients with cerebrovascular stenosis. Data are shown as number (n) and percentage (%) of individuals having a certain genotype; checked by Chi-square test.

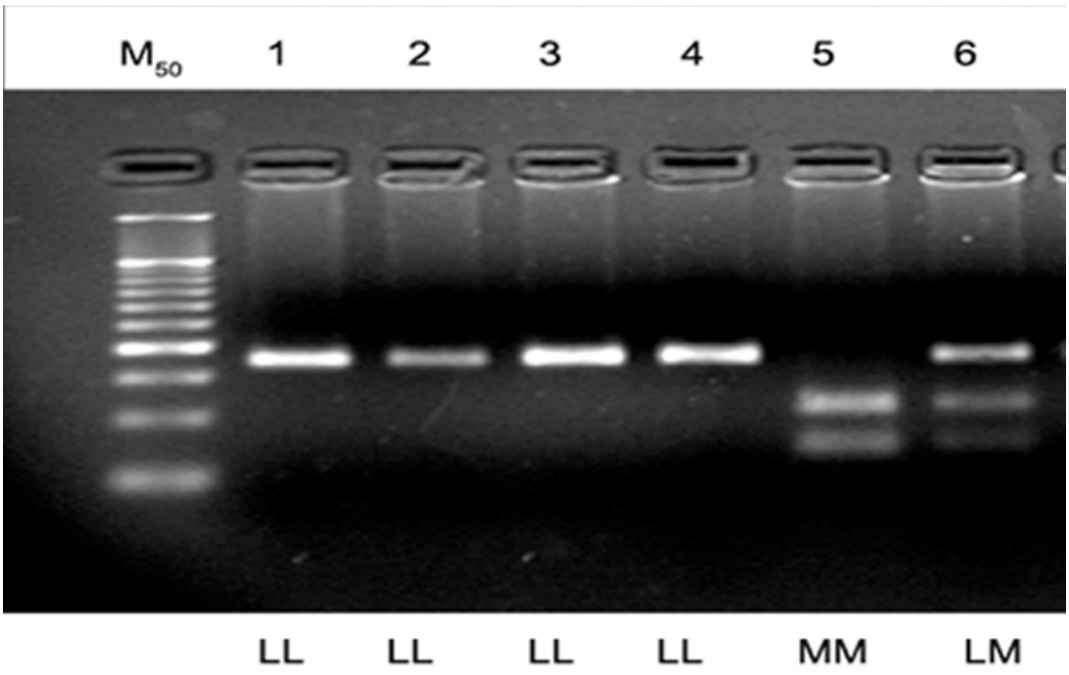

Fig. 1. Determination of L55M *pon1* gene polymorphism by the PCR-RFLP procedure using *Hin*1II restriction enzyme. Lines 1- 4 LL, line 5 MM, and line 6 LM genotype.

