„Prohlašuji, že tato práce je mým původním autorským dílem, které jsem vypracoval(a) samostatně. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpal(a), jsou uvedeny v seznamu použité literatury a v práci řádně citovány."

Dvořák R.

CHARLES UNIVERSITY IN PRAGUE
FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

DEPARTMENT OF BIOCHEMICAL SCIENCES

HUMAN PARAOXONASE (PON1) L55M ALLELIC POLYMORPHISM AND HEMODIALYSIS

MASTER THESIS

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

Hradec Králové 2008

Renata Dvořáková

UNIVERZITA KARLOVA V PRAZE
FARMACEUTICKÁ FAKULTA V HRADCI KRÁLOVÉ

KATEDRA BIOCHEMICKÝCH VĚD

POLYMORFISMUS L55M LIDSKÉ PARAOXONASY (PON1) A HEMODIALYSA

RIGORÓZNÍ PRÁCE

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

Hradec Králové 2008 Renata Dvořáková

CHARLES UNIVERSITY IN PRAGUE
FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ
DEPARTMENT OF BIOCHEMICAL SCIENCES

HUMAN PARAOXONASE (PON1) L55M ALLELIC POLYMORPHISM AND HEMODIALYSIS

RENATA DVOŘÁKOVÁ

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. PON 1 L55M polymorphism can take a part in the mechanism of development of CHD.

Aim: To map genotype distribution of PON 1 L55M allelic polymorphism in Croatia and to determine the relationship of PON1 L55M polymorphism and hemodialysis treatment.

Methods: HD-patients (N=51, M/F=28/23, age=60±12 years) and control (blood donors) subjects (N=95, M/F=48/47, age=49±17 years) from area of Slavonski Brod, Croatia were included in this study. PON1 L55M polymorphism was detected by polymerase chain reaction (PCR) and restriction fragments length polymorphism (RFLP) method.

Results: The distribution of PON1 L55M genotypes in hemodialysis patients (11% MM, 40% LM, and 49% LL) differs from control subjects (12% MM, 48% LM, and 40% LL). The allele frequency for PON1 L55M allelic polymorphism did differ in 5% in hemodialysis patients (69% L allele, 31% M allele) and control subjects (64% L allele, 36% M allele). The most common is L allele for both the HD and CS subjects.

Conclusion: There were approximately 55.5% PON1 L55M polymorphic subjects and 44.5% subjects with no PON1 L55M polymorphism in this study. L allele is the most common allele for PON 1 L55M polymorphism in hemodialysis patients as well as in group of control subjects. The allele frequency for PON1 L55M allelic polymorphism did differ in 5% of hemodialysis patients (69% L allele, 31% M allele) and control subjects (64% L allele, 36% M allele). This study suggested that hemodialysis is associated rather with M allele appearance than with MM genotype for PON 1 L55M allelic polymorphism.

(VII + 91 pages including 8 tables, 43 figures and 128 references; original in English)

Keywords: paraoxonase, genotype, polymorphism, hemodialysis

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

Reviewer: Mgr. Martina Gavelová, Ph.D.

ACKNOWLEDGEMENTS

This master thesis was done due to CEEPUS mobility HR-0046 in Zagreb, Croatia.

I would like to thank Prof. Dr. sc. Karmela Barišić and Prof. Dr. sc. Dubravka Juretić from University of Zagreb, Faculty of Pharmacy and Biochemistry, Division of medical Biochemistry and Haematology for their continued support and help with experimental part of my thesis and perceptive criticisms on the final manuscript.

I am also very grateful to Prof. MUDr. Jaroslav Dršata, Csc. from Charles University, Faculty of Pharmacy, Division of Biochemical Sciences for his expert and professional consultations.

Finally, my very sincere thanks go to Marija Grdić from University of Zagreb for her support and kindness during my stay in Zagreb.

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. 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.

Hemodialysis is the most common method used to treat advanced and permanent kidney failure. Serum PON 1 activity and/or serum concentration is significantly decreased in some clinical conditions, such as: atherosclerosis, diabetes mellitus, and chronic renal failure. Serum PON 1 activity is abnormally low in patients on maintenance hemodialysis. It is well known that these pathologies are associated with increased susceptibility of LDL and HDL to oxidation.

PON 1 gene possesses common allelic polymorphisms, M55L and Q192R. Genotype distribution in hemodialysis patients and control subjects may reveal prognosis of chronic renal failure, moreover, it could improve prevention of its later complications.

2 HEMODIALYSIS TREATMENT AND HUMAN PARAOXONASE

2.1 HEMODIALYSIS

Hemodialysis (Fig. 1) is the most common method used to treat advanced and permanent kidney failure. 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.

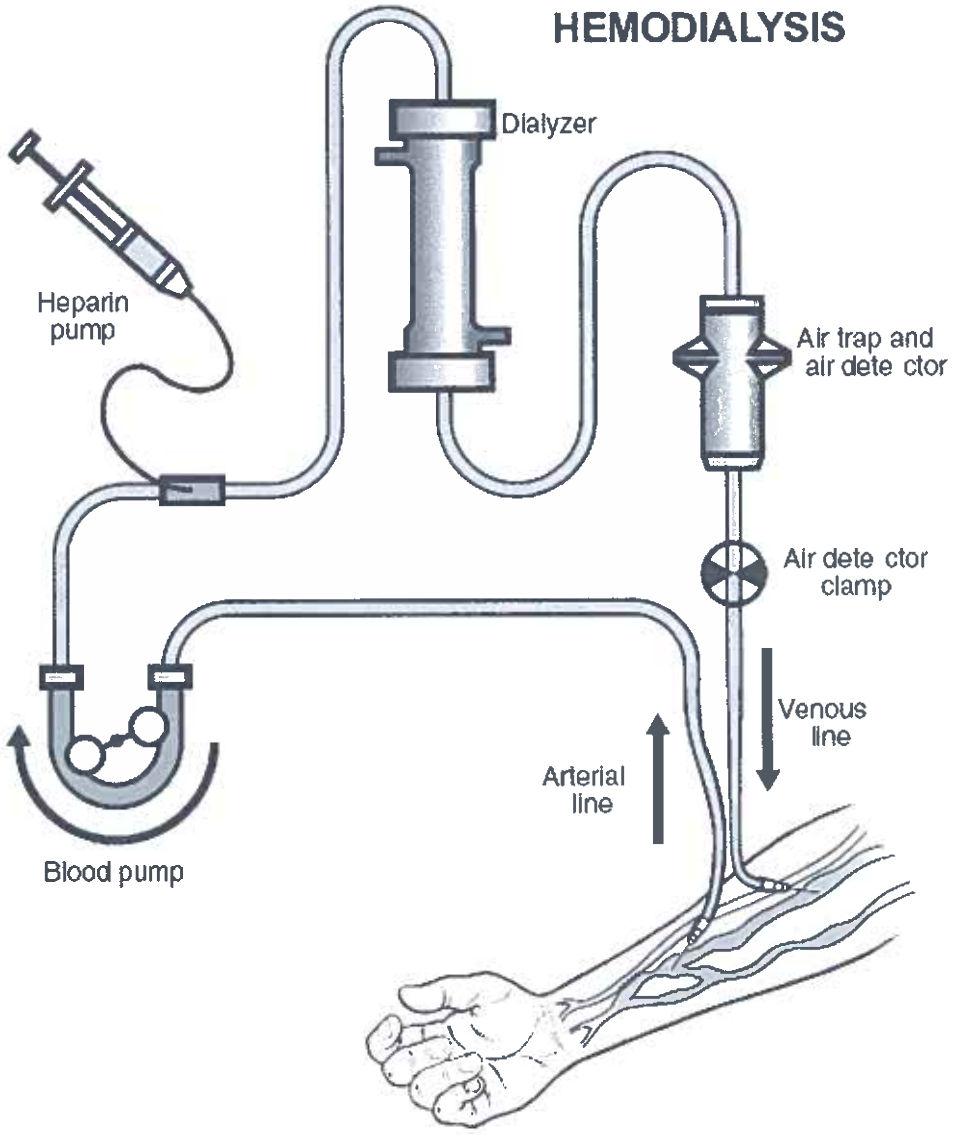

Fig. 1: Hemodialysis process [1].

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 cannot 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 [2].

A specific kidney disease causes sometimes kidney failure. In other cases, it's a complication of another condition, such as:

- Diabetes
- Hypertension
- Glomerulonephritis
- Vasculitis
- Polycystic kidney disease

2.1.1 Hemodialysis Equipment

Hemodialysis equipment (Fig. 2) is designed to filter the wastes from the blood of a patient who has renal failure [2]. The term dialysis refers to the separation of molecules on the basis of size by their ability to diffuse through an artificial semipermeable membrane [3]. The patient's blood is pumped through a tube from the radial artery and passes through a machine – dialyser (Fig. 3), 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 [2]. 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 cannot reabsorb Na^+, K^+, glucose, and other needed molecules.

These substances are prevented from diffusing through the membrane by including them in the dialysis fluid [2].

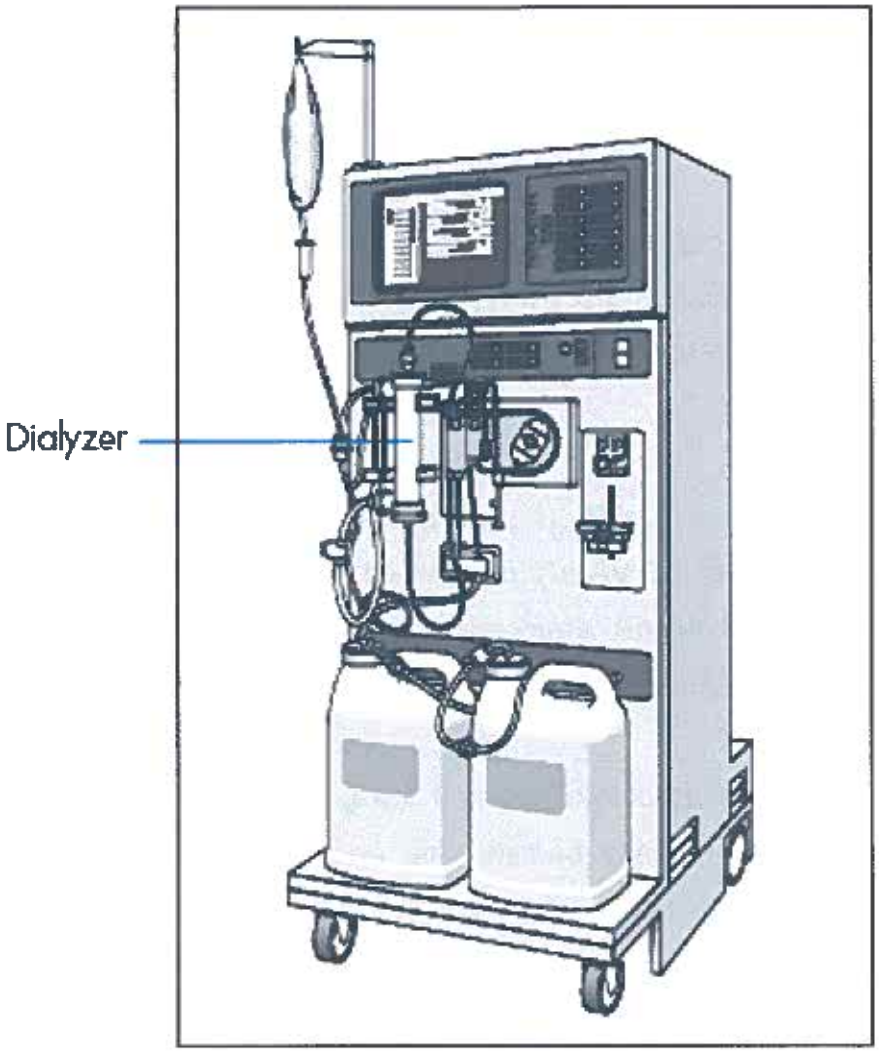

Fig. 2: Hemodialysis machine [4].

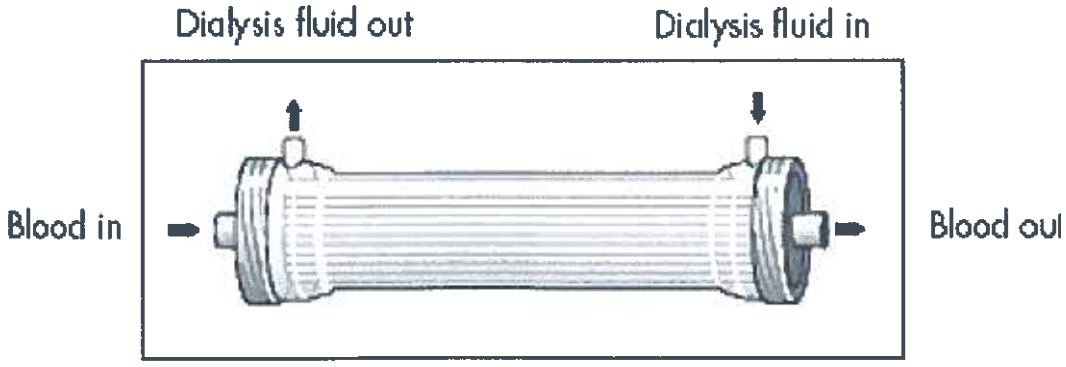

Fig. 3: Hemodialyser [4].

2.1.2 HEMODIALYSIS ACCESS POINTS

Before patient starts hemodialysis, a surgeon creates a vascular access point for blood to leave for cleansing and then re-enter patient's body during treatment. Ideally, the access point is created weeks or even months before patient needs hemodialysis.

There are three types of access points:

1. **Temporary access.** If patient needs emergency hemodialysis, the surgeon may insert temporary catheter. If it's left in place for too long, patients face a risk of infection, clotting in the catheter and stenosis of surrounding blood vessels.

2. **Arteriovenous (AV) fistula** (Fig. 4)**.** This is a connection between an artery and a vein, usually in the forearm. An AV fistula may take six weeks or longer to heal, but it can last for many years. An AV fistula is less likely than other types of access points to form clots or become infected.

3. **Arteriovenous (AV) graft.** If patient's blood vessels are too small to form an AV fistula, the surgeon may instead connect an artery and a vein with a synthetic tube. An AV graft often heals within two to three weeks. With proper care, an AV graft may last several years - but it's more likely to form clots and become infected than is an AV fistula [5].

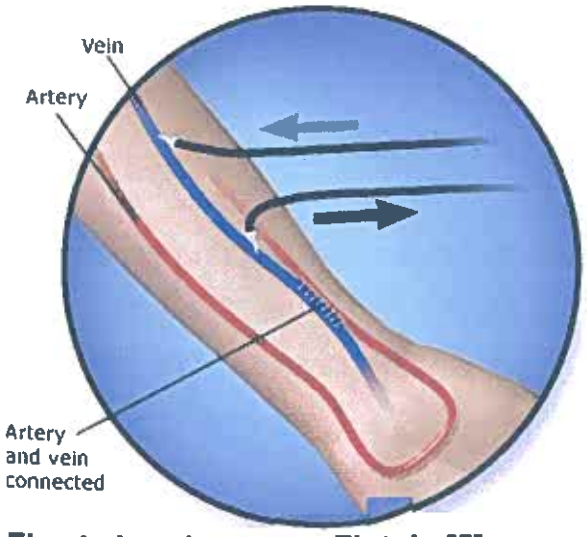

Fig. 4: Arteriovenous Fistula [5].

If created access point stops working, the surgeon can create a new access point in the other forearm, upper arm or groin. Or patient may consider peritoneal dialysis. Dialysis fluid is introduced into the peritoneal cavity (Fig. 5), 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 [2].

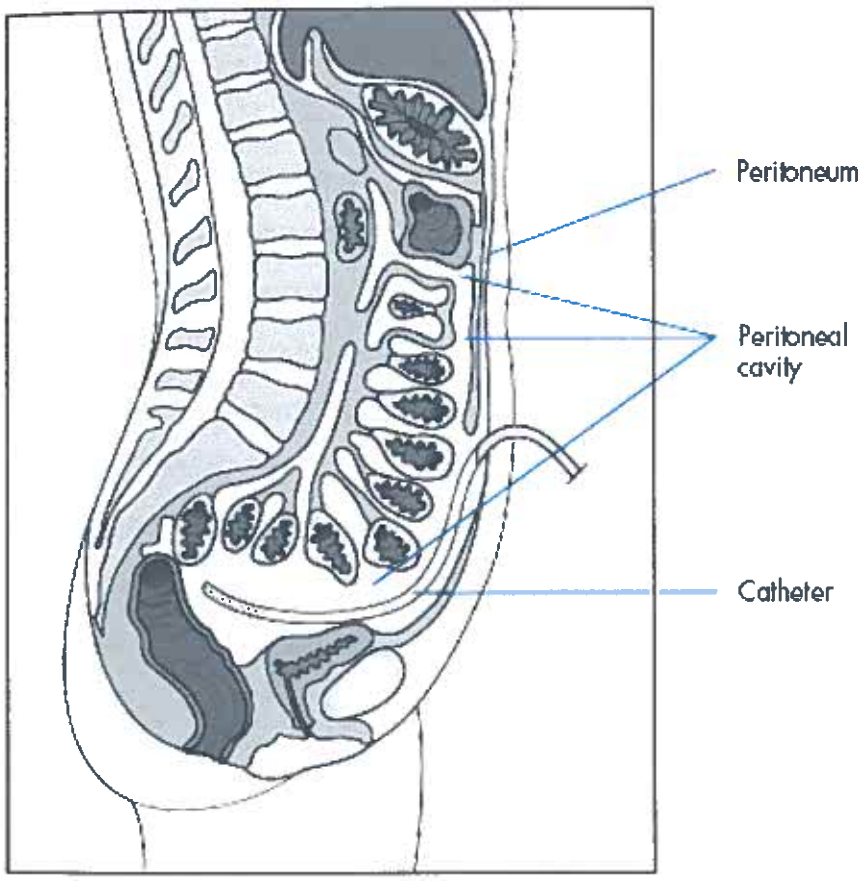

Fig. 5: Peritoneal cavity and peritoneal catheter [5].

2.1.3 Frequency Of Hemodialysis Treatment

Most people receive hemodialysis three times a week, about three to five hours at each session. This type of hemodialysis, known as conventional hemodialysis, is usually done in a dialysis centre.

At some dialysis centres, patient can choose shorter but more frequent treatments. This is known as daily dialysis. It's usually done six days a week for about two to two and a half hours. Although conventional hemodialysis is more common, people who

choose daily hemodialysis often report greater improvements in blood pressure and quality of life.

Researchers are exploring whether shorter daily sessions, or longer sessions performed overnight while the patient sleeps, are more effective in removing wastes [6].

2.1.4 HEMODIALYSIS MEDICATIONS

While patient is receiving hemodialysis, he will likely need various medications:

- Blood thinners to prevent clots in the hemodialysis machine and tubing
- Blood pressure medication to control blood pressure
- Erythropoetin to stimulate bone marrow to produce new red blood cells
- Calcium, iron and other nutritional supplements to control the level of certain nutrients in blood
- Phosphate binders to prevent the build-up of phosphorus in blood
- Stool softeners and laxatives to manage constipation

Doctor will do frequent blood tests to monitor patient's condition.

Blood tests for hemodialysis patients: albumin, urea and nitrogen, calcium, creatinine, ferritin, folate, glycated hemoglobin, hematocrit, hemoglobin, iron, parathyroid hormone, phosphorus, potassium, sodium level, vitamin B-12, prothrombin time, hepatitis B, hepatitis C, cholesterol and triacylglyceride tests [5].

2.2 HUMAN PARAOXONASE

2.2.1 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 [7]. The *pon* genes are similar: they include nine exons, and eight introns [8]. 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 [9]. The homologous paraoxonase family members across species exhibit a significantly higher degree of homology (80% – 90%) [7]. 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 [7].

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 [10, 11]. There is significant linkage disequilibrium (LD) across the cluster, between the *pon* 3 polymorphism and *pon* 1 and *pon* 2 alleles (Fig. 6) [11].

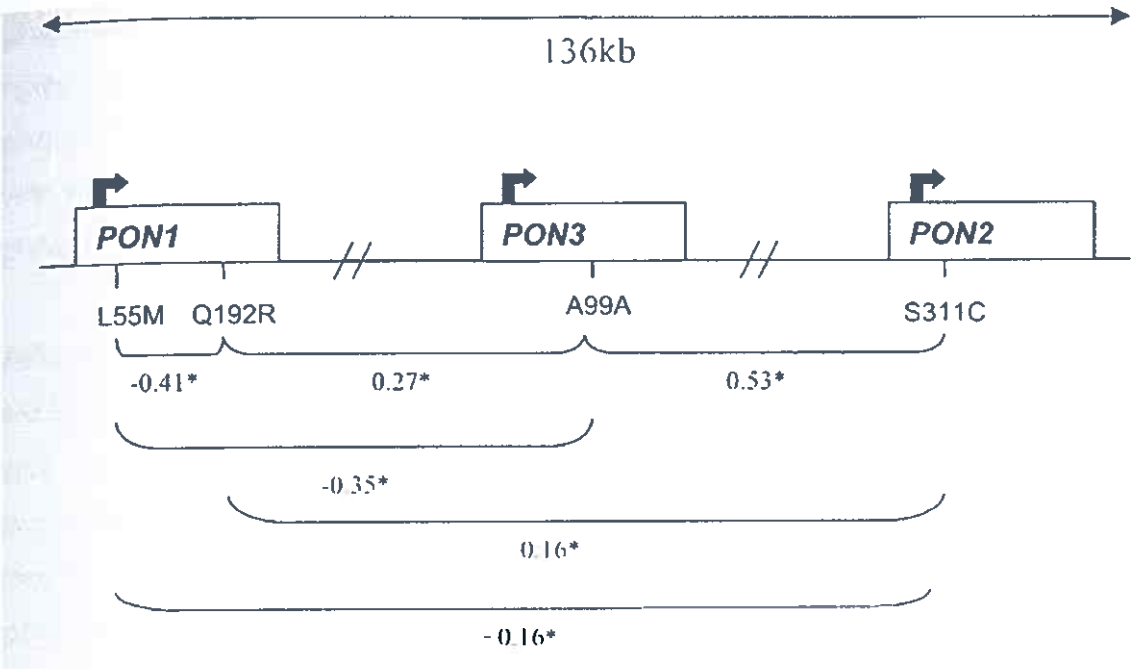

Fig.6: *pon* gene cluster linkage disequilibrium analysis. Linkage disequilibrium calculated as delta values. *P <0.00005; [11].

