

DISTURBANCES OF LIPID METABOLISM IN PATIENTS WITH
ATHEROSCLEROSIS; CLINICAL AND MOLECULAR GENETIC
INVESTIGATIONS

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2005

1. Introduction

Ross et al. found that on the surface of monocytes in the circulation, integrins appear, which bind to vascular adhesion molecules on the endothelial surface. The adherent monocytes penetrate the subendothelial space, where they become macrophages. Simultaneously, one of the circulating lipoproteins, mainly the small, dense LDL cholesterol gets into the subendothelial space, where it is oxidized by free radicals, which are produced during macrophage activation. Macrophages present in the subendothelial space take up the oxidatively modified LDL through their scavenger receptor, although this uptake is not able to mediate the negative feed back mechanism, which should be able to stop the cholesterol accumulation in the cells, resulting in macrophages becoming foam cells that initiate atherosclerosis.

The elements of the extracellular matrix (collagens, elastin, glucosaminoglicans) have a key role in the progression of the injury of vessel wall in the subendothelial space. With aging and in the progression of the atherosclerotic plaque the amount of the elastic fibers is decreasing and the activity of elastase increasing. The elastase degrades the elastin, resulting in increasing of the elastin degradation products which causes multiplex alteration in the biosynthesis of the extracellular matrix.

The association between low levels of high-density lipoprotein cholesterol (HDL-C) and an increased risk for cardiovascular disease has been well established through epidemiological and clinical studies. HDL protects LDL and cell membranes against lipid peroxide-induced damage thus potentially impeding the initiation and progression of atheromatous lesion.

During our studies several patient groups were examined, in whom the lipid metabolism-, oxidative environment- and extracellular matrix alterations observed are similar to those detected during the pathogenesis of atherosclerosis in spite of different etiology. Among the patients studied, those who suffered from Alzheimer's disease or vascular dementia represented more common diseases, while patients suffering from Pseudoxanthoma elasticum (PXE) represented a rare disease, of which the genetic background and pathomechanism is not well known in detail.

1.2. The role of LDL and oxidized LDL in atherosclerosis

The earliest stage of atherosclerotic process is fatty streak formation induced by oxidative modification of LDL. The fatty acid component of LDL is protected by alpha tocopherol, beta carotene and ubiquinol against the oxidative damage. The oxidized LDL is

taken up through CD 36 type scavenger receptors by macrophages, this uptake is not able to mediate the negative feed back mechanism, resulting in macrophages becoming foam cells that initiate atherosclerosis. Beside these the oxidized LDL has plenty of proatherogen properties: induces the expression of VCAM-1, ICAM-1, IL-1, IL-8, MMP1, and NF κ B-t, inhibits producing of NO, increases the aggregation of thrombocytes.

1.3. The protective role of HDL

The association between low levels of high-density lipoprotein cholesterol (HDL-C) and an increased risk for cardiovascular disease has been well established through epidemiological and clinical studies. The proteins associated to HDL determine the antiatherogenic properties of HDL. HDL can intervene at different stages of the atherosclerotic process; inhibition of monocyte adhesion molecules, VCAM-1, ICAM-1. HDL increases the activity of NOS, and the production of prostacyclin. The antioxidative effects of HDL are attributed to presence of several proteins, including paraoxonase (PON), platelet-activating factor acetylhydrolase (PAFAH), and lecithin-cholesterol acyltransferase (LCAT), glutathione peroxidase (GPX).

1.4. The antiatherogen paraoxonase

Paraoxonase can inhibit low-density lipoprotein (LDL) oxidation and prevents accumulation of lipidperoxides in LDL. This enzyme is a glycoprotein of 43000 Da molecular weight, its gene is located on chromosome 7, and the enzyme's activity and stability require the presence of Ca⁺⁺. PON1 hydrolyses organophosphate substrates such as paraoxon. Paraoxonase is synthesized in the liver, and is comprised of three phenotypes: BB with high activity, AA with low activity and AB type with intermediate activity. PON1 is decreased in disorders associated with a high risk of adverse cardiovascular events, including acute myocardial infarction, diabetes mellitus, familial hyperlipidaemia, chronic renal insufficiency. HDL from the PON1 knock-out mouse loses the capacity to impede the accumulation of lipid peroxides.

There are two polymorphism in the PON1 coding region Q192R and M55L which has effect on the PON1 activity and enzyme concentration. Differences in the ability of the polymorphic forms to protect LDL from oxidation and their contribution to the risk of developing cardiovascular heart disease was examined in several studies. The results were not conclusive. PON1 activity is modified by acquired factors, diet, lifestyle, disease.

1.5. The Alzheimer's disease and vascular dementia

The prevalence of dementia is increasing in developed countries. New therapeutic possibilities and the development of preventive measures have led to an increase in the life span of the population, thereby increasing the prevalence of vascular cerebral disease, and dementia with organic etiology.

One of the characteristic neuropathological features of AD is the presence of amyloid-containing senile plaques. The senile plaques comprise aggregates of β -amyloid, which is derived from the amyloid precursor protein.

Increasing evidence suggests that cholesterol plays a role in the pathophysiology of Alzheimer's disease, and elevated serum total-cholesterol level has been shown to be a risk factor for AD.

The presence of apoE₂ isoform inhibits the development of AD, while the presence of apoE₄ raises the risk of Alzheimer's disease 3-8 fold. Earlier studies suggest that apoE₂ and apoE₃ form a stable bond with tau protein, and this binding inhibits the phosphorylation of tau protein, thereby stabilizing microtubules and cytoskeleton in the neuron. On the other side, the connection between apoE₄ and tau protein is unstable, and can not protect against phosphorylation, and neuron degeneration. The aggregation of tau isoforms into intraneuronal filaments is an important pathological event in the pathogenesis of AD. Until recently, it was thought that an abnormal phosphorylation of tau proteins was responsible for the aggregation in AD. Buée et al. suggest that in addition to phosphorylation, other mechanisms may be involved in the formation of pathological tau filaments. In the pathogenesis of AD, the neuron degeneration is caused by a combination of beta amyloid production, oxygen deficiency, and lipid peroxidation.

