CHARLES UNIVERSITY IN PRAGUE
FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

DEPARTMENT OF BIOCHEMICAL SCIENCES

HUMAN PARAOXONASE (PON1) Q192R POLYMORPHISM IN HEMODIALYSIS PATIENTS

DIPLOMA THESIS

Supervisors:
Prof. Dr. sc. Karmela Barišić, and Prof. MUDr. Jaroslav Dršata, Csc.

Hradec Králové 2006

Renata Pšikalová

UNIVERZITA KARLOVA V PRAZE
FARMACEUTICKÁ FAKULTA V HRADCI KRÁLOVÉ

KATEDRA BIOCHEMICKÝCH VĚD

POLYMORFISMUS Q192R LIDSKÉ PARAOXONASY (PON1) U HEMODIALYSOVANÝCH PACIENTŮ

DIPLOMOVÁ PRÁCE

Vedoucí diplomové práce:
Prof. Dr. sc. Karmela Barišić a Prof. MUDr. Jaroslav Dršata, Csc.

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RENATA PŠIKALOVÁ

ABSTRACT

Background: Patients on maintenance hemodialysis (HD) are at high risk of coronary heart disease. Human paraoxonase (PON1, EC 3.1.8.1) is a high-density lipoprotein (HDL) associated enzyme that protects low-density lipoprotein (LDL) from oxidation and also protects against atherosclerosis. The decrease of PON1 activity is a possible mechanism of developing CHD.

Aim: To determine the relationship of PON1 Q192R polymorphism, atherogenic biochemical markers, CRP and PAF serum levels, and PON1 activity to hemodialysis treatment.

Methods: HD-patients (N=45, M/F=25/20, age=61±14years) and control (healthy) subjects (N=47, M/F=27/20, age=61±15years) from area of Split, Croatia were included in this study. PON1 Q192R polymorphism was detected by polymerase chain reaction (PCR) and restriction fragments length polymorphism (RFLP) method. Lipid profile, CRP and PAF serum concentrations were determined by methods routinely used in clinical biochemistry laboratories. Paraoxon was used as a substrate for measuring PON1 activity.

Results: The distribution of PON1 Q192R genotypes in HD-patients (4% RR, 16% RQ, and 80% QQ) did not differ significantly from control subjects (4% RR, 17% RQ, and 79% QQ). Atherogenic serum markers (TC, TAG, LDL, and oxLDL) were significantly higher in HD-patients. HDL and ApoA levels were lower as compared with control subjects independent of the PON1 genotype. Serum PON1 activities in both HD-patients and control subjects seemed to be regulated by PON1 Q192R polymorphism: RR> RQ > QQ.

Conclusion: The distribution of PON1 Q192R genotypes was similar in HD-patients as compared with control subjects. The lipid profile of HD-patients is more atherogenic as compared to control subjects independently of genotype. Serum PON1 activity is regulated by PON1 Q192R polymorphism.

(VII + 85 pages including 11 tables, 41 figures and 146 references; original in English)

Keywords: paraoxonase, polymorphism, genotype, hemodialysis

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CONTENTS

1 INTRODUCTION

Human serum paraoxonase (PON 1, arylalkylphosphatase [E.C. 3.1.8.1]) is the enzyme, whose biochemical and genetic characteristics have been extensively studied. The paraoxonase gene family consists of three genes: *pon* 1, *pon* 2, and *pon* 3. The *pon* genes are similar: they include nine exons, and eight introns.

Human serum paraoxonase (PON 1) is expressed primarily in the liver and its product is secreted into the bloodstream where it appears as a component of HDL. A number of PON–containing HDL subspecies, each of which has its own, unique apolipoprotein content, coexists in plasma. PON 1 hydrolyses organophosphates and possesses peroxidase, lactonase, as well as arylesterase activity. PON is also capable of hydrolyzing oxidized phospholipids as well as removing the oxidized fatty aldehydes. There is general agreement that PON 1 prevents the oxidation of lipids in low density lipoproteins (LDL) and HDL, and inactivates oxidized lipids in LDL.

Serum PON 1 activity and/or serum concentration was found to be significantly decreased in some clinical conditions, such as: atherosclerosis, diabetes mellitus, and chronic renal failure. It is well known that these pathologies are associated with increased susceptibility of LDL and HDL to oxidation.

It has been reported that PON 1 gene possesses common polymorphisms, Q192R and M55L. The enzyme PON 1 was originally found to be responsible for the hydrolysis of paraoxon. PON 1 R192Q polymorphism is involved in serum paraoxonase activity that is measured using paraoxon as a substrate [56]. The activity against paraoxon in subjects with the RR genotype is higher than in those with QQ genotype. The activity in heterozygote shows a middle level. The physiologic substrate(s), if any, of PON 1 has not been identified yet.

Serum PON 1 activity is abnormally low in patients with chronic renal failure on maintenance hemodialysis. Mapping of PON 1 activity and genotype distribution in hemodialysed patients and control subjects may reveal prognosis of chronic renal failure, moreover, it could improve prevention of its later complications.

2. PARAOXONASE RESEARCH

2.1 HUMAN PARAOXONASE

2.1.1 THE PARAOXONASE GENE FAMILY

The human paraoxonase (PON) multigene cluster is located on the long arm of the chromosome 7 at q21 – q22 and lies within a 136kb region of this chromosome. The mice cluster is located on chromosome 6.

The paraoxonase family consists of three genes: *pon* 1, *pon* 2, and *pon* 3 [1]. The *pon* genes are similar: they include nine exons, and eight introns [2]. In humans, *pon* 1, *pon* 2, and *pon* 3 share approximately 60% identity at the amino acid level, and about 70% identity at the nucleotide level [3]. The homologous paraoxonase family members across species exhibit a significantly higher degree of homology (80% – 90%) [1]. The *pon* 1 gene in both, human and mouse, encodes a 355 amino acid protein, however *pon* 2 and *pon* 3 encode proteins that are several amino acids shorter than PON 1 [1].

It has been reported that *pon* 1 gene possesses common polymorphisms, Q192R and M55L. Two common polymorphisms C311S and A148G have been found within *pon* 2 gene. Single nucleotide polymorphism A99A has been demonstrated to be often present in *pon* 3 gene [4, 5]. There is significant linkage disequilibrium (LD) across the cluster, between the *pon* 3 polymorphism and *pon* 1 and *pon* 2 alleles (Fig. 1) [5].

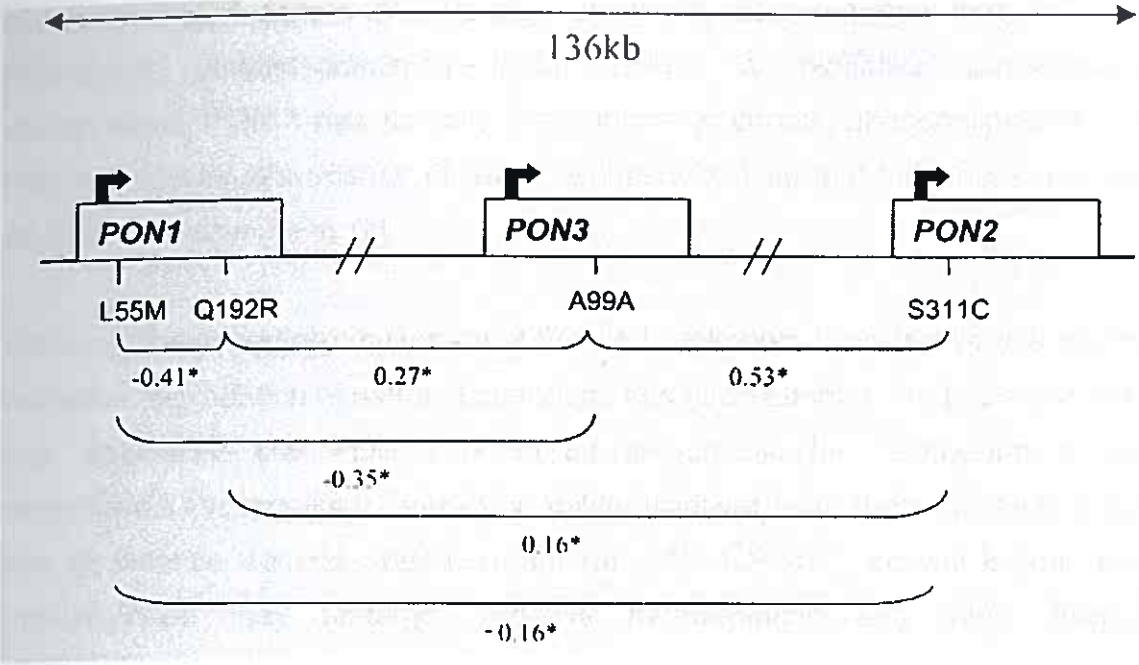

Fig.1: *pon* gene cluster linkage disequilibrium analysis. Linkage disequilibrium calculated as delta values. *P <0.00005; [5].

PON 1 and PON 3 are found to be associated with plasma high density lipoproteins (HDL, i.e. lipoproteins with high density 1,063 – 1,210 g/ml) and are mainly expressed by the liver. PON 2 is ubiquitously expressed but does not appear to be associated with HDL. All three proteins hydrolyse organophosphates and have peroxidase, lactonase, as well as arylesterase activity, although to different extents. All three proteins are also capable of hydrolyzing oxidized phospholipids, removing the oxidized fatty aldehydes. There is a general agreement that paraoxonase family members are capable of preventing the oxidation of lipids in low density lipoproteins (LDL) and HDL, and inactivating oxidized lipids in LDL. The exact function of the different family members across species suggests a strong evolutionary pressure to preserve these functional differences [1].

2.1.2 PARAOXONASE 1

Human serum paraoxonase (PON 1) is expressed primarily in the liver and secreted into the bloodstream where it appears tightly bound to HDL [6]. The PON 1 enzyme (arylalkylphosphatase, EC 3.1.8.1) is 43–45 kDa glycoprotein [4] with calcium–dependent esterase activity that is able to catalyze the hydrolysis of a broad range of substrates including organophosphates, aryl–esters, lactones and several

pharmacological agents [7]. Rodrigo et al. [8] demonstrated that PON 1 also hydrolyzes platelet–activating factor (PAF), a bioactive pro–inflammatory phospholipid. PON 1 has recently been shown to contain phospholipase-A_2 activity, with the subsequent release of lysophosphatidylcholine that influences macrophage cholesterol biosynthesis [9].

Although the physiologic substrate of PON 1 is unknown, a protective role against the oxidative degradation of serum lipoproteins has been attributed to this enzyme [10]. It was postulated that PON 1 could be responsible for inactivation of multi – oxygenated phospholipids present in mildly oxidized LDL, thus constituting the first line of defence against oxidative damage [11]. If PON 1 activity is low, oxidized phospholipids may undergo oxidative fragmentation and these fragmented phospholipids could then be scavenged by PAF–acetylhydrolase (PAF–AH; EC 3.1.1.47) [11].

PON 1 serum levels differ between individuals by as much as 13–fold [12]. This variation may be partially attributable to genotype as well as to a variety of pathophysiological factors as well. Recent studies have indicated that the status of PON 1 in an individual is determined by both PON 1 gene polymorphism and serum PON 1 activity [13, 14]. In humans, two genetic polymorphisms of PON 1 partially account for variations of serum PON 1 concentrations and activities: Q192R and M55L [15].

2.1.2.1 PON 1 MOLECULE: STRUCTURE, FUNCTION

The available structure-function analysis of PON 1 was summarized by Draganov et al. [16]: The enzyme has two calcium-binding sites; one of these is essential for its hydrolytic activity. The chelatation of calcium inactivates PON 1 activity, decreases its stability, and nevertheless interferes with its ability to inhibit LDL oxidation [17].

The presence of cysteine residues is of particular interest because it supports the hypothesis that PON 1 is the cysteine esterase, which uses a residue as a nucleophilic component at its catalytic centre, rather than the serine residue common to all serine esterases [18]. PON 1 has three cysteine residues: two forms an

intramolecular disulphide linkage, the third free cysteine residue 284 is required for optimal paraoxonase and arylesterase activities and is also essential for the action of PON 1 in protecting LDL from oxidation, it means that cysteine 284 is free in active PON 1 [19]. The PON 1 exists in two oxidation states which differ by the presence or absence of an intramolecular disulphide bond between cysteine 42 and cysteine 353 [18].

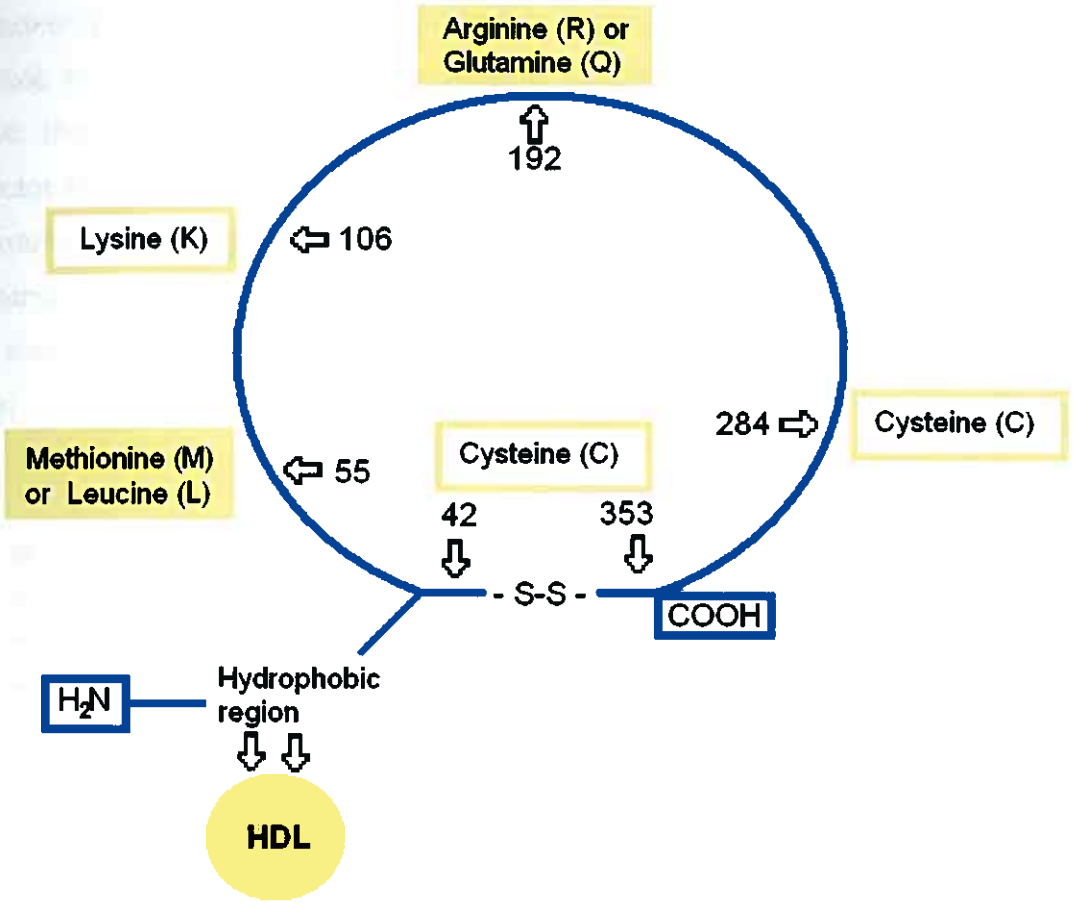

Fig. 2: The structure of human PON 1. Two polymorphic places at the positions 55 and 192 in PON 1 structure; [18].

The mature protein retains its hydrophobic signal sequence at N-terminus without the N-terminal methionine residue, which is followed by the alanine [20]. Five of the histidine residues and tryptophan 281, all are essential for arylesterase activity. Additionally, six aspartate/glutamate residues are also essential for arylesterase and paraoxonase activity [19].

All amino acids required for PON 1 activity are conserved between the human, rabbit, and mouse sequences [21].

PON 1 has a variety of substrates, and the relative activity towards different substrates varies by polymorphic forms [22].

2.1.2.2 PON 1 AND PARAOXON

The enzyme PON 1 was originally found to be responsible for the hydrolysis of paraoxon (O,O-diethyl-O–p–nitrophenylphosphate).This organo-phosporic compound is toxic to humans because it irreversibly inhibits acetyl cholinesterase that breaks down the neurotransmitter acetylcholine and thus leads to accumulation of this effector at synaptic junctions with consequent neuron overstimulation [23].
In mammals, paraoxon is a catabolite of oxidative desulphuration of the insecticide parathion; this reaction is catalyzed by the microsomal cytochrome P-450 system in the liver and other tissues [24]. The hydrolytic products resulting from the action of PON 1 on paraoxon are themselves relatively harmless.

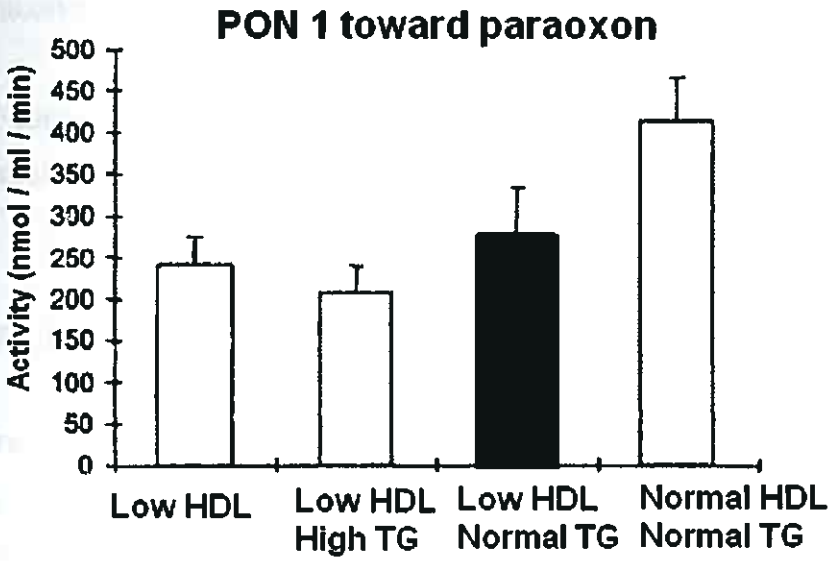

Fig. 3: PON 1 activity toward paraoxon in patients and control subjects. TG, triglycerides; [21].

Paraoxonase is equally able to hydrolyze other substrates, including phenyl acetate [25].

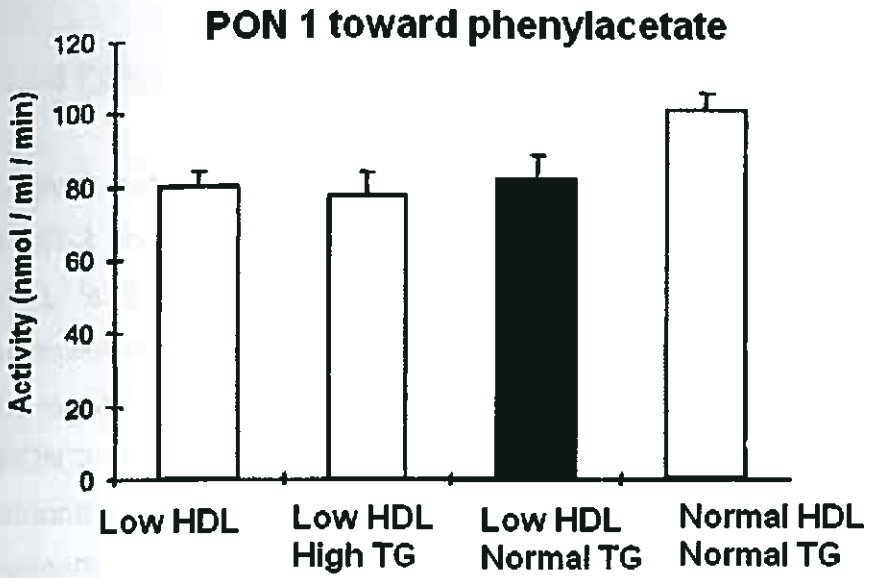

Fig. 4: PON 1 activity toward phenyl acetate in patients and control subjects. TG, triglycerides; [21].

PON 1 is therefore considered as a highly important detoxification enzyme. In addition, PON 1 hydrolyzes nerve gases such as sarin, soman and tabun, whose biochemical mechanism of action is similar to that of paraoxon [26].

Finally, it is highly important to note, that the physiologic substrate(s), if any, of PON has not been identified yet.

2.1.2.3 PON 1 AND CALCIUM

The enzyme activity of PON 1 is highly dependent on the presence of calcium. An essential consequence of such calcium dependency is that every attempt to measure PON activity must involve serum and not EDTA-plasma [27]. The EDTA-sensitive hydrolysis of paraoxon by normal human serum samples was studied and role of calcium in this reaction was dual [28]; calcium is needed both, to maintain an active site and to facilitate dissociation of diethylphosphate, the final product of hydrolysis of paraoxon, from the enzymatic molecule [29].

2.1.3 PON 2 AND PON 3

PON 3, but not PON 2, is also associated with HDL. However the signal peptides of PON 3 and PON 2 are very different from PON 1. Whether the effect of PON 3 on HDL is the same as for PON 1 remains to be determined. There are some differences in the structure of the members of the paraoxonase family. The conserved histidine at position 243 and tryptophan at position 281 in both PON 1 and PON 2 are replaced by lysine and leucine respectively in PON 3 of both human and murine sequences [1]. The functional differences between these proteins have been recently elucidated.