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 [7].

2.2.2 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 [12]. The PON 1 enzyme (arylalkylphosphatase, EC 3.1.8.1) is 43–45 kDa glycoprotein [10] 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 [13]. Rodrigo et al. [14] 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 [15].

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 [16]. 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 [17]. 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) [17].

PON 1 serum levels differ between individuals by as much as 13–fold [18]. 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 [19, 20]. In humans, two genetic polymorphisms of PON 1 partially account for variations of serum PON 1 concentrations and activities: Q192R and M55L [21].

2.2.2.1 PON 1 MOLECULE: STRUCTURE, FUNCTION

The available structure-function analysis of PON 1 was summarized by Draganov et al. [22]: 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 [23].

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 [24]. 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 [25]. 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 [24].

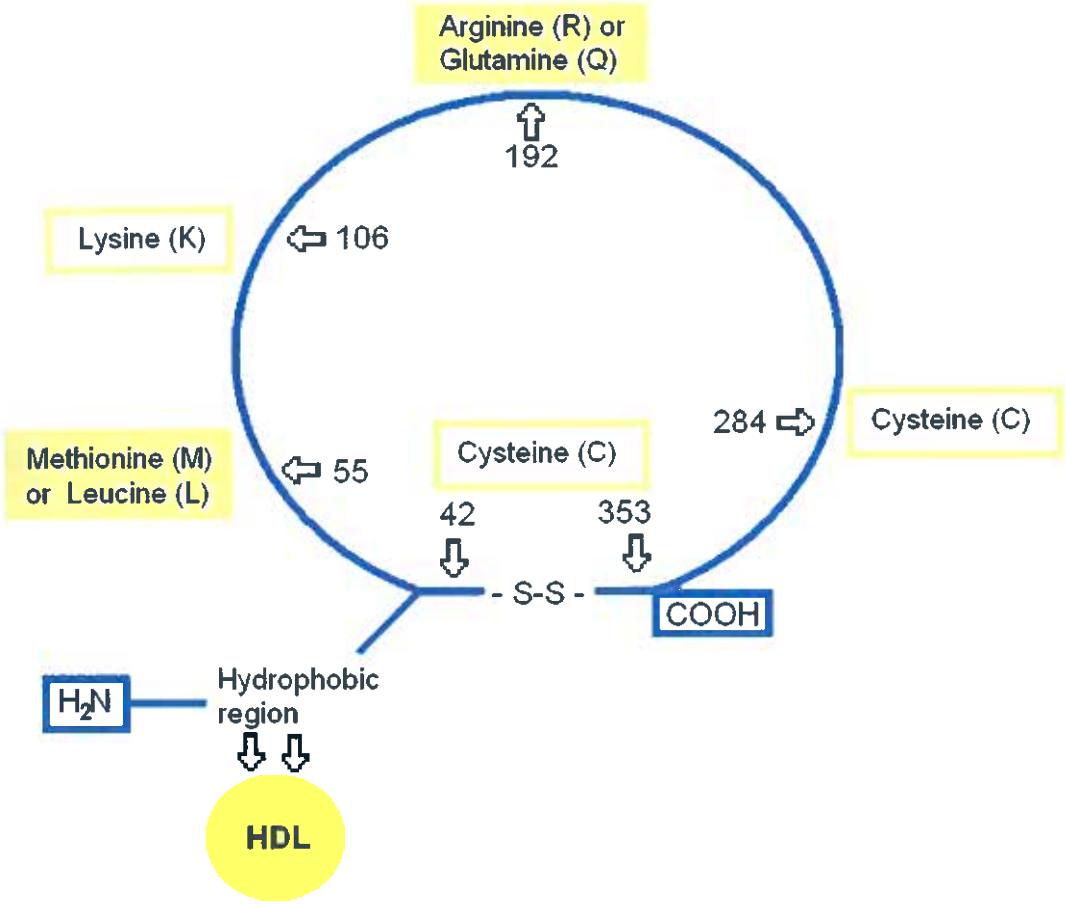

Fig. 7: The structure of human PON 1. Two polymorphic places at the positions 55 and 192 in PON 1 structure; [24].

The mature protein retains its hydrophobic signal sequence at N-terminus without the N-terminal methionine residue, which is followed by the alanine [26]. 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 [25].

All amino acids required for PON 1 activity are conserved between the human, rabbit, and mouse sequences [27].

PON 1 has a variety of substrates, and the relative activity towards different substrates varies by polymorphic forms [28].

2.2.2.2 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 [29].

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 [30]. The hydrolytic products resulting from the action of PON 1 on paraoxon are themselves relatively harmless.

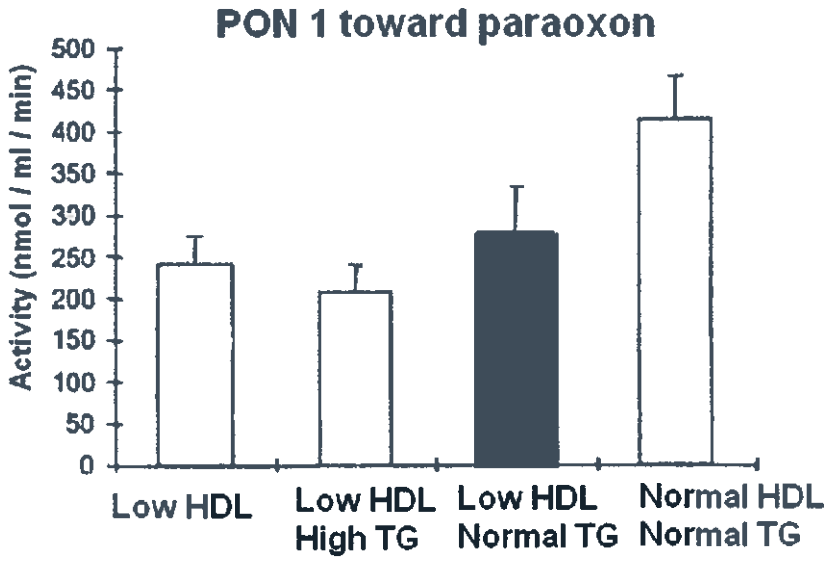

Fig. 8: PON 1 activity toward paraoxon in patients and control subjects. TG, triglycerides; [27].

Paraoxonase is equally able to hydrolyze other substrates, including phenyl acetate [31].

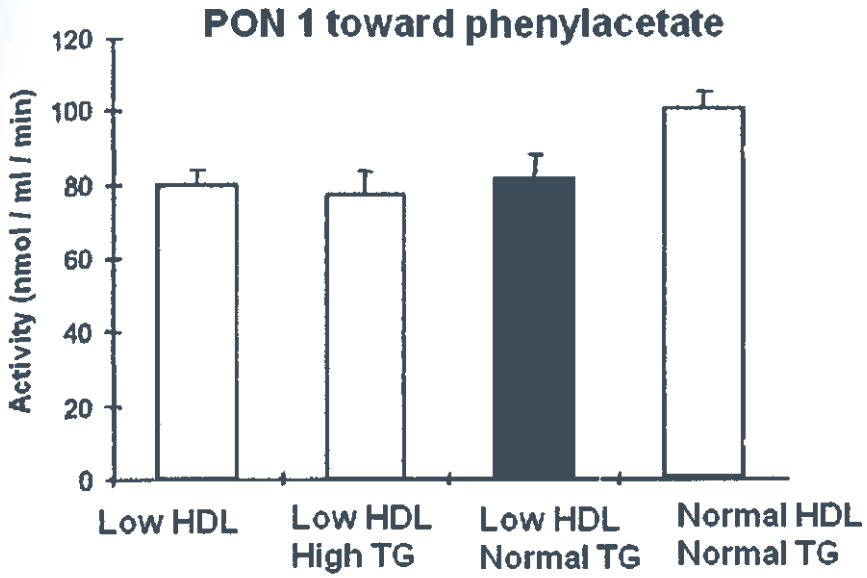

Fig. 9: PON 1 activity toward phenyl acetate in patients and control subjects. TG, triglycerides; [27].

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 [32].

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

2.2.2.3 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 [33]. The EDTA-sensitive hydrolysis of paraoxon by normal human serum samples was studied and role of calcium in this reaction was dual [34], 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 [35].

2.2.3 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) [36]. 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) [37]. 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 [38]. 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) [39] and only 22% of lipids [40]. This subclass also appears to contain the peptide hormone, gherlin [41]. Kelso et al. [42] 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 [43]. 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 [44].

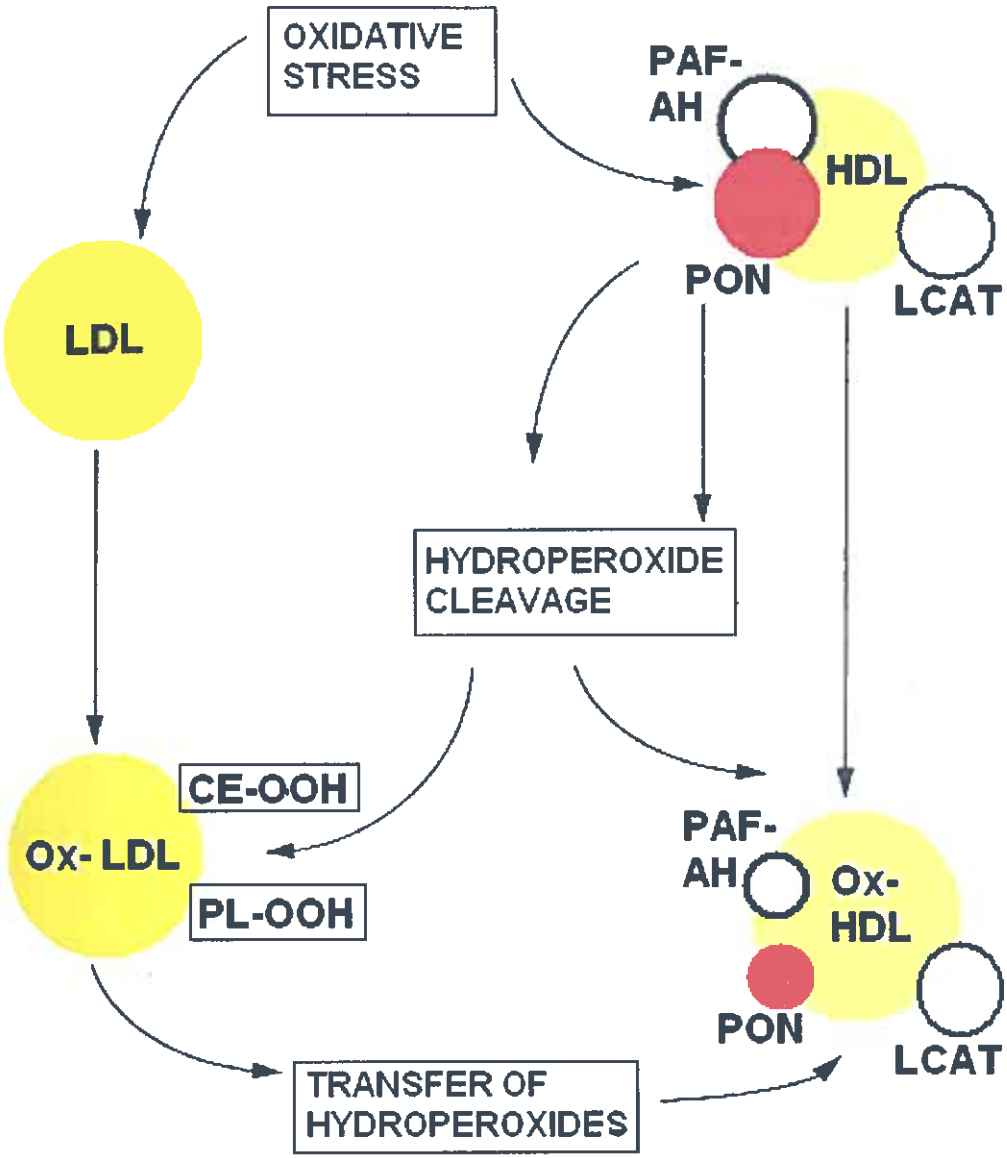

Fig. 10: Proposed scheme for the coordinated actions of HDL–associated enzymes in the hydrolysis of lipid hydroperoxides generated in lipoproteins upon oxidative stress. Abbreviations: PAF–AH, platelet activating factor acetylhydrolase; LCAT, lecithin-cholesterol–acyl-transferase; ox–LDL and ox–HDL, oxidized LDL and HDL, respectively; CE–OOH and PL–OOH, hydroperoxides of cholesteryl esters and phospholipids, respectively; [16].

A number of PON–containing HDL subspecies, each of which has its own, unique apolipoprotein content, coexists in plasma [36]. To what extent each of these entities participates in a protective effect against the oxidation of LDL (or other lipoprotein particles) remains unknown yet.

2.2.4 PON 1 And Protection of Lipoproteins Against Oxidation

Different authors have studied HDL ability to inhibit LDL oxidation *in vitro* [45] and some of them pointed out that PON 1 could play a key role in this protective function [46]. 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 [47] and/or lipid peroxidation products [48]. Aviram et al. [48] 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 [49, 17];
- PAF–AH [47], and
- PON 3 [50, 51].

The PON 1–mediated antioxidant mechanism is involved in hydrolysis of lipid peroxides and cholesteryl ester hydroperoxides [48]. 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 [52]. These enzymatic activities may help to explain the protection of HDL lipid oxidation since PON 1 is associated with HDL.

Shiavon et al. [53] 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* [54]. In contrast to the facts mentioned above, Zech et al. [55] 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.

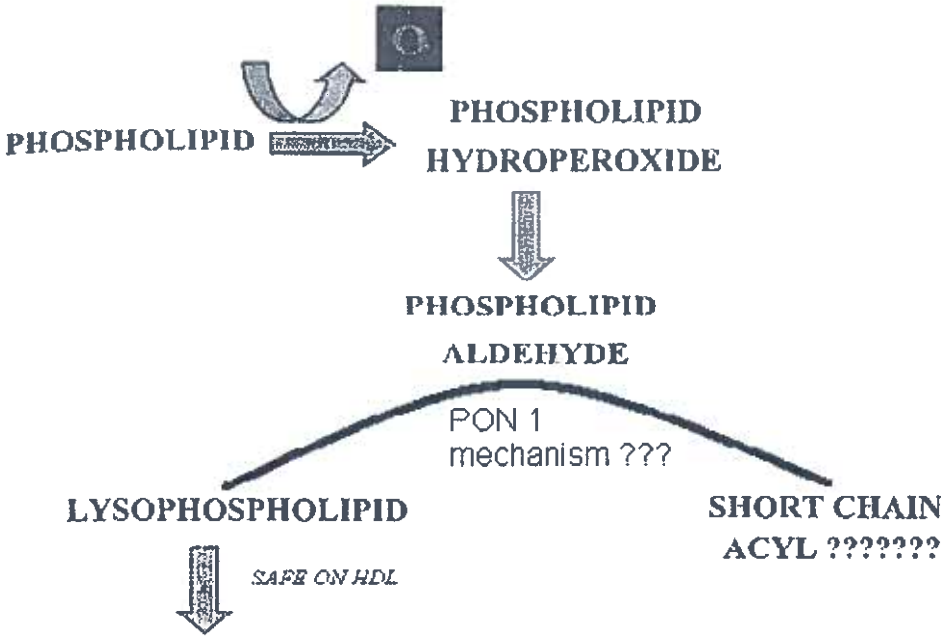

Fig. 11: Putative mechanism of action of PON 1 in destroying oxidized lipids in LDL; [56].

Experiments utilizing purified PON 1 [33, 24] 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 [48].

2.2.5 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 [57]. 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 [58]. 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 [24].

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 [59]. Exogenous PON 1 is able to reverse the oxidative stress in macrophages in aged apolipoprotein E deficient and PON 1 deficient mice [60].

2.2.6 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. 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 [7]. Murine macrophages express PON 2 and PON 3 [24], but their role in oxidative stress is not clear. 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 hydrolyses lactones, including several of the statines [7]. PON 2 is apparently unable to hydrolyse paraoxon [16].

2.2.6.1 PON 2

There is very little information available on this recently identified member of the PON family, PON 2. PON 2 mRNA is ubiquitously expressed in nearly every human tissue, with the highest expression in liver, lung, placenta, testis, and heart. The product of PON 2 has not yet been identified in biological tissue.

Several reports showed the association between genetic polymorphisms in PON 2 and variation in plasma lipoproteins (61, 62, 63). There are a few published reports of an association of PON 2 gene polymorphisms and CHD. Recently three reports (62, 64, 65) showed that the PON 2 C/S311 polymorphism is associated with CHD. One report (66) showed that PON 2 polymorphisms were not associated with CHD. Leus et al. (62) reported that the CHD risk associated with the PON2 S311 allele was confined to PON 1 Leu/Leu55-Gln/Gln192-PON2 Ser/Ser311 subjects. The

observation that in two studies (66, 61), despite different populations, the PON 2 Ser311 allele was associated with CHD makes it tempting to speculate that the PON 2 gene is the responsible gene rather than the PON1 gene for the association with CHD. However more data are needed to make definite conclusions about the associations between PON 2 polymorphisms and CHD.

There is such evidence of a link between low birth weight and elevated risk of cardiovascular disease, from both humans and experimental animals. A recent reports show association of PON 2 polymorphisms with birth weight (61, 67) but more independent studies are needed to make definite conclusions.

2.2.6.2 PON 3

PON 3 is interposed between PON 1 and PON 2 in the PON gene cluster and is the least studied compared to PON 1 and PON 2.

PON 3 is a 40-kDa protein associated with the HDL fraction of serum and is absent in LDL (68, 69). PON 3 is synthesized primarily in the liver. In contrast to PON 1, PON 3 has very limited arylesterase and no paraoxonase activities but rapidly hydrolyses lactones such as stain prodrugs.

Some studies (68, 69) suggest that PON 3 protein plays a role distinct from PON 1 in the lipoprotein metabolism of the kidney, and that therefore PON 1 and PON 3 may play distinct roles in the prevention of atherosclerosis.

2.3 PON 1 POLYMORPHISM AND HEMODIALYSIS

2.3.1 PON 1 AND HEMODIALYSIS

Serum PON 1 activity is abnormally low in patients with chronic renal failure on maintenance hemodialysis [70, 71, 72, 73]. Shiavon et al. [53] 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 [74]. Thus the low serum HDL concentration [70], and the different distribution of HDL subspecies [53, 73], rather than PON 1 genotype [73], 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 [70]. 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 [73]. The ratio PON 1 activity/HDL cholesterol is abnormally low in HD patients [70].

Since PON 1 is sensitive to oxidants and is inactivated by oxidized lipids [75], it can be postulated that increased oxidative stress may decrease serum PON 1 activity and impair the antioxidant activity of HDL in HD patients [76]. 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 [77]. Increased output of reactive oxygen species, including hydrogen peroxide, superoxide, and hypochlorous acid (HOCl), by activated neutrophils enhances oxidative stress in HD patients [74, 78]. 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 [79] and increase concentrations of low molecular

weight oxidants [101]. Both increased glycation [80] 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 [77].

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.

2.3.2 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 [81].

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 [82]. This modification has been demonstrated to play an important role in the pathogenesis of atherosclerosis [83] and uraemia [84].

There is evidence that low serum PON 1 activity may accelerate atherogenesis [86]. Recent studies [11, 85, and 86] 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.

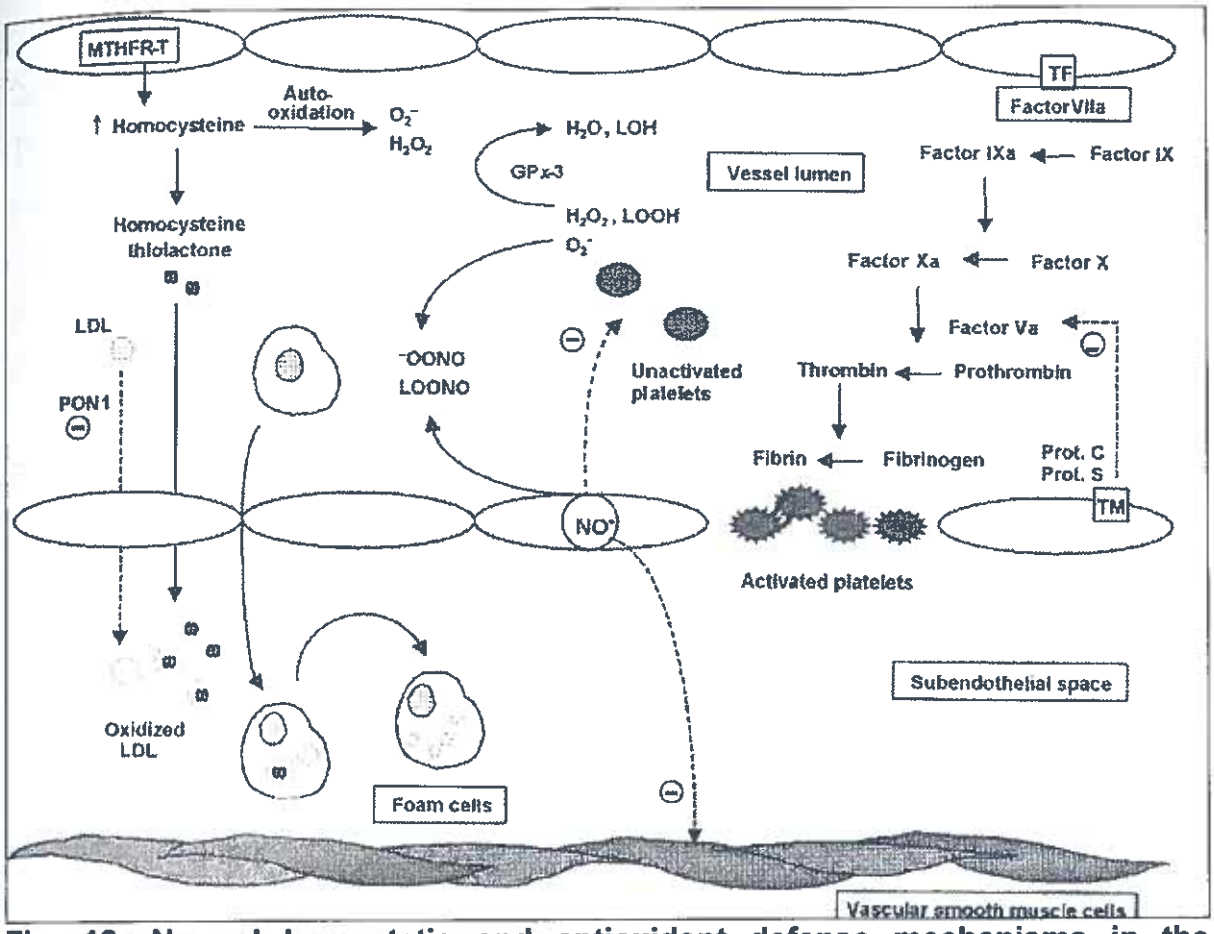

Fig. 12: Normal hemostatic and antioxidant defense mechanisms in the vasculature. LDL oxidation is prevented by PON 1 [87].