Oxidative stress is an important noxious agent; Behl et al. established that amyloid produces free radicals, which damage the neurons. Dyrks et al demonstrated that oxidative stress is responsible for transforming soluble amyloid into the insoluble fibril form. Troncoso et al found a strong association between oxidative stress and the polymerisation of tau protein. These findings suggest that beside beta amyloid, oxidative stress also plays a role in the development of the disease. In this respect, there are some similarities between atherosclerosis and AD, because oxidative stress and lipid metabolism also play important roles in atherosclerosis. VAD is caused by vessel occlusion related to arteriosclerosis leading to progressive white matter degeneration. This similar pathomechanism raises the possibility of

the protective HDL-cholesterol playing an important role in the development of AD and vascular dementia, since HDL inhibits oxidative modification and atherosclerosis. This protective effect is partly the result of HDL's reverse-cholesterol transport function, and partly due to HDL associated paraoxonase (PON).

The aim of our study was to determine the lipid parameters and paraoxonase activity in 30 AD and 40 VAD patients, compared to that of healthy controls. We would like to study the connection between lipid abnormalities and HDL-associated oxidative changes in AD and VAD.

1.6. ABC transporter protein family, ABCA1, ABCC6

The human ABC (ATP-binding cassette) gene family consist of 48 members divided into seven subgroups. Genetic lesions in several of these genes have been disclosed in heritable diseases and in general, based on the tissue-specific expression of these genes and the nature of the respective transport substrates, mutations can result in a spectrum of diseases: ABCA1 (Tangier disease), ABCA4 (Stargardt disease, retinitis pigmentosa), ABCC2 (Dubin-Johnson syndrome), ABCC6 (pseudoxanthoma elasticum), ABCC7 (cystic fibrosis). The ABCA1 has critical role in the reverse cholesterol transport, and in the regulation of HDL metabolism, and macrophage differentiation.

Recently the mutations of *ABCC6* were identified as genetic background of Pseudoxanthoma elasticum. The *ABCC6* was shown to have an efflux transport function, involved in the Mg^{++} ATP-dependent transport of the glutathione S-conjugates leukotriene C_4 , the S-(2,4-dinitrophenyl) glutathione and the anionic cyclopentapeptide (BQ123). In vitro studies showed impaired transport activity of *ABCC6* in the case of three *ABCC6* mutant forms associated with PXE. The precise function of MRP6 (*ABCC6*), the physiologic substrate, and the correlation between the alteration of this protein and the development of the symptoms are still unknown. *ABCC6* is mainly expressed in the liver and kidney whereas only limited expression was detected in the affected tissues. The protein for *ABCC6* is localized in the basolateral plasma membrane.

1.7. A Pseudoxanthoma elasticum

Pseudoxanthoma elasticum (PXE) is an inherited disorder, characterized by dermal, ocular and cardiovascular lesions. The clinical presentation of the disease is associated with the accumulation of morphologically abnormal and mineralized elastic fibers in these tissues.

Mutations in the ATP-binding cassette transporter C6 (*ABCC6*), also known as multidrug resistance-associated protein 6 (MRP6) gene (MIM #603234), that has been mapped to the chromosomal locus 16p13.1, have been identified to cause for PXE (OMIM 264800). The *ABCC6* gene has 31 exons, spanning ~73 kb of genomic DNA. So far at least 80 mutations have been published in patients with PXE, of which only three are larger exonic deletions (deletion of exon 15, exons 23-29, exons 1-31). The majority of the mutations are localized in the region encoding for the Nucleotide binding domains (NBDs). This domain is highly conserved and it is thought to be essential for the function of the *ABCC6* protein. The most common mutations are R1141X and a deletion from exon 23 to exon 29, with the latter being present in 13% of PXE cases.

The characteristic symptoms of PXE are associated with alterations of elastic fibers and calcification. The histopathologic hallmark of the disease is the accumulated pleiomorphic elastotic material with altered calcified elastic fibers in the skin. The abnormalities that are found in the different organs are caused by mineralization and calcification of elastic fibers in the connective tissue.

The earliest manifestations of PXE are skin lesions including yellowish papules, inelastic, loose and sagging skin and accumulation of elastin-like material, predominantly located on the neck, axilla, antecubital fossa, groin, and periumbilical area. The major findings in the eye consist of angioid streaks, which result from a pathological fracture of the Bruch's membrane, the elastin-rich layer of the retina. The damage of choroid and retinal vessels can induce neovascularization, hemorrhage and scarring, that leads to severe visual impairment. The cardiovascular signs include early myocardial infarction, intermittent claudication, rupture of blood vessels, particularly within the gastrointestinal tract. In addition mitral prolapse can often be found in PXE patients and in their heterozygotic relatives.

The clinical course of PXE patients shows high variability like differing age of onset and genetic heterogeneity. Heterozygous carriers in families with autosomal recessive PXE can have various minor symptoms of PXE. A precise histopathologic examination of the skin is a prerequisite for the diagnosis of PXE. In 1992, a classification system based on the clinical and histological features was proposed.

The purpose of this report was to characterise the genetic defect in a Hungarian PXE family. The presence of a novel genomic deletion in combined heterozygosity with a frequently occurring genomic deletion emphasizes the need to specifically look for genomic deletions in patients with PXE.

2. Specific aims

As demonstrated in the previous sections, lipid metabolism, antioxidant systems and the extracellular matrix might change in a similar manner contributing to the development and progression of atherosclerosis, and the mechanisms related to atherosclerosis might even contribute to the pathomechanism of these different disease states.

With these studies we wanted to provide more information and additional data on the pathomechanism of dementias of different origin and on the development of a rare genodermatosis.

Our aims were:

I. To investigate in Alzheimer's disease (AD) and in vascular dementia (VD)

1. certain lipid parameters, and the polymorphism of ApoE,
2. serum paraoxonase activity,
3. establish correlation between the lipid alterations, paraoxonase activity and the development and severity of these dementias.

II. In pseudoxanthoma elasticum (PXE)

1. routine clinical examination of affected families, pedigree analysis, determination of the pattern of inheritance,
2. detection of mutations by the genetic analysis of family members,
3. to prove the causative role of new mutations and to demonstrate the importance of these new mutations in the development of the disease,
4. culturing and functional examination of fibroblasts from PXE patients to evaluate the role of elastase activity and IL-1 β in the pathologic derangement of extracellular matrix.