PON 2 and PON 3 have more limited aryl esterase and paraoxonase activities than PON 1 but PON 3 efficiently hydrolyzes lactones, including several of the statines [1]. PON 2 is apparently unable to hydrolyze paraoxon [10].

Murine macrophages express PON 2 and PON 3 [30], but their role in oxidative stress is not clear.

2.2 PON 1 AND LIPOPROTEINS

2.2.1 PON 1 AND HDL

PON 1 circulates in blood as a component of HDL (i.e. lipoproteins with high density 1,063 – 1,210 g/ml) [31]. HDL is active in promoting reverse cholesterol transport from the vessel wall to the liver. It also acts as an anti-inflammatory agent – a property that is a composite of many factors including its content of oxidized phospholipids, lipid hydroperoxides, paraoxonase, platelet–activating factor acetylhydrolase (PAF–AH), and lecithin cholesterol acyl transferase (LCAT) [32]. The quantity and the composition of HDL particles are both, important for its antioxidative and anti-inflammatory properties.

Active PON 1 produced by transfected cells is present on the cellular plasma membrane, from which it can be desorbed by a high affinity saturable mechanism,

involving HDL and protein–free phospholipid complexes [33]. The preferential association of PON 1 with HDL is mediated by desorption and by its signal peptide.

PON 1 is not present in all HDL particles, but in a small dense subclass containing apolipoprotein A–I (apoA–I), apolipoprotein J (apoJ; clusterin) [34] and only 22% of lipids [35]. This subclass also appears to contain the peptide hormone, gherlin [36]. Kelso et al. [37] demonstrated a specific association between apoJ and PON 1 in HDL particles of relatively low Mr (90 – 140 kDa).

PON 1 retains its N–terminal peptide sane that is important for its binding to HDL as well as for its association with phospholipids. PON 1 is tightly bound to apoA–I, as a result of this hydrophobic N–terminal domain of the enzyme. The apoA–I is not necessary for the association of PON 1 with HDL or phospholipids, but the stability of its arylesterase activity is increased in the presence of apoA–I [38]. In the absence of either murine apoA–I or apoE, lipid–free PON 1 constituted half of the total plasma paraoxonase activity and almost 90% in the absence of both apolipoproteins [39].

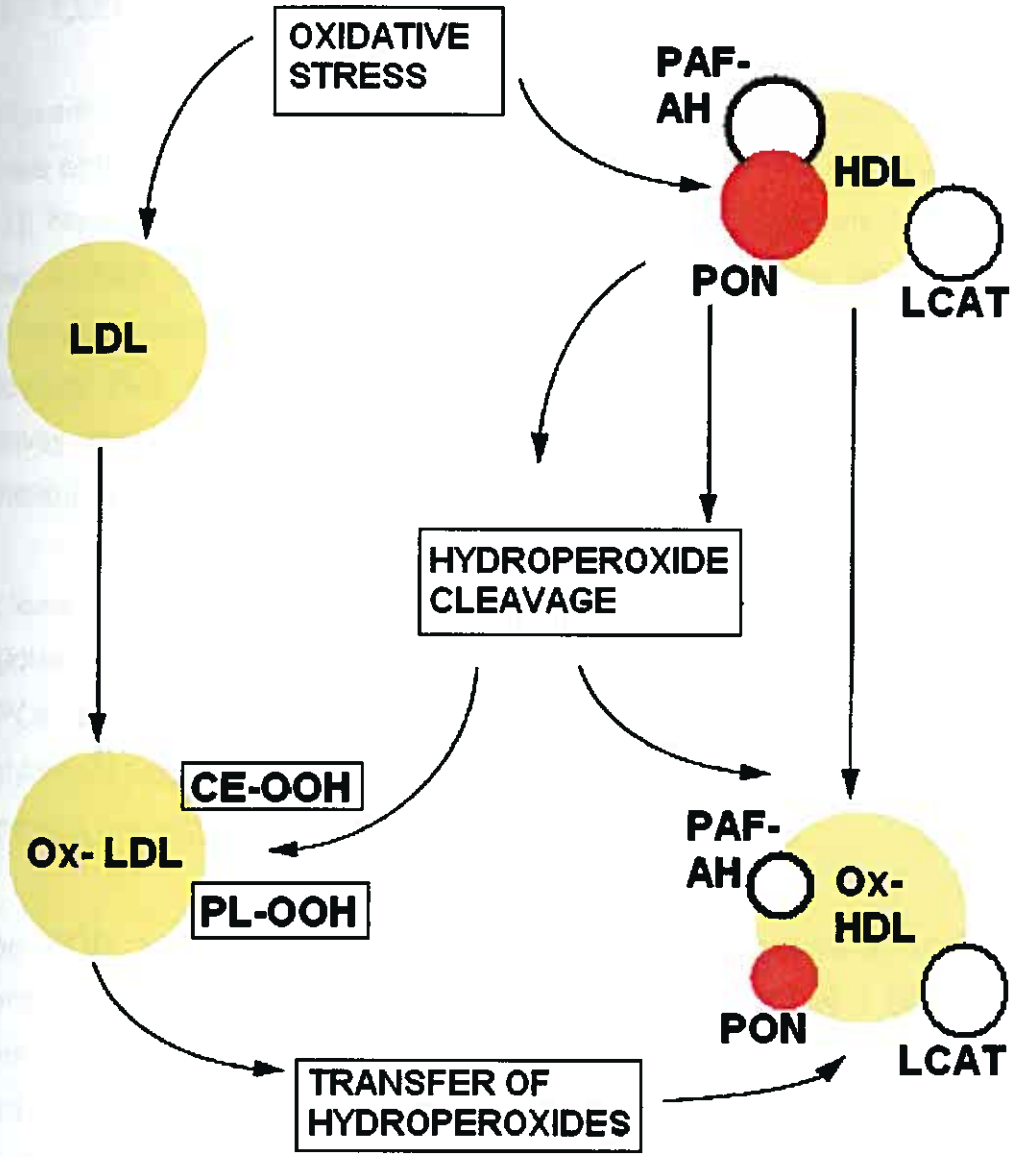

Fig. 5: Proposed scheme for the coordinated actions of HDL–associated enzymes in the hydrolysis of lipid hydroperoxides generated in lipoproteins upon oxidative stress. Abbreviations: PAF–AH, platelet activating factor acetylhydrolase; LCAT, lecithin-cholesterol–acyl-transferase; ox–LDL and ox–HDL, oxidized LDL and HDL, respectively; CE–OOH and PL–OOH, hydroperoxides of cholesteryl esters and phospholipids, respectively; [10].

A number of PON–containing HDL subspecies, each of which has its own, unique apolipoprotein content, coexists in plasma [31]. To what extent each of these entities participates in a protective effect against the oxidation of LDL (or other lipoprotein particles) remains unknown yet.

2.2.2 PON 1 And Protection of Lipoproteins Against Oxidation

Different authors have studied HDL ability to inhibit LDL oxidation *in vitro* [40] and some of them pointed out that PON 1 could play a key role in this protective function [41]. Nevertheless PON 1 antioxidant effect is not exclusively confined to LDL but also to HDL protection. This protection is most probably related to the PON 1 hydrolyzing activity of some activated phospholipids [42] and/or lipid peroxidation products [43]. Aviram et al. [43] showed an inverse correlation between PON 1 activity and HDL oxidability. The same authors have also reported that PON 1 inhibitory effect upon HDL oxidation is concentration–dependent.

At least three enzymes present on HDL have been shown to prevent the formation of oxidized LDL (ox–LDL) *in vitro*:

- PON 1 [44, 11];
- PAF–AH [42], and
- PON 3 [45, 46].

The PON 1–mediated antioxidant mechanism is involved in hydrolysis of lipid peroxides and cholesteryl ester hydroperoxides [43]. PON 1 has the capacity to hydrolyze phosphatidylcholine isoprostane and phosphatidylcholines containing C9 and C5 core aldehydes or acids in the sn–2 position, liberating the free aldehydes or acids and lysophosphatidylcholine [47]. These enzymatic activities may help to explain the protection of HDL lipid oxidation since PON 1 is associated with HDL.

Shiavon et al. [49] have found that PON 1 enzymatic activity of HDL was due to HDL–3 subspecies fraction (about 95% of PON 1 activity carried by HDL is found in the HDL–3). One of important functions of HDL–3 is connected to antioxidant activity of PON 1. There is also evidence that HDL–3 has a stronger antioxidant action than HDL–2 in preventing LDL–oxidation *in vitro* [50]. In contrast to the facts mentioned above, Zech et al. [51] have suggested that PON 1–specific activities significantly increased during conversion from HDL–3 to HDL–2. HDL–2 has a larger proportion of triglycerides and phospholipids, two lipids highly susceptible to undergo oxidation, than HDL–3; therefore higher PON 1 activity could be required for HDL–2 protection.

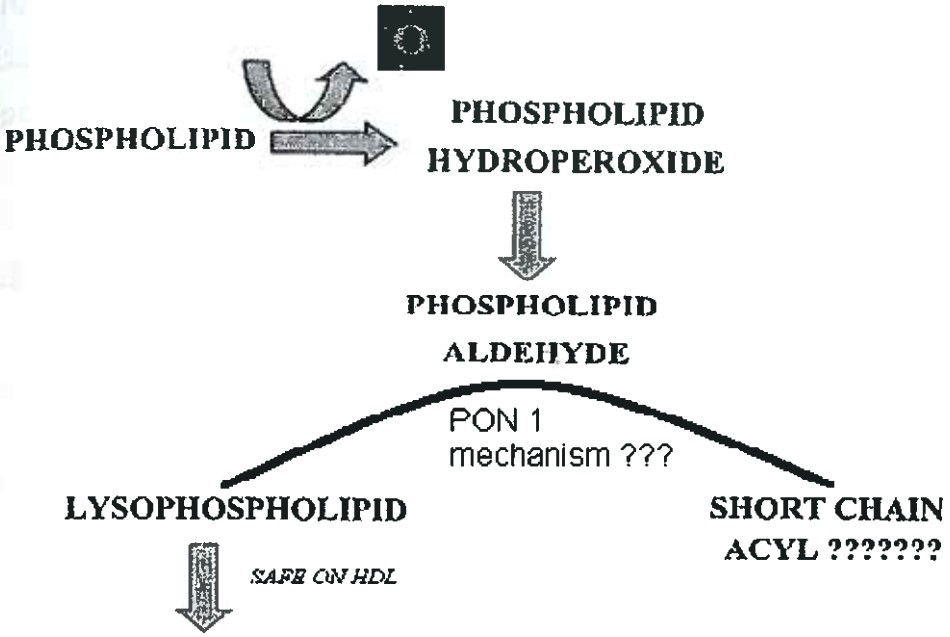

Fig. 6: Putative mechanism of action of PON 1 in destroying oxidized lipids in LDL; [48].

Experiments utilizing purified PON 1 [38, 30] suggest that the lipid–free form of protein is capable of protecting LDL against oxidation. It remains to be determined whether this antioxidative effect of PON 1 depends on transient HDL–LDL association or on transfer of oxidized phospholipids between LDL and HDL [43].

2.2.3 PON 1, Macrophages And Oxidative Stress

The antioxidative property of PON 1 has anticipated many secondary consequences. For example, PON 1 reduces monocyte adhesion to endothelial cells and macrophage chemotaxis attributable to oxidized phospholipids [52]. Peritoneal macrophages and LDL from PON 1 gene–knockout mice contain more peroxidized lipids. These macrophages showed an increased capacity to oxidize LDL and to release superoxide anion [53]. Since macrophages do not express PON 1, the phenotype of these macrophages is probably the indirect result of reduced exposure to PON 1 or of the increase of oxidized LDL and lipid peroxide levels in the plasma of deficient mice [30].

Additionally, there is an adaptation to the oxidative stress resulting from PON 1 deficiency such as the elevated hepatic expression of hemoxygenase, peroxisome

proliferator–activated receptors - γ, scavenger receptor A, and CD 36 [54]. Exogenous PON 1 is able to reverse the oxidative stress in macrophages in aged apolipoprotein E deficient and PON 1 deficient mice [55].

2.3 MODULATION OF SERUM PON 1 LEVELS

2.3.1 GENETIC FACTORS

2.3.1.1 PON 1 R192Q POLYMORPHISM

PON 1 R192Q polymorphism is involved in serum PON 1 concentrations or paraoxonase activity that is measured using paraoxon as a substrate [56]. The activity against paraoxon in subjects with the RR genotype is higher than in those with QQ genotype. The activity in heterozygote shows a middle level. There is no difference between the two allozymes in activity toward other substrates such as phenyl acetate [57].

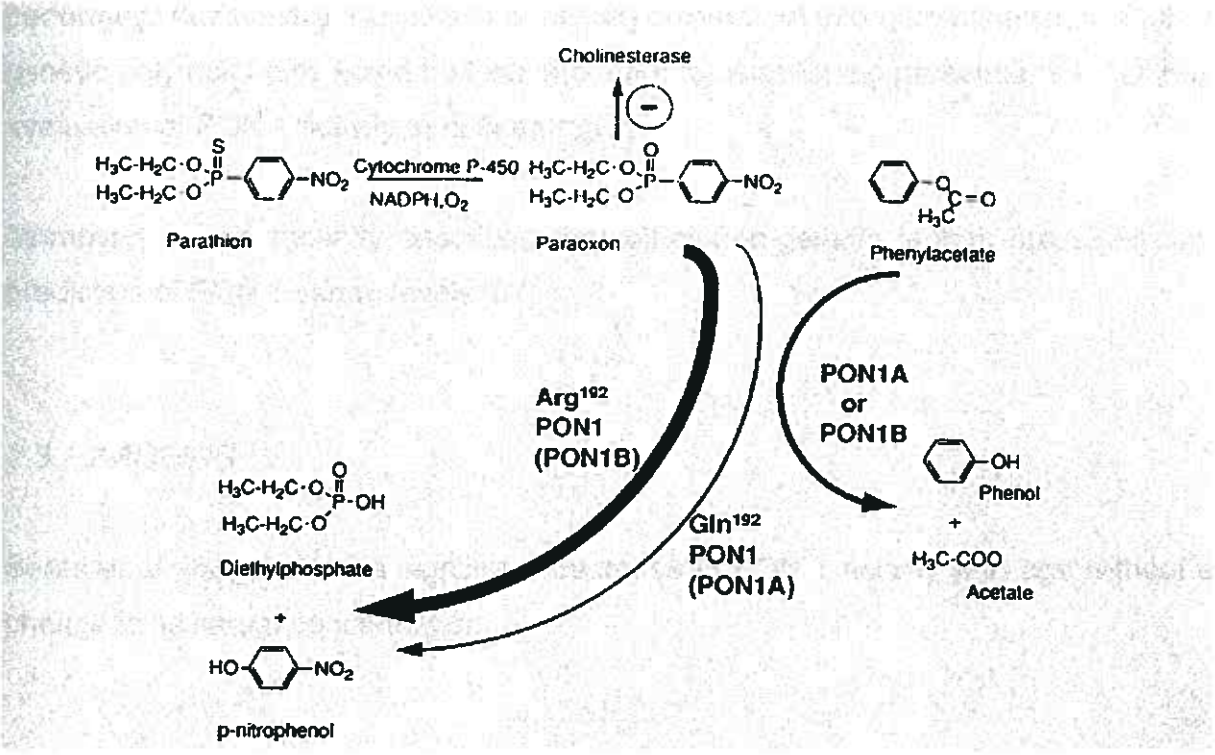

Fig. 7: Enzymatic action of PON 1. Arg192 isoform of PON 1 possesses markedly higher hydrolytic activity against paraoxon than the Gln192 isoform; [10].

The QQ genotype (low activity) is the most common and RR genotype (high activity) is the least common [58]. A series of studies (listed in [56]) progressively revealed that populations of European ancestry generally exhibit 50% homozygotes for a low PON 1 activity allozyme, 10% homozygotes for the corresponding high–activity allozyme, and 40% heterozygotes.

Mackness et al. [59] suggest that HDL containing PON 1 products of the QQ genotype are significantly more efficient in protecting LDL against copper–induced oxidative modification than those containing either the enzyme product from RQ or RR genotype.

More recently, epidemiologic studies on relatively large population groups have been appeared [60, 61, 62, 63, 64, and 65]. RR genotype (leading to a higher activity against paraoxon) was thus found in 18% of patients with coronary artery disease (CAD), and only in 11% of controls, a highly significant difference. The R allele was found mostly associated with increased risk for CAD in the above mentioned studies..

Since the allele frequency differs among ethnic groups, further studies need to be performed. Additionally, Mackness et al. [66] pointed out that determination of PON 1 genetic polymorphism would be less important in establishing presence of CAD than evaluation of PON 1 activity or concentration.

Moreover, it was recently described that other non-genetic factors are significant predictors of PON 1 serum levels [67].

2.3.1.2 AGEING

Seres et al. [68] showed a significant decrease in PON 1 activity with age without a change in its serum concentration.

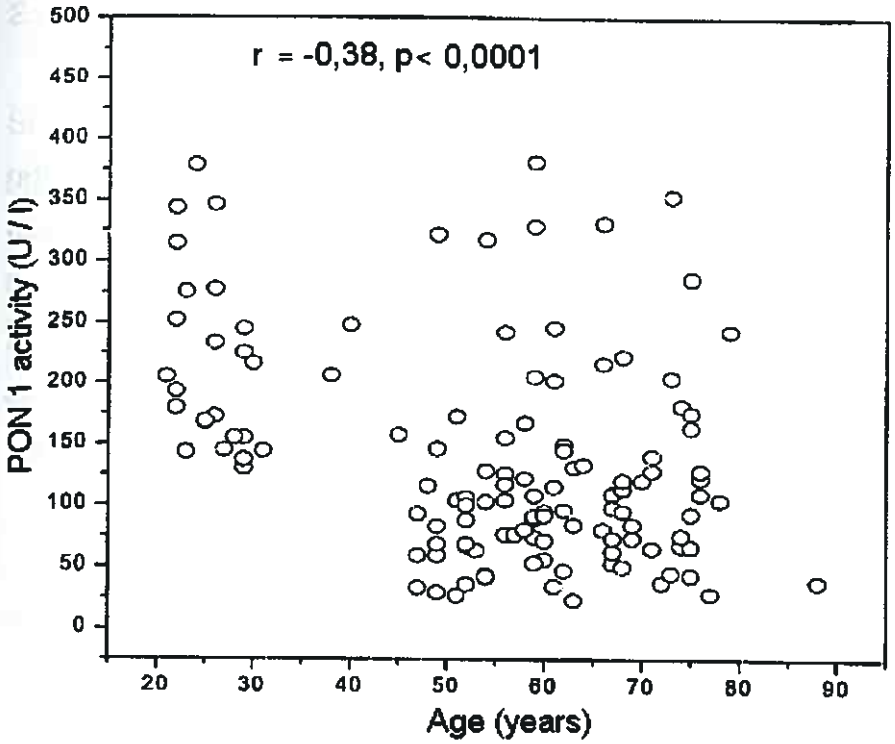

Fig. 8: Correlation of plasma PON 1 paraoxonase activity with age. Paraoxonase activity was determined by the rate of paraoxon hydrolysis; [68].

The decrease of PON 1 activity could not be explained by a decrease in HDL concentration. However, their results suggest that the development of oxidative stress conditions with aging could explain, observed reduction in PON 1 activity.

2.3.2 Non–Genetic Factors

Several non–genetic factors seem to contribute to PON 1 serum levels:

- Diet [69];
- Acute phase reactants [70];
- Pregnancy and hormonal factors [71, 72];
- Cigarette smoking [73];
- Simvastatin therapy [74];
- Regular exercise [75];
- Chemical substances (hypochlorous acid [76], and polyphenols [77]).

2.3.2.1 DIET

Shih et al. [69] have showed that HDL from fatty streak–susceptible mice, but not fatty streak–resistant mice, lose their capacity to protect homologous LDL against oxidation when the animals were maintained on a diet high in fat and cholesterol. Loss of such protective activity was associated with a reduction in both PON 1 activity and mRNA levels, thereby indicating some degree of genetic–dietary control on PON 1 production. Moreover, PON mRNA levels were strongly and inversely correlated with the development of aortic lesions.

2.3.2.2 ACUTE PHASE REACTANTS

PON 1 activity was found to be reduced in acute phase response [70, 78] as well as in acute infections such as influenza [79]. PON 1 synthesis could be decreased in the liver by the effect of the inflammatory cytokine IL–6 either directly or through biologically active oxidized lipids [80].

2.3.2.3 CIGARETTE SMOKING

Nishio and Watanabe [73] have recently demonstrated that a cigarette smoke extract is able to inhibit PON 1 activity in a dose– and time–dependent manner. Interestingly, these data suggest that compounds other than free radicals are responsible for the quenching of PON 1 activity, and point toward α, β-unsaturated aldehydes and possibly aromatic hydrocarbons. The ability of PON 1 to prevent lipid oxidation, and the fact that smoking reduces PON 1 activity, implies that variant with low PON 1 activity may play a role in modulating smoking associated coronary heart disease risk. When effects of smoking and genotype on CHD risk were examined, there was evidence for higher smoking–associated risk for man carrying the PON1 55M allele [5]. This fact requires confirmation in other prospective studies.