Patients on long-term HD have decreased PON 1/arylesterase activity [71, 77]. The uraemia or dialysis probably induces changes in the PON 1 enzyme activity, enzyme concentration, or its connection with HDL.

2.3.3 DIABETES MELLITUS AND HEMODIALYSIS

Diabetes mellitus (DM) is an increasingly important cause of end-stage renal disease [88]. Diabetic patients on dialysis have increased mortality and morbidity, mainly because of cardiovascular disorders [89].

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 [90].

Diabetic nephropathy occurs only in 25% to 40 % of the diabetic patients [90]. Therefore a genetic risk factor for this complication is very likely. Araki et al. [91] postulated that no polymorphism of PON genes is associated with diabetic nephropathy. In contrast, Murata et al. [92] 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 [90]. 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 [90]. Several studies revealed that PON 1 activity is one of the significant factors for retinopathy [93].

A large number of studies [listed in 90] 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 [94].

DM patients on HD have significantly lower HDL levels and serum PON 1 activities than non–DM patients on HD [95, 96]. 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 [70, 97].

Therefore, many authors have studied the association of the HDL-associated enzyme, PON 1, with chronic renal disease [77] and DM [70, 98]. However, whether or not the PON 1 status associates with DM in HD-patients has rarely been studied [99]. 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.

2.4 POLYMORPHISM

2.4.1 RESTRICTION FRAGMENTS LENGTH POLYMORPHISM

Most restriction fragments length polymorphisms (RFLPs) are created by a change in a single nucleotide in the gene, and so these are called single nucleotide polymorphisms (SNPs). Single nucleotide polymorphisms or SNPs are DNA sequence variations that occur when a single nucleotide (A, T, C, or G) in the genome sequence is altered. For example a SNP might change the DNA sequence AAGGCTAA to ATGGCTAA.

For a variation to be considered a SNP, it must occur in at least 1% of the population. SNPs, which make up about 90% of all human genetic variation, occur every 100 to 300 bases along the 3-billion-base human genome. Two of every three SNPs involve the replacement of cytosine (C) with thymine (T). SNPs can occur in both coding (gene) and non-coding regions of the genome. SNPs are also evolutionary stable (not changing much from generation to generation) making them easier to follow in population studies [100].

Many SNPs have no effect on cell function, but scientists believe others could predispose people to disease or influence their response to a drug. Although more than 99% of human DNA sequences are the same across the population, variations in DNA sequence can have a major impact on how humans respond to:

- disease;
- environmental insults (bacteria, viruses, toxins, and chemicals);
- and drugs and other therapies.

2.4.2 SNP AND DISEASE DEVELOPMENT

SNPs do not cause disease. They can help to determine predisposition to the development of particular disease [fig. 13].

There is great example of the role of SNPs in the development of disease, Alzheimer's disease. One of the genes associated with Alzheimer's, gene coding apolipoprotein E (ApoE), contains two SNPs that result in three possible alleles for this gene: E2, E3, and E4. Each allele differs by one DNA base, and the protein product of each gene differs by one amino acid. Each individual inherits one maternal copy of ApoE and one paternal copy of ApoE. Research has shown that an individual who inherits at least one E4 allele will have a greater chance of getting Alzheimer's. Apparently, the change of one amino acid in the E4 protein alters its structure and function enough to make disease development more likely. Inheriting the E2 allele, on the other hand, seems to indicate that an individual is less likely to develop Alzheimer's. Of course, SNPs are not absolute indicators of disease development. Someone who has inherited two E4 alleles may never develop Alzheimer's, while another who has inherited two E2 alleles may. ApoE is just one gene that has been linked to Alzheimer's disease[100].

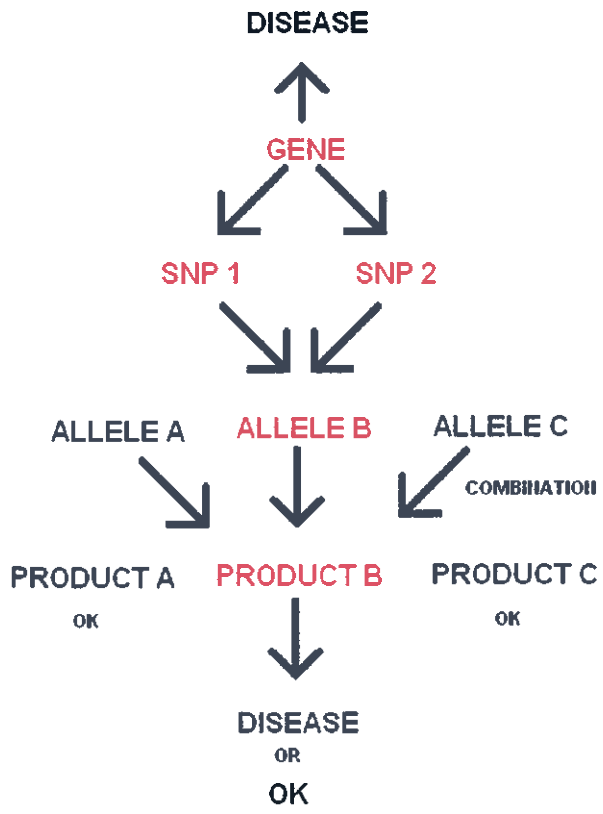

Fig. 13: Example of affection of disease by SNPs (predisposition to disease development). Mechanism is described in the text above.

Many common chronic disorders such as heart disease, diabetes, or cancer can be caused by variations in several genes. The polygenic nature of these disorders is what makes genetic testing for them so complicated.

2.4.3 SNP MAPS

Scientists believe SNP maps will help them identify the multiple genes associated with such complex diseases as cancer, diabetes, vascular disease, and some forms of mental illness. These associations are difficult to establish with conventional gene-hunting methods because a single altered gene may make only a small contribution to the disease.

Several groups worked to find SNPs and ultimately create SNP maps of the human genome. Among these groups were the U.S. Human Genome Project (HGP) and a large group of pharmaceutical companies called the SNP Consortium or TSC project [101].

2.5 HUMAN PARAOXONASE PON 1 L55M ALLELIC POLYMORPHISM

2.5.1 PON 1 L55M POLYMORPHISM AND PON 1 ACTIVITY

Paraoxon hydrolytic activity is greatest with HDL and with purified PON 1 55LL individuals and least with PON 1 55MM individuals [102, 103]. Heterozygotes have intermediate level. Thus, PON 1 55MM individuals have HDL and PON 1 associated with the greatest protective capacity. This alloenzyme is also most active in hydrolysing diazoxon and the nerve gases serin and soman [102].

2.5.2 PON 1 L55M POLYMORPHISM AND PLASMA LIPOPROTEINS PROFILE

Many studies have suggested that variation in serum PON activity is associated with variations in plasma lipoprotein concentrations including the serum ApoAI, LDL cholesterol, and HDL cholesterol. Hegele et al. [104] found that PON 1 polymorphisms were associated with variations in most plasma lipoproteins. There were detected significant differences in mean total cholesterol and LDL cholesterol levels between subjects with PON 1 LL55 and MM55 genotype, PON 1 55MM had a better lipoprotein profile [62]. Fanella et al. [105] found that carriers of PON 1 55MM have higher mean plasma concentrations of total and LDL cholesterol and ApoB than noncarriers. More recently Malin et al. [106] found that the 55LL has increased concentration of HDL and also tended to have higher ApoAI concentrations.

2.5.3 PON 1 L55M POLYMORPHISM AND CORONARY HEART DISEASE

PON has become a new risk factor for CHD in the past decade, yet the risk associated with PON 1 L55M polymorphism remains unclear. Regarding the PON 1 L55M polymorphism there have been just few studies. In the study of insulin non-dependent diabetics was reported that the homozygosity for the L allele is an independent risk factor for CHD for the first time [107].

Recently five promoter region polymorphisms of the human PON 1 gene have been reported. These have variable impact on promoter activity, which leads to differences in gene expression and serum concentration and activity of PON 1. The coding region M55L polymorphism also has a significant contribution to variation in serum PON concentration, whereas no significant contribution has been observed for the R192Q polymorphism [108]. This could be attributable in part to a strong association between the promoter polymorphisms and the L55M polymorphism.

Murphy et al. [109] found that there were significant interactions between the apoE and PON55 polymorphism that affected PON activity in different ways. A combination of the LL genotype with the presence of the ε4 allele was associated with a significant decrease in PON activity compared with ε3 individuals. Therefore the apoe genotype should be considered when interpreting the association between PON 1 L55M polymorphism and CHD [108].

2.5.4 PON 1 L55M Polymorphism And PAF-AH Activity

Two enzymes associated with HDL express PAF-AH itself and paraoxonase-1 (PON1). Several lines of evidence suggest that the HDL-PAF-AH activity may substantially contribute to the antioxidant and anti-inflammatory effects of HDL, thus this activity may be important component of the multiple mechanisms by which HDL slows the progression of atherosclerosis [110].

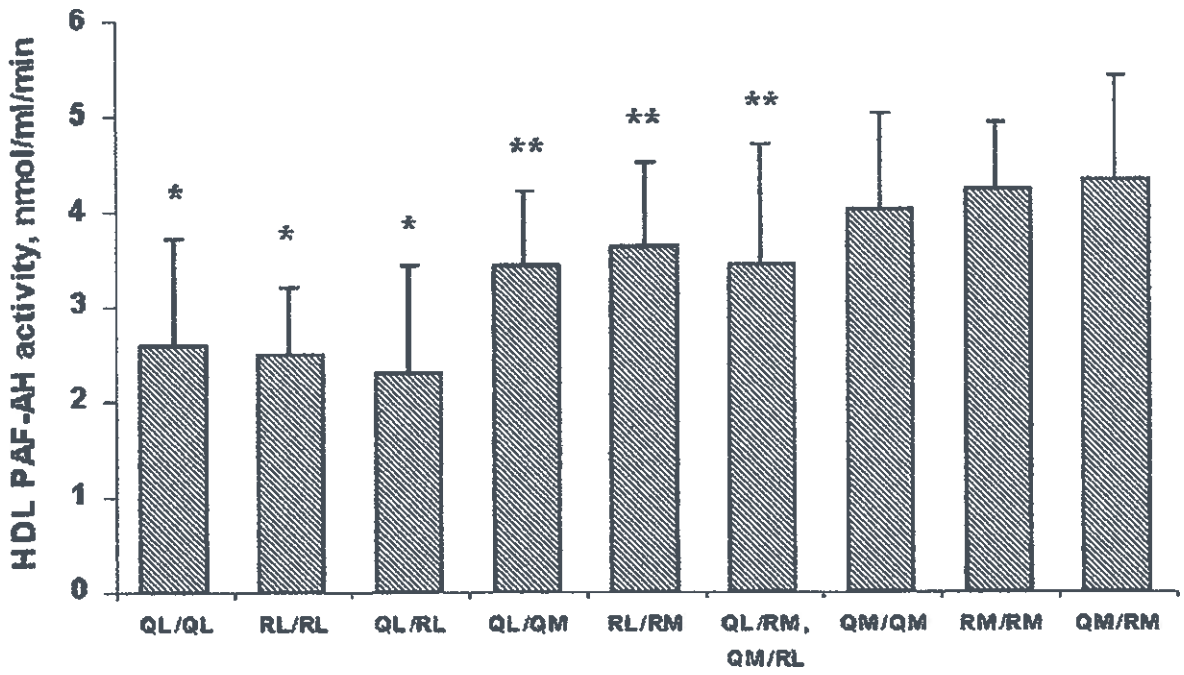

Fig. 14: Influence of PON 1 haplotypes on PAF-AH activity associated with HDL in dislipidemic patients. Values represent the mean ± SD. *P<0.03 compared with the PON 1 55MM. **P<0.05 compared with the homozygote haplotypes for either the L or the M allele [111].

Recent study showed that the PON 1 M55L polymorphism significantly affects the HDL-PAF-AH activity in all studied subjects, the PON 1 55LL individuals having lower enzyme activity compared to those having 1 M and 2 M alleles. These results provide evidence that PON 1 significantly contributes to the pool of HDL-PAF-AH activity in human plasma, and suggest that the low PAF-AH activity in HDL carrying the PON 1 L alloenzyme may be an important factor contributing to the low efficiency of this HDL in protecting LDL against lipid peroxidation [111].

3 AIM OF THESIS

The aim of thesis is:

A. To determine genotype for PON1 L55M allelic polymorphism in population of hemodialysis patients (HD).

B. To determine genotype for PON1 L55M allelic polymorphism in population of control subjects (CS).

C. To compare genotype distribution for PON1 L55M allelic polymorphism in HD and CS.

D. To compare allele frequency for PON1 L55M allelic polymorphism in HD and CS.

E. To map age and gender of studied subjects.

F. To compare age and gender distribution in HD and CS.

4.1 METHODS

4.1.1 INSTRUMENTS

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

Fig. 15 : Centrifuge, Heraeus Instruments, Germany.

Fig. 16: PCR machine, Applied Biosystems, USA.

4.1.2 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:

50mM Magnesium Chloride	Invitrogen	USA
10x PCR Buffer, Minus Mg	Invitrogen	USA
Taq DNA Polymerase (5U/µl)	Invitrogen	USA
10 mM dNTP Mix, PCR Grade	Invitrogen	USA
PRIMER PON 1 R192Q *R* (5'– TGA AAG ACT TAA ACT GCC AGT C – 3')		
	Microsynth	Switzerland
PRIMER PON 1 R192Q *F* (5' - CCT GCA ATA ATA TGA AAC AAC CTG - 3')		
	Microsynth	Switzerland
Deoxynucleoside Triphosphates (dNTPs): dATP, dTTP, dCTP, dGTP (10Mm)		
	Invitrogen	USA

ELECTROPHORESIS:

Agarose	SIGMA	USA
Ethidium Bromide	SIGMA	USA
Bromophenol Blue	SIGMA	USA
Natrium Acetate	Kemika	Croatia
100bp DNA Ladder (0.1µg/µl)	Fermentas	USA

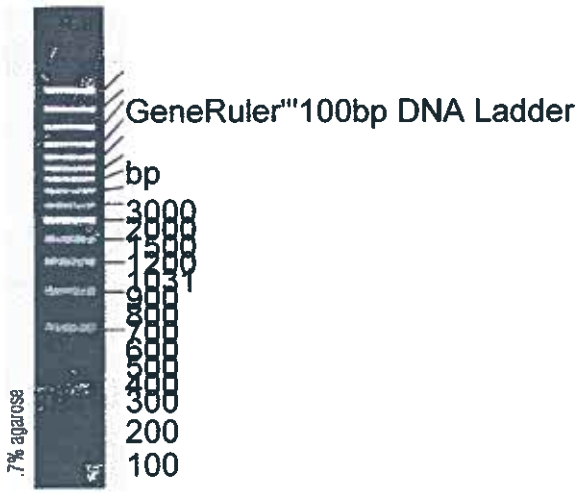

Fig. 17: 100bp DNA Ladder. This DNA Ladder is prepared from different plasmids, containing pUC, pBR322, λ phage and yeast genome sequences. Analysis of 0.5µg (5µl) of the DNA Ladder on agarose gel by ethidium bromide staining generates following 14 discrete bands pattern (in base pairs): 3000, 2000, 1500, 1200, 1031, 900, 800, 700, 600, 500, 400, 300, 200, 100.

RFLP:

Hin 1II (5U/µl)	Fermentas	USA
10x Buffer G	Fermentas	USA

4.1.3 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 $MgCl_2$

Dissolve all the components in 400ml of autoclaved water, add autoclaved water to the total volume of 500ml and sterile filtrate in the vacuum. Store it in 4 - 8 °C.

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 autoclaved water to the total volume of 500ml and autoclave it. Store it in the room temperature.

NLB, 100 ml: 500 µl 2 M Tris
8.04 ml 5 M NaCl
400 µl 0.5 M EDTA

Mix all the components together, add autoclaved water to total volume of 100ml and autoclave it. Store it in 4 -8°C.

10% SDS, 20 ml:

Dissolve 2 g of SDS in 20 ml of autoclaved water. Store it in 4 -8°C.

2 M Tris pH 8.2, 50 ml:

Dissolve 12.114 g of Tris base in 40 ml of autoclaved water. Add HCl (cca 3ml) to establish pH 8.2. Add autoclaved water to total volume of 50 ml and autoclave it. Store it in the room temperature.

2 M $MgCl_2$, 50 ml:

Dissolve 10.165 g of $MgCl_2x\ 6H_2O$ in 50 ml of autoclaved water (exothermic reaction!) and autoclave it. Store it in the room temperature.

0.5 M EDTA, 50 ml:

Dissolve 9.306 g of $Na_2EDTAx2H_2O$ in 30 ml of autoclaved water. Add NaOH to establish pH 8.2. Add autoclaved water to total volume of 50 ml and autoclave it. Store it in the room temperature.

5M NaCl, 50 ml:

Dissolve 14.6 g of NaCl in 50 ml of autoclaved water and autoclave it. Store it in the room temperature.

Saturated solution of NaCl, 50 ml:

Dissolve 20 g of NaCl in 50 ml of distilled water and autoclave it. Store it in the room temperature.

TAE-buffer pH 7.9, 1000 ml: 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

Store it in the room temperature.

TE-buffer pH 8.0, 1000 ml: 1.21 g Tris-HCl (10 mM)
0.37 g Na_2EDTA (1 mM)

Add autoclaved water 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) saccharose in water

Store it in 4 -8°C.

Store it in 4 -8°C.

4.1.4 DNA ISOLATION

Blood samples were collected in tubes containing ethylene diamine tetraacetate (EDTA), and DNA was isolated from the whole blood using the alternative of Miller's method. This three-day procedure involves osmotic cell lysis with several centrifugations (using CLB, SLR, and NLB-solutions), and overnight leucocytes lysis by serine protease from *Tritirachium album* (proteinase K) and SDS, salting out of the cellular proteins by saturated NaCl solution, DNA precipitation by absolute ethanol and DNA resuspendation. This method offers non-toxicity, DNA yield and purity, reliability and low price, and it does not inhibit PCR or digestion by restrictases.

Procedure:

1st day of DNA isolation:

- Defrost the whole blood in the room temperature;
- Pipette 2.5 ml of the whole blood into sterile 15 ml plastic tube;
- Add 10 ml of CLB solution, mix it manually to total resuspendation, leave it on t ice on shaker (200rpm) for 20 min;
- Centrifuge it for 20 min (6000 rpm, +4°C);
- Pour out the supernatant, mix the pallet with 8ml of CLB solution, and leave it on ice on shaker (200rpm) for 20 min;
- Centrifuge it for 20 min (6000 rpm, +4°C);
- Pour out the supernatant, mix the pallet with 10ml of SRL solution, and leave it on ice on shaker (200rpm) for 20 min;
- Centrifuge it for 20 min (6000 rpm, +4°C);
- Pour out the supernatant, mix the pallet with 1.5 ml of cold NLB solution, 100 µl of SDS and 250 µl of proteinase K;
- Store it in hybridization oven (gentle rotation, 42°C) during the night.

2nd day of DNA isolation:

- Take out the sample from Hybridization oven, add 500 µl of saturated solution of NaCl, and mix it well;
- Centrifuge it for 30 min (6000 rpm, +4°C);
- Pour the supernatant into new 15 ml plastic 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 this medusa of DNA, and transfer it into sterile Eppendorf tube;
- Centrifuge it for 5 min (5000 rpm, room temperature);
- Pour out the ethanol phase;
- Store the Eppendorf tube with the DNA-pallet opened on the dark and sterile place (room temperature) during the night to dry out all the ethanol.

3rd day of DNA isolation:

- Add 100 µl of autoclaved water;
- Incubate it in 37°C, 300rpm during the night. The DNA is isolated now. Store it in +4°C for at least two days before PCR process.

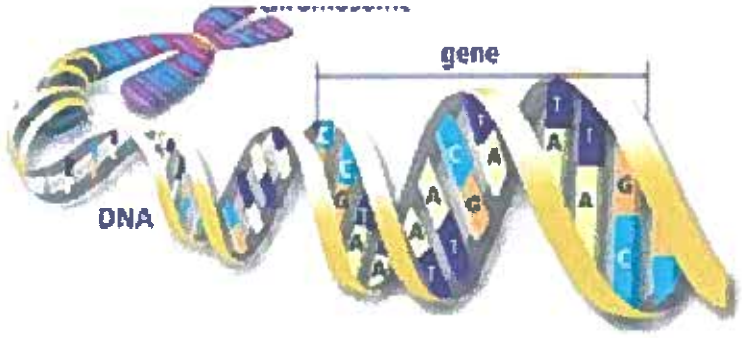

Fig. 18: Schematic structure of DNA [112].