3. Materials and methods

Blood sampling

Blood sampling was taken after an overnight fast. From these samples, haemoglobin, haematocrit, leukocyte count, liver enzymes, urea, creatinine, CK, fibrinogen, C-reactive protein, bilirubin, uric acid, serum glucose, total serum cholesterol, HDL-cholesterol, triglyceride, apoA1, apoB100, lipoprotein(a) and activity of serum paraoxonase were measured.

Lipid measurements

Serum cholesterol and triglycerides were assayed with a Boehringer Mannheim GmbH Diagnostic enzyme kit, while the HDL-cholesterol was measured by the phospho-tungstic-magnesium precipitation method. The LDL-cholesterol fraction was calculated indirectly using the Friedewald equation ($Tg < 4.5$ mmol/l). Apolipoprotein examination was performed with immuno-nephelometric assay (Orion Diagnostic kit).

Paraoxonase (PON) activity

Paraoxonase activity was determined, using paraoxon (O,O-diethyl-O-p-nitrophenylphosphate; Sigma Chemical Co.) as the substrate, by measuring the increase in the absorbance at 412 nm due to formation of 4-nitrophenol. Activity was measured by adding 50 μ l serum to 1 ml Tris/ HCl buffer (100 mmol/l, pH=8.0) containing 2 mmol/l $CaCl_2$ and 5.5 mmol/l paraoxon. The rate of generation of 4-nitrophenol was determined at 412 nm, 25°C, by the use of a Hewlett-Packard 8453 UV-Visible Spectrophotometer. Enzymatic activity was calculated from the molar extinction coefficient $17100\text{ M}^{-1}\text{ cm}^{-1}$. One unit of paraoxonase activity is defined as 1 nmol of 4-nitrophenol formed per minute under the above assay conditions. Salt-stimulated PON activity was measured in the presence of 1M NaCl.

Arylesterase assay

Arylesterase activity was also measured spectrophotometrically. The assay contained 1 mM phenylacetate in 20 mM Tris/HCl (pH 8.0). The reaction was started by the addition of serum, and the increase in absorbance was read at 270 nm. Blanks were included to correct the spontaneous hydrolysis of phenylacetate. Enzyme activity was calculated using the molar extinction coefficient $1310\text{ M}^{-1}\text{ cm}^{-1}$. Arylesterase activity was expressed in units per liter. One unit is defined as 1 μ mol phenylacetate hydrolyzed per minute.

ApoE polymorphism

Determinations were carried out from peripheral blood lymphocytes. The polyacrylamide gel electrophoresis was performed on fragments of genomic DNA, obtained by salt extraction, by PCR technique.

Statistical methods

The SAS TM for Windows TM 6.11 computer program was used to perform the statistical analysis. Data were presented by descriptive analysis (case number, mean, standard deviation). The comparisons between groups were performed by *t* -test and ANOVA. The $p < 0.05$ probability was accepted as the significance level.

Materials

All chemicals were purchased from SIGMA-Aldrich (Taufkirchen, Germany) unless otherwise specified.

Genetic analysis

Genomic DNA was extracted from whole blood EDTA samples using Qiagen Midi Kit (Qiagen, Hilden, Germany). The sequence information of the 31 exons of *ABCC6* was compared to the published cDNA sequence (GenBank accession number AF076622) and a BAC clone CIT987SK-A-962B4 (GenBank accession number U91318).

The primers for amplification of all exons of *ABCC6* were a generous gift from Charles D. Boyd (Pacific Biomedical Research Center, University of Hawaii, Honolulu). All 31 exons were amplified using the Qiagen Taq PCR Core Kit (Qiagen, Hilden, Germany) on a Perkin Elmer Thermocycler under the following conditions: 2 min 94 °C, 40 sec 94 °C, 1 min 55 °C 10-31 exons (1 min 60 °C 1-9 exons) for 35 cycles, and 5 min 72 °C. The PCR products were purified with QIAquick PCR purification Kit (Qiagen, Hilden, Germany), and were visualized on ethidium bromide-stained 2% agarose gels. Cycle-sequencing reactions were performed with Big-Dye terminators (DNA sequencing kit, PE Applied Biosystems, Darmstadt, Germany), afterwards the amplified genomic segments were purified with DyeEx 96 kit, and both strands were sequenced on an ABI Prism Genetic Analyzer 3100 capillary sequencer (PE Applied Biosystems, Darmstadt, Germany) according to the protocol of the manufacturer.

In order to identify the causing mutations leading to PXE we screened all 31 exons of the *ABCC6* gene in the DNA sample of the index patient. The primer pairs used for the detection of the deletions can be seen in Table 1. The PCR reaction conditions were similar with the ones described above.

ABCC6ex22F	5'-CAT CTG CCA TGG GCA TGT TT-3'	Boyd CD
ABCC6ex23F	5'-GGG TGG CCA AGC CAT AAG AT-3'	Boyd CD
ABCC6ex23FR	5'-TAG AAT TCC CAG GGA CAG GG-3'	Boyd CD
ABCC6ex26FR	5'-AAC CTT TTC TGG GAG GCC AG-3'	Boyd CD
ABCC6ex26R	5'-GCC TGT AGC AGA TGT CAA CA-3'	Boyd CD
ABCC6ex31a	5'-CGT GTG GAG CTA TCG ATG AC-3'	Boyd CD
23int1F	5'-CAA GTA GCT GGG ACT ACA GG-3'	-
23int2F	5'-AAT TCC TGG CCC AAG TGA T-3'	-
25int1R	5'-TCA CAC CTA TAA TCT CAG CA-3'	-
25int2R	5'-TCC TTA AGC TTA GCA GCC TT-3'	-
25int3R	5'-TAG CAG CTC TAG CCC TGC CA-3'	-
IVS22F	5'-TCC CCT AAA GAT GGA GAG AT-3'	Le Saux 2001 [16]
IVS29R	5'-CTG TAG GCA GGT CAT TCA AA-3'	Le Saux 2001 [16]