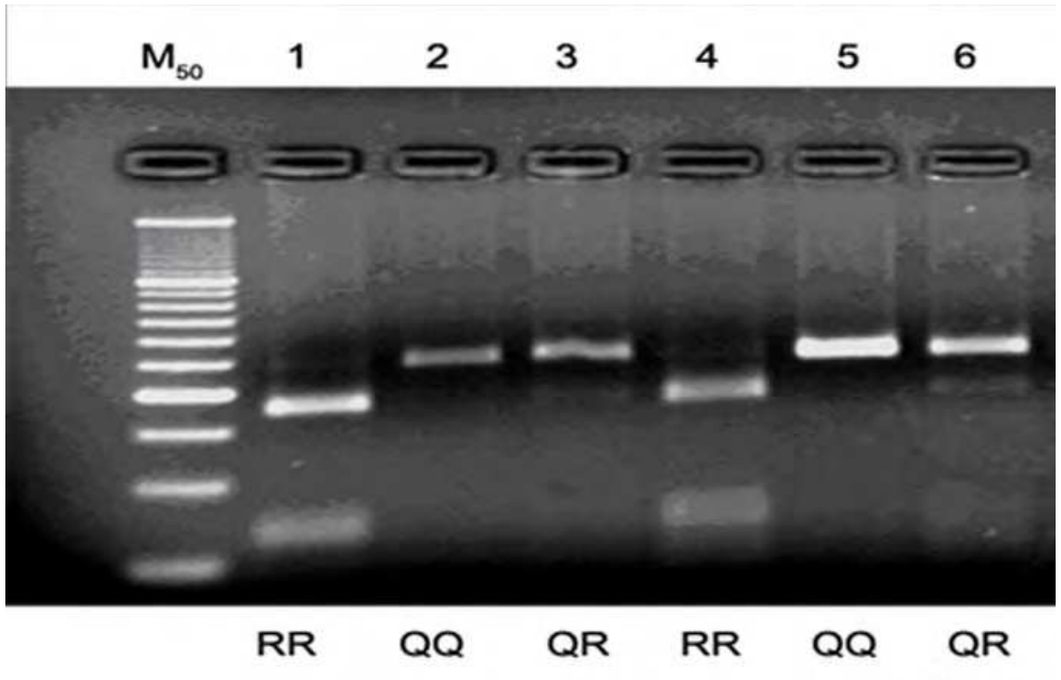

Fig. 2. Determination of Q192R *pon1* gene polymorphism by the PCR-RFLP procedure using *Bsp*PI restriction enzyme. Line 1 RR, line 2 QQ, line 3 QR, line 4 RR, line 5 QQ, and line 6 QR genotype.

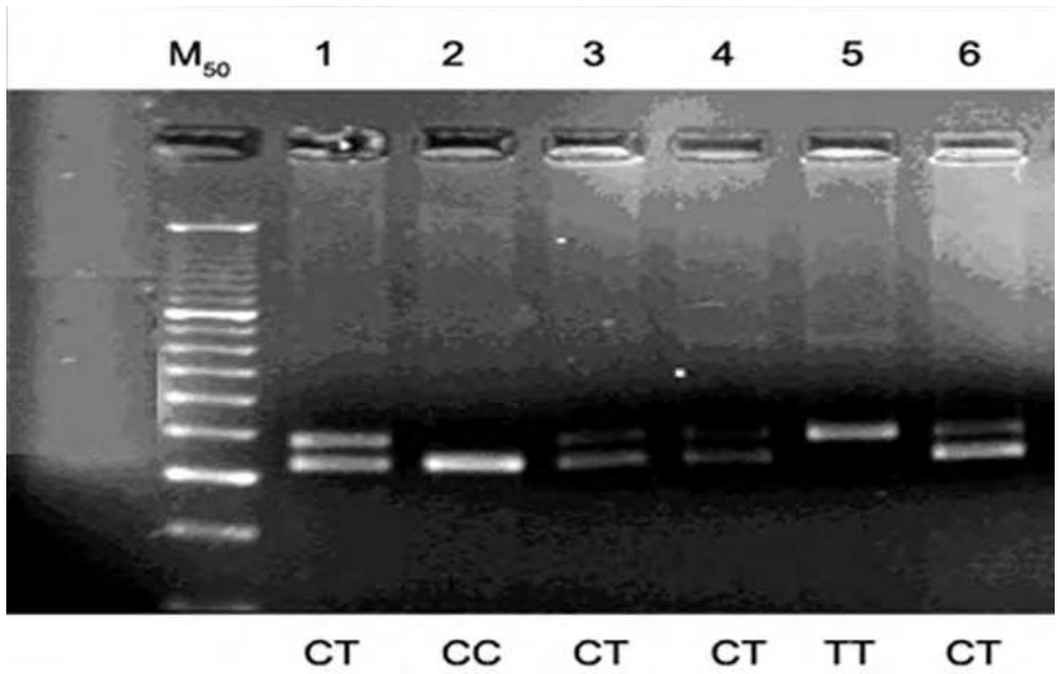

Fig. 3. Determination of -108C>T *pon1* gene polymorphism by the PCR-RFLP procedure using *Bsr*BI restriction enzyme. Line 1 CT, line 2 CC, lines 3, 4 CT, line 5 TT and line 6 CT genotype.