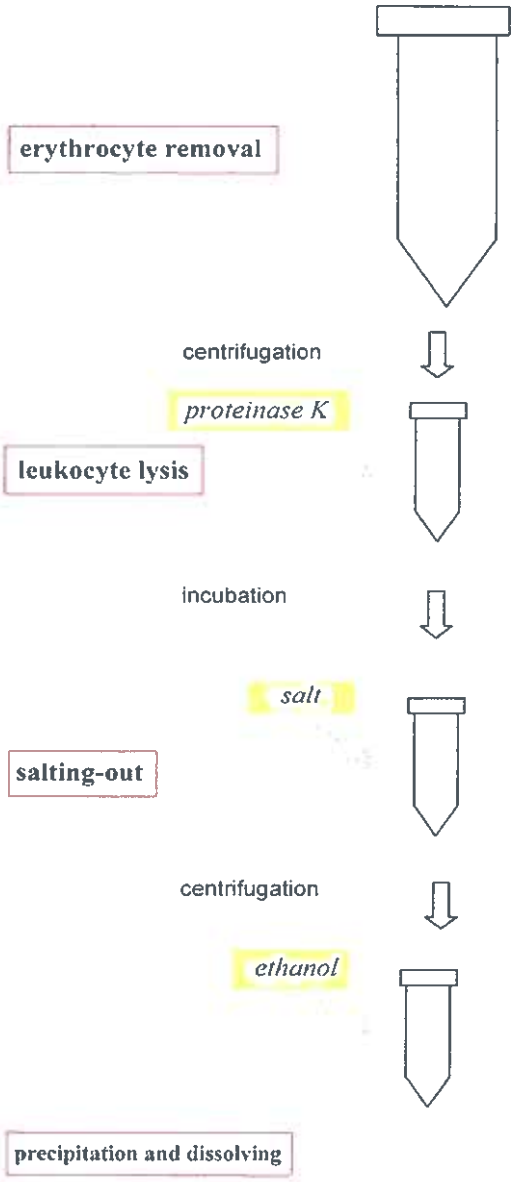

Fig. 19: Schema of proteinase K salting-out method of DNA isolation.

4.1.5 DNA Quality And Quantity Tests

Quality and quantity of isolated DNA was established by UV-spectrophotometry.

Isolated DNA was diluted in the ratio 1:50 by TE-buffer to total volume of 500 µl (it means: 10 µl of isolated DNA and 490 µl of TE-buffer). Eppendorf tube with isolated DNA must be kept at least 30 min in the room temperature before diluting.

Absorbance was measured for two different wavelengths, λ_1=260 nm and λ_2=280 nm, for each sample. TE-buffer was used as the blank.

Quality of isolated DNA is determined by A_1 (260 nm)/A_2 (280 nm) ratio: if A_1/A_2 ratio lies in the interval from 1.8 to 2.0 than the quality of isolated DNA is the best suitable for further PCR and RFLP procedure.

Quantity of isolated DNA was presented as concentration [µg/ml]. The absorbance A_1 (260 nm) of sample containing 50 µg/ml of DNA is equal one (A_1=1.000).

The concentration was calculated using formula:
c [µg/ml] = A (260 nm) x 50* x 50;**
*50 µg/ml DNA has A_{260}=1.0;
**dilution factor is 50.

4.1.6 POLYMERASE CHAIN REACTION (PCR)

The polymerase chain reaction is a relatively simple technique that amplifies a DNA template to produce specific DNA fragments in vitro. Traditional methods of cloning a DNA sequence into a vector and replicating it in a living cell often require days or weeks of work, but amplification of DNA sequences by PCR requires only hours. PCR offers a fast and convenient method of amplifying a specific DNA segment. These features make the technique extremely useful, not only in basic research, but also in commercial uses, including genetic identity testing, forensics, industrial quality control and in vitro diagnostics.

The PCR process was originally developed to amplify short segments of a longer DNA molecule [113]. A typical amplification reaction includes the target DNA, a thermostable DNA polymerase, two oligonucleotide primers, deoxynucleotide triphosphates (dNTPs), reaction buffer and magnesium. The reaction is placed in a thermal cycler, an instrument that subjects the reaction to a series of different temperatures and time periods. This series of temperature and time adjustments is referred to as one cycle of amplification. Each PCR cycle theoretically doubles the amount of targeted sequence (amplicon) in the reaction. Each step of the cycle

should be optimized for each template and primer pair combination. After 20-40 cycles, the amplified product may then be analyzed for size, quantity, sequence, etc., or used in further experimental procedures.

Procedure:

Each cycle of PCR includes steps for template denaturation, primer annealing and primer extension (Fig. 20). The initial step denatures the target DNA by heating it to 94°C for 30 seconds. In the denaturation process, the two intertwined strands of DNA separate from one another, producing the necessary single-stranded DNA template for replication by the thermostable DNA polymerase. In the next step of a cycle, the temperature is reduced to 63°C. At this temperature, the oligonucleotide primers can form stable associations (anneal) with the denatured target DNA and serve as primers for the DNA polymerase. This step lasts 30 seconds. Finally, the synthesis of new DNA begins as the reaction temperature is raised to the optimum for the DNA polymerase. For most thermostable DNA polymerases, this temperature is in the range of 70–74°C; it means 72 °C for *Taq* DNA polymerase used in this research. The extension step lasts 1 minute. The next cycle begins with a return to 94°C for denaturation (Tab. 1).

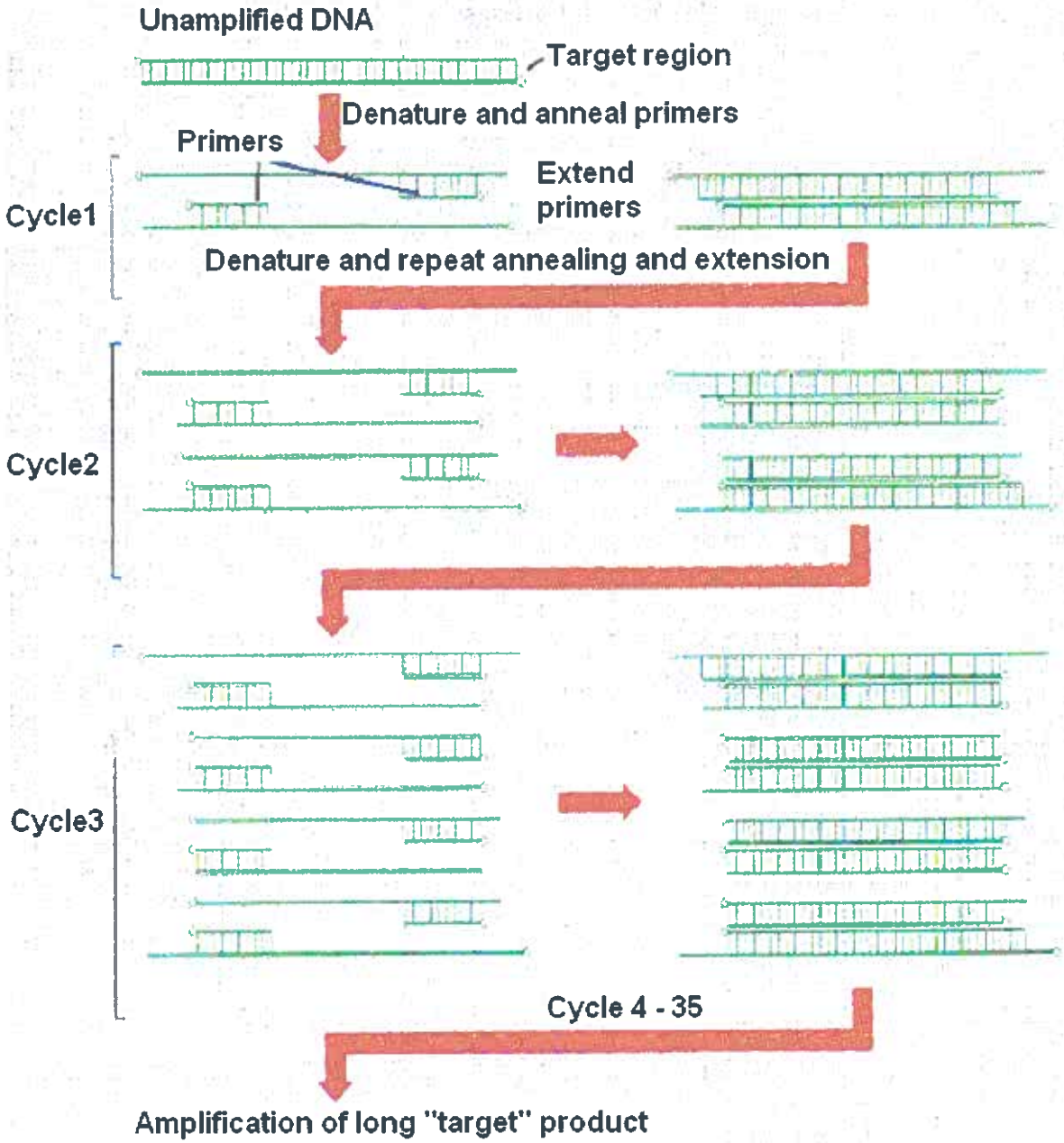

Fig. 20: The polymerase chain reaction (PCR). In each cycle of the reaction, the strands of the duplex DNA are separated by heat denaturation, the reaction mixture is cooled to the temperature at which synthetic DNA primers could anneal to a complementary segment on each strand, and then, the primers are extended by DNA polymerase. The process is repeated for 35 cycles in this case.

Tab. 1: PCR conditions for PON1 L55M polymorphism analysis.

Number of cycles	Time	Temperature [°C]
1	12min	95
35	30s 30s 60s	94 (**denaturation**) 63 (**annealing**) 72 (**extension**)
1	7min	72

DNA polymerase used in this research is Platinum® *Taq* DNA Polymerase (Invitrogen). It is recombinant *Taq* (*Thermophilus aquaticus*) DNA polymerase complexed with a proprietary antibody that blocks polymerase activity at ambient temperatures. Activity is restored after the denaturation step in PCR cycling at 94°C, thereby providing an automatic "hot start" for *Taq* DNA polymerase in. Hot starts in PCR provide increased sensitivity, specificity, and yield, while allowing assembly of reactions at room temperature. The use of this antibody helps reduce PCR optimization requirements, reaction set-up time and effort, handling of reaction components, and contamination risk, thereby improving PCR results [114].

Protocol:

Isolated target DNA must be kept at least 0.5 h in the room temperature before application of PCR. PCR was done using 0.5 µg of target DNA.

Reagent mixture was prepared according to tab. 2. Reagent mixture (total volume 23 µl) was mixed with isolated DNA (2.0 µl of solution containing 0.5 µg of target DNA dissolved in ddH_2O).

Tab. 2: Components of PCR mixture. * One unit of Platinum® *Taq* DNA Polymerase incorporates 10 nmol of dNTP into acid-precipitable material in 30 min at 74°C.

Components	Volume	Final Concentration
10x PCR-buffer	2.5 µl	1x
50mM $MgCl_2$	2.0 µl	4.0 mM
dNTPs mix	2.5 µl	1.0 mM
2 µM primer F	2.5 µl	0.2 µM
2 µM primer R	2.5 µl	0.2 µM
DNA polymerase	0.1 µl	1.0 unit*
ddH_2O	10.9 µl	Not applicable

Negative control must be prepared for each PCR procedure. This negative control sample consists of 23.0 µl of PCR mixture and 2.0 µl of ddH_2O.

PCR products are stored in +4°C. These products are prepared for analysis by agarose gel electrophoresis and visualisation by ethidium bromide staining. PCR product length is 172 bp.

4.1.7 CONTROL ELECTROPHORESIS

There are many **techniques used to fractionate nucleic acids** according to size, composition, and sequence:

- Hydroxyapatite chromatography (separates single-stranded DNA from double-stranded DNA);
- Pulsed-field gel electrophoresis on agarose gels (PFGE; separates large pieces of DNA ranging from 50,000 to 5 million bp);
- CsCl density gradient ultracentrifugation (separates DNAs according to their base composition);
- Polyacrylamide gel electrophoresis (PAGE; separates DNAs according to their size and charge);
- Agarose gel electrophoresis (separates DNAs and other large molecular mass compounds according to their size and charge).

In **electrophoresis**, charged molecules are separated according to their rates of migration in an electric field on a solid support such as cellulose acetate, paper, cross-linked polyacrylamide, or agarose. Gel electrophoresis employs cross-linked polyacrylamide or agarose gel support, so that molecules are separated according to size by gel filtration as well as according to size.

Nucleic acids <200kD may be separated by polyacrylamide gel electrophoresis because their electrophoretic mobility in such gels vary inversely with their molecular masses. However, DNAs of more than a few thousand base pairs are too large to penetrate even a weakly cross-linked polyacrylamide gel. This difficulty is overcome through the use of **agarose gels**. By using gels with appropriately low agarose content, relatively large DNAs in various size ranges may be fractionated.

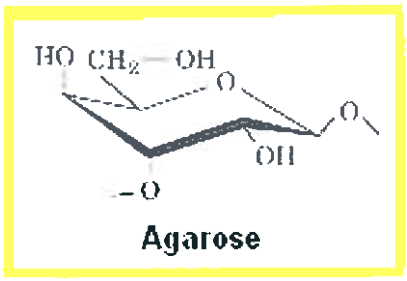

Fig. 21: Agarose subunit.

Migration of DNA fragments in agarose gel: Fragments of linear DNA migrate through agarose gels with a mobility that is inversely proportional to the $\log_{10}$ of their molecular weight. Circular forms of DNA migrate in agarose distinctly differently from linear DNAs of the same mass (typically uncut plasmids will appear to migrate more rapidly than the same plasmid after linearization).

Several factors have important effects on the mobility of DNA fragments in agarose gels. The main factors are:

- Agarose concentration: Higher concentrations of agarose facility separation of small DNAs, while low agarose concentration allows resolution of larger DNAs.

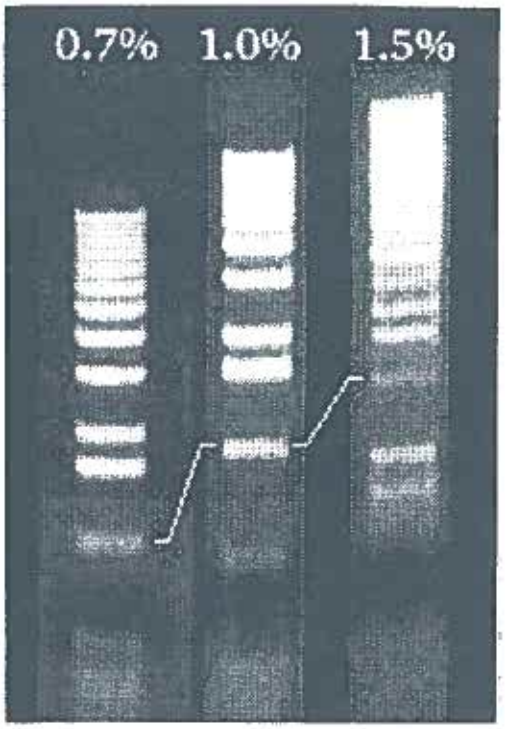

Fig. 22: Migration of a set of DNA fragments in three concentrations of agarose. All these fragments were in the same gel tray and electrophoresed at the same voltage for identical time period. The 1000bp fragment is indicated in each lane. The larger fragments are much better resolved in the 0.7% agarose gel, while the small fragments are separated well in 1.5% agarose gel.

• Voltage: As the voltage applied to a gel is increased, larger fragments migrate proportionally faster than small fragments.

• Electrophoresis buffer: The most used buffers for double-stranded DNA are TAE (Tris-acetate-EDTA) and TBE (Tris-borate-EDTA). DNA fragments migrate at different rates in these two buffers due to differences in ionic strength. Buffers do not only establish pH, but also provide ions to support conductivity.

• Effects of ethidium bromide: Binding of ethidium bromide to DNA alters its mass and rigidity, and therefore its mobility. It can be incorporated into agarose gels as well as added to samples of DNA before loading to enable visualization of DNA fragments within the gel.

DNA fragments detection: Gel bands resulting from an agarose gel electrophoresis can be detected by variety of techniques. Among the chief techniques is involved staining, radioactive counting, or immunoblotting. The double stranded DNA is detected by selective staining with intercalation agents. These intercalation agents are aromatic cations such as ethidium ion, acridine orange, or proflavin. These dyes bind to duplex DNA by intercalation (slipping in between the stacked base pairs),

where they exhibit 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. Single stranded DNA and RNA also stimulate the fluorescence of ethidium ion but to a lesser extent than does duplex DNA.

Fig. 23: Ethidium ion structure.

Procedure

The result of the amplification with the pair of PON1 L55M primers is the product of 172 bp. Therefore horizontal agarose gel electrophoresis was used.

Agarose gel preparation: There was prepared 2% agarose gel for electrophoresis of 172 bp PCR products: 2g agarose powder was mixed with 100 ml of TAE buffer and heated until the agarose was completely melted. After cooling the solution to approximately 60°C, 5 µl of ethidium bromide was added to the gel, than it was poured into the casting tray containing a sample comb. The agarose gel has solidified at the room temperature.

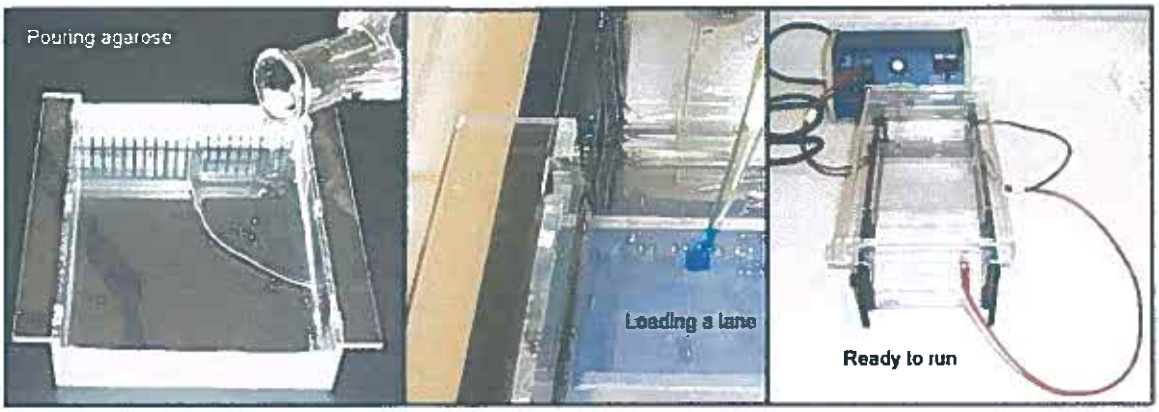

Fig. 24: Preparing and running standard agarose gel for DNA fragments electrophoresis. Equipment necessary to run agarose gel electrophoresis is relative simple and includes: electrophoresis chamber and power supply, gel casting trays, sample combs, electrophoresis buffer, loading buffer, tracking dyes, fluorescent dye, and transilluminator (UV-box).

After the gel has solidified, the comb was removed. The gel was inserted horizontally into the electrophoresis chamber and covered with TAE buffer. Samples containing DNA mixed with loading buffer (5 µl of PCR product + 2 µl of loading buffer) were inserted into the sample wells. In the first well of the gel was always applied marker mix (1 µl DNA-ladder + 1 µl LB-buffer + 5 µl ddH_2O). The negative control (5 µl ddH_2O + 2 µl LB-buffer) was applied into the last well of the gel.

Conditions of control electrophoresis: U = 60 V, and t = 45 min.

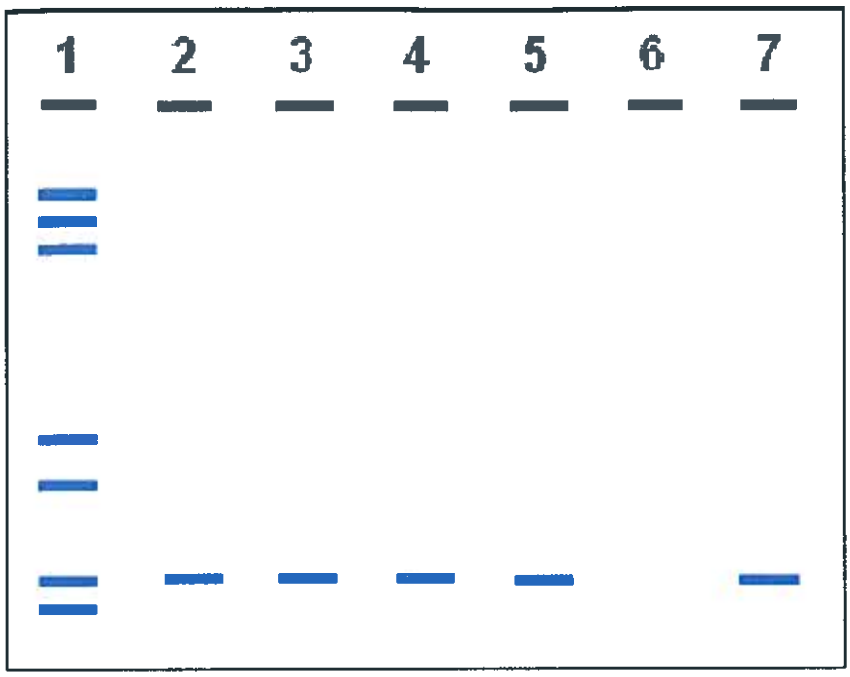

Fig. 25: Example of gel electrophoresis after UV-detection:

1 marker mix;

2-5 DNA-fragments=5 µl PCR product+2 µl LB-buffer;

6 empty well;

7 negative control=5 µl ddH_2O, 2 µl LB-buffer.

The distance of DNA on the gel was visually monitored by migration of added tracking dye, bromophenol blue (component of loading buffer). DNA fragments were visualized by staining with ethidium bromide solution (5 µl EtBr/100 ml TAE-buffer) after adequate migration. To visualize DNA products, the gel was placed on an ultraviolet transilluminator (UV light of λ = 254 nm). Examination or photography should be taken shortly after end of electrophoresis because the DNAs diffuse out of the gel over time.

4.1.8 Restriction Fragments Length Polymorphism (RFLP)

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.

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.