Table 1. List of primers used for deletion analysis

Detection of selected SNPs and point-mutations

Detection of single nucleotide polymorphisms (SNP) or point-mutations was performed using TaqMan real-time PCR assays. In brief, a TaqMan assay involves the use of two detection probes, each recognizing a specific allele. Each probe is labeled with a fluorophore on its 5' end (VIC or 6-FAM) and a non-fluorescent quencher (NFQ) attached to a minor-groove-binder (MGB) on its 3' end. The minor-groove-binder increases the melting temperature of the probe thus allowing a more stringent annealing. The NFQ quenches the fluorescence of the fluorophore as long as both are attached to the same oligonucleotide. During PCR, a probe with a perfect match will be cut into pieces by the 5'-3' exonuclease activity of the Taq polymerase releasing unquenched fluorophore, while a mismatched probe will be displaced. After PCR, which was carried out in a standard thermocycler (MWG Primus) in microtiter plates (384 wells), end-point fluorescence intensity was measured in an ABI Prism 6700HT Analyser (TaqMan). TaqMan probes were designed by ABI's assay-by-design service and are presented in. Standard concentrations (0.9 μ M each primer, 0.2 μ M each probe) and standard PCR conditions (after 10 min initial denaturation 40 cycles of 15 sec 92°C and 1 min at 60°C) were employed. The total reaction volume was 5 μ l, 5 ng of DNA were used in each reaction. The sequenced samples were used as controls.

Culture of fibroblasts and in vitro incubations/stimulations

Primary human skin fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal calf serum (FCS)+ 5% MEM, at 5% CO₂, and 95% O₂ atmosphere at 37°C. The cells were used between the 5-10th passage. The cells were seeded in 100 mm polystyrene Petri dishes (300000 cells/dish), and in 6-well polystyrene plates (40000 cells/well), and used for functional examinations and enzyme assays as they were semi-confluent. For in vitro stimulation the culture medium was replaced for DMEM containing 0,2% BSA (bovine serum albumin) and Human IL-1 β (100 U/ml) (R&D Systems) or or PBS (as control) for further 48 or 72 hours.

After finishing the in vitro stimulation the cells were washed 2x with ice-cold PBS, and harvested by scraping in 1 ml PBS. The cells were homogenized by ultrasonication and then centrifuged at 5000 g for 10 min and the supernatant was used for determination of elastase activity (Bieth et al).

Elastase enzyme assay

The assay was performed as Szendrői *et al* described with some minor modifications. Succinoyl-trialanine paranitroanilide (Suc(Ala)₃pNA, SIGMA) was used as a synthetic substrate for the determination of elastase activity. 125 mM solution of Suc(Ala)₃NA in N-ethyl-pyrrolidone (Fluka) was diluted 1:10 in 100 mM Tris-HCl buffer, pH:8,0, with 0,1% Brij 35 (Calbiochem). 20 μ l from the substrate solution was added to the mixture of 30 μ l porcine pancreas elastase solution (SIGMA) (0,286 mU/ml diluted in 1:100 in 100 mM Tris-HCl buffer, pH:8,0, with 0,1 % Brij 35) and 220 μ l 100 mM Tris-HCl buffer, pH:8,0, with 0,1 % Brij 35. The reaction mix was incubated at 37 °C and changes in optical densities were recorded at 405 nm as a function of time at 37 °C. One unit of elastase activity was defined as 1 nmol of nitroaniline released per h (a molar extinction coefficient $\epsilon_M=8800$ was used) as described Bieth *et al*.

The elastase activity of serum samples was measured as described by Bizbiz L *et al*. with minor modifications. 100 μ l from the serum was added to 100 μ l 100 mM Tris-HCl buffer (pH:8,0, with 0,1 % Brij 35). The reaction was started by adding of the 20 μ l 12,5 mM Suc(Ala)₃N solution. The samples were incubated at 37 °C for 24 hours, and the optical density was recorded at 405 nm with a spectrophotometer (Sunrise, Tecan, Crailsheim, Germany).

Statistics

Data are presented as mean $\pm$ S.E.M., unless otherwise stated. Statistical significance was determined by the Student *t*-test for unpaired samples. $P < 0.05$ has been considered as statistically significant.

4. Results

4.1. Activity of PON1 and lipid disturbances in AD and VD patient groups

30 patients with AD (20 females, 10 males, mean age 64.3 ± 11.7 years), 14 of whom had coronary artery disease (CAD); and 40 patients with VAD (27 females, 13 males, mean age 76.1 ± 12.4 years), 34 of whom had CAD; 40 voluntary, healthy, control subjects from the same age group (26 females and 14 males, mean age 72.3 ± 9.6 years) were included in the study. The patients were selected from the in and outpatient units of the Psychiatric Department, Medical and Health Science Center University of Debrecen. Patients were diagnosed according to the ICD-10 (and DSM-IV) diagnostic criteria system.

The serum cholesterol levels were significantly higher both in AD and VAD patients compared to the controls (C: 4.71 ± 0.89 ; VAD: 6.3 ± 0.8 ; AD: 6.52 ± 0.7 mmol/l; $p < 0.001$). Similar differences were found in serum LDL-cholesterol levels (C: 2.6 ± 0.6 ; VAD: 3.96 ± 0.8 ; AD: 3.84 ± 0.6 mmol/l; $p < 0.001$). The serum triglyceride level was higher in both patient groups compared to the controls, but not significantly (C: 1.06 ± 0.52 ; VAD: 1.47 ± 0.8 ; AD: 1.68 ± 0.1 mmol/l). The protective HDL's level was significantly higher in AD patients compared to the controls and to the VAD patients (C: 1.47 ± 0.1 mmol/l; VAD: 1.43 ± 0.31 ; AD: 1.95 ± 0.1 ; $p < 0.001$). The decrease in HDL-associated paraoxonase activity in both patient groups was significantly lower compared to the control group (C: 188 ± 55 U/l; VAD: 151 ± 47 ; AD: 131 ± 40 ; $p < 0.05$). The NaCl stimulated paraoxonase activity decreased significantly in both patient groups compared to the control group (C: 422 ± 120 ; VAD: 343 ± 89 ; AD: 272 ± 100 U/l; $p < 0.05$). No difference was observed in the arylesterase activity of the three groups (C: 130 ± 35 U/l, VAD: 128 ± 40 ; AD: 123 ± 34). To determine the connection between paraoxonase activity and HDL level, we corrected the enzyme activity for HDL concentration. The PON/HDL ratio in both patient groups decreased significantly compared to the healthy control group (C: 194 ± 79 ; VAD: 98.4 ± 40 ; AD: 88.4 ± 34 ; $p < 0.001$). In the AD patient's group apoE3/4 isoform was found in 44% of the samples, the frequency of apoE3/3 was 33%, and the apoE4/4 isoform's frequency was 19%, apoE2/3 isoform was found in 4% of samples, while in VAD patients, the distribution of apoE genotype was 3/3:56%, 3/4:38%, 2/3:6%.