2.3.2.4 REGULAR EXERCISE

Regular exercise is associated with a decrease in oxidized LDL levels, and an increase in PON 1 R192Q activity in R carriers [75]. These results suggest that the effect of regular exercise on PON 1 activity is modulated by PON 1–192 polymorphism. Changes were less evident for the PON 1–55 polymorphism.

2.3.2.5 HYPOCHLOROUS ACID

As was mentioned, the active site of PON 1 contains a cysteine thiol group that is essential for PON 1-mediated protection of LDL against oxidation, and tryptophan residues that are essential for PON 1 arylesterase activity [81]. Hypochlorous acid (HOCl) severely oxidizes serum proteins. Both, the cysteine and tryptophan are susceptible to oxidation by HOCl. PON 1 activity decreases linearly in healthy serum incubated at room temperature with increasing HOCl concentration. A decrease of 40% in serum PON 1 activity is achieved at the highest concentration of HOCl (32.9 mmol/l) [76].

2.3.2.6 POLYPHENOLS

Hayek et al. [77] have reported the effects of red wine or polyphenols (quercetine and catechine), which are known to exert antagonistic action against LDL oxidation. Their data revealed that consumption of either quercetine or red wine results in increased PON 1 activity compared with the control (by 113% and 75%, respectively). However, such values may actually result from partial prevention of the oxidative stress by the polyphenolic compounds.

2.4 PON 1 Q192R POLYMORPHISM AND PATHOLOGIC PROCESS

PON 1 activity and/or serum concentration is significantly decreased in some clinical conditions, such as:

- Atherosclerosis [82];
- Diabetes mellitus [83, 84];
- Myocardial infarction [85];
- Uraemia [86];
- Familial hypercholesterolemia [87];
- Alzheimer's disease [88];
- Chronic renal failure [89];
- Fish–eye disease [90];
- Tangier disease [91].

It is well known that these pathological conditions are associated with increased susceptibility of LDL and HDL to oxidation.

2.4.1 Renal Failure And Hemodialysis

Normally functioning kidneys remove waste products from the blood [92]. When nephrons are destroyed – as in chronic glomerulonephritis, infection of the renal pelvis and nephrons (pyelonephritis), or loss of a kidney – or when kidney function is reduced by damage caused by diabetes mellitus, arteriosclerosis, or blockage by kidney stones, a condition of renal insufficiency may develop. This can cause hypertension, due primarily to the retention of salt and water, and uraemia [93]. Uraemia is a condition in which substances ordinarily excreted in the urine accumulate in the blood [94]. Patients with uraemia or the potential patients for developing uraemia are often placed on dialysis machines [93].

An output of 50 to 60 cm^3 of urine per one hour is considered normal; an output of less than 30 cm^3 per one hour may indicate renal failure. Glomerulonephritis, an autoimmune inflammation in the glomeruli preceded by an upper respiratory tract streptococci infection, may permanently change the glomeruli and figure significantly in the development of chronic renal diseases and renal failure [94].

The most common causes of renal failure:

- Nephropathy,
- Pyelonephritis,
- Glomerulonephritis,
- Diabetes mellitus,
- Polycystic kidneys, and other degenerative diseases.

Renal failure means the loss of the kidney ability to maintain fluid and electrolyte balance and to excrete waste products. It can be either acute or chronic.

Acute renal failure is the sudden loss of kidney function caused by shock and haemorrhage, thrombosis, or other physical trauma to the kidneys. The kidneys may sustain a 90% loss of their nephrons through tissue death and still do not have a serious loss of function. If a patient suffering of acute renal failure is stabilized, the nephrons have an excellent capacity to regenerate.

A person with chronic renal failure can not sustain life independently. Chronic renal failure is the final result of kidney disease in which the kidney tissue is progressively destroyed. As renal tissue continues to deteriorate, the only options for sustaining life are hemodialysis or kidney transplantation [94].

Hemodialysis equipment is designed to filter the wastes from the blood of a patient who has chronic renal failure [94]. The term dialysis refers to the separation of molecules on the basis of size by their ability to diffuse through an artificial semipermeable membrane [93]. The patient's blood is pumped through a tube from the radial artery and passes through a machine, where it is cleaned and then returned to the body through a vein. The cleaning process involves pumping the blood past a semipermeable cellophane membrane that separates the blood from an isotonic solution [94]. Urea and other wastes in patient's blood can easily pass through the membrane pores, whereas plasma proteins are left behind (just as occurs across glomerular capillaries). Unlike the tubules, however, the dialysis membrane can not reabsorb Na^+, K^+, glucose, and other needed molecules. These substances are prevented from diffusing through the membrane by including them in

the dialysis fluid. Hemodialysis is commonly performed three times a week for several hours each session [93].

More recent hemodialysis techniques include the use of the patient's own peritoneal membranes (which line the abdominal cavity) for filtering. Dialysis fluid is introduced into the peritoneal cavity, and then, after a period of time, discarded after wastes have accumulated. This procedure, called continuous ambulatory peritoneal dialysis (CAPD), can be performed several times a day by the patients themselves on an outpatient basis [94].

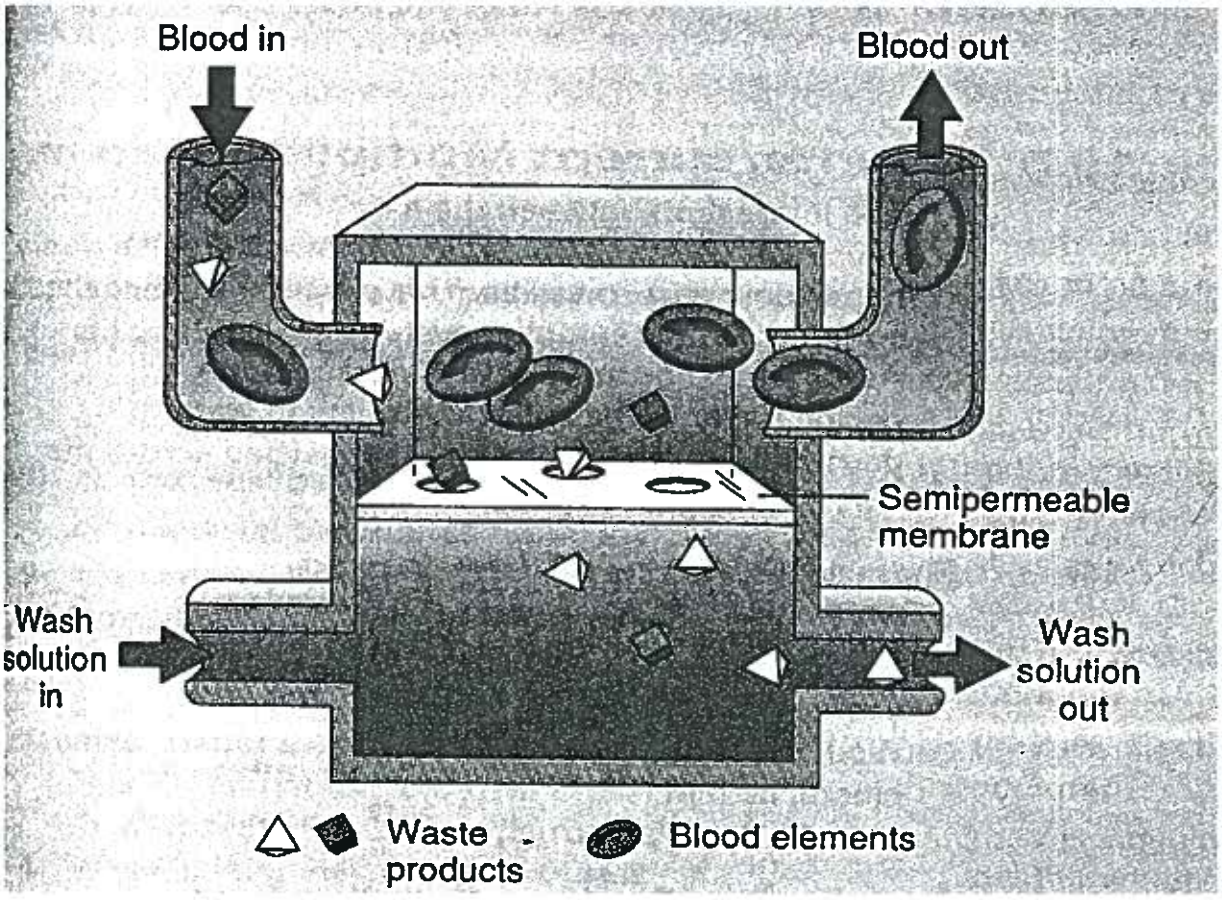

Fig. 9: The hemodialysis process; [67].

2.4.1.1 PON 1 AND HEMODIALYSIS

Serum PON 1 activity is abnormally low in patients with chronic renal failure on maintenance hemodialysis [86, 95, 96, 97]. Shiavon et al. [49] demonstrated that PON 1 activity was significantly decreased (about 30%), independently of the genotype, in HD patients compared with controls. In patients, each component of

HDL (HDL-C, apoA–I, and apoA–II) concentration is significantly lower than that in control subjects [101]. Thus the low serum HDL concentration [86], and the different distribution of HDL subspecies [49, 97], rather than PON 1 genotype [97], seem to be the main contributory factors reducing PON 1 enzyme activity in HD patients. There is evidence that other factors may also contribute to low serum PON 1 activity in these patients [86]. For example, metabolic change in the uremic milieu may modify HDL composition or structure, which may possibly affect the active site of the enzyme resulting in the decrease in its activities [97]. The ratio PON 1 activity/HDL cholesterol is abnormally low in HD patients [86].

Since PON 1 is sensitive to oxidants and is inactivated by oxidized lipids [98], it can be postulated that increased oxidative stress may decrease serum PON 1 activity and impair the antioxidant activity of HDL in HD patients [79]. Serum from HD patients contains low concentrations of antioxidants, including ascorbate, protein thiols, and albumin. Serum from HD patients contains high concentrations of severely oxidized proteins, additionally; basal dien levels of HD patients are higher than these of non-HD controls [99]. Increased output of reactive oxygen species, including hydrogen peroxide, superoxide, and hypochlorous acid (HOCl), by activated neutrophils enhances oxidative stress in HD patients [100, 101]. In these patients, overproduction of HOCl leads to high concentrations of severely oxidized proteins, and increased oxidants in plasma might also contribute to low serum PON 1 arylesterase activity [76].

Serum of HD patients also contains increased concentrations of low molecular weight compounds that increase protein glycation during prolonged incubation with high molecular weight fraction of serum [102] and increase concentrations of low molecular weight oxidants [101]. Both increased glycation [103] and oxidation of HDL and PON 1 can lead to a decrease in PON 1 activity.

The main factors that contribute to an increase in oxidative stress in patients with CRF under HD treatment are activation of oxidative metabolism in leucocytes by the dialysis membrane, increase in the formation of protein carbonyl and glycation products, and depletion of antioxidants [99].

It is still unclear how to prevent the decrease in PON 1 paraoxonase or arylesterase activity in HD patients. Further investigations are required to elucidate the causes of this decrease, and to find the tools to prevent it.

2.4.2 Atherosclerosis And Chronic Renal Failure

Patients with chronic renal failure (CRF) on maintenance hemodialysis are at high-risk of developing atherotrombotic events. The early mortality rate for cardiovascular disease is 11.6 %, accounting for a half of all cases of death of HD-patients [104].

The bio incompatible renal replacement therapies result in enhanced oxidative stress and insufficient antioxidant enzyme activities such as superoxide dismutase (SOD), catalase (CAT) and PON 1, together with the abnormalities with lipid parameters, especially in increased oxidative modification of LDL [105]. This modification has been demonstrated to play an important role in the pathogenesis of atherosclerosis [106] and uraemia [107].

There is evidence that low serum PON 1 activity may accelerate atherogenesis [82]. Recent studies [5, 60, and 108] have indicated that PON 1 is a strong independent factor for coronary heart disease and have gone some way in elucidating the mechanism of action of this important anti-atherosclerotic enzyme, however, there is much more to learn.

Ruiz et al. [60] demonstrated that the Q192R polymorphism of PON 1 might be involved in CHD, and that the RR is related to an increased risk of CHD. However, PON 1 activity in the RR genotype is higher than that in the QQ genotype.

Mackness et al. [59] showed that the PON 1 QQ-HDL was the most efficient at protecting LDL against oxidative modification and PON 1 RR-HDL was the least efficient.

Patients on long-term HD have decreased PON 1/arylesterase activity [95, 99]. The uraemia or dialysis probably induces changes in the PON 1 enzyme activity, enzyme concentration, or its connection with HDL.

2.4.3 Diabetes Mellitus And Hemodialysis

Diabetes mellitus (DM) is an increasingly important cause of end-stage renal disease [109]. Diabetic patients on dialysis have increased mortality and morbidity, mainly because of cardiovascular disorders [110].

Hyperglycaemia occurs in every patient with DM. It is the most important factor in the development of diabetic complications. Manifestation in certain families and the lack of complications in some diabetics with poor diabetic control indicate a genetic predisposition to develop diabetic complications like nephropathy, neuropathy and angiopathy [111].

Diabetic nephropathy occurs only in 25% to 40 % of the diabetic patients [111]. Therefore a genetic risk factor for this complication is very likely. Araki et al. [112] postulated that no polymorphism of PON genes is associated with diabetic nephropathy. In contrast, Murata et al. [113] demonstrated that among the gene polymorphisms tested, the Q192R polymorphism of PON 1 was associated with prevalence of nephropathy.

Peripheral diabetic neuropathy occurs in up to 66% of all diabetics [111]. Therefore and because of the possible pathological mechanisms, genetic risk factors like variations in the Na/K–ATPase–gene and in the aldose-reductase gene are discussed.

Up to 75% of the diabetic patients suffer for retinopathy after 15 years of suffering DM [111]. Several studies revealed that PON 1 activity is one of the significant factors for retinopathy [114] and that PON 1 Q192R genotype is an independent predictor of retinopathy [113].

A large number of studies [listed in 111] have been reported about an association between diabetic macroangiopathy and genetic variations in the paraoxonase-gene.

Increased ox-LDL levels in DM patients may result, at least partly, from reduced serum antioxidant capacity in the diabetic state, including the attenuation of PON 1 action. Increased ox-LDL could be a significant marker for susceptibility to vascular complications in diabetic patients [115].

DM patients on HD have significantly lower HDL levels and serum PON 1 activities than non–DM patients on HD [116, 117]. Serum PON 1 activities in both DM and non–DM patients are regulated by PON 1 Q192R polymorphism: RR>QR>QQ [117]. The finding that reduced PON 1 activity is associated with DM in patients on HD is consistent with the results of other studies in non-dialyzed subjects [86, 118].

Therefore, many authors have studied the association of the HDL-associated enzyme, PON 1, with chronic renal disease [99] and DM [86, 119]. However, whether or not the PON 1 status associates with DM in HD-patients has rarely been studied [120]. Moreover, it has not been clear whether the reduced PON 1 activity in patients with DM is due to the reduced quantity of HDL, yet. In conclusion, there is a fact that PON 1 status and HDL levels are independently associated with DM in patients on HD and may contribute to the increased risk of CAD in diabetic nephropathy.

3 AIM OF THESIS

The aim of thesis is:

A. To determine genotype and compare genotype distribution in population of hemodialysed (HD) patients and control healthy subjects (CS).

B. To compare the lipid and lipoprotein profile (TC, TAG, HDL, LDL, oxLDL, ApoA, ApoB, and LP(a)) in HD-patients and CS in order to consider the potential risk of atherosclerosis in studied subjects.

C. To compare CRP and PAF serum values in HD-patients and CS.

D. To compare PON1 activity and PON1 serum concentration in HD-patients and CS.

E. To examine the effect of PON1 Q192R genotype on lipid profile, CRP and PAF serum values, and PON1 activity

F. To determine factors (involved in this study) associated with hemodialysis treatment.

4 MATERIALS AND METHODS

4.1 SUBJECTS

4.1.1 HEMODIALYSIS PATIENTS

Studied subjects consisted of 45 patients (25 male / 20 female) from The Clinical Hospital Split, Croatia. All patients were treated on dialysis unit of The Department of Nephrology. They suffered from the end-stage of renal disease, main causes of their health condition were: diabetic nephropathy, chronic glomerulonephritis, chronic pyelonephritis, vasculitis, vascular diseases. Frequency of dialysis was three times per week and each dialysis lasted from four to five hours.

Tab. 1: Age and gender of HD-patients.

HD-PATIENTS	Age (years)		Total number
	Mean ± SD	Min - max	
All subjects (M+F)	61 ± 14	30 – 87	45
Male	60 ± 15	30 – 85	25
Female	63 ± 13	39 - 87	20

4.1.2 CONTROL SUBJECTS

Control subjects consisted of 47 healthy blood donors (27 male / 20 female) from The Clinical Hospital Split, Croatia.

Tab. 2: Age and gender of control samples.

CONTROL SAMPLES	Age (years)		Total number
	Mean ± SD	Min - max	
All subjects (M+F)	61 ± 15	27 – 88	47
Male	57 ± 15	27 – 80	27
Female	66 ± 14	42 – 88	20

4.2 SAMPLES

Blood samples were collected by venepuncture from the fasting subjects. The samples were stored at -20°C and the isolation was usually done within eight weeks. Before the isolation of DNA, the samples were de-frozen and gently shaken in order to homogenize.

All the biochemical tests (lipid and lipoprotein profile, glycaemia, and PON-activity) were done by The Department of Laboratory Medicine of The Clinical Hospital Split. For these tests, the blood was collected in heparin-containing tubes and tests were done immediately after blood collection.

4.3 METHODS

4.3.1 INSTRUMENTS

Centrifuge	Heraeus Instruments	Germany
Centrifuge	Eppendorf	Germany
Centrifuge	Hettich	Germany
Vortex	Genie	USA
Shaker vibromix	Tehtnica	Slovenia
Hybridization oven	Hoefer Scientific Instruments	USA
PCR machine	Applied Biosystems	USA
Electrophoresis set	Pharmacia Biotech	Sweden
UV lamp	UV tec	EEC
Spectrophotometer	Pye Unicam	Germany
Camera	Kodak	USA
pH–meter	Iskra	Croatia

4.3.2 CHEMICALS

Chemicals In the order of use:

DNA ISOLATION:

Saccharose	Kemika	Croatia
Triton X – 100	SIGMA	USA
TRIS – BASE	SIGMA	USA
HCl	Kemika	Croatia
$MgCl_2$	Kemika	Croatia
NaCl	Kemika	Croatia
EDTA	SIGMA	USA
Na_2EDTA	SIGMA	USA
SDS	SIGMA	USA
Proteinase K (14U/mg)	SIGMA	USA
100% ethanol	Kemika	Croatia
CH_3COOH	Kemika	Croatia

PCR:

$MgCl_2$ solution	Applied Biosystems	USA
10x PCR Buffer II	Applied Biosystems	USA
Taq – polymerase (5U/µl)	Applied Biosystems	USA
PRIMER PON 1 R192Q *R* (5'– CCT GAG AAT CTG AGT AAA TCC ACT – 3')		
	Microsynth	Switzerland
PRIMER PON 1 R192Q *F* (5'– TAT TGT TGC TGT GGG ACC TGA G – 3')		
	Microsynth	Switzerland
Deoxynucleoside Triphosphates (dNTPs): dATP, dTTP, dCTP, dGTP (10Mm)		
	Applied Biosystems	USA

ELECTROPHORESIS:

Agarose	SIGMA	USA
Ethidium Bromide	SIGMA	USA

Bromophenol Blue	SIGMA	USA
Natrium Acetate	Kemika	Croatia
1kb DNA ladder (1µl/µg)	Life Technologies	USA

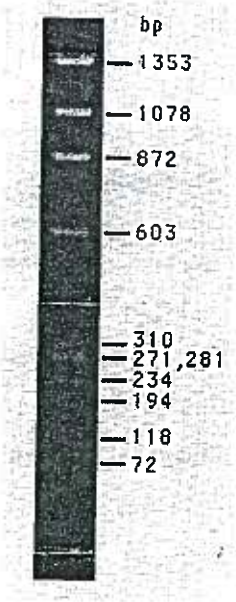

Fig. 10: 1kb DNA Ladder. This Ladder is suitable for sizing linear double-stranded DNA from 72 to 1353bp. These fragments can be visualized by ethidium bromide staining.

RFLP:

Alw I (5U/µl)	Bio Labs	UK
10x NEBbuffer 4	Bio Labs	UK

4.2.3 REAGENTS

CLB 500 ml: 54.8 g saccharose
5 g Triton X – 100
2.5 ml 2 M Tris pH=8.2
1.25 ml 2 M $MgCl_2$

Mix all the components together; add the autoclaved water to the total volume of 500ml and sterile filtrate.

SLR 500 ml: 3.5 ml 2 M Tris
1.75 ml 2 M $MgCl_2$
990 µl 5 M NaCl

Mix all the components together, add the autoclaved water to the total volume of 500ml and put it into the autoclave.

NLB 100 ml: 500 µl 2 M Tris
8.04 ml 5 M NaCl
400 µl 0.5 M EDTA
Add autoclaved water to total volume of 100ml and put it into autoclave.

10% SDS:
Dissolve 2 g of SDS in 20 ml of redistilled autoclaved water.