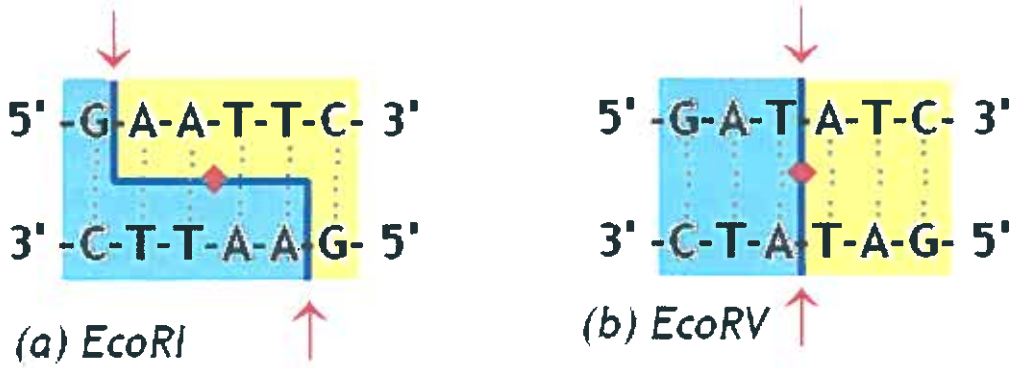

Fig. 26: Restriction sites. The restriction sequences of the restriction endonucleases (a) *Eco*RI and (b) *Eco*RV have two fold (palindromic) symmetry (red symbol). The cleavage sites are indicated (arrows). The *Eco*RI generates cohesive DNA fragments, whereas *Eco*RV generates blunt-ended fragments.

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.

Restriction endonucleases are classified into 11 subtypes as shown in tab. 3. Restriction endonuclease used in this thesis was *Hin1 II* and it is included in subtype P.

Tab. 3: Subtypes of restriction endonucleases.

SUBTYPE*	DEFINING FEATURE	EXAMPLES
A	Asymmetric recognition sequence	*Fok I* *Aci I*
B	Cleaves both sides of target on both strands	*Bcg I*
C	Symmetric or asymmetric target. R and M function in one polypeptide	*Gsu I* *Hae IV* *Bcg I*
E	Two targets; one cleaved, on an effector	*EcoR II* *Nae I*
F	Two targets, both cleaved co-ordinately	*Sfi I* *SgrA I*
G	Symmetric or asymmetric target. Affected by AdoMet	*Bsg I* *Eco57 I*
H	Symmetric or asymmetric target. Similar to Type I gene structure	*Bcg I* *Ahd I*
M	Subtype IIP or IIA. Require methylated target	*Dpn I*
P	Symmetric target and cleavage sites	*EcoR I* *Hin 1II* *Bsl I*
S	Asymmetric target and cleavage sites	*Fok I* *Mme I*
T	Symmetric or asymmetric target. R genes are heterodimers	*Bpu10 I* *Bsl I*

*Note that not all subtypes are mutually exclusive. E.g. *Bsl I* is of subtype P and T.

Hin1 II:

• Concentration: 5 units / µl (one unit is defined as the amount of *Hin1 II* required to digest 1 µl of DNA in 1 hour at 37°C in 50 µl of Buffer G)

• Source: *Haemophilus influenzae*

• Recognition site: **5'... C A T G ↓ ...3'**
3'... ↑ G T A C ...5'

• Supplied with: 10x Buffer G (10mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 50mM NaCl, 0.1mg/ml BSA)

• Enzyme activity in Buffer G: 100 %.

Protocol for digestion of PCR products: 1.0 µl *Hin1 II*;
1.5 µl Buffer G;
2.5 µl ddH_2O;
10.0 µl PCR-product (about 1 µg of DNA).

All components must be mixed gently and spin down for a few seconds in PCR-tube. This mixture is incubated at 37°C during the night (approximately 16 hours). RFLP product was electrophoresed directly after incubation.

4.1.9 RFLP ELECTROPHORESIS

RFLP products are prepared for analysis by agarose gel electrophoresis, visualisation by ethidium bromide staining, and UV detection. Restriction by *Hin1 II* of 172 bp PCR product with M allele results in 106 bp and 66bp DNA fragments, L allele is not digested by *Hin1II* (Fig. 27).

Procedure:

Agarose gel preparation: Protocol for RFLP electrophoresis is the same as for Control electrophoresis with one exception: there was prepared 4% agarose gel for electrophoresis.

Samples containing RFLP product mixed with loading buffer (15 µl of RFLP product + 2 µl of loading buffer) were inserted into the sample wells. In the first well of the gel was always applied marker mix (1 µl DNA-ladder + 1 µl loading buffer + 5 µl ddH_2O). In the second well of the gel was applied PCR product (5 µl of PCR product + 2 µl of loading buffer). The negative control (5 µl ddH_2O + 2 µl loading buffer) was applied into the last well of the gel.

Conditions of RFLP electrophoresis: U = 55 V, and t = 75 min.

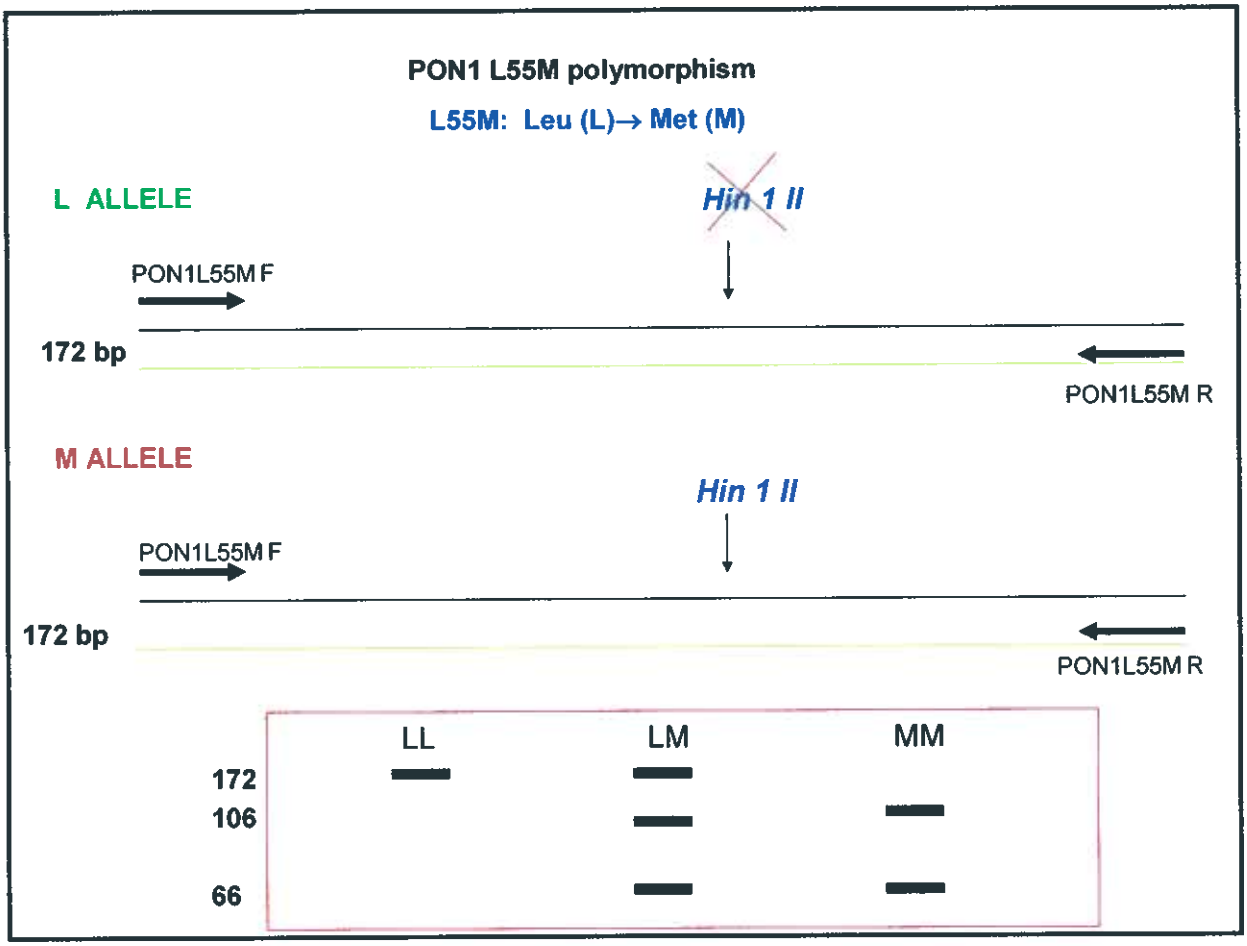

Fig. 27: PON1 L55M polymorphism. Digestion of 172 bp PCR product by restriction endonuclease *Hin1II* results in 2 DNA fragments: 106 bp and 66 bp.

4.2 SUBJECTS

There were included 146 subjects in this research. These subjects were divided into two groups, control subjects and hemodialysis patients. The place of origin of these subjects is Slavonski Brod, Croatia.

4.2.1 HEMODIALYSIS PATIENTS

Studied subjects consisted of 51 hemodialysis patients (28 male / 23 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: vascular diseases, diabetic nephropathy, chronic glomerulonephritis, chronic pyelonephritis, vasculitis. Frequency of dialysis was three times per week and each dialysis lasted from four to five hours.

Tab. 4: Age and gender of hemodialysis (HD) patients.

HD PATIENTS	Age (years)		Total number
	Mean ± SD	Min - max	
All subjects (male, female)	60 ± 12	22 – 77	51
Male	57 ± 15	22 – 76	28
Female	62 ± 10	43 - 77	23

4.2.2 CONTROL SUBJECTS

Control subjects consisted of 95 healthy blood donors (48 male / 47 female) from The Clinical Hospital Split, Croatia.

Tab. 5: Age and gender of control subjects.

CONTROL SUBJECTS	Age (years)		Total number
	Mean ± SD	Min - max	
All subjects (male, female)	49 ± 17	11 – 80	95
Male	49 ± 17	11 – 80	48
Female	66 ± 18	17 – 78	47

4.3 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.

Macroscopic appearance: There were significant differences between the samples from blood donors (control subjects) and patients treated on maintenance hemodialysis (hemodialysis patients). The differences are probably caused by different lipoprotein profile of studied subjects.

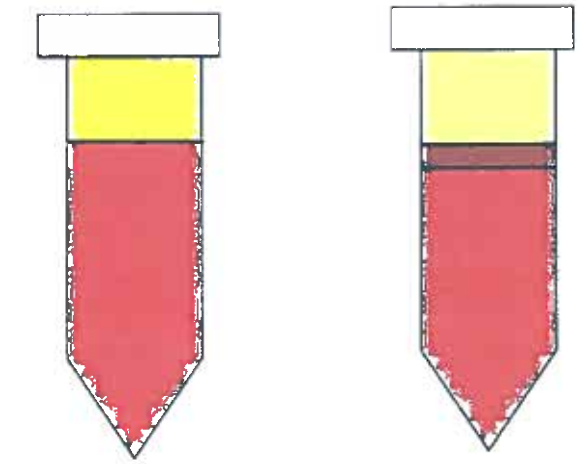

Fig. 28: Macroscopic structure of blood samples. Contrast between samples from control subjects and HD patients.

5 RESULTS

5.1 DNA QUALITY AND QUANTITY TEST

DNA was isolated from 92 control samples and 48 samples from HD patients by Miller's method. The quality 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 1.185 to maximum 3.720. These results (tab. 6) of DNA quality were acceptable for our further experiments.

The quantity of isolated DNA was calculated as concentration of DNA in each sample. The minimal amount isolated from one blood sample was 80 µg/ml; maximum of isolated DNA was 1995 µg/ml. There was no DNA isolated of three blood samples from HD patients and three blood samples from control subjects.

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

C [µg/ml] = A (260nm) x50xdilution.

It is necessary to know the amount of DNA isolated from each sample for our further procedures, especially for calculation of volume of sample for PCR. The amount of 0.25 µg DNA was used for PCR.

Tab. 6: Results of DNA quality (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		p
	Control subjects (n=92)	HD patients (n=48)	
DNA [µg/ml]	492 ± 300 / 80 – 1600	937 ± 448 / 233 – 1995	0.0007
A_1/A_2	1.773 ± 0.147 / 1.185 – 2.212	1.934 ± 0.145 / 1.404 – 3.720	0.0003

5. 2 ELECTROPHORESIS OF PCR PRODUCTS

The result of the amplification with the pair of PON1 L55M primers is the product of 172 bp. In order to be sure that the amplification of isolated DNA by PCR was correct each PCR product was analysed by agarose gel electrophoresis. All PCR products from 92 control subjects and 48 HD subjects were 172 bp long DNA fragments suitable for RFLP.

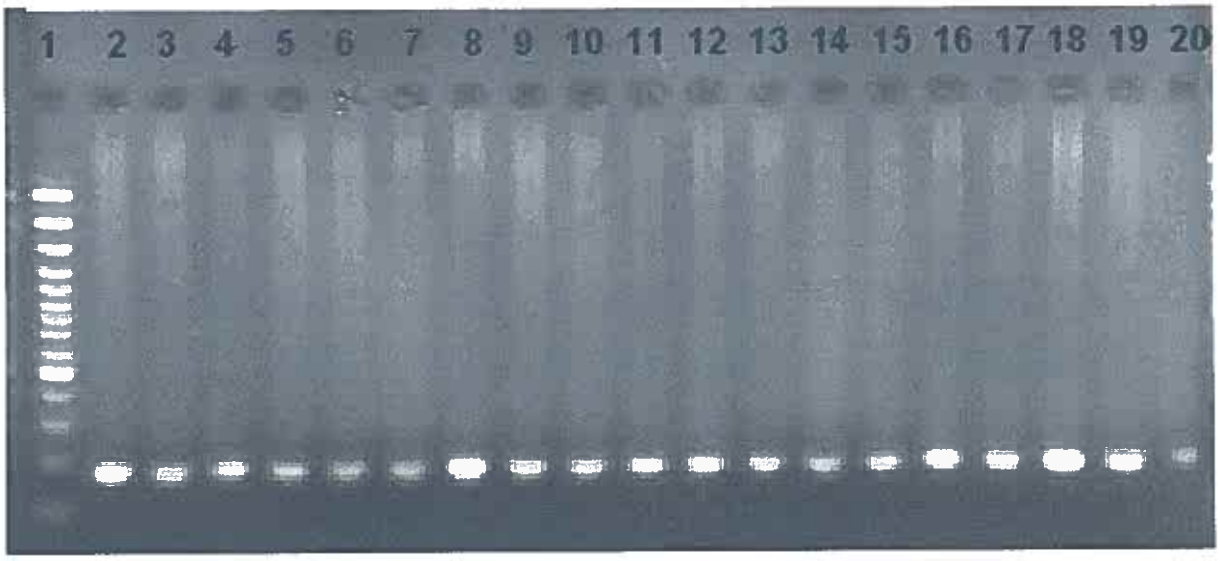

Fig. 29: Example of result of electrophoresis of PCR products, all DNA fragments are of the same length 172 bp. The marker is in line number 1. There were detected 19 DNA products in lines 2 to 20.

5. 3 RESTRICTION FRAGMENTS LENGTH POLYMORPHISM (RFLP)

PCR products (172 bp) were digested with *Hin1 II* enzyme at 37°C for 12 hours. The final agarose gel electrophoresis was done after digestion of all PCR products. According to the position of RFLP product on the gel it would be possible to estimate product's size and to determine genotype (MM genotype - homozygote, LM genotype - heterozygote, LL genotype - wild type for PON1 L55M polymorphism) of each sample.

There are three diagnostic bands for PON1 L55M polymorphism after digestion by restriction enzyme *Hin1 II*, these bands are fragments of 172 bp, 106 bp, and 66 bp (Fig. 30, 31).

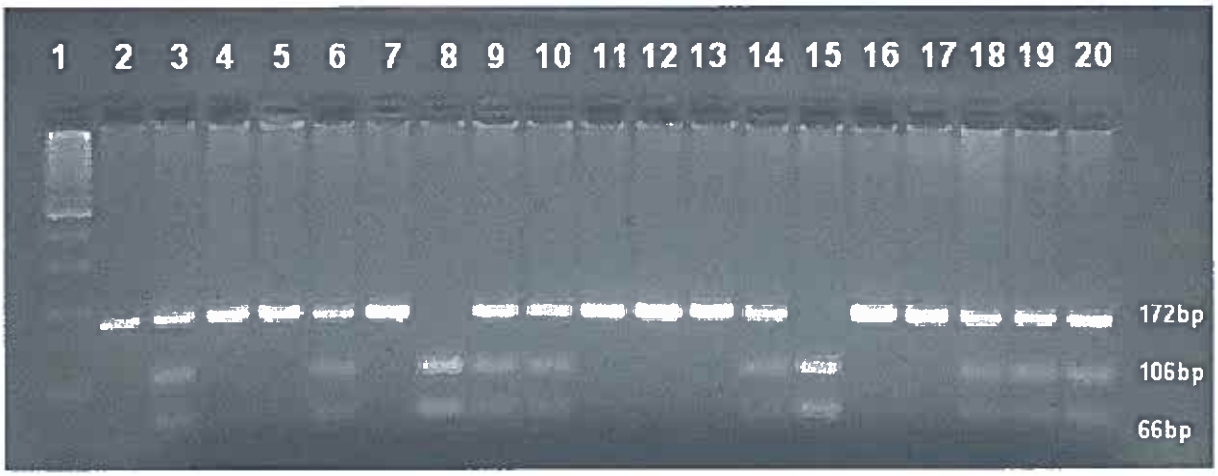

Fig. 30: Restriction patterns of the PCR amplified 172 bp fragment after digestion with *Hin1 II*. Fragment sizes of 172 bp correspond to L allele, whereas 106 bp and 66 bp fragments are diagnostic bands for M allele.
Line 1 Marker. Line 2 PCR product undigested. Lines 3, 6, 9, 10, 14, and 18-20 LM genotype. Lines 4, 5, 7,11-13, 16, and 17 LL genotype. Lines 8, and 15 MM genotype.

Fig. 31: Restriction patterns of the PCR amplified 172 bp fragment after digestion with *Hin1 II*. Fragment sizes of 172 bp correspond to L allele, whereas 106 bp and 66 bp fragments are diagnostic bands for M allele.
Line 1 Marker. Line 2 PCR product undigested. Lines 3, 6-9, 13, 14, 17 and 20 LM genotype. Lines 5, 10, 11, 15, 16, 18, and 19 LL genotype. Lines 4, and 12 MM genotype.

5.4 HEMODIALYSIS PATIENTS

5.4.1 GENOTYPE DISTRIBUTION

On 51 hemodialysis patients PON1 L55M allelic polymorphism was studied.

Genotype distribution is shown in fig. 32.

The most common is LL genotype (49%), followed by LM genotype (40%), and MM genotype is the least common (11%) in HD-patients.

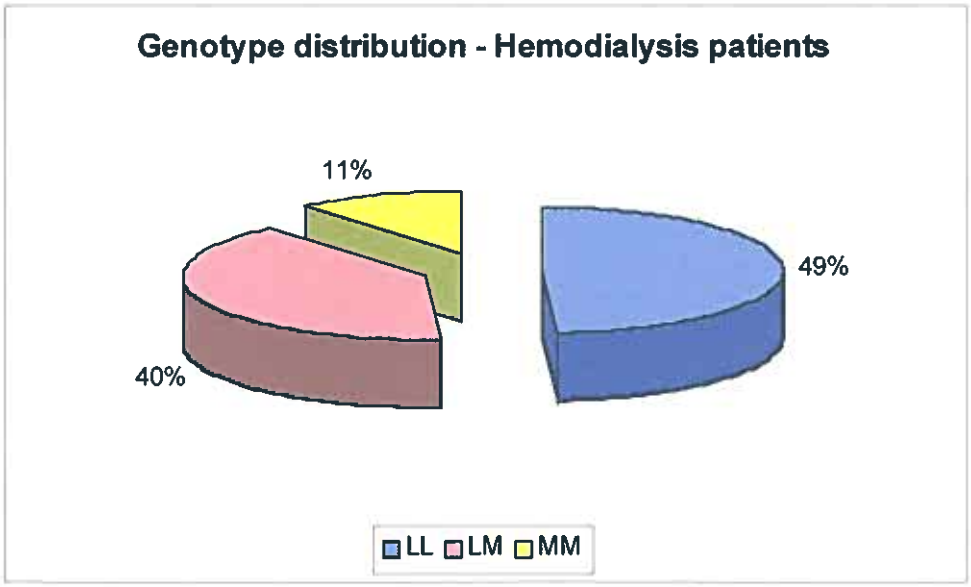

Fig. 32: Genotype frequency in hemodialysis patients.

5.4.2 ALLELE DISTRIBUTION

Allele frequency in control subjects is shown in fig. 33.

The allele L is more frequent (69%), M allele occurs just in 31% of hemodialysis patients.

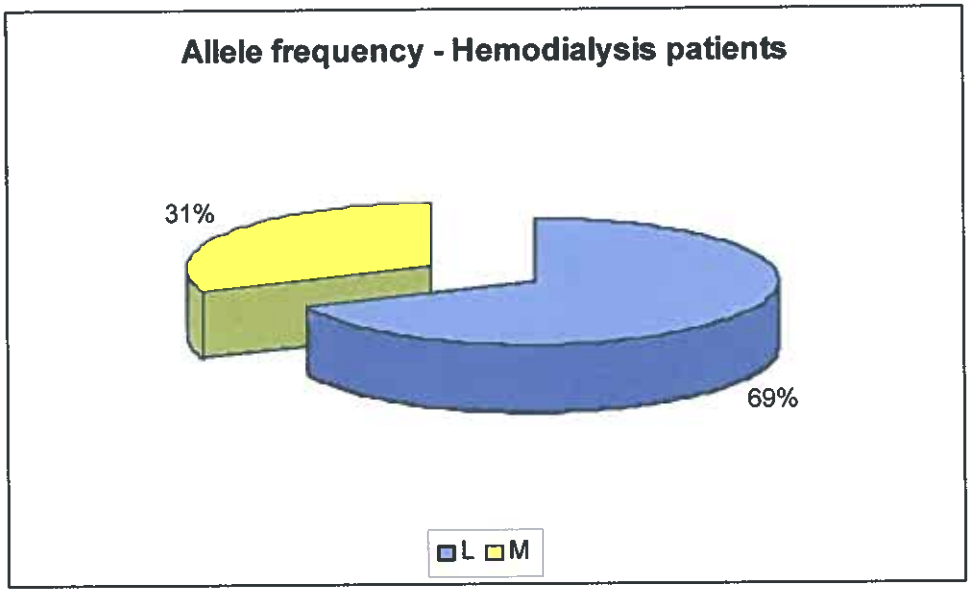

Fig. 33: Allele frequency in hemodialysis patients.

5.5 CONTROL SUBJECTS

5.5.1 GENOTYPE DISTRIBUTION

On 95 control subjects PON1 L55M allelic polymorphism was studied.

Genotype distribution is shown in fig. 34.