4.2. Clinical features of PXE patients

We have analyzed the genomic sequence of ABCC6 in 6 well-characterized patients with PXE, and we analyzed the family members also to make clear the pattern of inheritance. The diagnosis of PXE was established by using a PXE classification system (Lebwohl et al 1994) and confirmed by dermatological, ophthalmological, cardiological findings as well as histologic examination of the skin biopsy. The unaffected family members of the PXE patient were also examined. EDTA-blood samples for genetic analysis were obtained from all family members after informed consent had been given. Skin biopsy were obtained from 3 PXE patients from unaffected skin area (2,3,4 probands see at Table 2), and from 6 healthy controls which underwent to abdominal surgery. The study has been approved by the local ethical committee. Our patients: P1-P5 had similar clinical picture and PXE history like described in the literature. The details of the patients can be seen in the Table 2. In the families of our probands there was no another affected person.

In the case of P6 we found more serious clinical picture like in P1-P5 patients. P6 showed the characteristic features of PXE. The dermal symptoms of the index patient began at the age of seven with typical skin lesions: yellowish papules of the abdomen in the periumbilical area, on the lateral aspect of the neck, axillae and shoulders. Nowadays she has extended skin lesions. She suffered from hypertension since she was one year old. After renovascular origin had been revealed, surgery was performed at the age of eight. Since then her blood pressure appeared normal. At the age of twelve a skin biopsy confirmed the diagnosis of PXE. Ocular complications have been first discovered at the age of seventeen, including angioid streaks, bilateral macular degeneration and reduced visus on the left side. Subsequently mitral valve prolapse and mild mitral insufficiency have been found. There is no consanguinity in the pedigree. None of the relatives presented PXE skin lesions. Both parents have hypertension, and the mother of the patient had transient ischemic attack with hemiparesis. Among the family members no angina pectoris, myocardial infarction, ischemic heart disease, gastrointestinal bleeding, claudicatio, dyslipidaemia or ocular complications have yet been detected.

	Proband	Mutation	Exon	Aminoacid	Genotype	Age of onset	Skin, skin biopsy	Eye	Cardiovascular
1.	21 year old Hungarian woman	C3490T	24.	R1164X	Homozygous	12 year	Characteristic +	-	-
2.	49 year old Hungarian woman	C3421T C4015T	24. 28.	R1141X R1339C	Compound heterozygous	8 year	Characteristic +	Angioid streak, Choroidea atrophy	-
3.	23 year old Germanman	<i>T1484A</i> C4015T	12. 28.	<i>L495H</i> R1339C	Compound heterozygous	4 year	Characteristic +	Pepper and salt fundus	-
4.	53 year old German man	C3421T	24.	R1141X	Homozygous	22 year	Characteristic +	Angioid streak, Serious visual impairment	-
5.	19 year old Hungarian woman	C1552T	12.	R518X	Homozygous	10 year	Characteristic +	-	Mitral prolapse
6.	27 year old Hungarian woman	del23-29 del24-25	23-29 24-25		Compound heterozygous	7 year	Serious, disseminated, +	Angioid streak, bilateral macular degeneration, decreased visus (17 year)	Hypertension (1 y), art. renalis stenosis, mitral prolapse, mitral insuffitinetia

Table 2. Summary of the analysed PXE patients

4.3. Genetic examinations

We have analyzed the genomic sequence of ABCC6 in 6 well-characterized patients with PXE, and we analyzed the family members also to make clear the pattern of inheritance.

We found 5 different mutations in our patients, three of them were homozygous, and two of them were compound heterozygous. Among the mutations there were missens (R1339C, L495H) and nonsense (R1164X, R1141X, R518X) mutations. In all the 5 family our analyzing supported the recessive inheritance. In the case of proband 4. we found one mutant allele which known as autosomal recessive PXE causing mutation, the C4015T nucleotide substitution, but the status of our proband was heterozygous. Besides this already published mutation we found two unidentified nucleotid changes, the R1064W (C3190T) in the 23. exon and the L495H (T1484A) in the 12. exon. With Taqman Genotyping Assay in the case of C3190T change we found the C allele 98% and the T allele 2% allele frequency in 464 german healthy controls. This mutation results amino acid change from leucine to histidine. This mutation located in the fifth intracellular loop between the ninth and tenth

transmembrane domain. The change T1484A was not found in any healthy controls from 464, this suggested that this allele is pathogen and with the already known mutation can cause the PXE phenotype. With analyzing the family members we found the origins of these alleles. In all the 5 family our analyzing supported the recessive inheritance.

Screening for mutations in P6 (Table 2.) in the *ABCC6*-gene was done by PCR-amplification and sequencing of all exons of the patient. While exons 1-23 and exons 26-31 were amplifiable, no PCR products were generated from exons 24 and 25. This finding implicated that the corresponding genomic region was absent from both alleles. The DNA sequencing of the amplifiable *ABCC6* exons did not reveal any mutations (exons 1-23 and 26-31). In order to determine whether genomic deletions on both *ABCC6*-alleles are responsible for the absence of exon 24 and 25 and to determine the breakpoints of the deletions, long range PCR across exon 24 and 25 was performed (primers can be seen in table 1) and revealed a shorter PCR fragment in the patient as compared to controls (Figure 1). The mother was found to be heterozygous for this mutation (exon 24-25 deletion), since amplification of her genomic DNA showed the shorter deletion PCR product as well as a PCR product with the normal size.

We hypothesized that the father also has a genomic deletion on one allele encompassing exon 24 and exon 25. Detailed long range PCR analysis of his genomic DNA with a variety of intronic primers showed that he is heterozygous for a frequently occurring larger deletion encompassing exons 23-29. Amplification of his DNA with primers located in intron 22 and intron 29 (Table 1) resulted in a small size PCR product indicating a mutation. The breakpoint was shown to be in intron 22 and 29. This was demonstrated by amplification and sequencing of the breakpoint as described by Le Saux *et al.*. Taken together the family analysis revealed that the index patient inherited two alleles with genomic deletions from her parents. The exon 24-25 deletion has not been described so far in the literature, while the exon 23-29 deletion can frequently be found in the population.