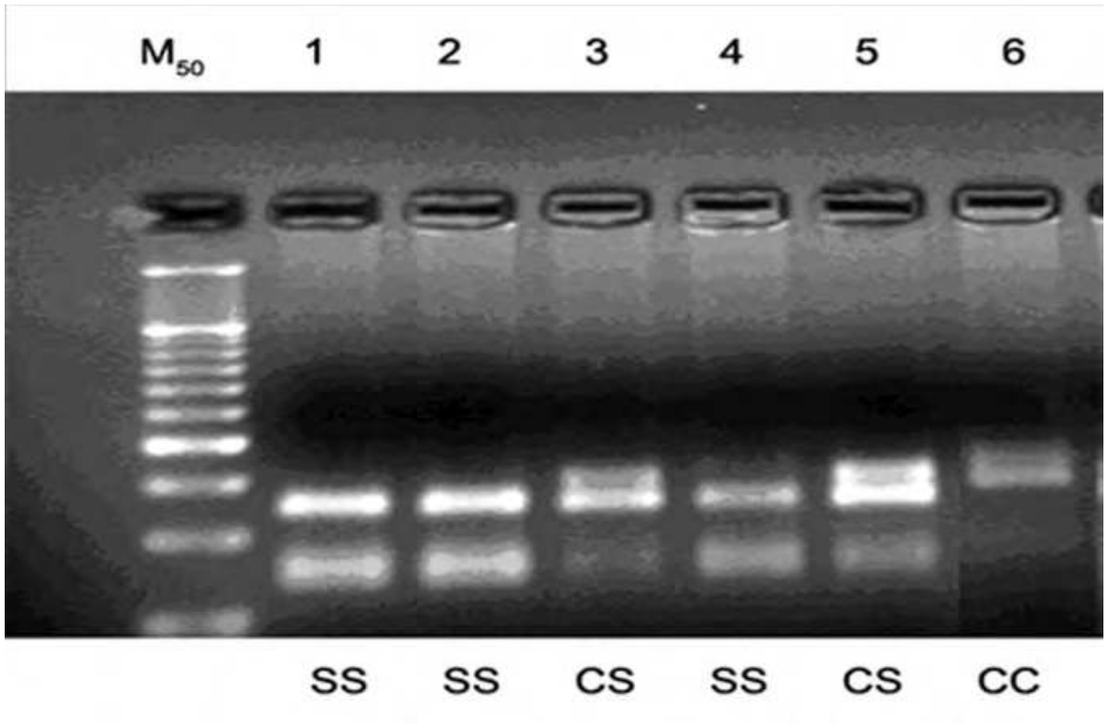

Fig. 4. Determination of S311C *pon2* gene polymorphism by the PCR-RFLP procedure using *Dde*I restriction enzyme. Lines 1- 2 SS, line 3 CS, line 4 SS, line 5 CS, and line 6 CC genotype.

Allele	**Control no-stenosis group**		**Patients with cerebrovascular stenosis**		**p**
	n	**%**	**n**	**%**	
pon1 L55M					
L	107	62	89	63	*P = 0,9744*
M	65	38	53	37	
pon1 Q192R					
Q	115	66	139	70	*P = 0,8477*
R	47	34	61	30	
pon1 -108C>T					
C	91	56	95	48	*P = 0,1246*
T	71	44	105	52	
pon2 S311C					
S	125	77	160	80	*P = 0,5980*
C	37	23	40	20	

Table 6. Allele frequencies of *pon1* and *pon2* polymorphisms in control no-stenosis group and patients with cerebrovascular stenosis. Data are shown as number (n) and percentage (%) of individuals having a certain allele; checked by Chi-square test.