The most common is LM genotype (48%), followed by LL genotype (40%), and MM genotype is the least common (12%) in control subjects.

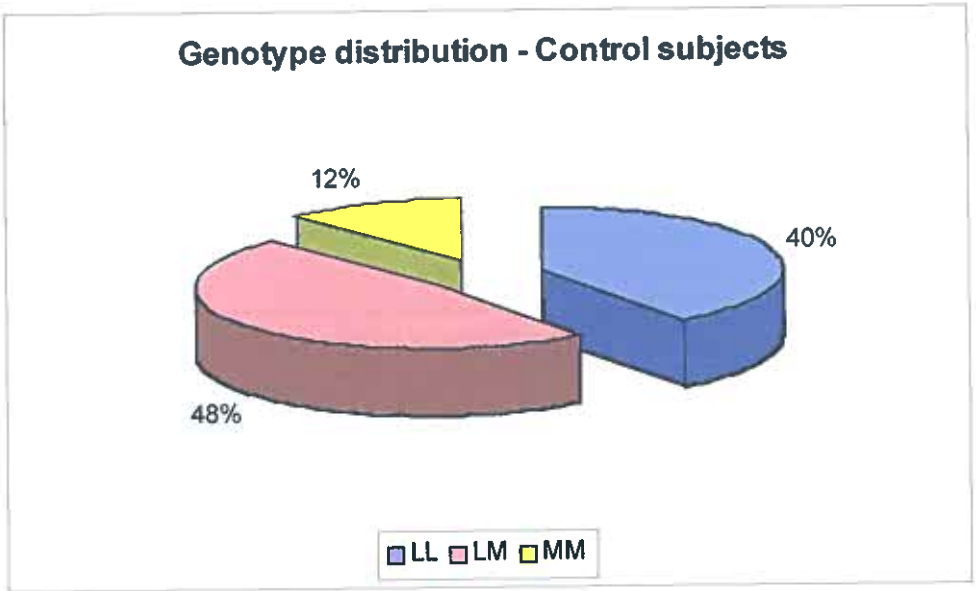

Fig. 34: Genotype frequency in control subjects.

5.5.2 ALLELE DISTRIBUTION

Allele frequency in control subjects is shown in fig. 35.

The allele L is more frequent (64%), M allele occurs just in 36% of control subjects.

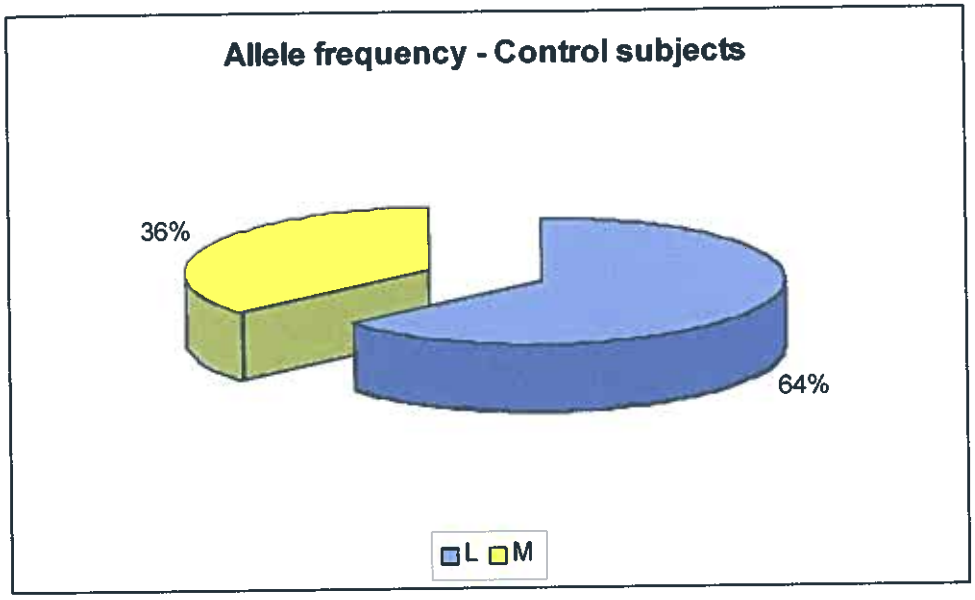

Fig. 35: Allele frequency in control subjects.

5.6 COMPARISON OF STUDIED SUBJECTS

5.6.1 AGE

There were studied 51 hemodialysis patients and 95 control subjects for PON1 L55M allelic polymorphism.

Age distribution of both studied groups is shown in fig. 36.

The most frequent is age from 51 to 75 years in both the hemodialysis patients (55%) and control subjects (50%).

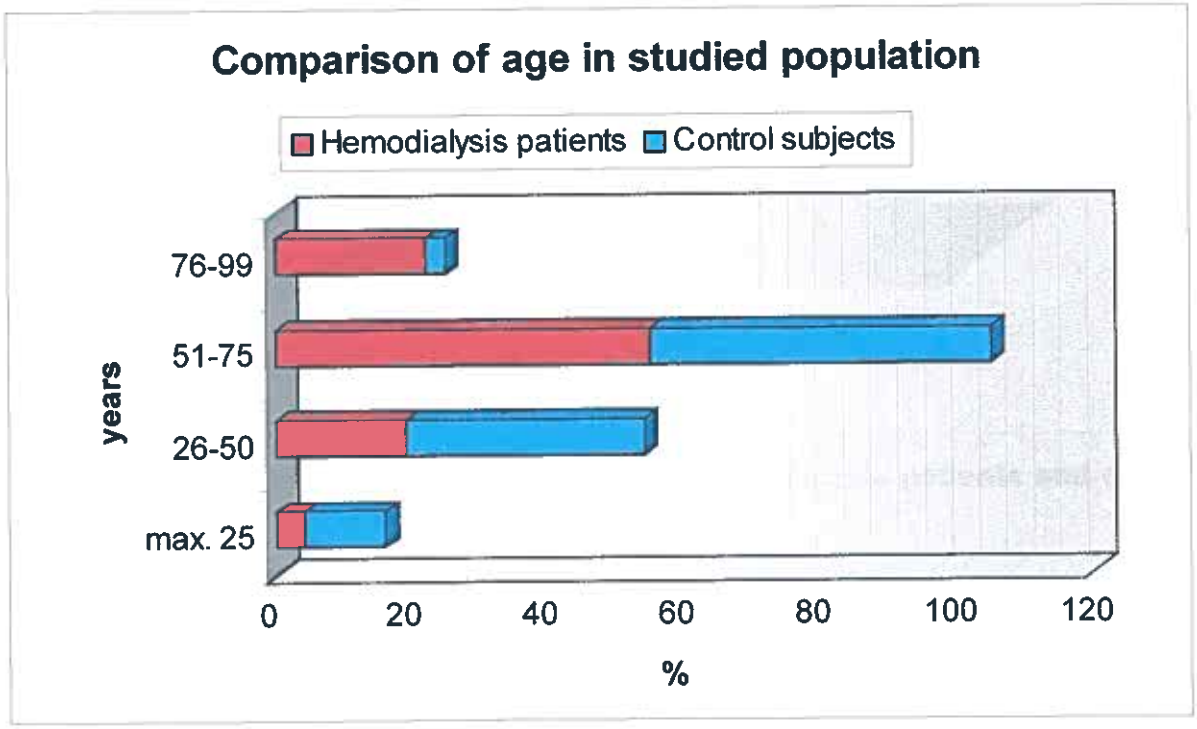

Fig. 36: Comparison of age of hemodialysis patients and control subjects divided into four groups and presented as frequency.

5.6.2 GENDER

Comparison of gender frequency of hemodialysis patients and control subjects is shown in fig. 37.

The most frequent are males (53%) in hemodialysis patients. Females are most frequent (51%) in control subjects.

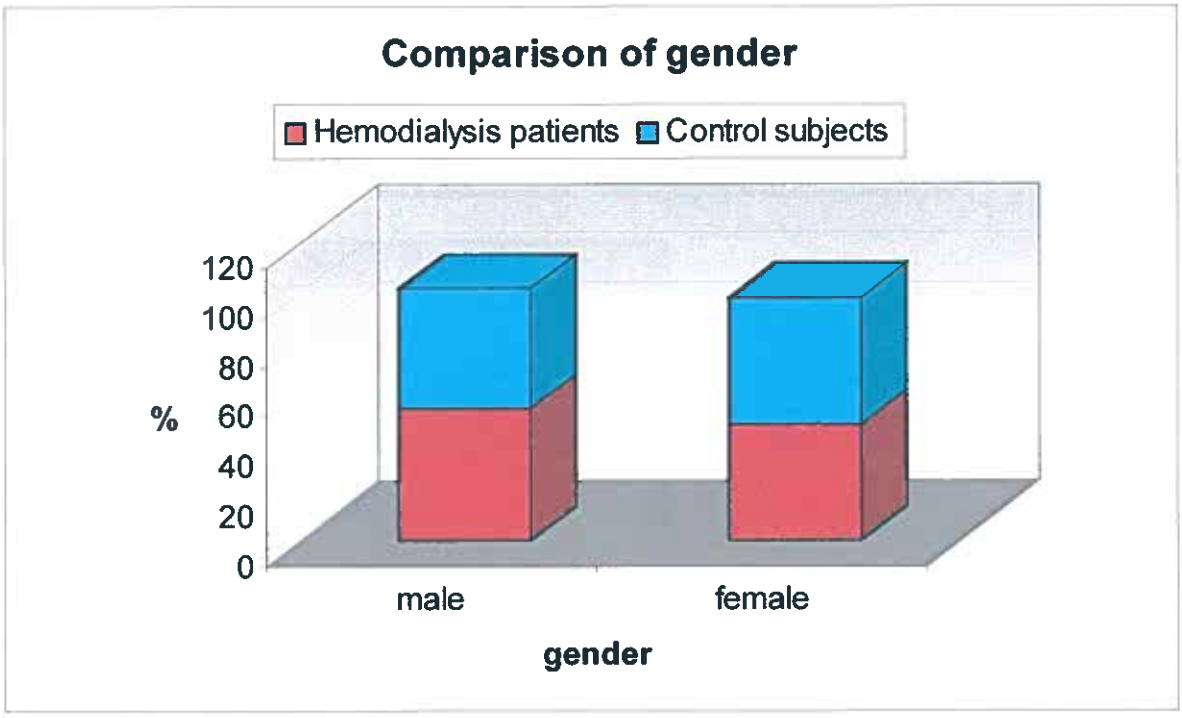

Fig. 37: Comparison of gender frequency in hemodialysis patients and control subjects.

5.6.3 AGE AND GENDER

Age and gender distribution in studied population is shown in fig. 38 and fig. 39. The most frequent is age from 51 to 75 years in both the male and female hemodialysis patients as well as in control subjects.

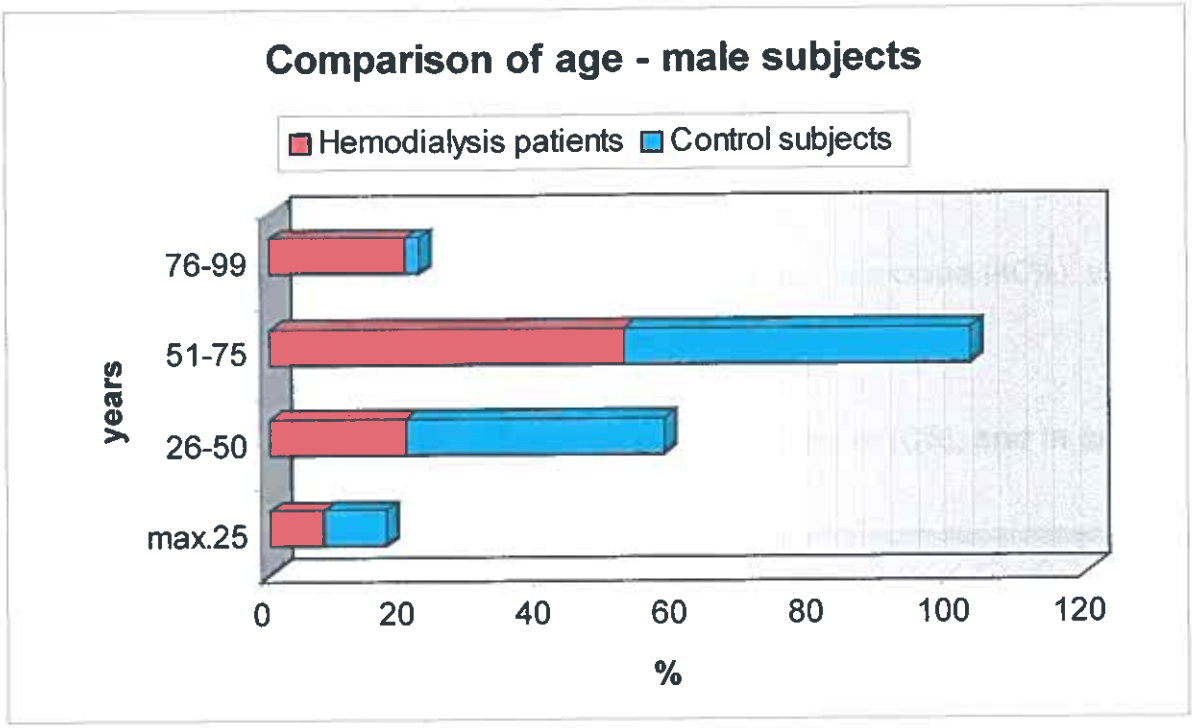

Fig. 38: Comparison of age of male hemodialysis patients and male control subjects divided into four groups and presented as frequency.

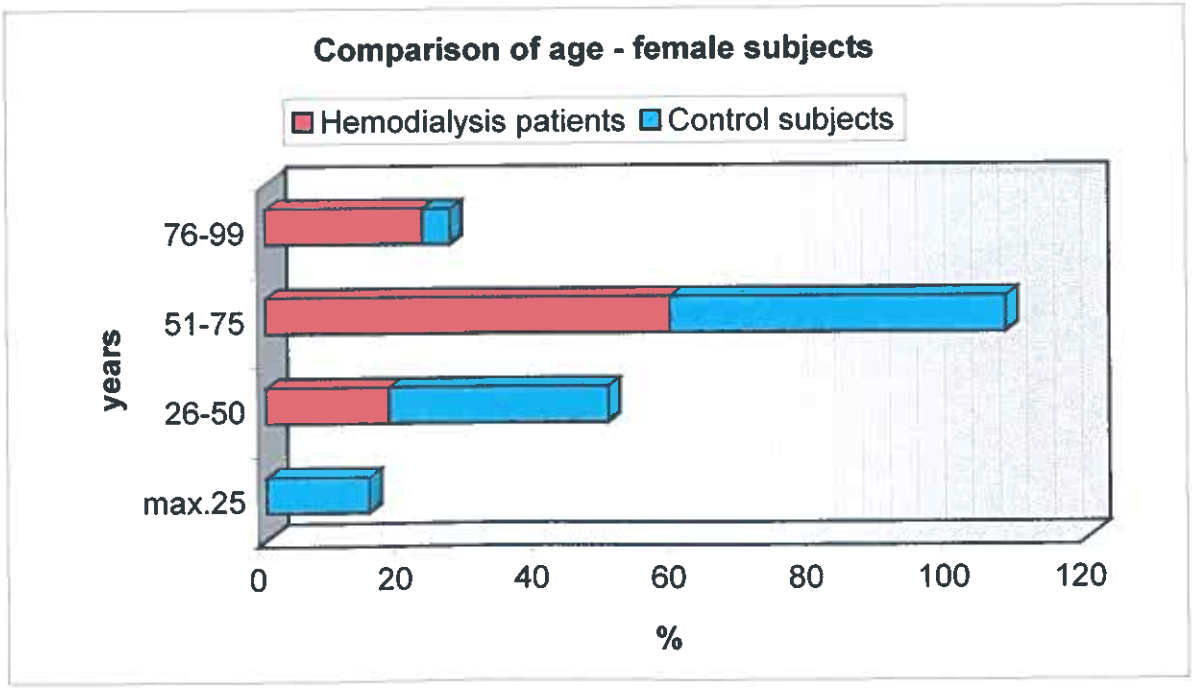

Fig. 39: Comparison of age of female hemodialysis patients and female control subjects divided into four groups and presented as frequency.

5.6.4 GENOTYPE

On 51 HD-patients and on 95 control subjects PON1 L55M allelic polymorphism was studied.

Genotype distribution is shown in tab.7 and fig.40.

The most common is LL genotype (49%), followed by LM genotype (40%), and MM genotype is the least common (11%) in hemodialysis patients.

The most common is LM genotype (48%), followed by LL genotype (40%), and MM genotype is the least common (12%) in control subjects.

Tab. 7: Genotype distribution in group of control subjects (CS), and in group of hemodialysis patients (HD).

Genotype	Subject	Total number	Frequency [%]
LL	CS	37	40.22
	HD	23	48.93
LM	CS	44	47.83
	HD	19	40.43
MM	CS	11	11.95
	HD	5	10.64

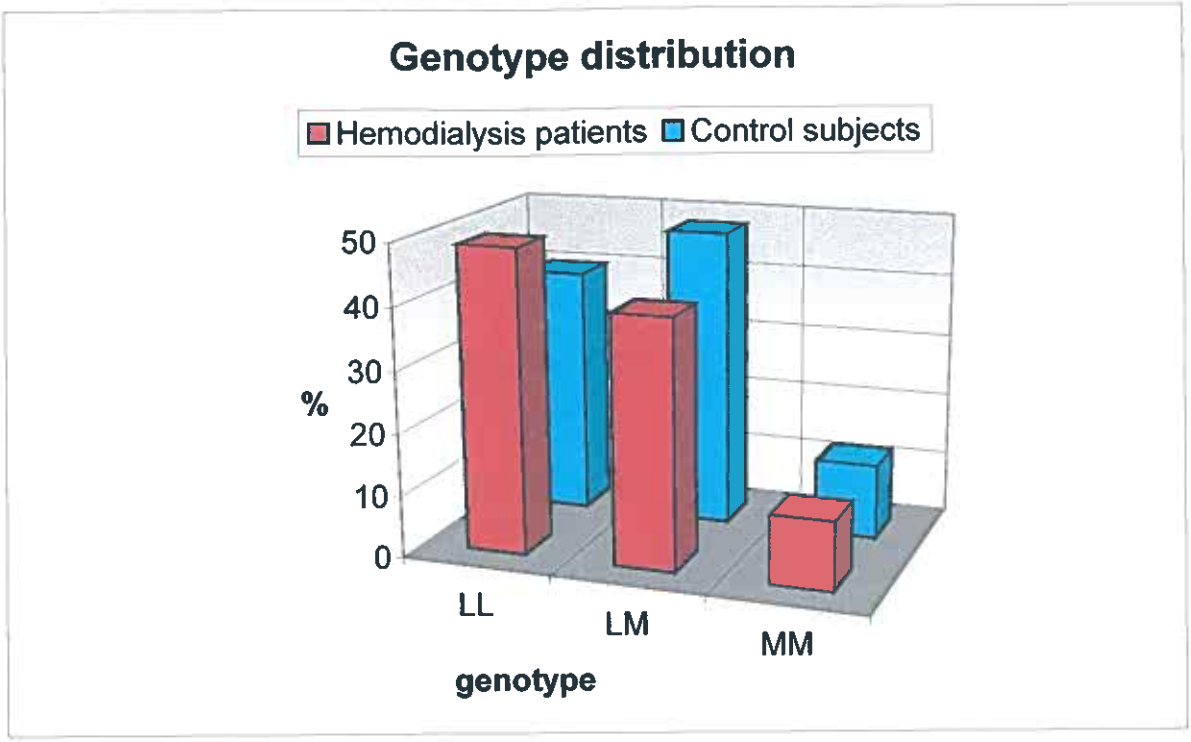

Fig. 40: Comparison of genotype distribution of hemodialysis patients and control subjects.

5.6.5 GENOTYPE AND GENDER

Genotype and gender distribution in studied population is shown in fig. 41 and fig. 42.

The most common is LL genotype, followed by LM genotype, and MM genotype is the least common in male subjects.

The most common is LM genotype, followed by LL genotype, and MM genotype is the least common in female subjects.

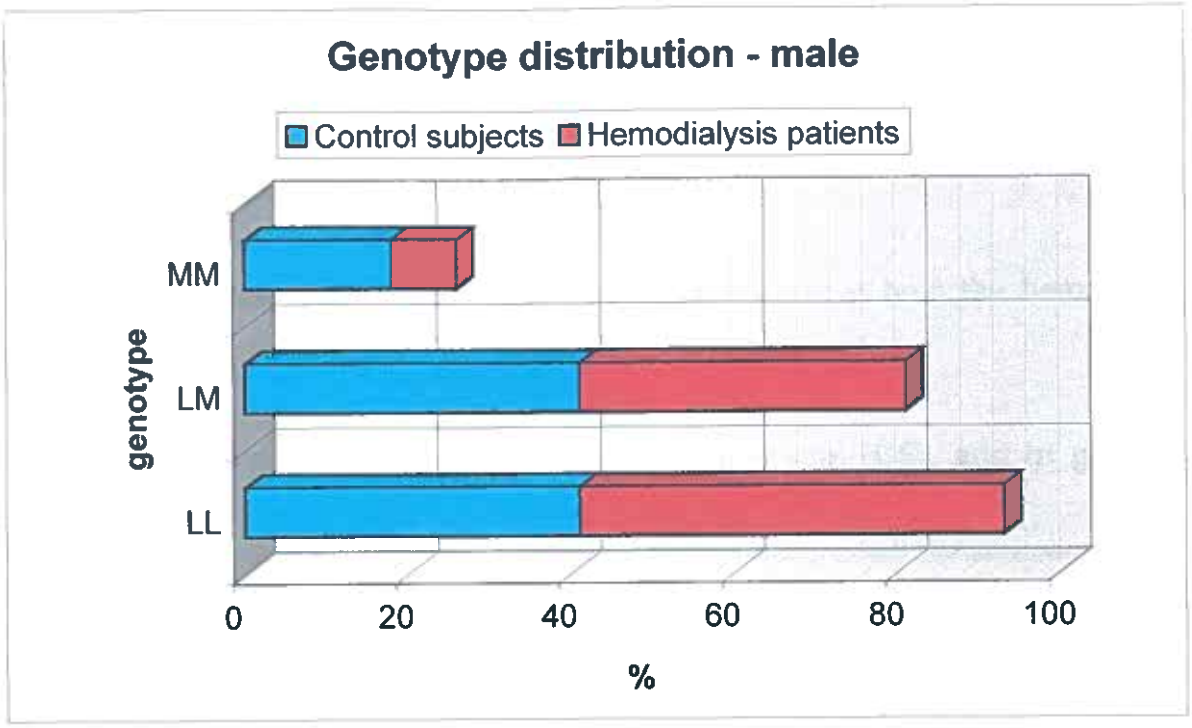

Fig. 41: Comparison of genotype distribution of male hemodialysis patients and male control subjects.