Finally we have identified the breakpoint for the novel small deletion involving exons 24 and 25. We have performed DNA-sequencing on the 2.03 kb large fusion PCR-product from the patient, which was generated with primers 23F and 26R, using sequencing primers from the introns encompassing exons 24 and 25. The 23F and also 23FR (the exon 23 reverse primer in the opposite orientation) resulted in a readable sequence. The 23int1F primer gave a readable sequence until position +359 in Figure 2. This region consists of a short track of Ts, followed by some Ts and As. The next primer (23int2F) did not give any sequence. Accordingly, this part of the sequence is not present in the fusion PCR-product. None of the

exon 24 and 25 exon primers worked in the DNA-sequencing of the fusion product, showing that these sequences are missing in the fusion PCR product as well.

On the other site, DNA-sequencing of the fusion PCR-product with the exon 26 reverse primer resulted in readable DNA sequence demonstrating its presence on the fusion product. The antisense version of the exon 26 forward primer (primer 26FR) also resulted in good sequences. This was also the case for the primers 25int3R and 25int2R. The primer 25int1R gave clear sequencing results until the position -1,074 in Figure 2, this region consists of a long stretch of Ts, followed by superimposed, thus not-readable, signals due to the frequently seen inability of Taq polymerase to sequence the long T-stretch (stuttering). The roughly 700 bp proximally located primer 25R did not give readable sequencing results indicating that it is not part of the 2.03 kb fusion PCR-product.

As mentioned before the mother was heterozygous for the deletion, so amplification resulted in two PCR-products of the regular size (6 kb) and the smaller size (2.03 kb). We performed direct sequencing using the primers named above. In contrast to the approach outlined above, we designed two parallel experiments. In the first experiment we used the mixture of the two PCR-products. The fusion PCR-product yielded a strong band on an agarose gel, the larger fragment (regular size) gave a weaker band (Figure 1). With primer 23int1F readable DNA sequence was available until the breakpoint. Downstream an overlap phenomenon was seen because of the presence of the larger PCR-product. Primer 23int2F gave a good sequence because the sequence derived from the regular 6 kb fragment only. In reverse orientation the primer 25int1R gave good sequencing results until the breakpoint, where the overlap phenomenon occurred (see above). The overlap phenomenon is due to two processes. The presence of the wild type PCR-product as well as the stuttering of the Taq polymerase caused by the many As. Primers 25int2R and 3R gave nice results until the proximity of the breakpoint, but did not reach the breakpoint because of their distance to that point.

In the second experiment the 2.03 kb deletion product was cut out of the gel, purified and sequenced. Primer 23int1F gave good sequences until the breakpoint. Thereafter we obtained overlapping sequences. Primer 23int2F gave no signal (like the patient). Primer 25int1R gave good results until the breakpoint. Then overlapping occurred that is due to stuttering. Sequencing fragments of primers 25int2R and 25int3R did not reach the breakpoint.

After identification of the breakpoint we confirmed the deletion by performing a PCR reaction on the 2.03 kb fusion product using the two primers closest to the breakpoint. These were the proximal primer 23int1F and the distally located primer 25int1R. With these primers a 172 bp PCR product was generated verifying the genomic breakpoint (Figure 1). DNA sequencing of

this PCR product revealed the same results as seen by DNA-sequencing of the 2.03 kb fragment with the same primers.

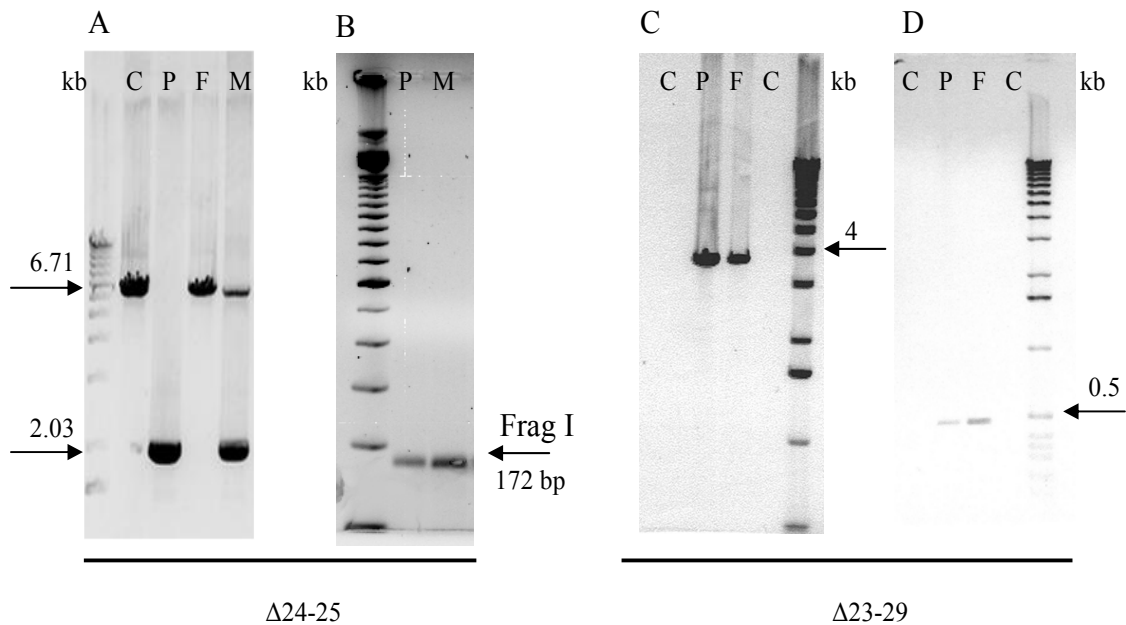


Figure 1.

Characterization of the deletions of the index patient by analysis of PCR products on agarose gels.