Proteinase K:
Add 10 ml of autoclaved water to 10 mg of proteinase K, mix it in the laminar box, pipette needed volume to the sterile Eppendorf-tube.

2 M Tris pH=8.2:
Dissolve 12.114 g of Tris base in 40 ml of autoclaved water. Mix it with HCl (cca 3ml) to pH=8.2. Add autoclaved water to total volume of 50 ml and autoclave it.

2 M $MgCl_2$:
Dissolve 10.165 g of $MgCl_2x\ 6H_2O$ in 50 ml of water and autoclave it.

0.5 M EDTA:
Dissolve 9.306 g of $Na_2EDTAx2H_2O$ in 30 ml of distilled water. Mix with NaOH to pH=8.2. Add distilled water to total volume of 50 ml and autoclave it.

5M NaCl:
Dissolve 14.6 g of NaCl in 50 ml of distilled water and autoclave it.

Saturated solution of NaCl 100ml:
Dissolve 20 g of NaCl in 50 ml of distilled water and autoclave it.

TAE-buffer pH=7.9: 4.84 g Tris HCl (0.04 M)
0.41 g CH_3COONa (0.005 M)
0.01 g EDTA (0.04 mM)

1.0 l ddH_2O

TE-buffer pH=8.0: 1.21 g Tris-HCl (10 mM)

0.37 g Na_2EDTA (1 mM)

Add ddH_2O to the total volume of 1.0 l. Mix to pH=8 with HCl. Store in the room temperature.

LB-buffer: 0.25 % bromophenol blue

40.0 % (w/v) sucrose in water

Primers:

Dilute primers in water to the final concentration 100 µmol/l:

Add 482.8 µl ddH_2O to primer PON1 R192Q F, and add 446.2 µl ddH_2O to primer PON1 R192Q R.

4.3.4 DNA ISOLATION

Genomic DNA was isolated from the whole blood by the **Miller's method**. The osmotic lysis, centrifugation, salting out and ethanol precipitation of DNA is involved in this three-day procedure;

1st day:

- Defrost the whole blood in the room temperature;
- Pipette 2.5 ml of the whole blood into 15 ml plastic tube;
- Add CLB-solution to total volume of 12 ml, gently mix, leave on the ice for 20 min;
- Centrifuge for 20 min (6000 rpm, +4°C);
- Pour out the supernatant, mix the pallet with 8ml of CLB-solution, mix it gently and leave it on ice for 20 min;
- Centrifuge for 20 min (6000 rpm, +4°C);
- Pour out the supernatant, add 10ml SRL-solution, mix gently, put it on ice on the shaker, shake it gently for 20 min;
- Centrifuge for 20 min (6000 rpm, +4°C);

- Pour out the supernatant, add 1.5 ml of cold NLB-solution, 100 µl of cold SDS and 250 µl of proteinase K;
- Mix it well till the pallet is dissolved put it on the shaker through the night and keep the temperature of 42°C.

2nd day:

- Add 500 µl of saturated solution of NaCl into plastic tubes with sample, mix well, centrifuge for 30 min (6000 rpm, +4°C);
- Prepare a new 15 ml plastic tube, mark it and pour the supernatant into this new tube;
- Add absolutely concentrated ethanol (deep frozen before use, it means-20°C) to total volume of 8ml, mix it gently until the medusa of DNA appears;
- Pipette 500 µl of this sample and catch the medusa of DNA in this volume, transfer it into marked Eppendorf tube;
- Centrifuge it for 5 min (4000 rpm, room temperature);
- Pour out the ethanol phase;
- Keep Eppendorf tube with the DNA-pallet on the dark and sterile place through the night to dry out all the ethanol.

3rd day:

- Add 100 µl of ultra clean autoclaved water;
- Keep it for 2 hours in the room temperature and then keep it in the refrigerator in +4°C for at least two days before PCR process.

4.3.5 DNA PURITY TEST

The purity of isolated DNA was estimated by measuring of absorbance at 260 nm and 280nm. Concentration of DNA for each sample was calculated using the equation:

c [µg/ml] = A (260 nm) x 50* x dilution factor.

***50 µg/ml DNA has A_{260}=1.0**

Isolated DNA was diluted 1:50** (10 µl of isolated DNA and 490 µl of TE-buffer). The absorbance was measured at 260 nm and 280 nm, TE-buffer was used as the blank. If the ratio A (260 nm) / A (280 nm) lies in the interval from 1.8 to 2.0 purity of DNA is acceptable for further PCR and RFLP. The concentration of isolated DNA (c [µg/ml]) was calculated:

$$c\ [\mu g/ml] = A\ (260\ nm) \times 50^{*} \times 50^{**}.$$

4.3.6 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) offers a fast and convenient method of amplifying a specific DNA segment. In this technique a heat denaturated (strand-separated) DNA sample is incubated with DNA polymerase, dNTPs, and two oligonucleotide primers that direct the DNA polymerase to synthesize new complementary strands. Multiple cycles of this process, each doubling the amount of DNA present, exponentially amplify the DNA.

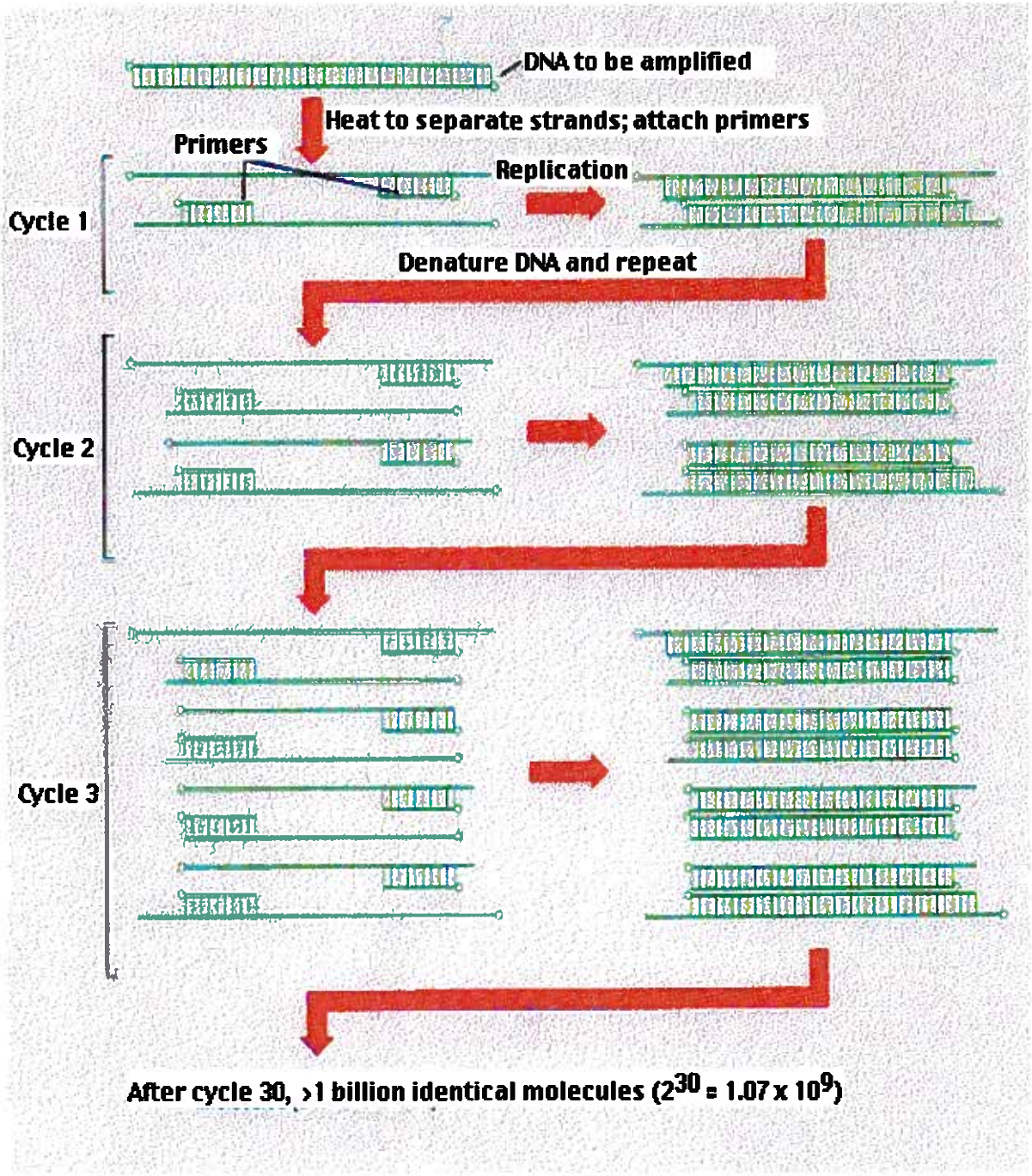

Fig. 11: **The polymerase chain reaction (PCR).** In each cycle of the reaction, the strands of the duplex DNA are separated by heat denaturation, the reaction mixture is cooled to the temperature at which synthetic DNA primers could anneal to a complementary segment on each strand, and then, the primers are extended by DNA polymerase. The process is repeated (for example 30 – 35 cycles). The number of "unit-length" strands doubles with every cycle after the second cycle.

Procedure

PCR reagents mix for PON1 R192Q polymorphism: total volume 25µl

11.9 µl ddH_2O

2.5 µl PCR-buffer

2.0 µl $MgCl_2$

2.5 µl ddNTPs mix

2.5 µl primer F

2.5 µl primer R

0.1 µl Taq-polymerase

Add 1.0 µl of isolated DNA (1 µl / ml). Prepare one negative control sample with 1 µl of ddH_2O instead of DNA.

PCR conditions:

Number of cycles	Time	Temperature [°C]
1	12min	95
35	30s 30s 60s	94 60 72
1	7min	72

4.3.7 CONTROL ELECTROPHORESIS

Electrophoresis, the migration of ions in an electric field, is widely used for the analytical separation of biological molecules. Gel electrophoresis is among the most powerful and yet conveniently used methods for macromolecular separation. The gels in common use, polyacrylamide and agarose, have pores of molecular dimensions whose sizes can be specified. The molecular separations are therefore based on gel filtration as well as the electrophoretic nobilities of the molecules being separated.

The result of the amplification with the pair of PON1 R192Q primers is the product of 234 bp. The very large pores needed for the polyacrylamide gel electrophoresis

(PAGE) of large molecular mass compounds (>200kD) requires gels with such low polyacrylamide concentration (<2.5) that they are too soft to be usable. This difficulty is circumvented by using agarose. Therefore horizontal submarine agarose gel electrophoresis was used.

Separated DNA fragments can be identified using fluorescence dyes, commonly used ethidium bromide (EtBr). PCR products were detected by staining of a gel with the solution of EtBr (5 µl EtBr/100 ml TAE-buffer), and its illumination with UV light (λ=254nm).

Procedure

Preparation of agarose gel (2%): melt 1 g of agarose in 50 ml TAE-buffer on the fire, add 5 µl of EtBr. Pour the gel. Add TAE-buffer (100-200 ml) after gel polymerization.

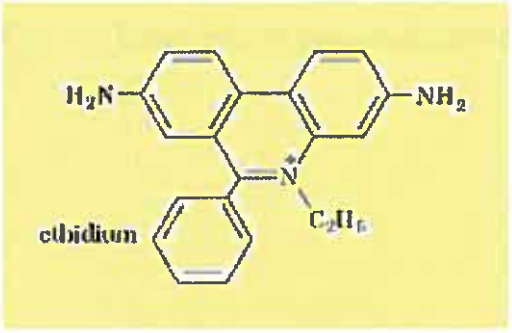

Fig. 12: **Ethidium structure.** Ethidium is planar aromatic cation that binds to duplex DNA by intercalation, and exhibits a fluorescence under UV light that is far more intense than that of the free dye. As little as 50 ng of DNA may be detected in a gel by staining it with ethidium bromide.

Preparation of marker mix: 1 µl DNA-ladder;
1 µl LB-buffer;
5 µl ddH_2O.

Conditions of electrophoresis: U=100 V;
t=30 min.

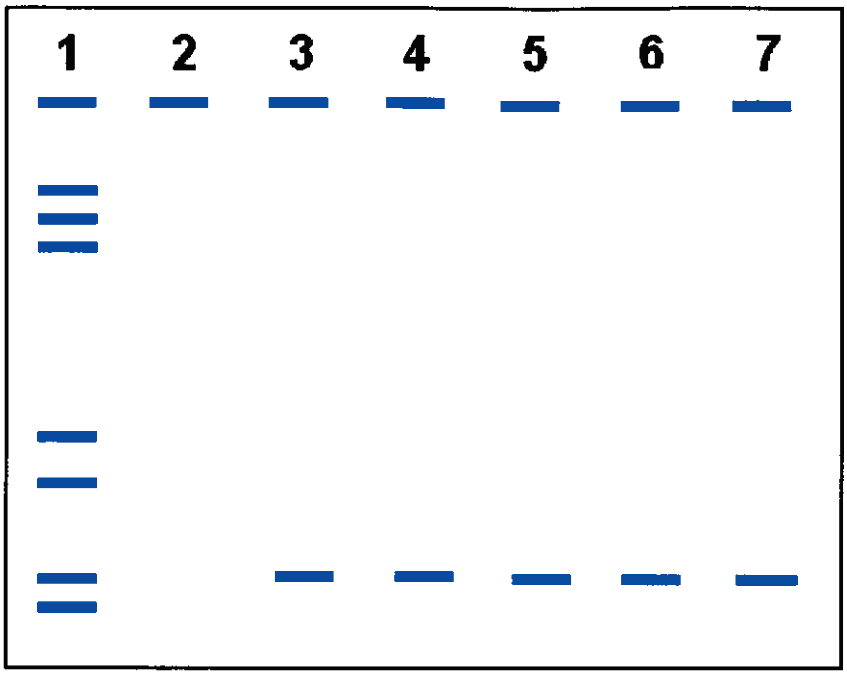

Fig. 13: **Example of gel electrophoresis:** 1 marker mix;
2 blank=5 µl ddH_2O, 2 µl LB-buffer;
3-7 DNA-fragments=5 µl PCR product,
2 µl LB-buffer.

4.3.8 RESTRICTION FRAGMENTS LENGTH POLYMORPHISM (RFLP)

Individuality In humans and other species derives from their genetic polymorphism.

There are three main types of polymorphisms:

- RFLP;
- VNTR (Variable Number of Tandem Repeats);
- STR (Short Tandem Repeats).

RFLP is a technique in which organisms may be differentiated by analysis of patterns derived from cleavage of their DNA. If two organisms differ in the distance between sites of cleavage of a particular restriction endonuclease, the length of the fragments produced will differ when the DNA is digested with a restriction enzyme.

Most RFLPs are created by a change in a single nucleotide in the gene, and so these are called single nucleotide polymorphisms (SNPs).

RFLPs have provided valuable information in many areas of biology, including:

- Screening human DNA for the presence of potentially deleterious genes;
- Providing evidence to establish the innocence of (or a probability of the guilt of), a crime suspect by DNA "fingerprinting";
- Diagnostics of inherited diseases for which the molecular defect is known;
- Medical research.

Restriction endonucleases are enzymes that cleave DNA molecules at specific nucleotide sequences depending on the particular enzyme used. Enzyme recognition sites are usually 4 to 6 base pairs in length. Cleavage by restriction enzyme produces either cohesive (having either a 5' or 3' single-stranded protrusion) or blunt-ended (no single-stranded protrusion) fragments. Cohesive fragments can be subsequently ligated to other restriction fragments if their single-stranded protrusions or "overhangs" are compatible. All blunt-ended fragments can be ligated to each other.

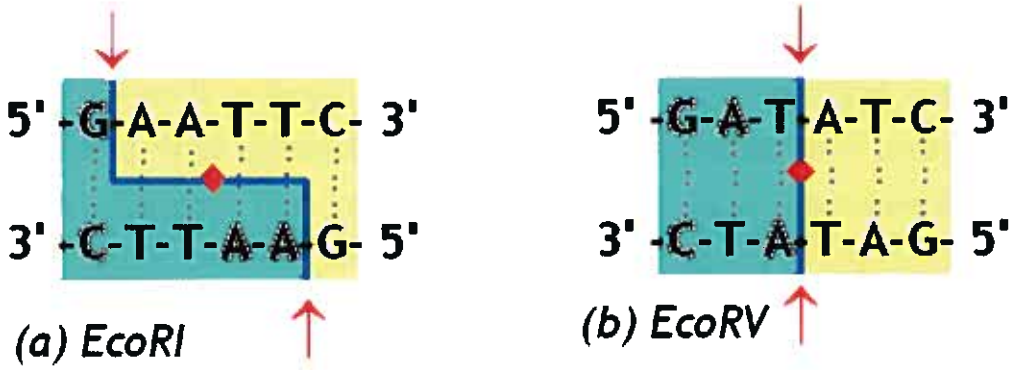

Fig. 14: **Restriction sites.** The restriction sequences of the restriction endonucleases (a) *Eco*RI and (b) *Eco*RV have two fold (palindromic) symmetry (red symbol). The cleavage sites are indicated (arrows). The *Eco*RI generates cohesive DNA fragments, whereas *Eco*RV generates blunt-ended fragments.

Restriction enzymes were discovered about 30 years ago during investigations into the phenomenon of host-specific restriction and modification of bacterial viruses. These enzymes are named by using the first letter of the genus, the first two letters of the species, and the order of discovery. Restriction enzymes are exceedingly varied;

they range in size from the diminutive *Pvu*II (157 Amino acids) to the giant *Cje*I (1250 amino acids) and beyond. Among over 3,000 activities that have been purified and characterized, more than 250 different sequence-specificities have been discovered. Restriction enzymes protect bacteria from infections by viruses, and it is generally accepted that this is their role in nature. They function as microbial immune systems. These enzymes were found to cleave DNA at specific sites, generating discrete, gene-size fragments that could be re-joined in the laboratory. Researchers were quick to recognize that restriction enzymes provided them with a remarkable new tool for investigating gene organization, function and expression.

Restriction enzymes are traditionally classified into three types (type I, type II, type III) on the basis of subunit composition, cleavage position, sequence-specificity and cofactor-requirements. Type II enzymes cut DNA at defined positions close to or within their recognition sequences. They produce discrete restriction fragments and distinct gel banding patterns, and they are the only class used in the laboratory for DNA analysis and gene cloning. The most common type II enzymes are those like *Hha*I, *Hind*III and *Not*I that cleave DNA within their recognition sequences.

The next most common type II enzymes are those like *Alw*I that cleave outside of their recognition sequence to one side. These enzymes are intermediate in size, 400-650 amino acids in length, and they recognize sequences that are continuous and asymmetric. They comprise two distinct domains, one for DNA binding, and the other for DNA cleavage. They are thought to bind to DNA as monomers for the most part, but to cleave DNA cooperatively, through dimerization of the cleavage domains of adjacent enzyme molecules.

***Alw*I:**

- Source: an *E. coli* strain that carries the cloned *Alw*I gene from *Acinetobacter lwoffii;*
- Recognition site: 5'...GGATC $(N)_4$...3'
  3'... CCTAG $(N)_5$...5'.

Procedure:

For PON1-192 polymorphism, PCR products and isolated DNA were digested with *Alw*I enzyme for 12 hours at 37°C. RFLP mix for PCR-product and RFLP mix for isolated DNA was prepared:

- RFLP mix for PCR-product: 0.5 µl *Alw*;
  1.5 µl NEB buffer;
  8.0 µl ddH_2O;
  5.0 µl PCR-product.
- RFLP mix for isolated DNA: 0.5 µl *Alw*I;
  1.5 µl NEB buffer;
  11.0 µl ddH_2O;
  2.0 µl isolated DNA.

After RFLP were the samples electrophoresed.

4.3.9 FINAL ELECTROPHORESIS

Large variety of alleles (polymorphisms) may be present in the population. Some people will be homozygous and reveal a single band; others will be heterozygous with each allele producing its band.

Procedure:

The samples were electrophoresed in 3% agarose gel for 40 min at 100 V.

Preparation of agarose gel (3%): melt 1.5g of agarose in 50ml TAE-buffer on the fire, add 5µl of EtBr. Pour the gel. Add TAE-buffer (100-200ml) after gel polymerization.

Preparation of marker mix: 1 µl DNA-ladder;
1 µl LB-buffer;
5 µl ddH_2O.

The samples were electrophoresed as follows:

1...marker mix;

2...isolated DNA undigested;

3...PCR-product undigested;

4...no DNA;

5...PCR-product digested No. 1;

(n-5)... PCR-product digested No. n.

There was used the same detection as in control electrophoresis above.