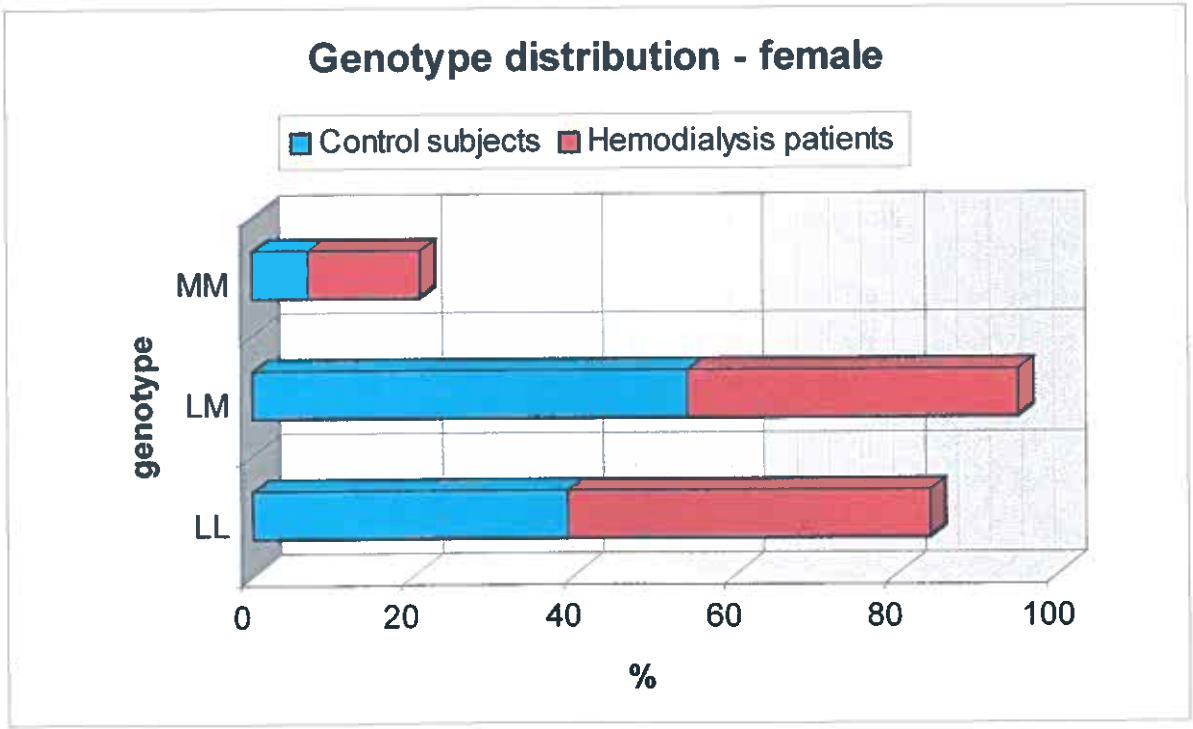

Fig. 42: Comparison of genotype distribution of female hemodialysis patients and female control subjects.

5.6.6 ALLELE

Allele distribution is shown in tab.8 and fig.43.

The most common is L allele, M allele is the least one in both the hemodialysis patients and control subjects.

Tab. 8: Allele frequency in group of control subjects (CS), and in group of hemodialysis patients (HD).

Allele	Subjects	Frequency [%]
L	CS	64.13
	HD	69.15
M	CS	35.87
	HD	30.85

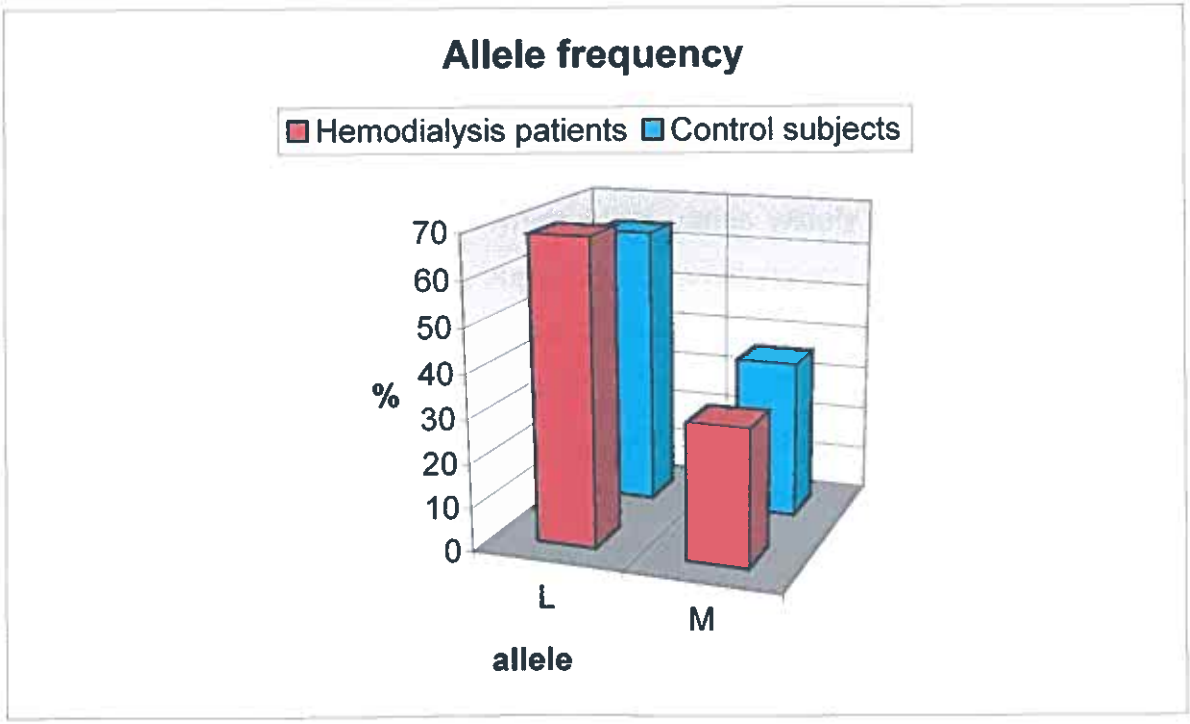

Fig. 43: Comparison of allele frequency of hemodialysis patients and control subjects.

6 DISCUSSION

This thesis is aimed to determine the frequency of L55M allelic polymorphism of human paraoxonase gene (PON 1 L55M) in population of patients undergoing hemodialysis treatment and blood donors (control subjects). These subjects were collected in The Clinical Hospital Split, Croatia. All studied subjects live in the region of Slavonski Brod, Croatia.

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 2004-2008 periods. My master thesis research was done due to CEEPUS mobility HR-0046 in Zagreb, Croatia.

Genomic DNA was isolated from whole blood by the salting out method of Miller, with some modifications. The polymerase chain reaction/restriction fragments length polymorphism (PCR/RFLP) technique was used to map PON1 L55M (Leu55Met) genotype distribution in observed population.

There was found approximately 55.5% subjects with PON1 L55M polymorphic (Met55 allele homozygote or heterozygote) and 44.5% subjects with no polymorphism (Leu55 homozygote - wild type) in studied population (HD and CS together). Frequency of L55M polymorphism varies widely among individuals and populations of different ethnic groups, such variations appearing to be related to PON1 serum levels and activity of this enzyme. Santos et al. [115] showed that PON 1 LL genotype is more frequent then MM haplotype in all Amerindians and Asian populations. Ahmed et al [116] have reported that the distribution of PON 1 L55M allelic polymorphism was significantly different between black and white women. However, Ferre et al. [117] and Van Lenten et al. [118] found no significant differences in PON 1 L55M 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.

It was investigated that PON 1 55L allele is relatively homogenous, with frequencies ranging from 93% for the Japanese population to 97% for the Amerindian population

[115]. Our research revealed PON 1 L55 allele in 96% of studied subject - Croatian population. We have studied the difference between the frequency for hemodialysis patients and control subjects. We have determined that L allele is the most common allele for PON 1 L55M polymorphism in hemodialysis patients as well as in group of control subjects. The allele frequency for PON1 L55M allelic polymorphism did differ in 5% of hemodialysis patients (69% L allele, 31% M allele) and control subjects (64% L allele, 36% M allele).

Hemodialysis (HD) patients are in a high risk of atherosclerosis and coronary heart disease. It is becoming increasingly apparent that metabolic changes, abnormalities in oxidative stress, are central to the pathogenesis of coronary heart disease. Therefore it is possible for antioxidant enzymes such as PON 1 to play an important role in the development of cardiovascular disease (CVD).

CVD is the most common cause of mortality in HD-patients. Some studies have indicated a significant association of PON1 L55M polymorphism and CVD [119]. However, a number of other studies [61, 120] have not reported the presence of this association. We found that the distribution of PON 1 L55M genotypes significantly differes in frequency of LL (in 9% of cases) and LM (in 8% of cases) genotype in hemodialysis patients (HD) and control subjects (CS). MM genotype frequency did not differ significantly in HD and CS. This study suggested that hemodialysis is associated rather with M allele appearance than with MM genotype for PON 1 L55M allelic polymorphism.

Based on studies mentioned above, studies on the PON 1 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 PON 1 L55M 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 PON 1 L55M genotype, CVD and hemodialysis treatment.

The second possible factor is that the promoter polymorphisms of PON 1 gene can influence the association between the PON 1 L55M 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. [121] and Attman et al. [84] 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 [122]. In addition to the role of HDL in reverse cholesterol transport, it has the ability to protect LDL against oxidation [75, and 123, 124]. 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.

Many studies [104, 119, and 93] have suggested that variation in PON1 genotype in population is associated with variations in lipoprotein profile. However, there are studies that did not confirm this results [120, 125, and 126]. These contradictory results originated from studies conducted in various ethnicities and they could be explained partly by differences in PON 1 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 [127]. 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 [124]. These results are in agreement with the assertion that inflammation enhances cardiovascular risk and mortality in HD-patients [127].

Some reports [28, 125, and 128] suggested that PON1 activity and PON 1 concentration are better predictors of CVD than PON 1 genotype. Finally, PON 1 genotypes remain important in the study of PON 1 role in CVD as potential death danger for hemodialysis patients. Provided that researchers clearly understand the physiological substrates of PON 1, we can determine exactly the activity of PON and than explore the relationship between PON gene polymorphisms and hemodialysis and its mechanisms, which will redound to the therapy of hemodialysis and even other related diseases.

In conclusion, further studies are necessary to estimate the role of PON 1 allelic polymorphism in pathogenesis of CVD. Furthermore, it is important to determine how PON 1 enzyme concentration and activity are involved in the development of atherosclerosis in patients undergoing hemodialysis treatment. Both functional and genetic studies are needed to test the nature of the association described in this study and to detect new mutations in the gene cluster, acting as susceptible loci for vascular disorders.

7 SUMMARY

This research takes part in a complex genotypisation for human paraoxonase PON 1 allelic polymorphisms in Croatian population of blood donors. Some results of this thesis are summarised in following paper „Genetic Frequencies of Paraoxonase 1 Gene Polymorphisms in Croatian Population", authors Marija Grdić, Karmela Barišić, Lada Rumora, Ilza Salamunić, Milena Tadijanović, Tihana Žanić Grubišić, Renata Pšikalová, Zlata Flegar-Meštrić, and Dubravka Juretića; published in Croatica Chemica Acta. The hemodialysis patients' genotypisation is used in research of comparison of human paraoxonase allelic polymorphism in hemodialysis patients and control subjects in Croatia (not published yet).

I have summarized almost all available information in the field of paraoxonase research in theoretical part of my Master 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 have done genotypisation for human paraoxonase PON 1 L55M allelic polymorphism of hemodialysis patients (HD) and control subjects (CS) 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 L55M allelic polymorphism and hemodialysis. I mapped gender and age distribution of studied population and compared it.

There were approximately 55.5% PON1 L55M polymorphic subjects and 44.5% subjects with no PON1 L55M polymorphism. The distribution of PON1 L55M genotypes in HD (11% MM homozygote, 40% LM heterozygote, and 49% LL wild type) did differ from control subjects (12% MM homozygote, 48% LM heterozygote, and 40% LL wild type).

I have determined that L allele is the most common allele for PON 1 L55M polymorphism in hemodialysis patients as well as in group of control subjects. The allele frequency for PON1 L55M allelic polymorphism did differ in 5% of hemodialysis patients (69% L allele, 31% M allele) and control subjects (64% L allele, 36% M allele).

This study suggested that hemodialysis is associated rather with M allele appearance than with MM genotype for PON 1 L55M allelic polymorphism.

8 CONCLUSION

A. The distribution of genotype for PON1 L55M allelic polymorphism was determined in hemodialysis patients (HD): 11% MM homozygote, 40% LM heterozygote, and 49% LL wild type. There are approximately 51% PON1 L55M polymorphic subjects and 49% subjects with no PON1 L55M polymorphism (LL wild type) in HD.

B. The distribution of genotype for PON1 L55M allelic polymorphism was determined in control subjects (CS): 12% MM homozygote, 48% LM heterozygote, and 40% LL wild type. There are approximately 60% PON1 L55M polymorphic subjects and 40% subjects with no PON1 L55M polymorphism (LL wild type) in CS.

C. There is significant difference in frequency of LL (9%) and LM (8%) in HD and CS. MM genotype frequency did not differ significantly in HD and CS. There are approximately 55.5% PON1 L55M polymorphic subjects and 44.5% subjects with no PON1 L55M polymorphism (LL wild type) in studied population (HD and CS together).

D. The allele frequency for PON1 L55M allelic polymorphism did differ in 5% in hemodialysis patients (69% L allele, 31% M allele) and control subjects (64% L allele, 36% M allele). The most common is L allele for both the HD and CS subjects.

E. The gender and age distribution was determined in studied subjects. The most common is male (51%). The average age in studied population is approximately 55 years.

F. The gender distribution did differ in 4% in hemodialysis patients (53% male, 47% female) and control subjects (49% male, 51% female). Age distribution differs significantly (24%) in the group not older 5O years in HD (23%) and CS (47%). The most frequent age is between 51 and 75 years in both the HD (55%) and CS (51%).

9 ABBREVIATIONS

A	adenine
Apo	apolipoprotein
Arg	arginine
ATP	adenosine triphosphate
AV	arteriovenous
AVF	arteriovenous fistula
AVG	arteriovenous graft
C	cytosine
CAD	coronary artery disease
CAPD	continuous ambulatory peritoneal dialysis
CHD	coronary heart disease
CRF	chronic renal failure
CRP	C-reactive protein
CS	control subjects
CVD	cardiovascular disease
DM	diabetes mellitus
EC	enzyme classification
EDTA	ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
Fig.	Figure
G	guanine
Glu	glutamine
HD	hemodialysis
HDL	high density lipoprotein(s)
HD-patient	hemodialysis patient
HOCl	hypochlorous acid
IL	interleukin
kb	kilo-base
L	leucine
LB-buffer	loading buffer
LCAT	lecithin-cholesterol acyltransferase
LD	linkage disequilibrium
LDL	low density lipoprotein(s)

LP	lipoprotein(s)
LP(a)	lipoprotein a
M	methionine
M/F	male/female
oxLDL	oxidized LDL
P	probability
PAF	platelet-activating factor
PAF-AH	PAF-acetylhydrolase
pb	base pair
PCR	polymerase chain reaction
PON	paraoxonase
pon	paraoxonase gene
RFLP	restriction fragments length polymorphism
SDS	sodium dodecyl sulphate
T	thymine
Tab.	Table
TAG	Triacylglycerides
Taq	*Thermus aquaticus*
TC	total cholesterol
U	uracil
WT	wild type

10 REFERENCES

[1] The Foundation of Kidney Care, Canada, 1998.

[2] Kent M. Van De Graaff, Stuart Ira Fox, Karen M. LaFleur; Synopsis Of Human Anatomy And Physiology; The Mc Graw-Hill Companies, Inc.; 1997; Unit IV, Chapter 19: 498-499.

[3] Stuart Ira Fox; Human Physiology; The Mc Graw-Hill Companies, Inc.; 1996; Chapter 17: 533.

[4] National Institute of Diabetes and Digestive and Kidney Diseases, NIH Publication No. 07 – 4666, December 2006.

[5] The Foundation of Kidney Care, Canada, 2006.

[6] National Kidney Foundation, USA, 1998.

[7] Godfrey S. Getz, Catherine A. Reardon; Paraoxonase, a cardioprotective enzyme: continuing issues; Current Opinoin in Lipidology 2004, 15:261-267.

[8] S. L. Primo – Parmo, R. C. Sorenson, J. Teiber, B. N. LaDu: The human serum paraoxonase / arylesterase gene (PON 1) is one member of a multigene family; Genomics 1996, 33: 498-507.

[9] S. Campo, A. M. Sardo, G. M. Campo, A. Avenoso, M. Castaldo, A. D'Ascola, E. Giunta, A. Calatroni, A. Saitta; Identification of paraoxonase 3 gene (PON 3) missense mutations in a population of Southern Italy; Mutation Research 2004, 546: 75-80.

[10] C. E. Furlong, L. G. Costa, C. Hassett, R. J. Richter, J. A. Sundstrom, D. A. Adler, C. M. Disteche, C. J. Omiecinsky, C. Chapline, J. W. Crabb; Human and rabbit paraoxonases: purification, cloning, sequencing, mapping, and role of polymorphism in organophosphate detoxication; Chem. Biol. Interact 1993, 87: 35-48.

[11] K. S. Robertson, et al.; Human paraoxonase gene cluster polymorphisms as predictors of coronary heart disease risk in the prospective Northwick Park Heart Study II; Biochemica et Biophysica Acta 2003, 1639: 203-212.

[12] B. Mackness, P. N. Durrington, M. I. Mackness; Human serum paraoxonase; Gen. Pharmacol. 1998, 31: 329-336.

[13] B. Mackness, M. I. Mackness, S. Arrol, W. Turkie, P. N. Durrington; Effect of the molecular polymorphisms of human paraoxonase (PON 1) in the rate of hydrolysis of paraoxon; Br. J. Pharmacol. 1997, 122: 265-268.

[14] L. Rodrigo, B. Mackness, P. N. Durrington, A. Hemandez, M. I. Mackness; Hydrolysis of platelet – activating factor by human serum paraoxonase; Biochem. J. 2001, 354: 1-7.

[15] O. Rozenberg, D. M. Shih, M. Aviram; Human serum paraoxonase 1 decreases macrophage cholesterol biosynthesis: possible role for its phospholipase-A_2-like activity and lysophosphatidylcholine formation; Arterioscler. Thromb. Vasc. Biol. 2003, 23: 461-467.

[16] P. N. Laplaud, T. Dantoine, M. J. Chapman; Paraoxonase as a risk marker for cardiovascular disease: facts and hypotheses; Clin. Chem. Lab. Med. 1998, 36 (7): 431-441.

[17] A. D. Watson, J. A. Berliner, S. Y. Hama, B. N. LaDu, K. F. Faull, A. M. Fogelman, M. Navab; Protective effect of high density lipoprotein associated with paraoxonase; J. Clin. Invest. 1995, 96: 2882-2891.

[18] L. G. Costa, T. B. Cole, G. P. Jarvik, C. E. Furlong; Functional genomics of the paraoxonase (PON1) polymorphisms: effects of pesticide sensitivity, cardiovascular disease, and drug metabolism; Annu. Rev. Med. 2003, 54: 371-392.

[19] L. G. Costa, W. F. Li, R. J. Richter, D. M. Shih, A. Lusis, C. E. Furlong; The role of paraoxonase (PON 1) in the detoxification of organophosphates and its human polymorphism; Chem. Biol. Interact. 1999, 119/120: 429-438.

[20] R. J. Richter, C. E. Furlong; Determination of paraoxonase (PON 1) status requires more than genotiping; Pharmacogenetics 1999, 9. 745-753.

[21] I. Leviev, R. W. James; Promoter polymorphisms of human paraoxonase PON 1 gene and serum paraoxonase activities and concentrations; Arterioscler. Thromb. Vasc. Biol. 2002, 20: 516-521.

[22] D. I. Draganov, B. N. LaDu; Pharmacogenetics of paraoxonase: a brief review; Naunyn Schmiedebergs Arch Pharmacol 2004, 369: 78-88.

[23] M. Aviram, S. Billecke, R. Sorenson, et al. ; Paraoxonase active site required for protection against LDL oxidation involves its free sulphhydryl group and is different from that required for its arylesterase / paraoxonase activities; Arterioscler. Thromb. Vasc. Biol. 1998, 18: 1617-1624.

[24] R. C. Sorenson, S. L. Primo – Parmo, C. L. kuo, S. Adkins, O. Lockridge, B. N. LaDu; Reconsideration of the catalytic centre and mechanism of mammalian paraoxonase / arylesterase; Proc. Nat. Acad. Sci. USA 1995, 92: 7187-7191.

[25] D. Josse, W. Xie, F. Renault, et al.; Identification of residues essential for human paraoxonase (PON 1) arylesterase / organophosphatase activities; Biochemistry 1999, 38: 2816-2825.

[26] C. Hassett, R. J. Richter, R. Humbert, C. Chapline, J. W. Crabb, C. J. Omiecinski, et al. ; Characterization of cDNA clones encoding rabbit and human serum paraoxonase: the mature protein retains its signal sequence; Biochemistry 1991, 30: 10141-10149.

[27] C. E. Furlong, W. F. Li, D. M. Shih, et al. ; Genetic factors in susceptibility: serum PON 1 variation between individuals and species; Hum. Ecol. Risk Assess. 2002, 8: 31-43.

[28] G. P. Jarvik, T. S. Hatsukami, C. Carlson, et al. ; Paraoxonase activity, but not haplotype utilizing the linkage disequilibrium structure, predicts vascular disease; Arterioscler. Thromb. Vasc. Biol. 2003, 23: 1465-1471.