In our proband no PCR product was generated when we amplified the exon 24 and 25 (data not shown). A, PCR analysis of the deletion mutation. The amplification of a region corresponding to exons 23-26 with long range PCR, using primers 23F and 26R (Table 1) with genomic DNA from control individual (lane C) and from the proband (lane P), her mother (lane M), her father (lane F), resulted in a PCR product shortened by 4.68 kb (2,03 kb in size) in the proband (lane P). Her mother (lane M) is heterozygous for this deletion, in her case amplification resulted in the 2.03 kb and the normal-size PCR product (size 6.71 kb). B, Reamplification from the 2.03 kb fusion PCR-product using the closely located PCR-primers 23int1F and 25int1R resulted in a 172 bp fusion product containing the breakpoint from the patient and the mother samples. C, Long range PCR using primers 22F and 31R (Table 1). Due to the large distance, a PCR product is not generated in controls (lane C). In the proband (lane P) and her father (lane F) a shorter PCR product (4 kb) was identified that was generated from the allele with the deletion. D, Using intronic primers IVS22F and IVS29R (Table 1) a PCR product across the breakpoint was generated (0.5 kb) in the proband (lane P) and the father (lane F), but not in the control (lane C).

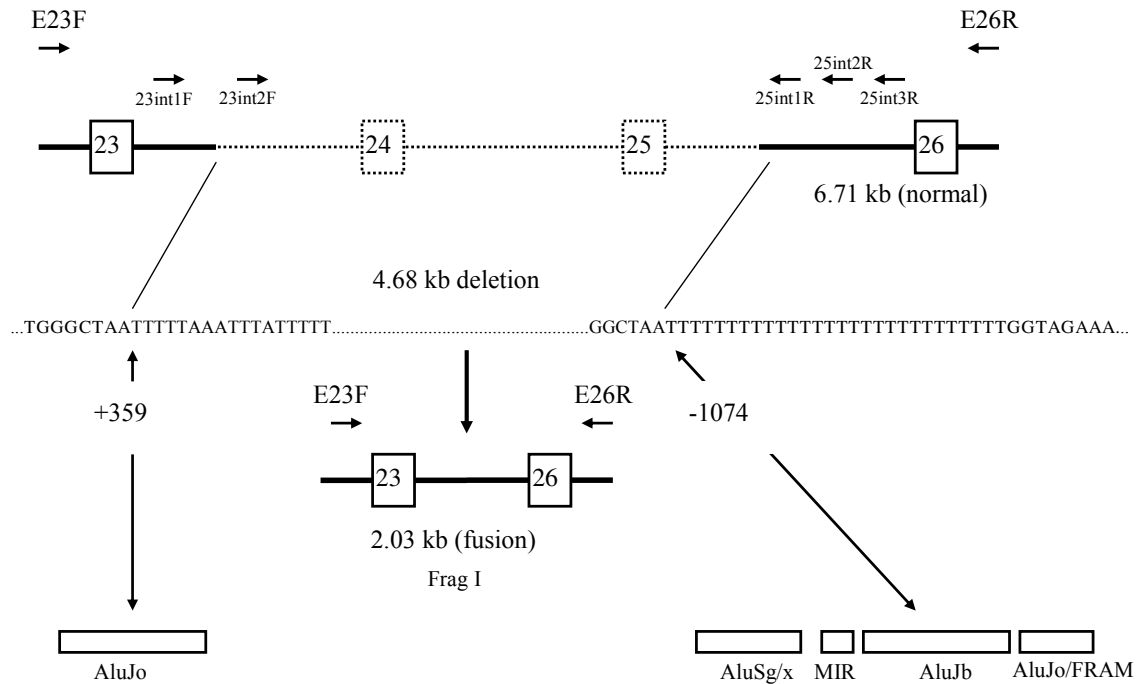


Figure 2.

The genomic organization and deletion breakpoints are shown for the ABCC6 exon 24-25 deletion.

The wild type PCR product generated with primers E23F and E26R is 6.71 kb long. The genomic deletion seen in the patient and her mother (carrier) is 4.68 kb large and results in a 2.03 kb PCR product when amplified with E23F and E26R primers. DNA sequencing of the 2.03 kb fragment from the patient revealed the deletion breakpoints within intron 23 and intron 25 originating in two sites with runs of Ts. The nucleotide position of the breakpoints is given as the distance to the 3'-end of exon 23 (+359 nt) and the distance to the 5'-end of exon 26 (-1,064 nt). The recombination leading to the deletion occurred in the middle of two Alu-repeats (indicated as boxes). Additional repeats are shown (Alu, MIR).

4.4. Elastase enzyme assays

Altered elastic fibers, changes in their size and shape, accumulation of Ca salt in them has been described in PXE. Elastases are a group of proteases produced by several cell types including polynuclear neutrophils, macrophages and fibroblasts. Elastic fibers are targets of dermak fibroblast elastase. These suggest the possible pathogenic role of elastase in PXE. Schwartz *et al.* showed increased elastase activity in fibroblasts of PXE patients. It has been described that interleukin 1 β , stimulates the activity of elastase in normal human fibroblasts.

Thus we performed elastase activity measuring in PXE and normal fibroblast with and without interleukin 1 β stimulation.

In our examinations the intracellular elastase activities of the fibroblasts samples derived from the skin biopsy of 4 PXE patients (proband 2,3,4,) compared with 6 normal control fibroblasts. We detected higher elastase activity after 72 h incubation then 48 incubation in the PXE samples and also in the normal samples. In the PXE patients group the basic elastase activity was higher than in the normal fibroblasts, after 48 h this difference we didn't found to be significant with T test, after 72 h incubation this difference was significant ($p<0,05$). (Figure 3.)

The incubation of subconfluent fibroblast cultures with IL-1 β (100 U/ml) resulted increase in the elastase activity in the both group ($p<0,05$). The IL-1 β stimulation caused elastase activity increasing in higher rate in the normal group (after 48 h incubation increased with 117%, after 72 h increased with 164%) compared with PXE group (after 48 h increased with 55%, after 72 h increased with 56%). (Figure 3.)