3.5 PAF-AH activity assay

The values of PAF-AH activity did not differ significantly between control no-stenosis group and group of patients with cerebrovascular stenosis (Table 7) while LDL standardized PAF-AH activity (U/mmol) showed significant difference. The PAF-AH activity showed significant relationship with total and LDL cholesterol in both groups studied (Table 8).

	Control no-stenosis group	Patients with cerebrovascular stenosis	
	Median (IQR)	Median (IQR)	p
PAF-AH activity (U/L)	405 (134)	414 (171)	0.769
LDL standardized PAF-AH activity (U/mmol)	99 (30)	119 (41)	<0.0001

Table 7. Serum Platelet-activating factor acetylhydrolase (PAF-AH) activity in groups studied. p values: significance level for difference between the group of patients with cerebrovascular stenosis and the control no-stenosis group tested by Mann-Whitney test; p <0.05 was considered as statistically significant.

	Correlation coefficient			
	Control no-stenosis group		Patients with cerebrovascular stenosis	
	r	p	r	p
Tryacylglicerol	0.353	0.0006	0.153	0.1018
Total cholesterol	0.417	<0.0001	0.591	<0.0001
HDL cholesterol	-0.360	0.0005	-0.006	0.9495
LDL cholesterol	0.459	<0.0001	0.5879	<0.0001

Table 8. Relationships between platelet-activating factor acetylhydrolase (PAF-AH) activity and serum lipids and lipoproteins levels. p <0.05 was considered as statistically significant.

4. Discussion

Atherosclerosis, a disease of large arteries, is the primary cause of heart disease and stroke (Lusis, JA. 2000). Epidemiological studies over the past 50 years have revealed various risk factors for atherosclerosis and cardiovascular disease, which can be grouped into factors with an important genetic component and those that are largely environmental (Gupta et al., 2009; Lusis, JA. 2000). The results of our study indicated that significant changes associated with cerebrovascular stenosis could be the result of the environmental factors and demographic characteristics of the examined population, which is in accordance with previous studies that have investigated the atherosclerosis and the severity and extent of cardiovascular disease (Mallat et al., 2010; Costa et al., 2005; Granér et al., 2006). All groups examined in our study were characterized by a high frequency of cigarette smoking and overweight, which is consistent with the results of a previous large cross-sectional epidemiological study of Croatian population (Turek et al., 2001), and could be considered

as the possible risk factors that contribute to the increased risk of cerebrovascular stenosis (Flegar-Meštrić et al., 2007; Vrhovski-Hebrang et al., 2002).

It has been reported that raised levels of atherogenic lipoproteins are a prerequisite for most forms of atherosclerotic disease (Mallat et al., 2010; Tsimikas et al., 2009; Lusis, JA. 2000).

In our study, the median values obtained in the groups of patients with different degrees of cerebrovascular stenosis were for total cholesterol, LDL-cholesterol and tryacilglycerols higher and for HDL-cholesterol lower than the recommended values for prevention of atherosclerotic disease (De Backer et al., 2004), indicating a possible contribution of dyslipidemia to the risk of developing future stenosis of cerebral arteries.

Today, the aim of cardiovascular risk prevention is to determine atherosclerotic disease activity and shift the present focus from identification of stenosis, which is a focal disease, to identification of patients with inflamed and rupture-prone plaque (Karabina et al., 2010). Numerous biomarkers have been proposed to better discern the vulnerability of plaque rupture, pathogenesis, or cardiovascular risk. Epidemiologic, genetic, and biochemical studies support an antiatherogenic role for paraoxonase (PON) 1. The two other members of the PON gene family, namely, PON2 and PON3, have also been reported to possess antioxidant properties and may exhibit antiatherogenic capacities as well (Shih, DM & Lusis, AJ. 2009). Previous studies have demonstrated that PON1 expression is down regulated by oxidative stress. In contrast, more recent studies have shown that PON2 expression is up regulated in response to oxidative stress-inducing agents, while PON3 expression remains unchanged (Ng et al., 2005). Although PON1 activity is determined genetically, various factors, such as diet, lifestyle and environmental factors, can influence PON1 activity (Ng et al., 2005; Gupta et al., 2009). Between individuals, there is an approximately 10- to 40-fold variation in PON1 activity (Gupta et al., 2009).