[29] W. N. Aldridge; A – esterases and B - esterases in perspective. In: E. Reiner, W. N. Aldridge, F. C. G. Hoskin, editors; Enzyme hydrolyzing organophosphorous compounds; Chichester (UK): Elis Horwood Limited;1989, p. 1-14.

[30] R. E. Poore, R. A. Neal; Evidence for extra hepatic metabolism of parathion; Toxicol. Appl. Pharmacol. 1972, 23: 759-768.

[31] F. D. Brites, J. Verona, L. E. Schreier, J. Ch. Fruchart, G. R. Castro, R. L. Wikinski; Paraoxonase 1 and platelet-activating factor acetylhydrolase activities in patients with low HDL-cholesterol levels with or without primary hypertriglyceridemia; Archives of Medical Research 2004, 35: 235-240.

[32] H. G. Davies, R. J. Richter, M. Keifer, C. A. Broomfield, J. Sowalla, C. E. Furlong; The effect of the human serum paraoxonase polymorphism is reversed with diazoxon, soman and sarin; Nat. Genet. 1993, 3: 73-76.

[33] M. I. Mackness; Why plasma should not be used to study paraoxonase; Atherosclerosis 1998, 136: 195-196.

[34] J. A. Vitarius, L. G. Sultatos; Kinetic mechanism of the detoxification of the organophosphate paraoxon by human serum A – esterase; Drug. Metab. Disp. 1994, 22: 472-478.

[35] J. A. Vitarius, L. G. Sultatos; The role of calcium in hydrolysis of organophosphate paraoxon by human serum A – esterase; Life Sci. 1995, 56: 125-134.

[36] M. C. Blatter, R. W. James, S. Messmer, F. Barja, D. Pometta; Identification of a distinct human HDL subspecies defined by a lipoprotein-associated protein, K-49; Eur. J. Biochem. 1993, 211: 871-879.

[37] M. Navab, J. A. Berliner, G. Suffanagounder, et al. ; HDL and the inflammatory response induced by LDL-derived oxidized phospholipids; Arterioscler. Thromb. Vasc. Biol. 2001, 21: 481-488.

[38] S. Deakin, I. Leviev, M. Gomaraschi, et al. ; Enzymatically active paraoxonase 1 is located at the external membrane of producing cells and released by a high affinity, saturable, desorption mechanism; J. Biol. Chem. 2002, 277: 4301-4308.

[39] G. J. Kelso, W. D. Stuart, R. J. Richter, C. E. Furlong, T. C. Jordan- Starck, J. A. K. Harmony; Apolipoprotein J is associated with paraoxonase in human plasma; Biochemistry 1994, 33; 832-839.

[40] D. E. Jenne, B. Lowin, M. C. Peitsch, et al. ; Clusterin (complement lysis inhibitor) forms a HDL complex with apolipoprotein A-I in human plasma; j. Biol. Chem. 1991, 266: 11030-11036.

[41] N. J. Beaumont, V. O. Skinner, T. M. Tan, et al. ; Ghrelin can bind to a species of HDL associated with paraoxonase; J. Biol. Chem. 2003, 278: 8877-8880.

[42] H. V. De Silva, W. D. Stuart, C. R. Duvic, J. R. Wetterau, M. J. Ray, D. G. Ferguson, et al. ; A 70-kDa apolipoprotein designated apoJ is a marker for subclasses of human plasma HDL; J. Biol. Chem. 1990, 265: 13240-13247.

[43] R. C. Sorenson, C. L. Bisgaier, M. Aviram, et al. ; Human serum paraoxonase / aryl esterase's retained hydrophobic N-terminal leader sequence associated with HDLs by binding phospholipids; Arterioscler. Thromb. Vasc. Biol. 1999, 19: 2214-2225.

[44] V. G. Cabana, C. A. Reardon, N. Feng, et al. ; Serum paraoxonase: effect of the apolipoprotein composition of HDL and the acute phase response; J. Lipid. Res. 2003, 44: 780-792.

[45] A. Graham, D. G. Hassall, S. Rafique, J. S. Qwen; Evidence for a paraoxonase – independent inhibition of LDL oxidation by HDL; Atherosclerosis 1997, 135: 193-204.

[46] L. Jaouad, C. Milochevitch, A. Khalil; PON 1 paraoxonase activity is reduced during HDL oxidation and is an indicator of HDL antioxidant capacity; Free Radic. Res. 2003, 37:77-83.

[47] A. D. Watson, M. Navab, S. Y. Hama, A. Sevanian, S. M. Prescott, D. M. Stafforini, et al. ; Effect of platelet activating factor – acetylhydrolase on the formation and action of minimally oxidized LDL; J. Clin. Invest. 1995, 95: 774-782.

[48] M. Aviram, M. Rosenblat, C. L. Bisgaier, R. S. Newton, S. L. Primo Parmo, B. N. LaDu; Paraoxonase inhibits HDL oxidation and preserves its function. A possible peroxidative role for paraoxonase; J. Clin. Invest. 1998, 101: 1581-1590.

[49] M. I. Mackness, S. Arol, P. N. Durrington; Paraoxonase prevents accumulation of lipoperoxides in LDL; FEBS Lett. 1991, 286: 152-154.

[5O] S.T. Reddy, D. J. Wadleigh, V. Grijalva, C. Ng, S. Hama, A. Gangopadhyay, et al.; Human paraoxonase-3 is an HDL-associated enzyme withbiological activity similar to paraoxonase-1 protein but is not regulated by oxidized lipids; Arterioscler. Thromb. Vasc. Biol. 2001, 21: 542-547.

[51] B. N. La Du; Is paraoxonase-3 another HDL-associated protein protective against atherosclerosis? ; Arterioscler. Thromb. Vasc. Biol. 2001, 21: 467-468.

[52] Z. Ahmed, A. Ravandi, G. F. Maguire, et al.; Multiple substrates for paraoxonase-1 during oxidation of phosphatidylcholine by peroxinitrite; Biochem. Biophys. Res. Commun. 2002, 290: 391-396.

[53] R. Schiavon, P. Battaglia, E. De Fanti, A. Fasolin, S. Biasioli, L. Targa, G. Guidi; HDL 3 related decreased serum paraoxonase (PON) activity in uremic patients: comparison with PON 1 allele polymorphism; Clinica Chemica Acta 2002, 324: 39-44.

[54] M. Yoshikawa, N. Sakuma, T. Hibino, T. Sato, T. Fujinami; HDL 3 exerts more powerful anti-oxidative, protective effects against copper-catalyzed LDL oxidation than HDL 2; Clin. Biochem. 1997, 30: 221-225.

[55] R. Zech, M. Rockseisen, K. Kluge, K. Dewald, V. W. Armstrong, J. M. Chemnitius; Lipoproteins and hydrolysis of organophosphorous compounds; Chem. Biol. Interact. 1993, 87: 85-94.

[56] B. Mackness, M. Mackness; Paraoxonase 1: biochemistry and contribution to atherosclerosis; International Congress Series 2004, 1262: 91-94.

[57] Z. Ahmed, S. Babaei, G. F. Maguire, et al.: Paraoxonase-1 reduces monocyte chemotaxis and adhesion to endothelial cells due to oxidation of palmitoyl, linoleoyl glycerophosphorylcholine; Cardiovasc. Res. 2003, 57: 225-231.

[58] O. Rozenberg, M. Rosenblat, R. Coleman, et al.; Paraoxonase (PON) deficiency is associated with increased macrophage oxidative stress: studies in PON-1 knockout mice; Free Radic. Biol. Med. 2003, 34: 774-784.

[59] D. M. Shih, Y. R. Xia, Y. P. Wang, et al.; Combined serum paraoxonase knockout/apolipoprotein E knockout mice exhibit increased lipoprotein oxidation and atherosclerosis; J. Biol. Chem. 2000, 275: 17527-17535.

[60] B. Fuhrman, N. Volkova, M. Aviram; Oxidative stress increases the expression of the CD36 scavenger receptor and the cellular uptake of oxidized LDL in macrophages from atherosclerotic mice: protective role of antioxidants and of paraoxonase; Atherosclerosis 2002, 161: 307-316.

[61] R. A. Hegele; Paraoxonase genes and disease; Ann Med 1999, 31: 217-224.

[62] Leus F. R.,et al.; PON 2 gene variants are associated with clinical manifestation of cardiovascular disease in familial hypercholesterolemia patients; Atherosclerosis 2001, 154: 641-649.

[63] Gunnarsdottir I., et al.: Size at birth and coronary artery disease in a population with high birth weight; Am J Clin Nutr 2002, 76: 1290-1294.

[64] Chen Q., et al.: Association between the severity of angiographic coronary artery disease and paraoxonase gene polymorphisms in the National Heart, Lung, and Blood Institute-sponsored WISE study; Am J Hum Genet 2003, 72: 13-22.

[65] Pan J. P., et al.; The risk of coronary artery disease in population in Taiwan is associated with C311S polymorphism of PON 2 gene; Zhonghua Yi Xue Za Zhi 2002, 65: 415-421.

[66] Pinizzotto M., et al.; PON 2 polymorphisms are associated with nephropathy in type II diabetes; Diabetologia 2001, 44: 104-107.

[67] Busch C. P., et al.; Association of PON 2 variation with birth weight in Trinidadian neonates of South Asian ancestry; Pharmacogenetics 1999, 9: 351-356.

[68] Draganov D. I., et al.; Rabbit serum PON 3 is a HDL-associated lactonase and protects LDL against oxidation; J Biol Chem 2000, 275: 33435-33442.

[69] Reddy S. T., et al.; PON 3 is an HDL-associated enzyme with biological activity similar to PON 1 protein but is not regulated by oxidized lipids; Arterioscler Thromb Vasc Biol 2001, 21: 542-547.

[70] R. Schiavon, E. De Fanti, D. Giavarina, S. Biasioli, G. Cavalcanti, G. Guidi; Serum paraoxonase activity is decreased in uremic patients; Clin. Chem. Acta 1996, 247: 71-80.

[71] T. F. Dantoine, J. Debord, J-P Charmes, et al. ; Decrease of serum PON activity in chronic renal failure; J. Am. Soc. Nephrol. 1998, 9: 2082-2088.

[72] O. Hasselwander, J. Mc Eneny, D. Mc Master, et al.; HDL composition and HDL antioxidant capacity in patients on regular haemodialysis; Atherosclerosis 1999, 143: 125-133.

[73] T. Itahara, T. Suehiro, Y. Ikeda, M. Inoue, T. Nakamura, Y. Kumon, M. Kawada, and K. Hashimoto; Serum paraoxonase and arylesterase activities in hemodialysis patients; J. Of Atheroscler. And Thromb. 2000, 3 (7): 152-158.

[74] M.Hu, S. Louie, C. E. Cross, P. Motchnik, B. Hallivell; Antioxidant protection against hypochlorous acid in human plasma; J. Lab. Clin. Med. 1993,121: 257-262.

[75] M. Aviram, M. Rosenblat, S. Billecke, et al.; Human serum paraoxonase (PON1) is inactivated by oxidized low density lipoprotein and preserved by antioxidants; Free Radic. Biol. Med. 1999, 26: 892-904.

[76] B. J. Van Lenten, A. C. Wagner, D. P. Nayak, S. Hama, M. Navab, A. M. Fogelman; HDL loses its anti-inflammatory properties during acute influenza infection; Circulation 2001, 103: 2283-2288.

[77] A. Gölcin, M. Özgőnül, E. Y. Sözmen, S. L. Aslan, B. Sözmen; Renal cortical thickness and PON 1 activity. Both decrease in chronic renal failure; J. Nephrol. 2002, 15: 144-149.

[78] J. Himmelfarb, E. Mc Monagle, E Mc Menamin; Plasma protein thiol oxidation and carbonyl formation in chronic renal failure; Kidney Int. 2000, 58: 2571-2578.

[79] M. F. Weiss, P. Erhard, F. A. Kader-Attia at al.; Mechanism for the formation of glycoxidation products in end-stage renal disease; Kidney Int. 2000, 57: 2571-2585.

[80] C. C. Hedrick, S. R. Thorpe, M.-X. Fu et al.; Glycation impairs high density lipoprotein function; Diabetologia 2000, 43: 312-320.

[81] United States Renal Data System. USRD 1998 Annual Data Report. U.S. Department of Health and Human Services. The National Institute of Diabetes and Digestive and Kidney Disease, Bethesda, MD, August1998.

[82] P. O. Attman, P. Alaupovic; Lipid abnormalities in chronic renal insufficiency; Kidney Int. 1991, 31:16-23.

[83] D. Steinberg, S. Parthasarathy, T. E. Carew, J. C. Khoo, J. Z. Witztum; Beyond cholesterol. Modification of Low-density lipoprotein that increase its atherogenicity; N. Engl. J. Med. 1989, 320: 915-924.

[84] P. O. Attman, O. Samuelson, P. Alaupovic; Lipoprotein metabolism and renal failure; Am. J. Kidney Dis. 1993, 21: 573-592.

[85] M. C. Blatter, R. W. James, P. Dussoix, et al. ; Paraoxonase polymorphism Met-Leu 54 is associated with modified serum concentrations of the enzyme. A possible link between the PON gene and increased risk of cardiovascular disease in diabetes; J. Clin. Invest. 1997, 99: 62.

[86] J. Ruiz, H. Blanche R. W. James, et al.; Gln-Arg 192 polymorphisms of PON and coronary heart disease in type 2 diabetes; Lancet 1995, 346: 869.

[87] Barbara Voetsch B., Loscalzo J.; Genetic Determinants of Arterial Thrombois; Arterioscler Thromb Vasc Biol 2004

[88] F. Chantrel, I. Enache, M. Bouiller, I. Kolb, K. Kunz, P. Petitjean, B. Moulin, T. Hannedouche; Abysmal prognosis of patients wizh type 2 diabetes entering dialysis; Nephrol. Dial. Transplant. 1999, 14: 129-136.

[89] T. Shinzato, S. Nakai, T. Akiba, C. Yamazaki, R. Sasaki, T. Kitaoka, K. Kubo, T. Shinoda, K. Kurokawa, F. Marumo, T. Sato, K. Maeda; Survival in long-term haemodialysis patients: result from the annual survey of the Japanese Society for Dialysis Therapy; Nephrol. Dial. Transplant. 1997, 12: 884-888.

[90] T. Klemm, R. Paschke; Possible genetic causes for late complications of diabetes mellitus; Med. Klin. 2000, 95: 31-39.

[91] S. Araki, Y. Makita, L. Canani, D. Ng, J. H. Warram, A. S. Krolewski; Polymorphisms of human paraoxonase 1 gene (PON1) and susceptibility to diabetic nephropathy in type I diabetes mellitus; Diabetologia 2000, 43: 1540-1543.

[92] M. Murata, T. Maruyama, Y. Suzuki, T. Saruta, Y. Ikeda; Paraoxonase 1 Gln/Arg polymorphism is associated with the risk ofmicroangiopathy in type 2 diabetes mellitus; Diabet. Med. 2004, 21: 837-844.

[93] Y. Ikeda, T. Suehiro, M. Inoue, Y. Nakauchi, T. Morita, K. Arii, H. Ito, Y. Kumon, K. Hashimoto; Serum paraoxonase activity and its relationship to diabetic complications in patients with non-insulin-dependent diabetes mellitus; Metabolism 1998, 47: 498-602.

[94] S. Tsuzura, Y. Ikeda, T. Suehiro, K. Ota, F. Osaki, K. Arii, Y. Kumon, K. Hashimoto; Correlation of plasma oxidized low-density lipoprotein levels to vascular complications and human serum paraoxonase in patients with type 2 diabetes; Metabolism 2004, 53: 297-302.

[95] A. Sanz Paris, L. M. Lou Arnald, J. A. Gutierrez Colon, A. Alvarez Lipe, R. Alvero Gamboa; Diabetes in hemodialysis: study of differential factors; Ann. Med. Intern. 1998, 15: 183-188.

[96] B. Zhang, S. Eto, P. Fan, C. Bian, E. Shimoji, T. Saito, K. Saku; Paraoxonase (PON1) Q192R polymorphism and serum PON1 activity in diabetic patients on maintenance hemodialysis; Clin. Nephrol. 2003, 60: 257-265.

[97] T. Sakai, B. Matsuura, M. Onji; Serum paraoxonase activity and genotype distribution in Japanese patients with diabetes mellitus; Intern. Med. 1998, 37: 581-584.

[98] M. Inoue, T. Suehiro, T. Nakamura, Y. Ikeda, Y. Kumon, K. Hashimoto; Serum arylesterase/diazoxonase activity and genetic polymorphisms in patients with type 2 diabetes; Metabolism 2000, 49: 1400-1405.

[99] M. Pinizzotto, E. Castillo, M. Fiaux, E. Temler,R. C. Gaillard, J. Ruiz; Paraoxonase 2 polymorphisms are associated with nephropathy in type II diabetes; Diabetologia 2001, 44: 104-107.

[100] www.genomics.energy.gov

[101] TSC Project; Eur. J. Hum. Genet. 2001, 9: 316-318.

[102] Watson C. E., Draganov D. I., et al.; Rabbits possess a serum paraoxonase polymorphism similar to the human Q192R; Pharmacogenetics 2001, 11: 123-134.

[103] Imai Y., Morita H., et al.; Evidence for association between paraoxonase gene polymorphism and atherosclerotic disease; Atherosclerosis 2000, 149: 435-442.

[104] R. A. Hegele; Paraoxonase genes and disease; Ann Med 1999, 31: 217-224.

[105] S. Fanella, S. B. Harris, T. K. Young, et al.; Association between PON1 L/M55 polymorphism and plasma lipoproteins in two Canadian aboriginal populations; Clin Chem Lab Med 2000, 38: 413-420.

[106] Malin R., et al.; Paraoxonase genotype modifies the effect of parvastatin on HDL cholesterol; Pharmacogenetics 2001, 11: 625-633.

[107] Garin M. C., et al.; Paraoxonase polymorphism Met Leu 54 is associated with modified serum concentrations of the enzyme; J Clin Invest 1997, 99: 62-66.

[108] Brophy V. H., et al.; Polymorpisms in the human paraoxonase (PON1) promoter; Pharmacogenetics 2001, 11: 77-84.

[109] Murphy M. M., et al.; The MTHFR C677T, APOE, and PON55 gene polymorphisms show relevant interactions with cardiovascular risk factors; Clin Chem 2002, 48: 372-375.

[110] Mertens A., and Holvoet P.; Oxidized LDL and HDL: antagonists in atherothrombosis; FASEB J. 2001, 15: 2073-2084.

[111] Kakafika A. I. et al.; The PON 1 L55M gene polymorphism is associated with reduced HDL- associated PAF-AH activity; J Lipid Res 2003, 44: 1919-1926.

[112] http://www.ornl.gov/hgmis

[113] Saiki R. et al.; Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia; Science1985, 230: 1350–1354.

[114] www.invitrogen.com

[115] Santos S. E .B., Ribeiro-dos-Santos A. K. C., Carneiro dos Santos N. P.; Frequency of the Q192R and L55M polymorphisms of the human serum paraoxonase gene (PON1) in ten Amazonian Amerindian tribes; Gen and Molec Biol 2005, 28, 1: 36-39.

[116] Z. Ahmed, A. Ravandi, G. F. Maguire, et al.; Apolipoprotein A-I promotes the formation of phosphatidylcholine core aldehydes that are hydrolysed by paraoxonase (PON-1) during high density lipoprotein oxidation with a peroxynitrite donor; J Biol Chem 2001, 276: 24473-24481.

[117] N. Ferre, M. Tous, A. Paul, et al.; Paraoxonase Gln-Arg (192) and Leu-Met (55) gene polymorphisms and enzyme activity in a population with a low rate of coronary heart disease; Clin Biochem 2002, 35: 197-203.

[118] B. J. Van Lenten, A. C. Wagner, M. Navab, A. M. Fogelman; Oxidized phospholipids induce changes in hepatic paraoxonase and ApoJ but not monocyte chemo attractant protein-1 via interleukin-6; J. Biol. Chem. 2001, 276: 1923-1929.

[119] S. Fanella, S. B. Harris, T. K. Young, et al.; Association between PON1 L/M55 polymorphism and plasma lipoproteins in two Canadian aboriginal populations; Clin Chem Lab Med 2000, 38: 413-420.

[120] N. Watzinger, H. Schmidt, M. Schumacher, et al.; Human paraoxonase1 polymorphisms and the risk of coronary heart disease: a community based study; Cardiology 2002, 98: 116-122.

[121] M. Dirican, R. Akca, E. Sarandol, K. Dilek; Serum paraoxonase activity in uremic predialysis and hemodialysis patients; J Nephrol 2004,17: 813-818.

[122] N. E. Miller; Associations of high density lipoprotein subclasses and apolipoproteins with ischemic heart disease and coronary atherosclerosis; Am Heart j 1987,113: 589-597.

[123] S. Parthasarathy, J. Barnett, L. G. Fong; High density lipoprotein inhibits the oxidative modification of low density lipoprotein; Biochem Biophys Acta 1990, 1044: 275-283.

[124] B. Mackness, M. I. Mackness, S. Arrol, et al.; Effect of the human serum paraoxonase 55 and 192 genetic polymorphisms on the protection by HDL against LDL oxidative modification; FEBS Lett. 1998, 423: 57.

[125] B. Mackness, G. K. Davies, W. Turkie, E. Lee, D. H. Roberts, E. Hill, C. Roberts, P. N. Durrington, M. I. Mackness; Paraoxonase status in coronary heart disease. Are activity and concentration more important than genotype?; Arterioscler. Thromb. Vasc. Biol. 2001, 21: 1451-1457.

[126] M. Bonafe, F. Marchegiani, M. Cardelli, et al.; Genetic analysis of paraoxonase (PON1) locus reveals an increased frequency of Arg192 allele in centenarians; Eur J Hum Genet 2002, 10: 292-296.

[127] J. L. Witztum; The oxidation hypothesis of atherosclerosis; Lancet 1994, 344: 793-795.

[128] R. W. James, I. Leviev, J. Ruiz; Promoter polymorphism T (-107) C of the paraoxonase PON1 gene is a risk factor for coronary heart disease in type2 diabetic patients; Diabetes 2000, 49: 1390-1393.