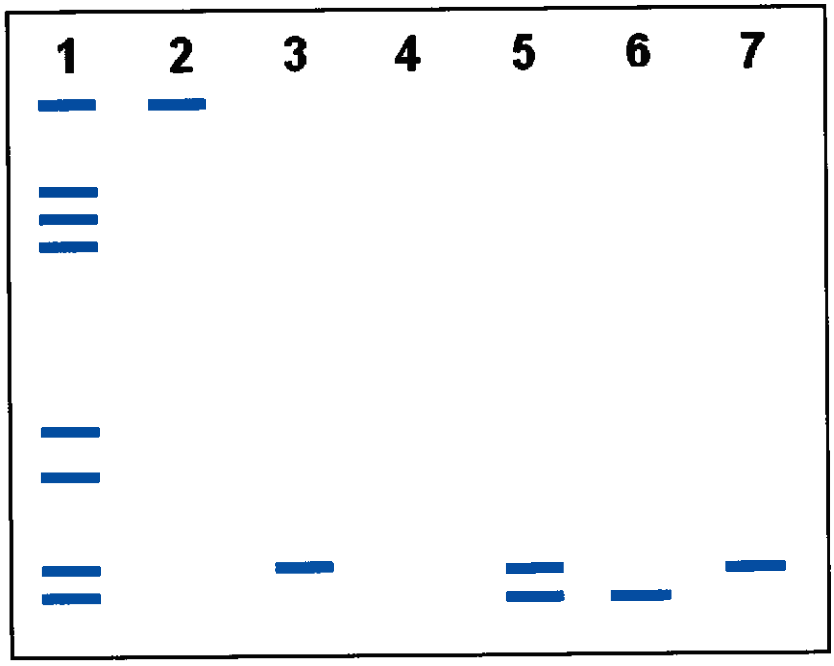

Fig. 15: **Example of final electrophoresis:**

1 marker mix;

2 isolated DNA undigested;

3 PCR-product undigested;

4 no DNA;

5 heterozygote (QR) for PON1-192;

6 homozygote (RR) for PON 1-192;

7 wild type (QQ).

5 RESULTS

5.1 DNA PURITY TEST

DNA was isolated from 47 control samples and 45 samples from HD patients by Miller's method. The purity of isolated DNA was estimated by measuring of absorbance A_1 [λ_1=260nm] and A_2 [λ_2=280nm]. The ratio A_1/A_2 lies in the interval from minimum 1.373 to maximum 2.180. These results (tab. 3) of DNA purity are acceptable for our further experiments.

The concentration of DNA (c [µg/ml]) in each sample was calculated as prescribed:

c [µg/ml] = A (260nm)x50xdilution.

Tab. 3: Results of DNA purity (A_1/A_2 ratio) and DNA quantity tests (concentration of isolated DNA [µg/ml]) in serum of hemodialysis (HD) patients and control subjects.

	Mean ± SD / min - max		
	Controls (n=47)	**HD patients (n=45)**	**P**
DNA [µg/ml]	1798 ± 478 / 915 – 2915	1478 ± 807 / 215 – 4265	0.0131
A_1/A_2	1.848 ± 0.191 / 1.373 – 2.180	1.841 ± 0.168 / 1.409 – 2.389	0.4246

5.2 CONTROL ELECTROPHORESIS

The result of the amplification with the pair of PON1 R192Q primers is the product of 238 bp. In order to be sure that the amplification by PCR was correct each PCR product was analysed by electrophoresis. All PCR products from 47 control samples and 45 HD-samples were 238 bp long DNA fragments suitable for RFLP.

Fig. 16: Example of product of control electrophoresis, all DNA fragments are of the same length 238bp. The marker is in line number 1. There were detected 19 samples in lines 2 to 20.

5.3 RESTRICTION FRAGMENTS LENGTH POLYMORPHISM (RFLP)

PCR products were digested with *AlwI* enzyme at 37°C for 12 hours. The final electrophoresis was done after digestion of all PCR products was finished. According to the position of RFLP product on the gel it would be possible to estimate product's size and to determine genotype (RR genotype - homozygote, RQ genotype - heterozygote, QQ genotype - wild type) of each sample.

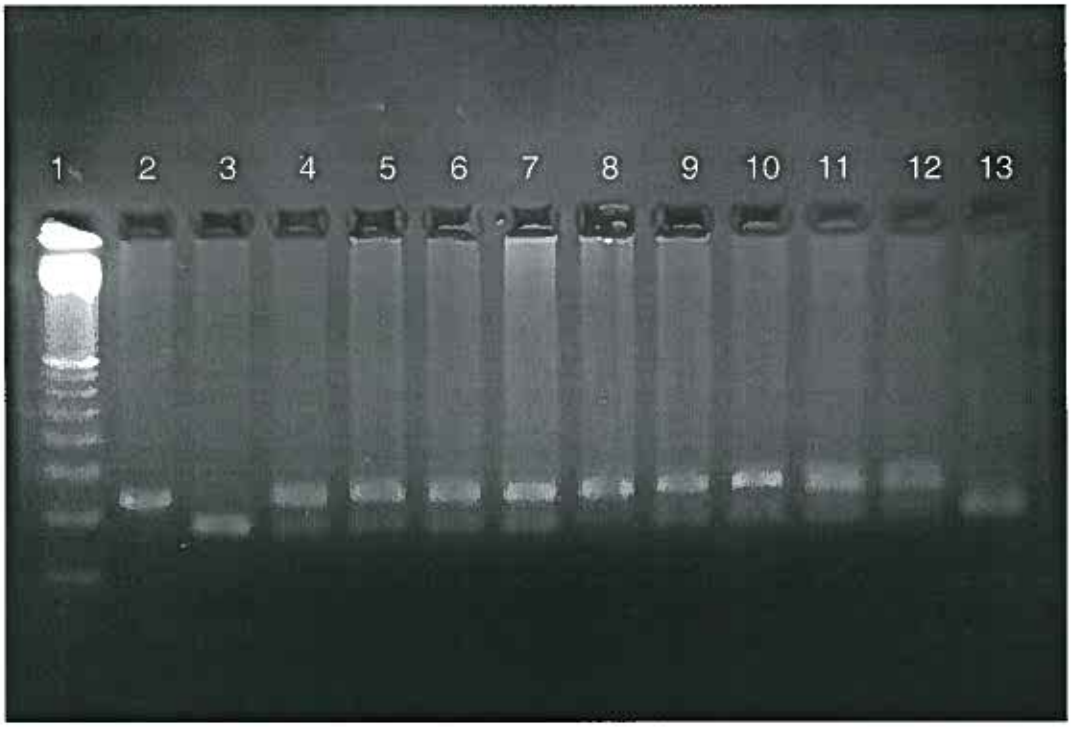

Fig. 17: Restriction patterns of the PCR-amplified 238bp fragment after digestion with *AlwI*. Fragment sizes of 238bp correspond to Q allele, whereas 172bp are diagnostic bands for R allele.

Line 1 Marker. Line 2 PCR product undigested. Lines 3, and 13 RR genotype. Lines 4-7, and 9-11 RQ genotype. Lines 8, and 12 QQ genotype.

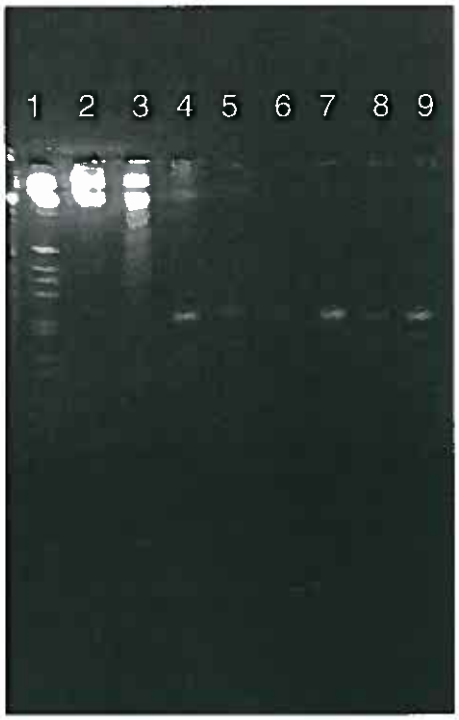

Fig.18: Restriction patterns of the PCR-amplified 238bp fragment after digestion with *AlwI*. Fragment sizes of 238bp correspond to Q allele, whereas 172bp are diagnostic bands for R allele. Line 1 Marker. Line 2 genomic DNA undigested. Line 3 genomic DNA digested. Line 4 PCR product undigested. Line 5-8 QQ genotype. Line 9 RQ genotype.

5.4 GENOTYPE DISTRIBUTION

5.4.1 Comparison of Control Subjects and Hemodialysed Patients

On 45 HD-patients and on 47 control subjects PON1 Q192R allelic polymorphism was studied.

Genotype distribution and allele frequency is shown in tab.4 and tab.5.

The most common is QQ genotype (78.72% of control subjects, and 80.00% of HD-patients), followed by RQ genotype (17.02% of control subjects, and 15.60% of HD-patients), and RR genotype is the least common (4.26% of control subjects, and 4.40% of HD-patients) in both control subjects and HD-patients.

Tab. 4: Genotype distribution in group of control subjects (CS), and in group of hemodialysis patients (HD).

Genotype	Subject	Total number	Frequency [%]
RR	CS	2	4.26
	HD	2	4.40
RQ	CS	8	17.02
	HD	7	15.60
QQ	CS	37	78.72
	HD	36	80.00

Tab. 5: Allele frequency in group of control subjects (CS), and in group of hemodialysis patients (HD).

Allele	Subjects	Frequency [%]
Q	CS	87.23
	HD	87.78
R	CS	12.77
	HD	12.22

5.5 SERUM CONCENTRATION OF TRIACYLGLYCERIDES, TOTAL CHOLESTEROL, HDL, LDL, oxLDL, ApoA, ApoB, AND LP(a)

5.5.1 SERUM CONCENTRATION OF TRIACYLGLYCERIDES, TOTAL CHOLESTEROL, HDL, LDL, oxLDL, APOA, APOB, AND LP(A) IN CONTROL SUBJECTS AND HD PATIENTS

The concentrations of TAG, TC, HDL, LDL, oxLDL, ApoA, ApoB, and Lp(a) were measured in serum of all control subjects (n=47) and patients on maintenance hemodialysis (n=45).

Serum values of HD-patients show bigger atherogenic risk in all the parameters so far measured than serum values of control subjects. The most statistically significant are the differences in TAG values (P=0.0014).

Tab. 6: Lipid and apolipoprotein profile in controls and hemodialysis (HD) patients.

	Mean ± SD / min - max		
	Controls (n=47)	**HD patients (n=45)**	**P**
TAG (mmol/l)	1.25 ± 0.32 / 0.59 – 2.03	1.92 ± 1.45 / 0.31 – 8.49	0.0014
TC (mmol/l)	4.59 ± 0.60 / 3.42 – 6.29	4.80 ± 1.23 / 3.42 – 6.29	0.1480
HDL (mmol/l)	1.21 ± 0.21 / 0.78 – 1.54	1.14 ± 0.32 / 0.78 – 1.54	0.0254
LDL (mmol/l)	2.91 ± 0.50 / 1.91 – 4.34	3.27 ± 1.00 / 1.91 – 4.34	0.0172
OxLDL (mg/l)	295 ± 220 / 77 - 1000	373 ± 341 / 39 – 1300	0.1009
ApoA (g/l)	1.26 ± 0.14 / 0.94 - 1.52	1.12 ± 0.17 / 0.79 – 1.53	0.0664
ApoB (g/l)	0.84 ± 0.19 / 0.51 – 1.16	0.97 ± 0.26 / 0.55 – 1.77	0.0048
Lp(a) (g/l)	0.246 ± 0.351 / 0.009 – 1.592	0.241 ± 0.260 / 0.031 – 0.970	0.0200

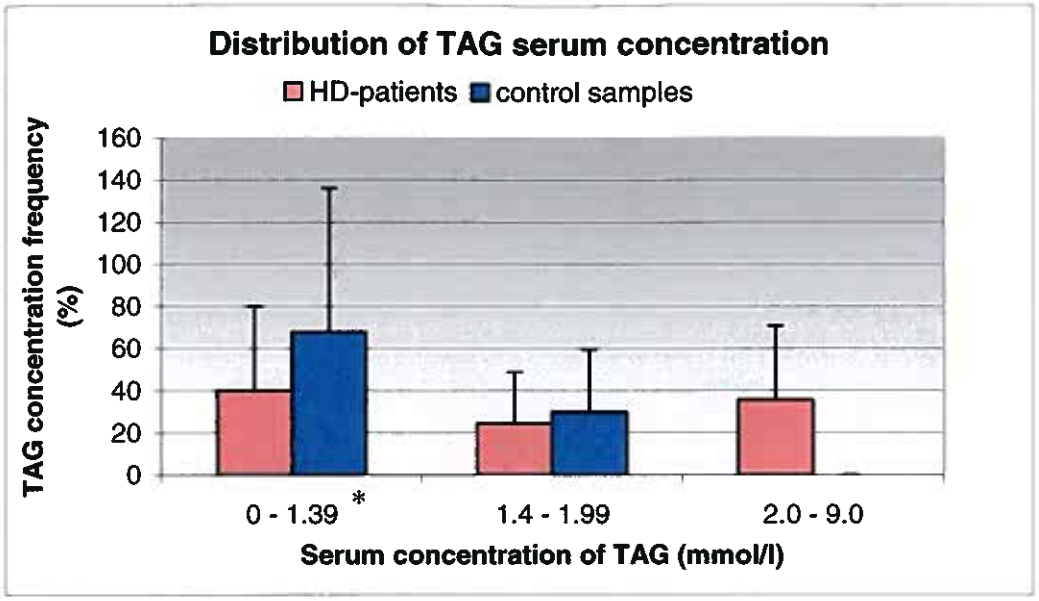

Fig. 19: Comparison of TAG serum concentration in the group of hemodialysis (HD) patients and in the group of control samples. * Significant difference between cases and controls: P<0.05.

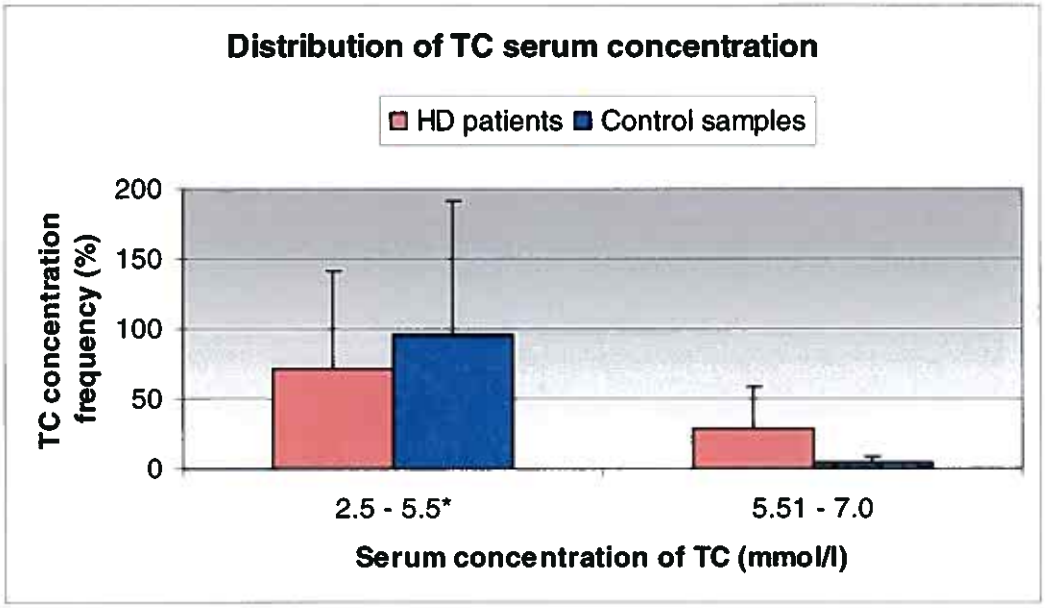

Fig. 20: Comparison of TC serum concentration in the group of hemodialysis (HD) patients and in the group of control samples. * Significant difference between cases and controls: P<0.03.

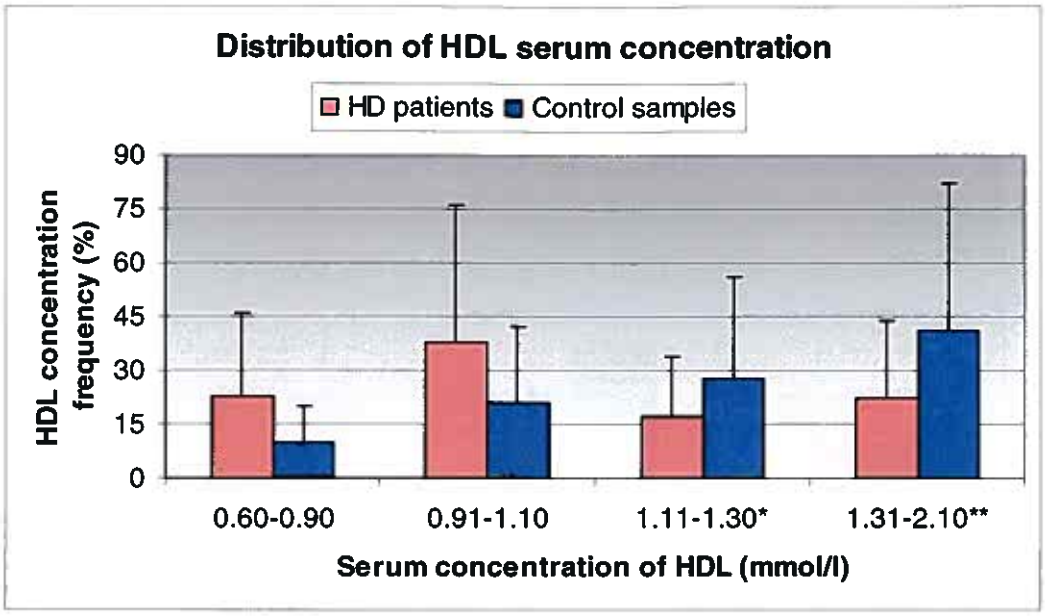

Fig. 21: Comparison of HDL serum concentration in the group of hemodialysis (HD) patients and the group of control samples. * Significant difference between cases and controls: $P<0.05$. ** Significant difference between cases and controls: $P< 0.03$.

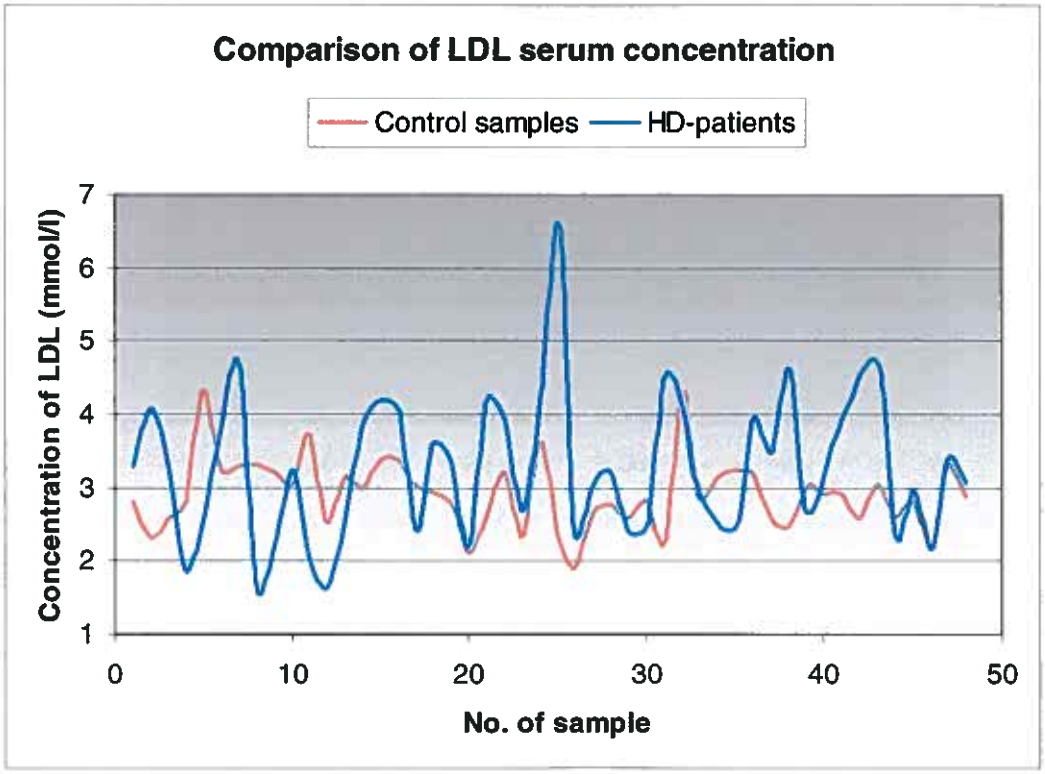

Fig. 22: Comparison of LDL serum concentration in the group of hemodialysis (HD) patients and the group of control samples.