Only a few studies have examined the relationship between PON1 activity and angiographically proven cardiovascular disease (Graner et al., 2006; Mackness et al., 2001). Our results indicated that basal and stimulated PON1 activities were significantly decreased in patients group with angiographically proven cerebrovascular stenosis (>50%) versus control no-stenosis group ($p<0.05$), and there were no statistically significant relationships between basal and stimulated PON1 activity and examined lipid parameters (total cholesterol, LDL-cholesterol, HDL-cholesterol and tryacilglycerols), $p>0.05$. Those results are in line with previous studies, indicating that PON1 activities toward paraoxon are lower in subjects with cardiovascular disease than in control subjects regardless of the PON1 genotype. This would suggest that the quality of the PON1 enzyme is a more important factor in cardiovascular disease than the PON1 gene (Mackness et al., 2001).

Polymorphisms in *pon1* and *pon2* genes (L55M and Q192R in *pon1*, and S311C in *pon2*) have been reported to be associated with the risk for the development of atherosclerosis as well as polymorphism in *pon1* promoter region (-108C>T) (Pasdar et al., 2006; Granér et al., 2006).

Paraoxonase-1 has several genetic polymorphisms that modify its activity and mass concentration. Hypothesized differences in the ability of the polymorphic forms to protect oxidation of LDL have led to numerous studies attempting to determine the relationship between *PON1* polymorphisms and cardiovascular disease. The results of meta-analysis of 88 studies on 4 *PON* polymorphisms [Q192R, L55M, and T(−107)C in the *PON1* and the S311C in the *PON2*] suggested an overall weak association between the R192 polymorphism and CHD risk. Despite these limitations, this meta-analysis suggests that Q192R polymorphisms may increase the risk of CHD, but no significant effect for L55M, T(−107)C

and S311C polymorphisms (Wang et al., 2011). Additionally, it has been reported that no significant genotypic or allelic frequency differences between stroke cases and controls for any of the structural polymorphisms of the *PON* genes tested were found (Pasdar et al., 2006).

In our study, there were no significant differences in genotype or allele frequencies of *pon1* and *pon2* genes between patients with stenosis of cerebral arteries and controls, indicating that there is no relationship between examined polymorphisms and reduced paraoxonase activity in patients group with angiographically proven cerebrovascular stenosis.

The platelet-activating factor acetylhydrolase (PAF-AH) or lipoprotein-associated phospholipase A_2 (Lp-PLA_2) is among the multiple biomarkers that have been associated with an increased CHD risk (Karabina et al., 2010; Garza et al., 2007; Tsimikas et al., 2009; Reddy et al., 2009; Wolfert et al., 2004). A recent meta-analysis of 14 prospective epidemiologic studies involving more than 20,000 patients established a high relative risk for cardiovascular events with high Lp-PLA2.(Garza et. al., 2007; Ballantyne et al., 2007). The LDL-associated PAF-AH activity increases in parallel with the severity of hypercholesterolemia, thus one of the major factors that determines plasma levels of PAF-AH is the rate of removal of LDL from the circulation (Karabina et al., 2010; Tsimikas et al., 2009). In our study, the PAF-AH activity shows the most significant linear relationship with total cholesterol and LDL cholesterol in the control no-stenosis group and the group of patients with cerebrovascular stenosis. It has been reported that increased Lp-PLA_2 activity is significantly related to incident cardiovascular disease (cardiovascular death, myocardial infarction, stroke, and transient ischemic attack) (Tsimikas et al., 2009; Mallat et al., 2010). In our study, the median serum PAF-AH activity did not differ significantly between patients with cerebrovascular stenosis and control no-stenosis group (median values 414 U/L versus 405 U/L, p>0,05), which is consistent with results of our previous study (Flegar-Meštrić et al., 2003), while LDL standardized PAF-AH activity a showed significant difference between the patients with cerebrovascular stenosis and control group (median values 119 U/mmol versus 99 U/mmol, p<0,0001).