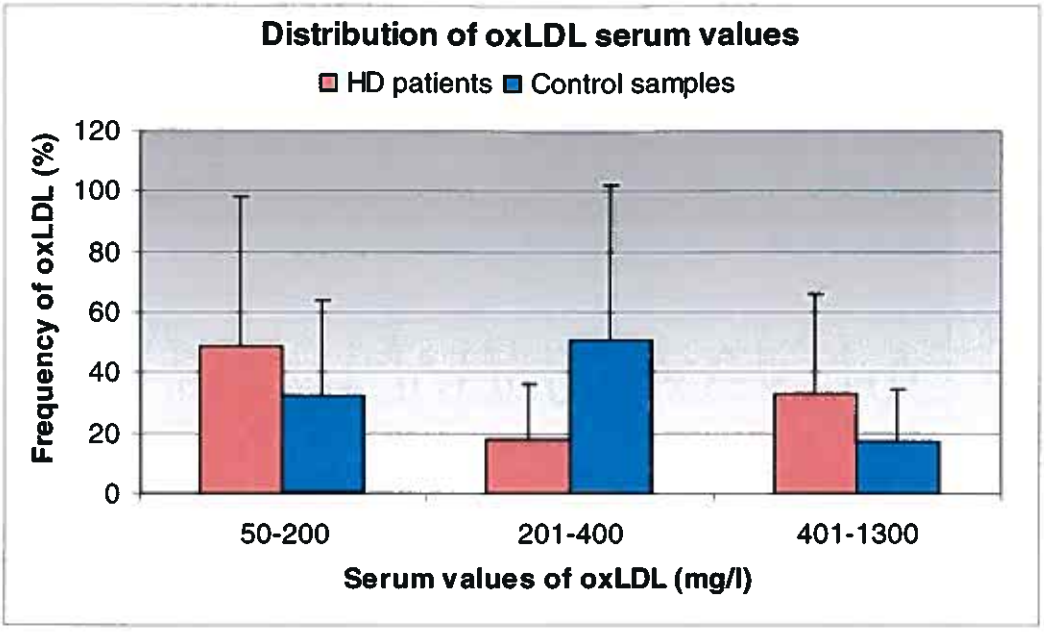

Fig. 23: Comparison of oxLDL serum values in the group of hemodialysis (HD) patients and the group of control samples.

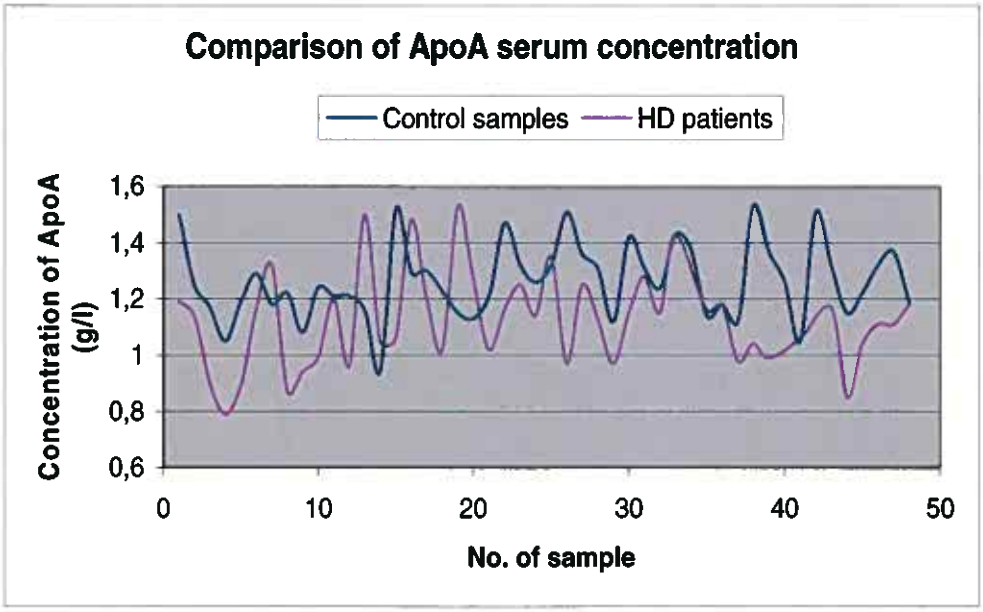

Fig. 24: Comparison of ApoA serum values in the group of hemodialysis (HD) patients and the group of control samples.

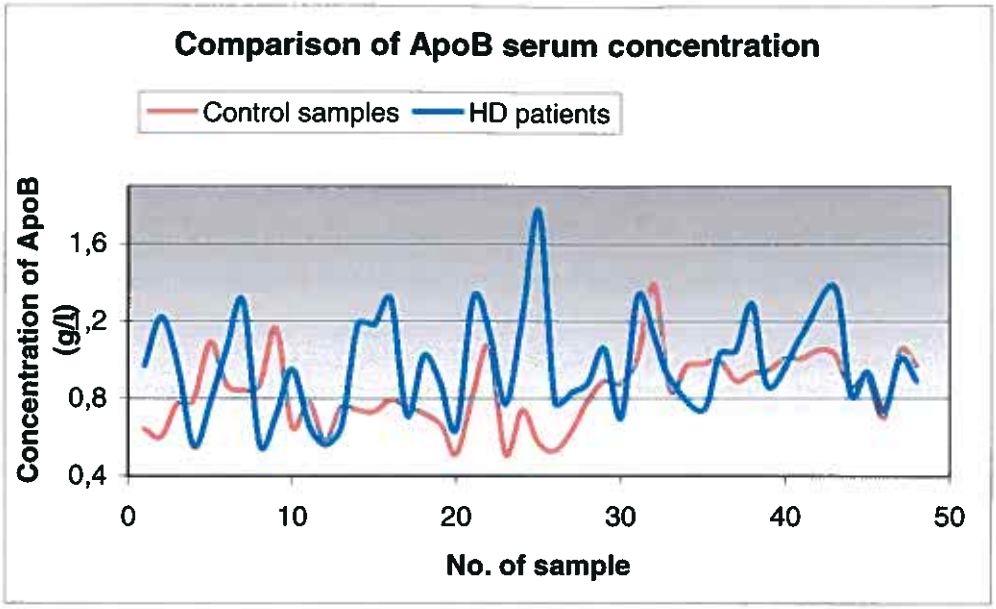

Fig. 25: Comparison of ApoB serum values in the group of hemodialysis (HD) patients and the group of control samples.

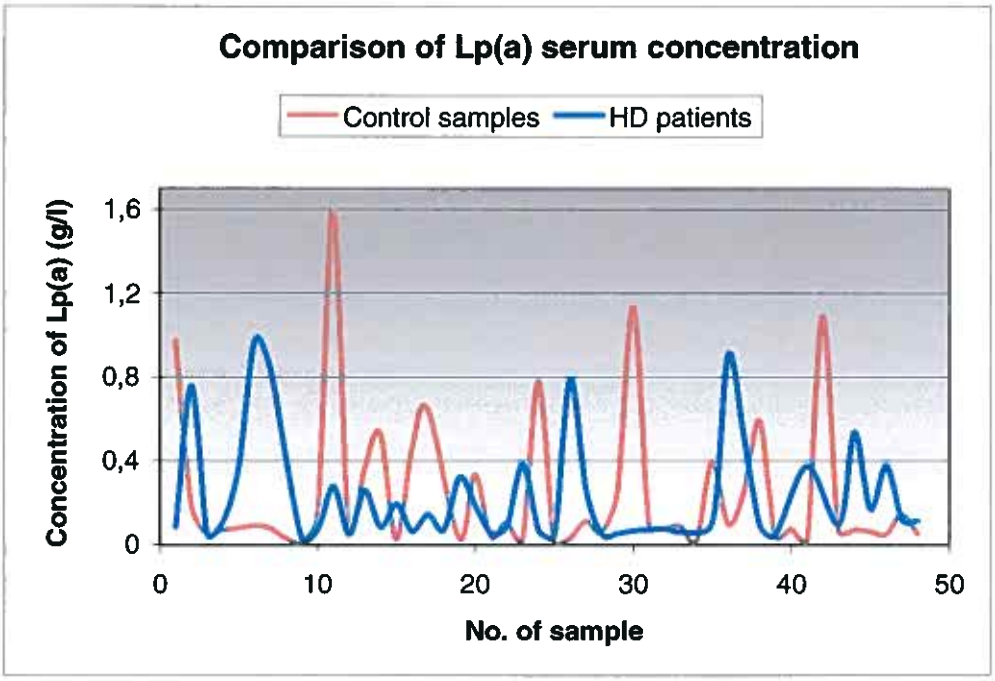

Fig. 26: Comparison of Lp(a) serum values in the group of hemodialysis (HD) patients and the group of control samples.

5.5.2 Serum Concentration Of Triacylglycerides, Total Cholesterol, HDL, LDL, oxLDL, ApoA, ApoB, And Lp(a) In Serum Of Control Subjects And HD patients According To R192Q PON1 Polymorphism

It is evident, however, that independently of the genotype atherogenic markers were significantly increased in HD patients compared with controls.

On the other hand, ApoB serum concentration could be comparable to genotype. Distribution of ApoB serum values was the same as the genotype distribution in both HD-patients and control subjects. ApoB serum concentration is falling down from the highest value in RR genotype to the lowest value in QQ genotype. Lp(a) concentrations seems to be genotype-dependent parameter too. Lp(a) serum concentration is falling down from the highest value in QQ genotype to the lowest value in RR genotype in both HD-patients and control subjects.

Characteristics of control subjects and HD-patients according to genotype distribution are presented in tab.7.

Tab. 7: Concentration of TAG, TC, HDL, LDL, oxLDL, ApoA, ApoB, and Lp(a) in serum of Controls and hemodialysis patients (HD-patients) according to PON1 genotype.

Genotype		Mean ± SD / min-max		
		Controls	HD patients	P
TAG (mmol/l)	RR	1.40 ± 0.28 / 1.2 – 1.6	1.30 ± 0.28 / 1.10 – 1.49	0.3716
	RQ	1.30 ± 0.34 / 0.81 – 1.89	2.17 ± 1.77 / 0.51 – 5.54	0.1242
	QQ	1.24 ± 0.33 / 0.59 – 2.03	1.91 ± 1.44 / 0.31 – 8.49	0.0036
TC (mmol/l)	RR	1.40 ± 0.28 / 4.93 – 5.13	5.33 ± 1.23 / 1.10 – 1.49	0.3966
	RQ	1.30 ± 0.34 / 3.55 – 6.10	4.90 ± 0.90 / 3.55 – 6.10	0,2106
	QQ	1.24 ± 0.33 / 3.42 – 6.13	4.75 ± 1.30 / 2.52 – 8.61	0,2298
HDL (mmol/l)	RR	5.03 ± 0.15 / 1.25 – 1.44	1.29 ± 0.08 / 1.23 – 1.35	0,3365
	RQ	4.53 ± 0.80 / 0.88 – 1.34	1.16 ± 0.30 / 0.75 – 1.62	0,4702
	QQ	4.58 ± 0.57 / 0.78 – 1.54	1.09 ± 0.27 / 0.65 – 2.01	0,0164
LDL (mmol/l)	RR	1.34 ± 0.13 / 3.05 – 3.14	3.80 ± 1.03 / 3.07 – 4.53	0,2558
	RQ	1.15 ± 0.18 / 2.2 – 4.34	3.31 ± 0.81 / 2.18 – 4.3	0,1371
	QQ	1.22 ± 0.21 / 1.91 – 4.32	3.23 ± 1.05 / 1.62 – 6.58	0,0510
OxLDL (mg/l)	RR	3.09 ± 0.06 / 161 – 227	331.50 ± 129.40 / 240 – 423	0,1468
	RQ	2.88 ± 0.67 / 97 – 1000	340.43 ± 415.16 / 39 – 993	0,4710
	QQ	2.91 ± 0.48 / 74 - 1000	382.17 ± 340.95 / 46 - 1300	0,0840
ApoA (g/l)	RR	194.35 ± 46.17 / 1.32 – 1.38	1.23 ± 0.07 / 1.18 -1.28	0,0879
	RQ	353.74 ± 275.36 / 0.94 – 1.53	1.13 ± 0.09 / 1.04 – 1.25	0,0755
	QQ	288.94 ± 213.23 / 1.05 – 1.52	1.12 ± 0.19 / 0.79 – 1.53	0,0001
ApoB (g/l)	RR	1.35 ± 0.04 / 0.97 – 1.03	1.11 ± 0.31 / 0.89 – 1.33	0,3536
	RQ	1.24 ± 0.18 / 0.57 – 1.39	0.98 ± 0.21 / 0.74 – 1.23	0,0879
	QQ	1.27 ± 0.13 / 0.51 – 1.16	0.96 ± 0.27 / 0.55 – 1.77	0,0085
Lp(a) (g/l)	RR	1.00 ± 0.04 / 0.02 – 0.07	0.09 ± 0.03 / 0.07 – 0.12	0,1259
	RQ	0.88 ± 0.25 / 0.02 – 0.65	0.22 ± 0.13 / 0.07 – 0.38	0,3931
	QQ	0.83 ± 0.17 / 0.01 – 1.59	0.25 ± 0.28 / 0.03 – 0.97	0,4870

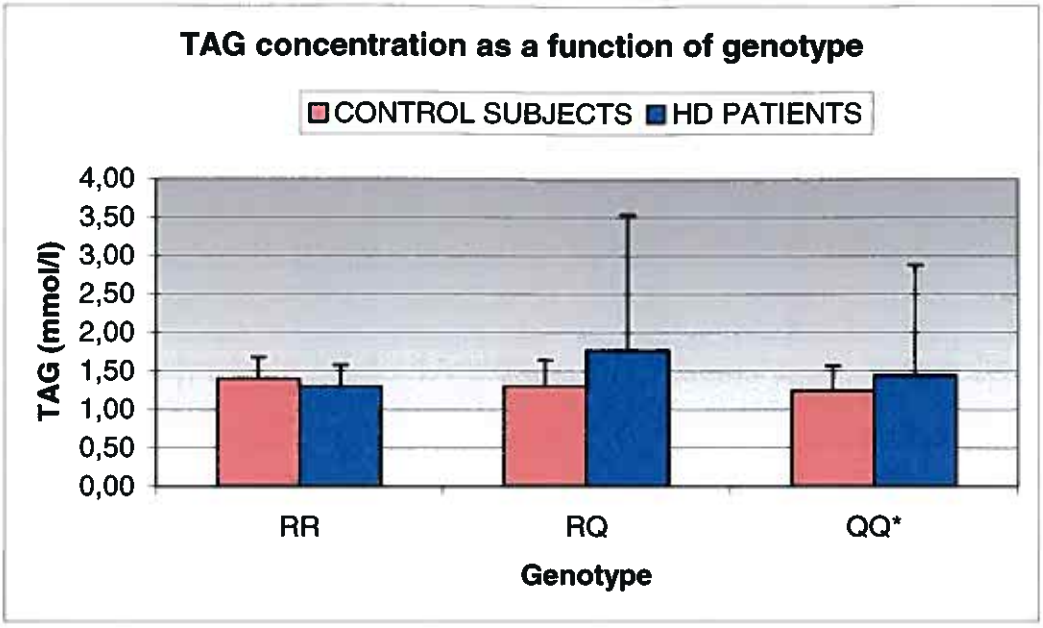

Fig. 27: Comparison of TAG mean value of serum concentration of samples from hemodialysis (HD) patients and samples from control subjects according to genotype distribution. *Significant difference between cases and controls: P< 0.004.

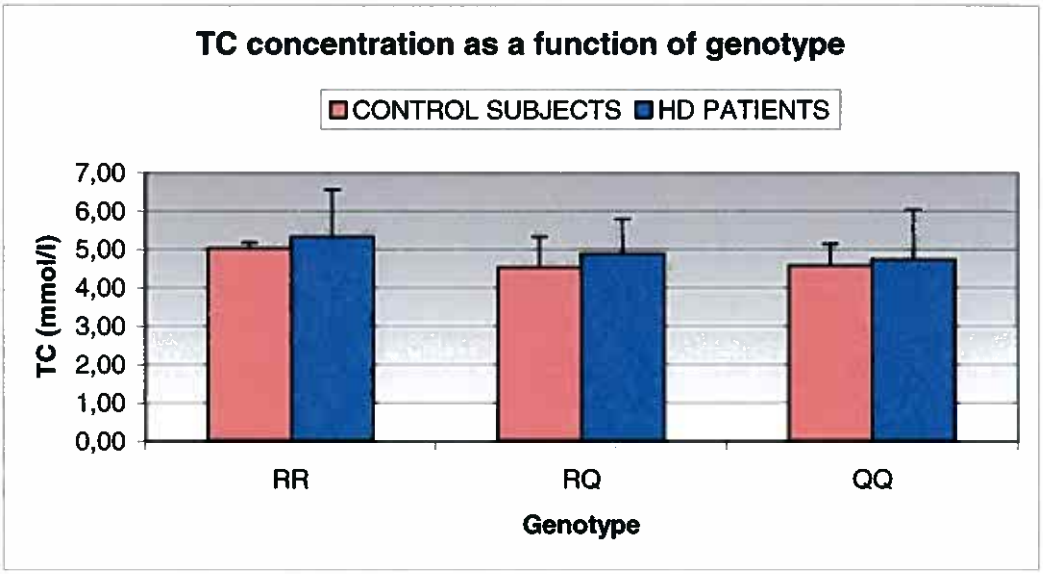

Fig. 28: Comparison of TC mean value of serum concentration of samples from hemodialysis (HD) patients and samples from control subjects according to genotype distribution.

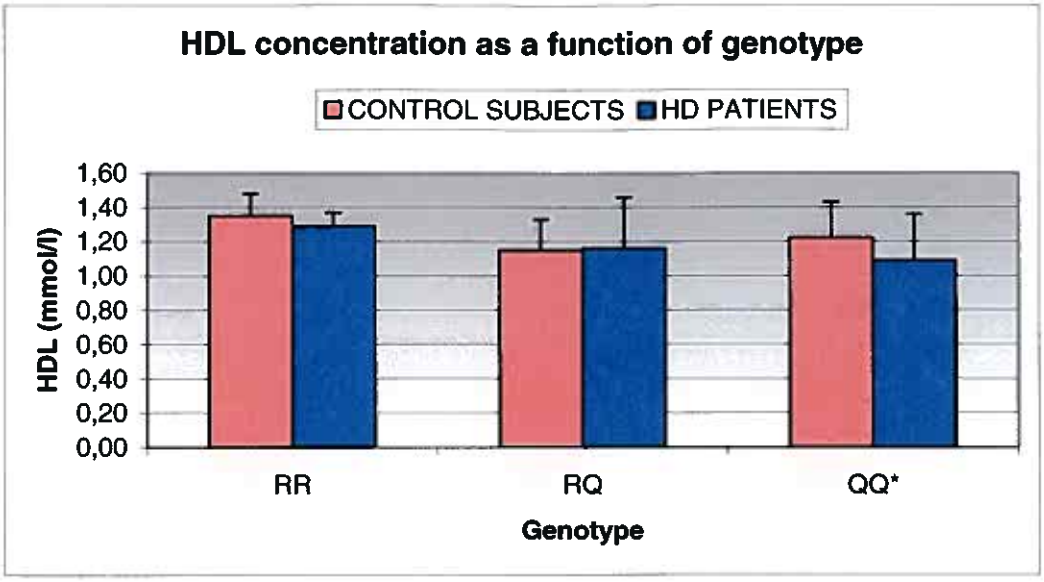

Fig. 29: Comparison of HDL mean value of serum concentration of samples from hemodialysis (HD) patients and samples from control subjects according to genotype distribution. *Significant difference between cases and controls: P=0.016.

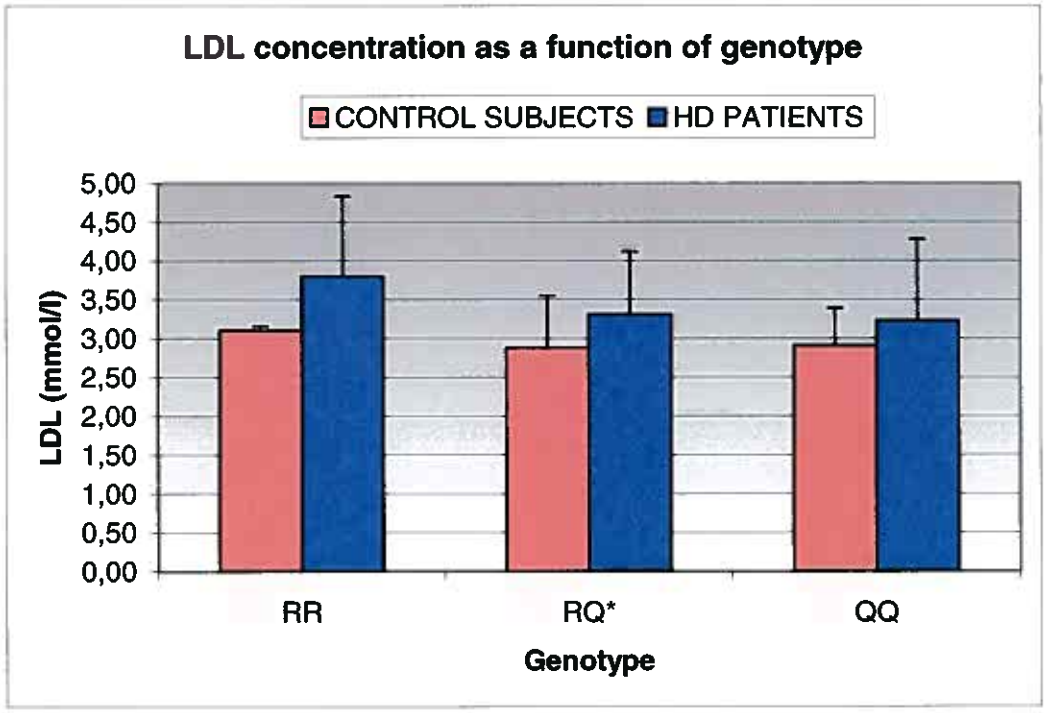

Fig. 30: Comparison of LDL mean value of serum concentration of samples from hemodialysis (HD) patients and samples from control subjects according to genotype distribution. * Significant difference between cases and controls: P< 0.05.

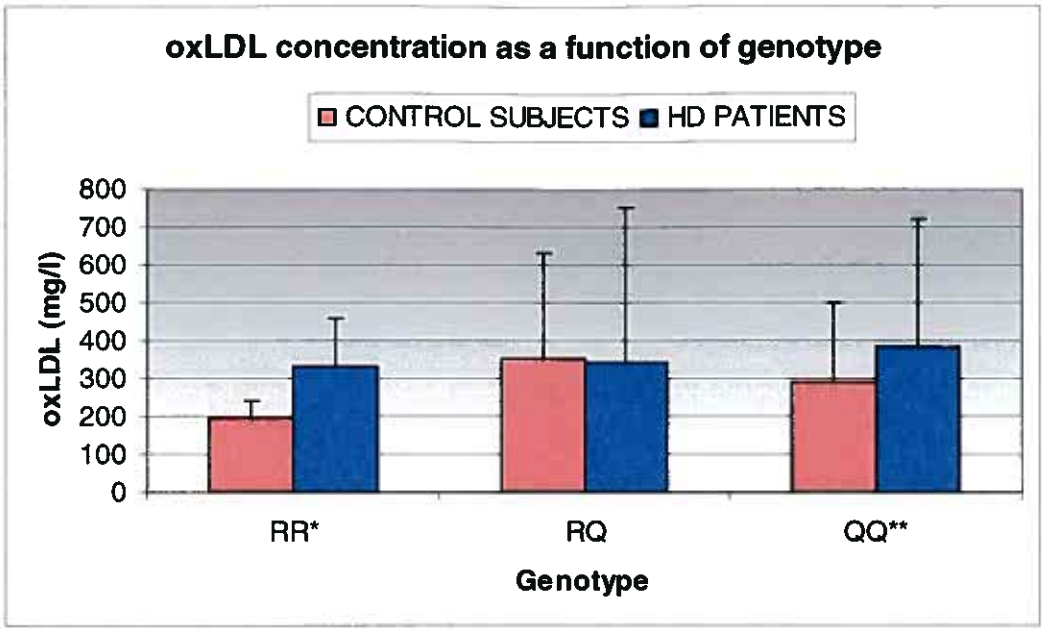

Fig. 31: Comparison of oxLDL mean value of serum concentration of samples from hemodialysis (HD) patients and samples from control subjects according to genotype distribution. * Significant difference between cases and controls: P< 0.05. ** Significant difference between cases and controls: P< 0.1.

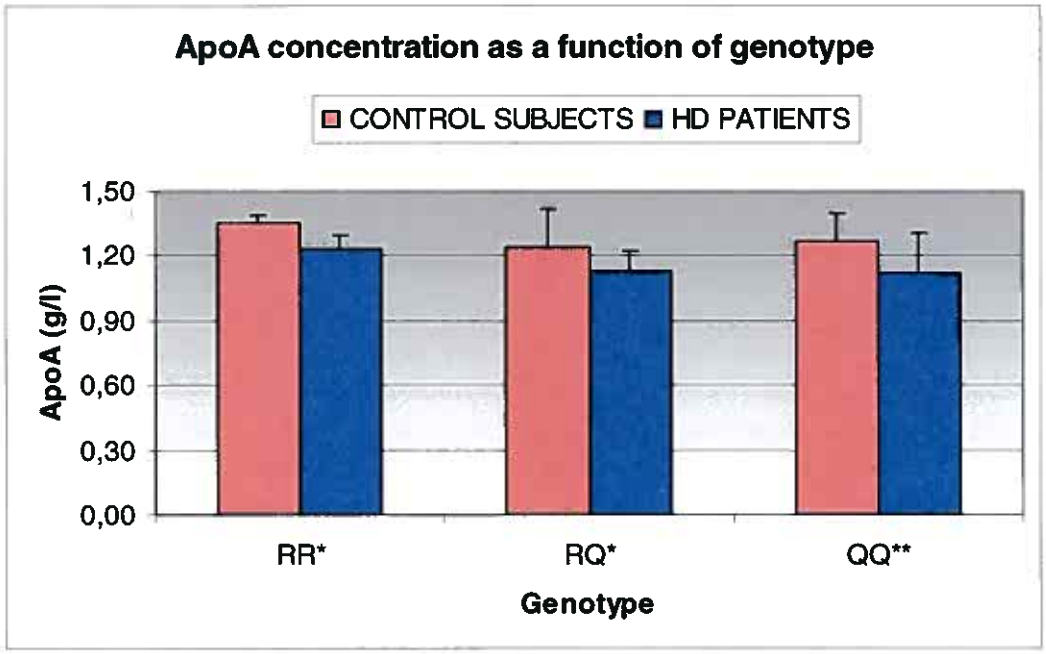

Fig. 32: Comparison of ApoA mean value of serum concentration of samples from hemodialysis (HD) patients and samples from control subjects according to genotype distribution. * Significant difference between cases and controls: P< 0.1. ** Significant difference between cases and controls: P=0.0001.

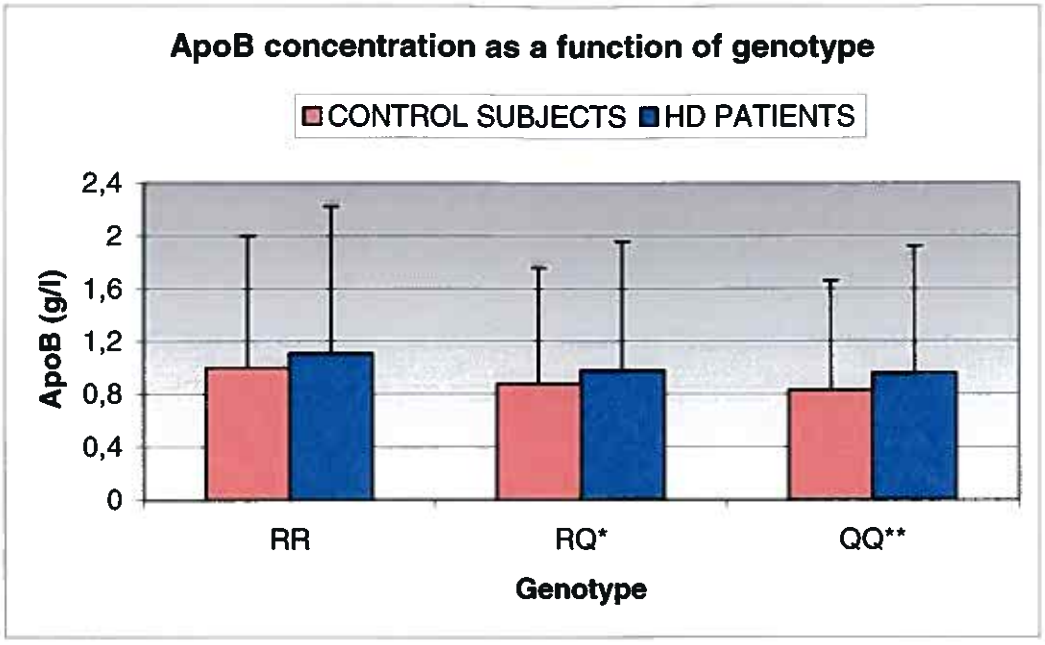

Fig. 33: Comparison of ApoB mean value of serum concentration of samples from hemodialysis (HD) patients and samples from control subjects according to genotype distribution. * Significant difference between cases and controls: P< 0.1. ** Significant difference between cases and controls: P< 0.01.

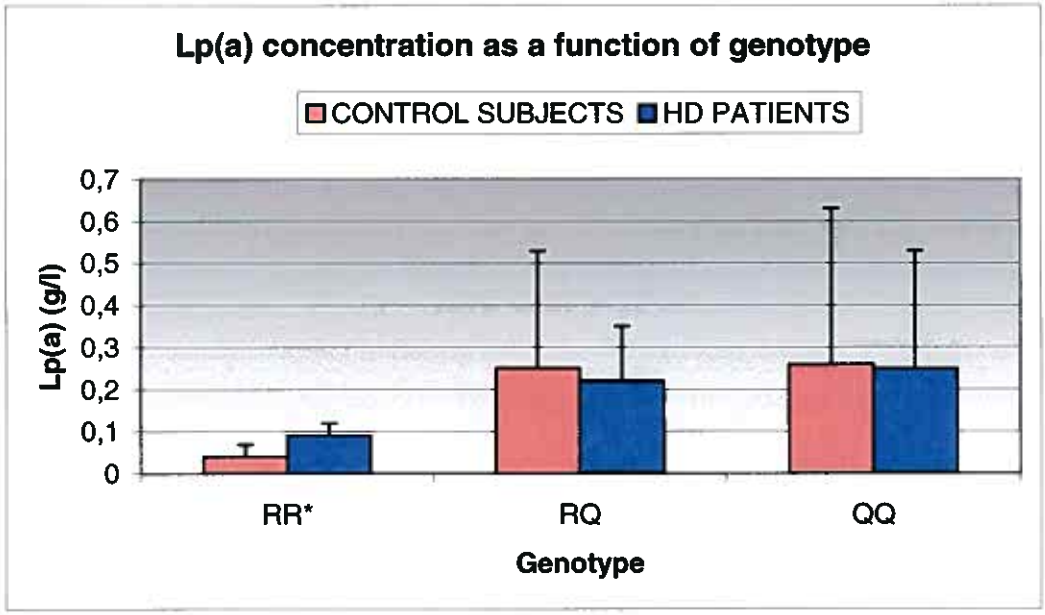

Fig. 34: Comparison of Lp(a) mean value of serum concentration of samples from hemodialysis (HD) patients and samples from control subjects according to genotype distribution. * Significant difference between cases and controls: P< 0.1.

5.6 SERUM CONCENTRATION OF C-REACTIVE PROTEIN, AND PLATELET ACTIVATED FACTOR

5.6.1 Concentration Of CRP And PAF In Serum Of Control Subjects And HD Patients

The concentration of CRP and PAF were measured in serum of all the control subjects (n=47) and patients on maintenance hemodialysis (n=45).

There are significant differences between CRP serum concentration between group of control subjects and HD patients. The mean value of serum concentration of CRP in the group of HD patients is 771% over than mean value of control subjects. There is no significant difference according to PAF serum concentration in both groups.

Tab. 8: Concentration of CRP and PAF in serum of hemodialysis (HD) patients and control subjects.

	Mean ± SD / min - max		
	Controls (n=47)	**HD patients (n=45)**	**P**
CRP (mg/l)	2.05 ± 2.92 / 0.0 – 12.9	17.86 ± 32.28 / 1.1 – 173.6	0.0001
PAF (µg/l)	323.73 ± 54.88 / 217.20 – 509.46	357.05 ± 94.26 / 223.79 – 726.05	0.0205

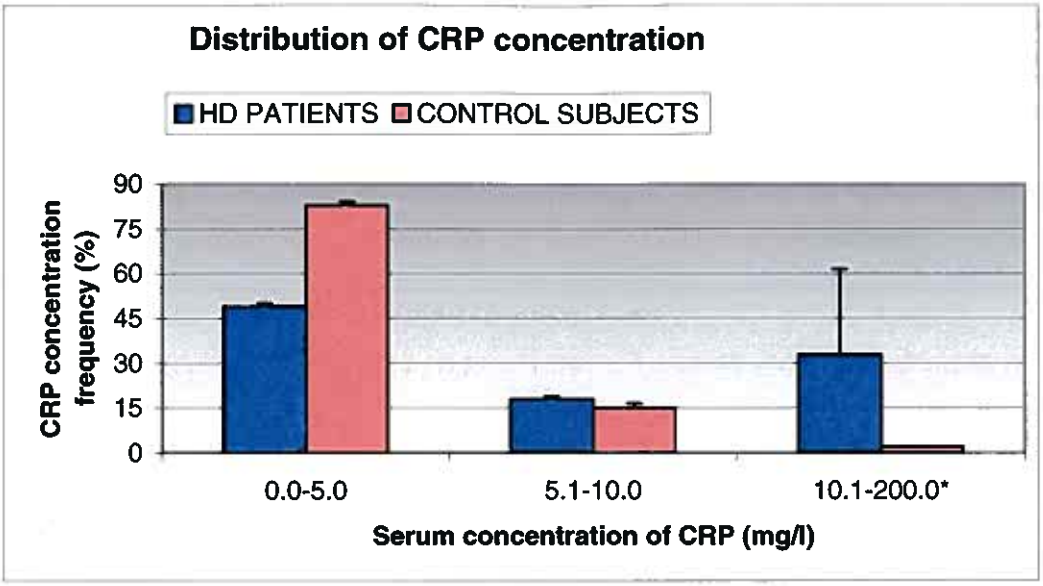

Fig. 35: Comparison of CRP serum concentration in the group of HD patients and the group of control samples. * Significant difference between cases and controls: P<0.005.

5.6.2 Concentration Of CRP And PAF In Serum Of Control Subjects And HD Patients According To R192Q PON1 Polymorphism

CRP serum values were significantly increased in HD-patients compared with control subjects independently of the genotype.

On the other hand, PAF serum concentration seems to be genotype –linked parameter: distribution of PAF serum values was the same as the genotype distribution in both HD-patients and control subjects. PAF serum concentration is falling down from the highest value in homozygotes to the lowest value in wild-type.

Characteristics of control subjects and HD-patients according to genotype distribution are presented in tab.9.

Tab. 9: CRP and PAF serum concentration of Controls and hemodialysis patients (HD-patients) according to PON1 genotype.

Genotype		Mean ± SD / min-max		
		Controls	HD patients	P
CRP (mg/l)	RR	1.40 ± 0.28 / 1.2 – 1.6	2.5 ± 0 / 2.5 – 2.5	0.4278
	RQ	1.30 ± 0.34 / 0.81 – 1.89	33.99 ± 63.21 / 2.7 – 173.6	0.0969
	QQ	1.24 ± 0.33 / 0.59 – 2.03	15.57 ± 23.53 / 1.1 – 108.9	0.0003
PAF (µg/l)	RR	1.40 ± 0.28 / 4.93 – 5.13	447.00 ± 5.00 / 443.46 – 450.54	0.0042
	RQ	1.30 ± 0.34 / 3.55 – 6.10	361.04 ± 67.00 / 302.21 – 493.82	0.2763
	QQ	1.24 ± 0.33 / 3.42 – 6.13	351.28 ± 99.50 / 223.79 – 726.05	0.0446

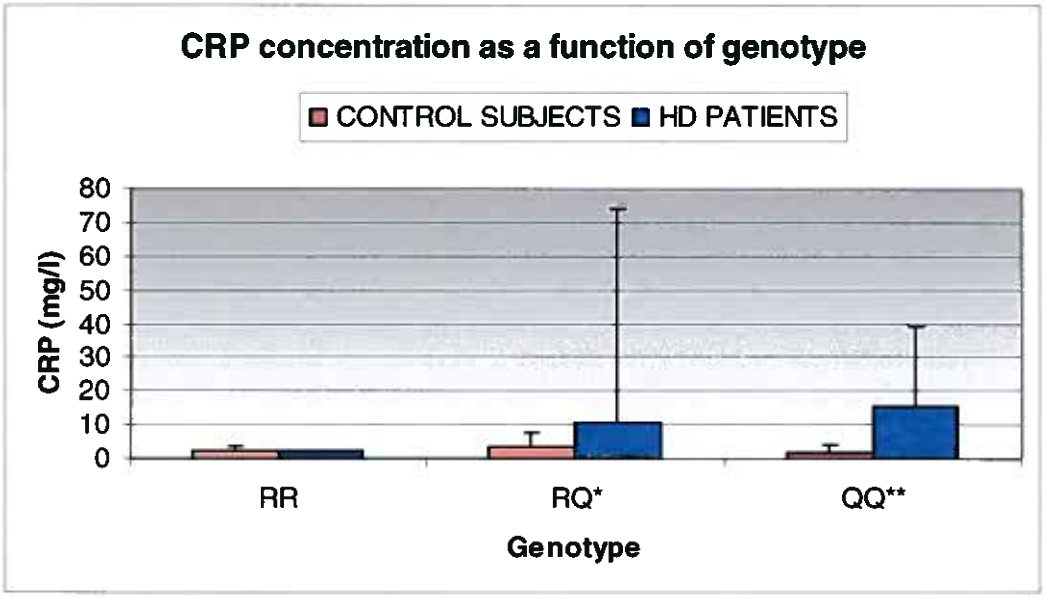

Fig. 36: Comparison of CRP mean value of serum concentration of samples from HD-patients and samples from control subjects according to genotype distribution. * Significant difference between cases and controls: P< 0.1. ** Significant difference between cases and controls: P< 0.0003.

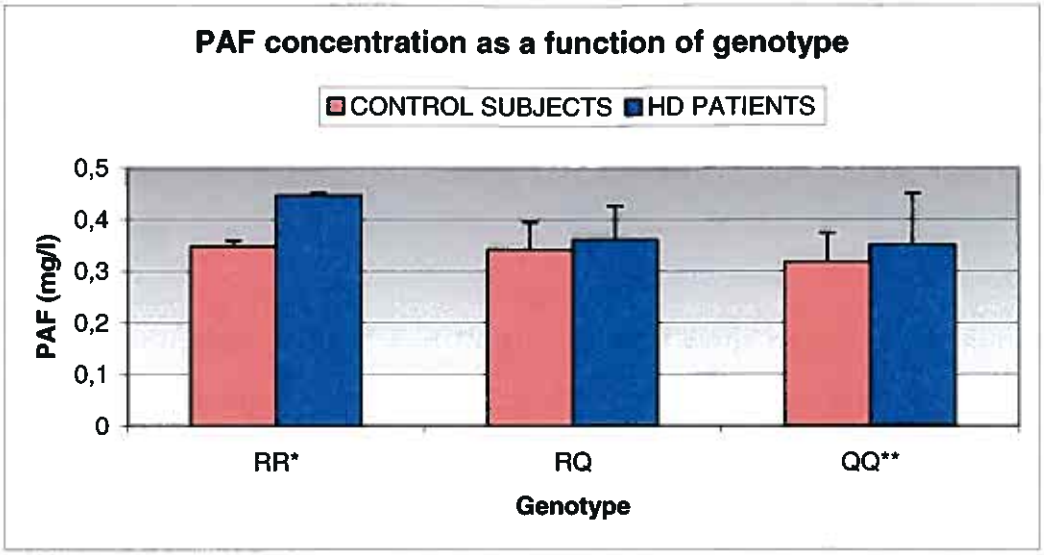

Fig. 37: Comparison of PAF mean value of serum concentration of samples from HD-patients and samples from control subjects according to genotype distribution. * Significant difference between cases and controls: $P < 0.005$. ** Significant difference between cases and controls: $P < 0.05$.

5.7 SERUM CONCENTRATION OF PARAOXONASE AND PARAOXONASE-1 ACTIVITY

5.7.1 SERUM CONCENTRATION OF PON AND PON 1 ACTIVITY IN CONTROL SUBJECTS AND HD PATIENTS

The concentration of PON and PON 1 activity were measured in serum of all the control subjects (n=47) and patients on maintenance hemodialysis (n=45).

PON serum values are significantly lower in serum of HD patients. The mean value of PON serum concentration of control subjects is 55% over than mean value of HD patients. The mean value of serum PON 1 activity of control subjects is 48% over than mean value of HD patients.

Tab. 10: Concentration of PON and PON 1 activity in serum of HD patients and control subjects.

	Mean ± SD / min - max		
	Controls (n=47)	**HD patients (n=45)**	**P**
PON (mg/l)	126.13 ± 93.42 / 27 – 393	81.44 ± 63.56 / 19 – 237	0.0100
PON 1 (IU/l)	267.51 ± 208.27 / 55 – 880	181.02 ± 63.56 / 38 – 543	0.0122

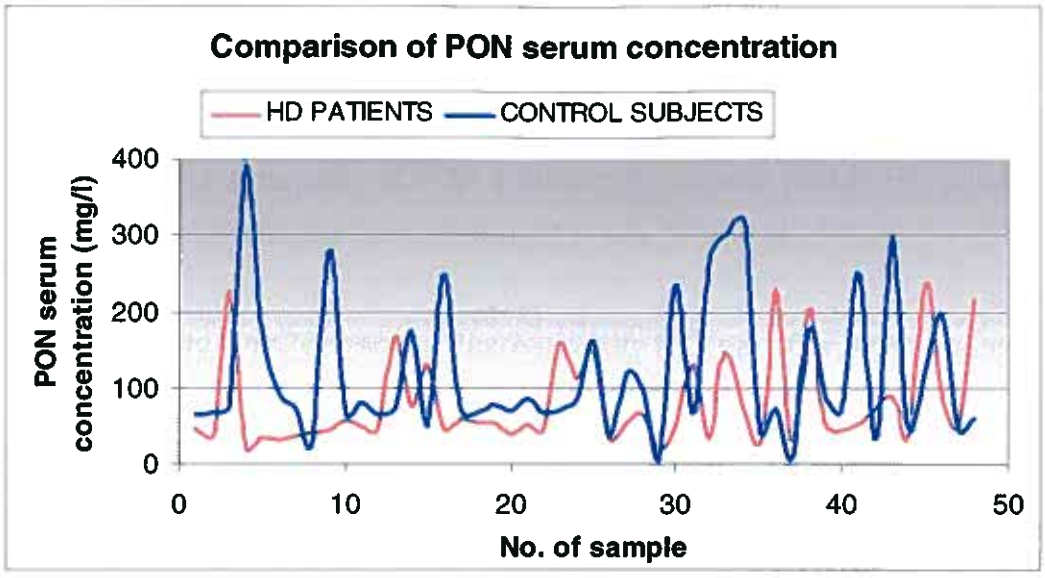

Fig. 38: Comparison of PON serum concentration in the group of HD-patients and the group of control samples.