Previous studies show that Lp-PLA_2 is a unique inflammatory biomarker that plays a critical role in the development of atherosclerosis and may be involved in the causal pathway of plaque inflammation and plaque rupture (Munzel, T. & Gori, T. 2009; Cariquist et al., 2007). The association of Lp-PLA_2 with cardiovascular risk among different population studies independent of classical risk factors makes the premise even stronger that Lp-PLA_2 is involved in progression of atherosclerosis to advanced rupture-prone unstable plaques (Reddy et al., 2009) . As Lp-PLA_2 is produced by macrophages and foam cells of atherosclerotic plaques that are numerous in unstable plaque, the differentiation between stable versus unstable plaque could be established by the presence of elevated Lp-PLA_2 (Reddy et al., 2009; Munzel, T. & Gori, T. 2009; Hiramoto et al., 1997; Zalewski, A. & Macphee, C. 2005). However, the clinical utility of Lp-PLA_2 activity for prediction of cardiovascular risk has to be explored in future studies.

5. Conclusion

The results of the present study show that basal and stimulated PON1 activities were significantly decreased in the patients group with cerebrovascular stenosis (group of patients with symptoms of cerebrovascular insufficiency and stenosis of carotid artery more than 50% of the lumen) versus control no-stenosis group ($p<0.05$). There were no statistically

significant relationships between PON1 activity and lipid parameters (total cholesterol, LDL-cholesterol, HDL-cholesterol and tryacilglycerols), $p>0.05$. According to the results obtained, we assume that decreased PON1 activities in patients with cerebrovascular stenosis may cause a decreased HDL antioxidant capacity and therefore contribute to the increased risk of the development of cerebrovascular atherosclerosis. However, there were no significant differences in genotype or allele frequencies of *pon1* and *pon2* genes between patients with stenosis of cerebral arteries and no-stenosis control group, indicating that changes in paraoxonase activity are determined by both genetic and environmental factors.
Our results show the most significant linear relationship between PAF-AH activity and total cholesterol and LDL-cholesterol ($p<0.001$) in the control no-stenosis group, as well as in the group of patients with cerebrovascular stenosis. The median serum PAF-AH activity did not differ significantly between the patients with cerebrovascular stenosis and control no-stenosis group ($p>0,05$), while LDL standardized PAF-AH activity showed significant difference between both examined groups ($p<0.0001$). According to our results, the LDL-standardized PAF-AH activity could be used as an additional discriminating biochemical indicator of cerebrovascular stenosis.

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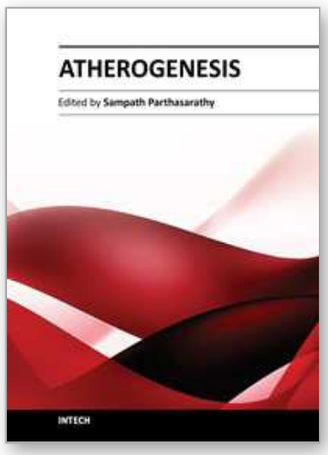

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This monograph will bring out the state-of-the-art advances in the dynamics of cholesterol transport and will address several important issues that pertain to oxidative stress and inflammation. The book is divided into three major sections. The book will offer insights into the roles of specific cytokines, inflammation, and oxidative stress in atherosclerosis and is intended for new researchers who are curious about atherosclerosis as well as for established senior researchers and clinicians who would be interested in novel findings that may link various aspects of the disease.

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