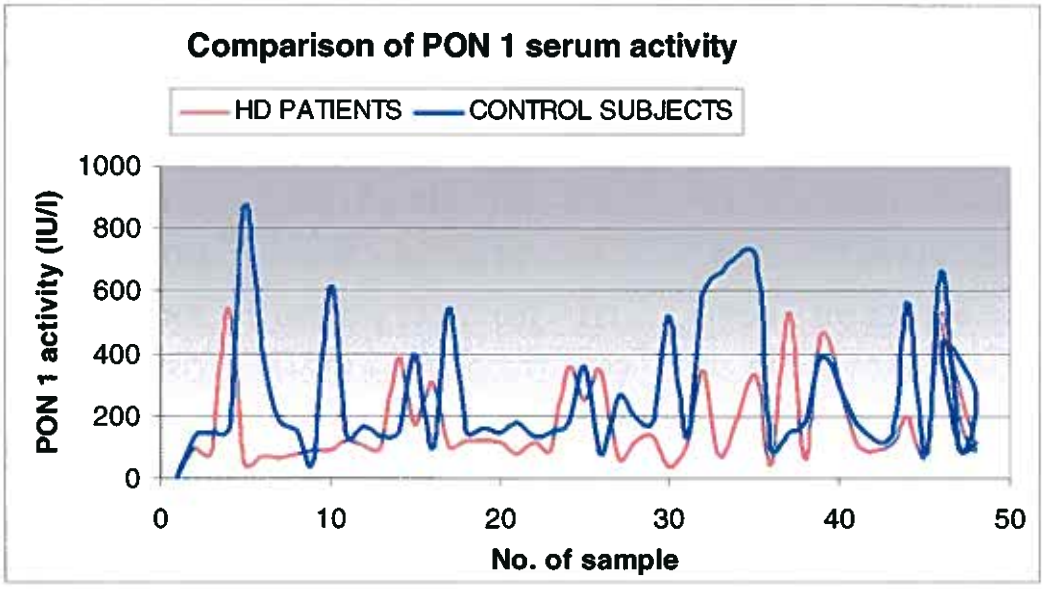

Fig. 39: Comparison of PON 1 serum activity in the group of HD-patients and the group of control samples.

5.7.2 Serum Concentration Of PON and PON 1 Activity In Control Subjects And HD Patients According To R192Q PON1 Polymorphism

The gene coding for PON 1 expresses an enzyme with different behaviour in the presence of paraoxon, depending on PON 1 R192Q polymorphism. This difference in

catalytic action is the result of amino acid substitution at position 192 where glutamine (Q allele) is replaced by arginine (R allele) in a high activity isoform.

Phenotypic characteristics of PON 1 of control subjects and of HD-patients compared with genotype distribution are presented in tab.16 and tab.17.

PON 1 activity was generally decreased in HD patients independent of genomic pattern. Both PON 1 activity and PON 1 serum concentration are increased in homozygotes (RR), and decreased in wild type (QQ, no polymorphism); the level of value of heterozygotes (RQ) reached the middle value.

Tab. 11: PON concentration, and PON1 activity in serum of Controls and hemodialysis patients (HD-patients) according to PON1 genotype.

Genotype		Mean ± SD / min-max		
		Controls	HD patients	P
PON (mg/l)	RR	310 ± 16.97 / 298 – 322	175 ± 60.81 / 132 – 218	0.0471
	RQ	202.12 ± 50.8 / 129 – 275	122.57 ± 61.60 / 55 – 237	0.0084
	QQ	98.25 ± 81.28 / 27 – 393	68.25 ± 57.49 / 19 – 231	0.0658
PON 1 (IU/l)	RR	692.00 ± 35.36 / 667 – 717	419.00 ± 103.23 / 346 – 492	0.0357
	RQ	446.62 ± 113.44 / 277 – 599	275.57 ± 139.73 / 118 – 531	0.0100
	QQ	205.84 ± 178.44 / 52 – 880	149.41 ± 136.72 / 38 – 543	0.0667

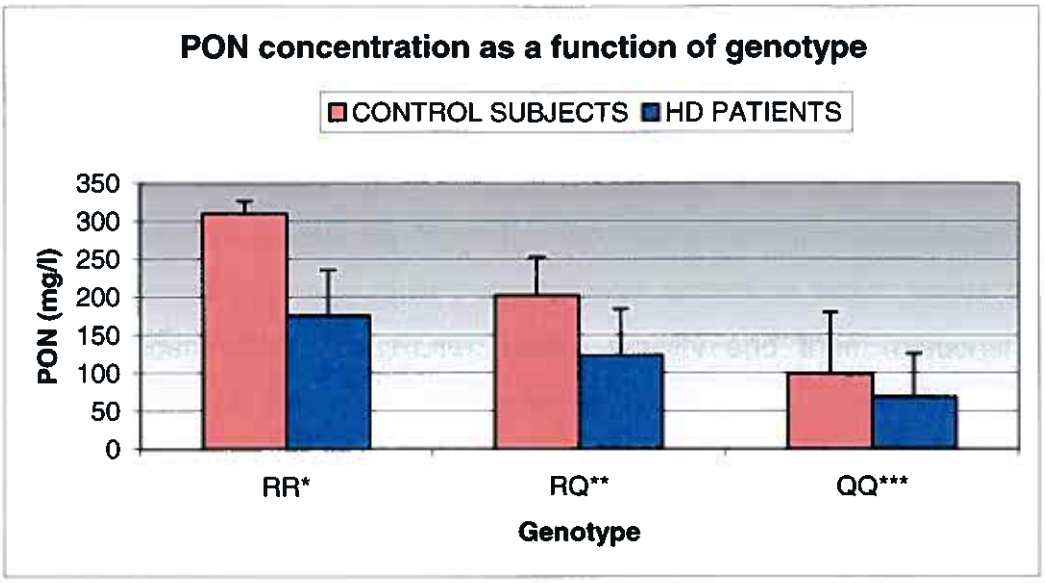

Fig. 40: Comparison of PON mean value of serum concentration of samples from HD-patients and control subjects according to PON1 genotype distribution. * Significant difference between cases and controls: P< 0.05. ** Significant difference between cases and controls: P< 0.01. *** Significant difference between cases and controls: P< 0.1.

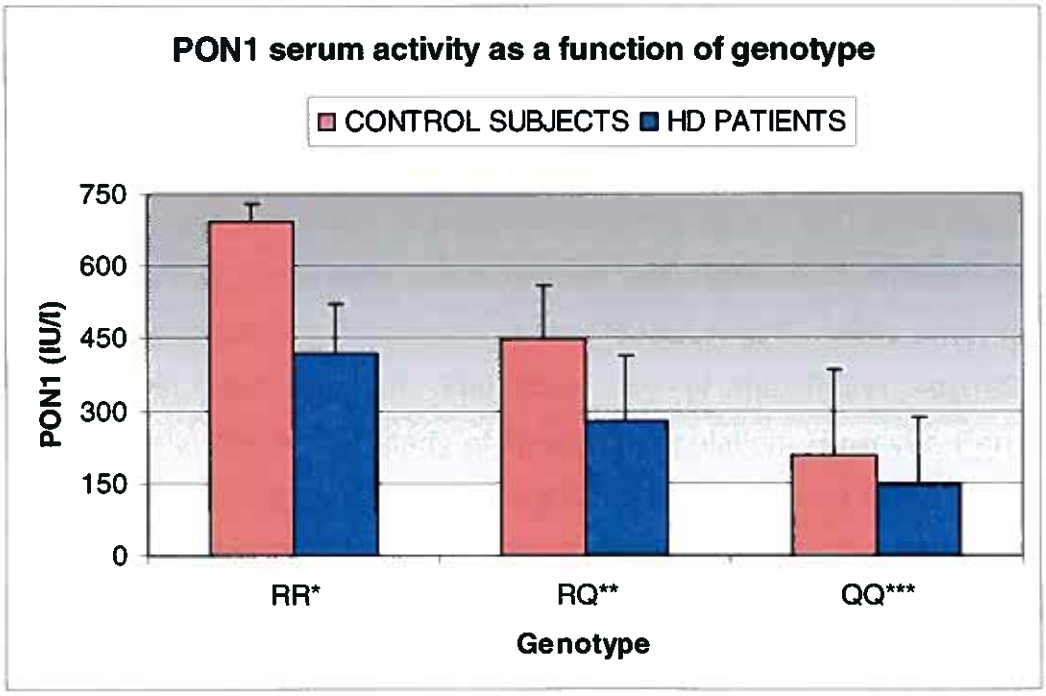

Fig. 41: Comparison of PON1 mean value of serum activity of samples from HD-patients and control subjects according to PON1 genotype distribution. * Significant difference between cases and controls: P< 0.05. ** Significant difference between cases and controls: P< 0.01. *** Significant difference between cases and controls: P< 0.1.

6 DISCUSSION

This thesis is aimed to determine the frequency of Q192R polymorphism of paraoxonase gene (PON1 Q192R polymorphism) in population of patients undergoing hemodialysis treatment. Hemodialysis (HD) patients are in a high risk atherosclerosis and coronary heart disease. Because of this well known fact, the lipid profile, C-reactive protein (CRP) and platelet activating factor levels (PAF), PON1 serum concentration, and PON1 serum activity and their relationship to PON1 Q192R polymorphism in HD-patients were studied.

This study is involved in an extensive research of polymorphisms of paraoxonase genes done by Marija Grdić on University of Zagreb, Faculty of Pharmacy and Biochemistry, Department of Medical Biochemistry in the years 2004-2006 periods. My diploma thesis research was done due to CEEPUS mobility HR-0045 in Zagreb, Croatia.

The polymerase chain reaction/restriction fragments length polymorphism (PCR/RFLP) technique was used to map PON1 Q192R (Gln192Arg) genotype distribution in observed population. There was found approximately 20% subjects with PON1 Q192R polymorphism (Arg192 allele homozygote or heterozygote) and 80% subjects without polymorphism (wild type). The frequencies of the PON1 alleles vary greatly across human populations. Ahmed et al [121] have reported that the distribution of PON1 Q192R polymorphism was significantly different between black and white women; the frequency of the PON1 Arg192 allele was lower in white women than black women. The frequency of the PON1 Arg192 allele was approximately 12% in our study of Croatian population. However, Ferre et al. [122] and Van Lenten et al. [80] found no significant differences in PON1 Q192R genotype distribution across population. These discrepancies may be related to the effects caused by other genes as PON2 or to posttranslational modifications of the enzymes.

Cardiovascular disease (CVD) is the most common cause of mortality in HD-patients. Some studies have indicated a significant association of PON1 Q192R polymorphism and CVD [123-125]. However, a number of other studies [126, 127] have not reported the presence of this association. We found that the distribution of PON1 Q192R genotypes in HD-patients [4 % homozygotes, 16 % heterozygotes, and 80 % wild types] did not differ significantly as compared with control healthy subjects [4 % homozygotes, 17 % heterozygotes, and 79 % wild types] in our study. These results suggested that no priority allele is connected with hemodialysis treatment.

Based on studies mentioned above, studies on the PON1 genotype as a predictor of CVD have obtained contrastive results, but generally speaking they suggest that PON 1 genotype is one of many predictors of CVD. These inconsistent associations between PON1 Q192R polymorphism and CVD may, at least partly, be well explained by the following possible factors. The most important one is the interaction between genes and environment. Disease develops as a consequence of interactions between the initial conditions coded by the genotype, and exposure to environmental agents that are integrated by time and space at levels above genome. Identification of relevant gene-environment interaction may reveal the relationship between PON1 Q192R genotype, CVD and hemodialysis treatment.

The second possible factor is that the promoter polymorphisms of PON1 gene can influence the association between the PON1 Q192R polymorphism and hemodialysis treatment.

The third possible factor is that PON1 is not involved directly in the disease which caused that the patient had to undergo hemodialysis treatment. If this hypothesis is true, further studies examining details connected with patient's diagnosis and hemodialysis process would be essential.

As was mentioned above, cardiovascular disease is the most common cause of morbidity and mortality in hemodialysis patients. The conventional risk factors such as smoking, diabetes mellitus and hypertension can only partially explain the accelerated development of atherosclerosis in these patients. Dirican et al. [128] and Attman et al. [107] reported that hemodialysis patients often show lipid and lipoprotein abnormalities characterized by reduced HDL-cholesterol and

hypertriglyceridaemia. HDL-cholesterol levels have been shown to be inversely correlated with the risk of atherosclerosis [129]. In addition to the role of HDL in reverse cholesterol transport, it has the ability to protect LDL against oxidation [59, 98, and 130]. LDL oxidation is currently considered to be an early key event in the development of atherosclerosis, leading to LDL uptake by the macrophage scavenger receptor, and therefore, to foam-cell formation.

These facts described above led us to study lipid and lipoprotein profile of hemodialysis patients. Triacylglycerides (TAG) and oxidized LDL (ox-LDL) levels were significantly increased in HD-patients as compared with control subjects (TAG and oxLDL were 34.9% and 20.9% over, respectively). ApoB was increased in HD-patients (13.4% over); on the other hand, ApoA was 11.1% lower in HD-patients as compared with control subjects. HDL-cholesterol levels were 5.8% lower in HD-patients, and LDL-cholesterol levels were 11.0% over in HD-patients as compared with control subjects. The difference of total cholesterol (TC) levels between HD-patients and control subjects was not statistically significant. As compared with other studies [107, 128], HDL-cholesterol and TC levels were not such as significantly decreased in HD-patients in our study. Schiavon et al. [49] showed HDL-3 fraction significantly decreased in HD-patients. Unfortunately, there was not measured HDL-3 fraction in our study to compare with the Schiavon. Lipoprotein(a) (LP(a)) levels were decreased in HD-patients as compared with control subjects (2.0% lower in HD-patients) in our study. In contrast with our results, LP(a) was found to be increased in HD-patients by several authors [89, 131-134]. Some authors [133-135] presented, that LP(a) levels correlated positively with acute phase proteins (such as CRP) and suggested that the enhanced LP(a) levels could be associated with the acute phase reaction in HD-patients, but this was not our case.

Many studies [126, 136, and 137] have suggested that variation in PON1 genotype in population is associated with variations in lipoprotein profile. However, our research revealed no association between PON1 Q192R polymorphism and plasma lipid and lipoprotein profile, and our results are in agreement with other studies [66, 127, and 138]. These contradictory results originated from studies conducted in various ethnicities and they could be explained partly by differences in PON1 genotype distribution between populations.

In addition to LDL oxidation pivotal role in foam-cell formation, ox-LDL possesses additional atherogenic properties that include the stimulation of thrombotic and inflammatory events [139]. The underlying mechanism by which HDL inhibits LDL oxidation is partly enzymatic. There is increasing evidence that PON1 enzyme could be involved in this process [59]. These hypotheses led us to involve CRP and PAF serum levels, as well as PON1 activity and PON serum concentration in this study. PAF levels were 9.3% over and CRP levels were 88.5% over in HD-patients as compared with control subjects, this CRP comparison represents the most significant difference among the biochemical markers presented in this study. These results are in agreement with the assertion that inflammation enhances cardiovascular risk and mortality in HD-patients [139].

In accordance with numerous reports [59, 95, 140, and 141], we found that paraoxonase activity as well as paraoxonase serum concentration were significantly decreased in HD-patients compared with control subjects (32.2% lower and 35.4% lower respectively). In agreement with other reports [89, 140, 142, and 143], serum PON1 activity and concentration were regulated by PON1 Q192R polymorphism in our study: RR > RQ > QQ. The relationship between PON1 activity, concentration respectively, and PON1 Q192R genotype distribution in HD-patients was similar to those of control subjects in our study. Controversially, several studies [49, 97, 144, and 145] have reported that PON1 activity was reduced in HD-patients independently of PON1 Q192R polymorphism. Some reports [22, 66, and 146] suggested that PON1 activity and PON1 concentration are better predictors of CVD than PON1 genotype. Finally, PON1 genotypes remain important in the study of PON1 role in CVD as potential death danger for HD-patients.

In conclusion, further studies are necessary to estimate the role of PON1 polymorphism in pathogenesis of CVD. Furthermore, it is important to determine how PON1 enzyme concentration and activity are involved in the development of atherosclerosis in patients undergoing hemodialysis treatment.

7 SUMMARY

I summarized almost all available new information in the field of paraoxonase research in theoretical part of my Diploma Thesis. Brief characteristics of hemodialysis are presented in theoretical part too. I used internet, especially database Medline, and Scientific Library in Hradec Králové to collect all information.

I did genotypization (PON1 Q192R) of hemodialysis (HD) patients and control subjects using polymerase chain reaction (PCR) and restriction fragments length polymorphism (RFLP) technique in experimental part of thesis. I tried to determine the relationship of PON1 Q192R polymorphism and available biochemical markers (TC, TAG, HDL, LDL, oxLDL, ApoA, ApoB, LP(a), CRP, PAF, PON1 serum activity, and PON1 serum concentration).

There were approximately 20% PON1 Q192R polymorphic subjects and approximately 80% subjects with no PON1 Q192R polymorphism. The distribution of PON1 Q192R genotypes in HD-patients (4% homozygote, 16% heterozygote, and 80% wild type) did not differ from control subjects (4% homozygote, 17% heterozygote, and 79% wild type).

There is no association between lipid profile, CRP and PAF serum concentration and PON1 Q192R polymorphism. The lipid and lipoprotein profile of HD-patients revealed that these patients have a high risk of atherosclerosis and cardiovascular disease.

This study suggested that decreased PON1 serum activity, PON1 serum concentration, ApoA and HDL serum levels, as well as increased atherogenic markers (TAG, TC, LDL, oxLDL), ApoB, CRP and PAF serum levels are associated rather with hemodialysis treatment than with PON1 Q192R polymorphism.

8 CONCLUSION

A. The distribution of PON1 Q192R genotypes in HD-patients (4% RR homozygote, 16% RQ heterozygote, and 80% QQ wild type) did not differ significantly from control subjects (4% RR homozygote, 17% RQ heterozygote, and 79% QQ wild type). There are approximately 20% PON1 Q192R polymorphic subjects and approximately 80% subjects with no PON1 Q192R polymorphism (QQ wild type) in studied population.

B. Atherogenic serum markers (TC, TAG, LDL, and oxLDL) were significantly increased in HD-patients, as well as ApoB levels; ApoA, Lp(a) and HDL levels were lower as compared with control subjects. The most significant difference between HD-patients and control subjects was in TAG levels and oxLDL levels. The lipid and lipoprotein profile revealed that HD-patients are in significantly higher risk of atherosclerosis as compared with control subjects.

C. CRP and PAF serum values were increased in HD-patients as compared with control subjects.

D. PON1 serum activity, as well as PON serum concentration was significantly decreased in HD-patients compared with control subjects.

E. The lipid and lipoprotein profile, CRP and PAF serum levels were independent on PON1 Q192R genotypes. Serum PON1 activities in both, HD-patients and control subjects, seemed to be regulated by PON1 Q192R polymorphism: RR homozygotes with biggest PON1 activity, RQ heterozygotes with middle PON1 activity, and QQ wild type (no PON1 Q192R polymorphism) with the lowest PON1 activity.

F. The results suggested that decreased PON1 activity, PON1 concentration, ApoA and HDL levels, as well as increased atherogenic markers (TAG, TC, LDL, oxLDL), ApoB, CRP and PAF levels are associated with hemodialysis treatment and that this association is not related to PON1 Q192R polymorphism.

9 ABBREVIATIONS

(In the order of use)

PON	paraoxonase
pon	paraoxonase gene
kb	kilo-base
LD	linkage disequilibrium
Fig.	Figure
Tab.	Table
P	probability
HDL	high density lipoprotein(s)
LDL	low density lipoprotein(s)
EC	enzyme classification
PAF	platelet-activating factor
oxLDL	oxidized LDL
PAF-AH	PAF-acetylhydrolase
TAG	Triacylglycerides
EDTA	ethylenediaminetetraacetic acid
LCAT	lecithin-cholesterol acyltransferase
Apo	apolipoprotein
LP	lipoprotein(s)
Arg	arginine
Glu	glutamine
CAD	coronary artery disease
IL	interleukin
CHD	coronary heart disease
HOCl	hypochlorous acid
CRF	chronic renal failure
HD	hemodialysis
HD-patient	hemodialysed patient
CS	control subjects
DM	diabetes mellitus
ATP	adenosine triphosphate
M/F	male/female
SDS	sodium dodecyl sulphate

PCR	polymerase chain reaction
Taq	*Thermus aquaticus*
LB-buffer	loading buffer
RFLP	restriction fragments length polymorphism
EtBr	Ethidium bromide
A	adenine
G	guanine
C	cytosine
T	thymine
U	uracil
WT	wild type (no polymorphism)
No.	Number (lat. *Numero*)
pb	base pair
LP(a)	lipoprotein a
n	number (quantity)
TC	total cholesterol
CRP	C-reactive protein
CVD	cardiovascular disease

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