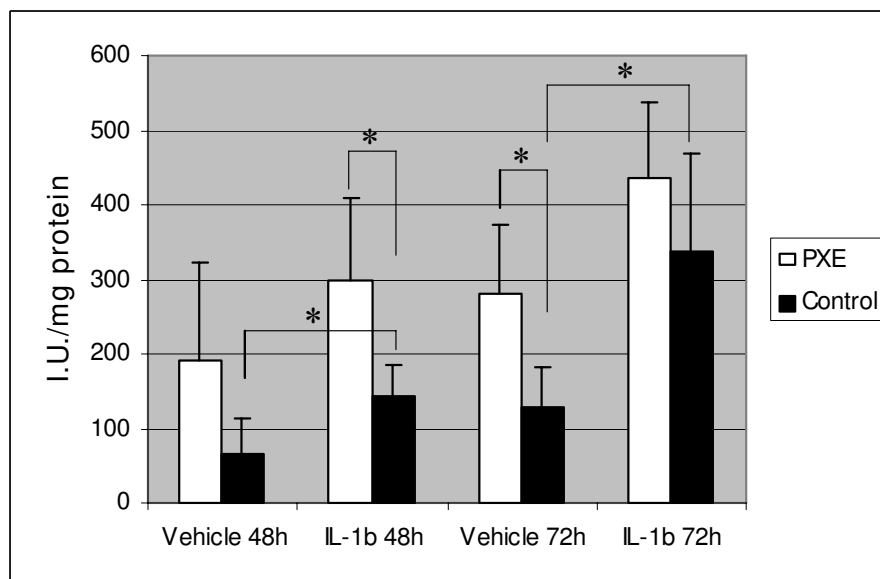


Figure 3.

The in vitro elastase activity measured in cultured fibroblast derived from control and PXE patients upon incubation with IL-1 β (100 U/ml; 48 and 72 h respectively). Vehicle represents culture medium supplemented with 0,2 % BSA. Data are presented as means $\pm$ S.D. from 4 PXE patients and 6 controls.

5. Discussion

5.1. Lipid alterations and activity of paraoxonase in patients with AD and VAD

There are many similar events in the pathomechanisms of AD and atherosclerosis. The altered lipid metabolism and the importance of oxidative stress are noticeable in both processes. Because of the increasing number of Alzheimer and vascular dementia cases, more investigations are required to determine the exact details of the pathomechanism. We found that occurrence of apoE4 isoform in the AD patients was 39.8% and in the VAD patients 19.7%, consistent with other studies.

The HDL particle has antiatherogenic function, due partly to its antioxidant effect. Mackness et al. found that HDL inhibits lipid peroxide accumulation in LDL particles in vitro. The activity of HDL associated paraoxonase has been reported to be significantly reduced in patients with diabetes mellitus, hypercholesterinaemia, after myocardial infarction, chronic uraemia, and after kidney transplantation.

In our present study, we found that in patients with AD and VAD, the serum triglyceride level was not significantly increased compared with healthy controls. In contrast, the serum cholesterol level in AD and VAD patients were significantly higher compared with the control subjects. We have found significant differences between the activities of HDL associated paraoxonase enzyme in the patients' groups and normal subjects. The NaCl stimulated paraoxonase activity was significantly decreased in the two patient groups.

These results suggest that paraoxonase in these patients is able to do less work in the presence of NaCl. The studies by Corrigan et al. raised the possibility that the altered components of HDL particle might cause decreased paraoxonase activity. Since paraoxonase is a HDL associated enzyme, and in the patients with AD we found significantly higher serum HDL level, the question has been raised about how paraoxonase activity correlates with one unit HDL in the patients' group compared with control subjects. We found significantly decreased PON/HDL ratio in both patients' group compared to control subjects. Thome et al. found significantly decreased superoxide dismutase in an AD patient group. This suggests that altered antioxidant capacity of the serum plays a role in the pathomechanism of Alzheimer's disease.

These results suggest that beside altered lipid metabolism and increased oxidative stress, a defect in antioxidant system capacity and altered paraoxonase activity play important roles in the above described pathomechanism.

5.2. Genetic alteration and clinical manifestation of our PXE patients

We performed DNA analysis in a patient suffering from a severe form of PXE with early onset and systemic symptoms. We identified genomic deletions on both alleles of the *ABCC6*-gene. These regions correspond functionally important, and frequently mutated parts of the *ABCC6* transporter.

Direct sequencing revealed that the index patient lacks the coding exons 24 and 25 on one *ABCC6*-allele and the exons 23-29 on the other *ABCC6*-allele. Thus she is compound heterozygous for two genomic deletions. We confirmed the pattern for autosomal recessive inheritance of PXE. The mother of the proband was heterozygous for the exon 24-25 deletion, and her father was heterozygous for the exon 23-29 deletion. A combination of two deleted alleles has not yet been described in the literature concerning PXE patients. The frequency of the 23-29 exon deletion in patients with PXE is very high. In the cohort-study of Le Saux *et al.* the *ABCC6*del23-29 showed a frequency of 12.9% among 122 unrelated PXE patients in the USA. In concordance, a cohort study in France obtained a frequency of 13%. Most of the mutations affect the C-terminal part of the protein, especially, the larger intracellular loop of the *ABCC6* protein (encoded by exon 24), and the NBD2 Walker motif (encoded by exons 28-30). Both regions are highly conserved and are thought to be functionally important either for ATP binding or transport function. The *ABCC6*del23-29 leads to the loss of the third transmembrane domain and the NBD2 sequence. This likely results in a non-functional protein.

In the case of the novel deletion including exons 24 and 25 we identified the breakpoints within intron 23 and intron 25. Their nucleotide position can be seen in Figure 2. The crossing-over occurred at the sites highly repetitive DNA sequences (Alu repeats). The proximal breakpoint is in the middle of a 295 bp Alu-repeat which is in inverse orientation (AluJo). The crossing over at the distal site occurred at the T-stretch in the middle of a 295 bp Alu-repeat (AluJb). This 295 bp Alu-repeat is flanked by a 143 bp AluJo/FRAM repeat (distal), a 65 bp mammalian interspersed repeat (MIR) and a 171 bp AluSg/x-repeat (proximal). Positions refer to the reverse strand of the whole chromosome 16 sequence (database entry as for May 2004).

PXE shows intra- and interfamilial heterogeneity in terms of clinical symptoms. In some cases skin manifestations are predominant with mild eye involvement, whereas in other cases eye involvement is dominant. Early cutaneous signs can appear in childhood, but mostly develop in young adulthood. In some cases dermatological symptoms can be serious with

minimal ophtalmologic and cardiovascular involvement, while in other cases the ocular and cardiovascular signs are dominant with minimal skin involvement. No clear genotype/phenotype correlation has been identified so far. Positive correlation was observed between patients' age and severity of the disease. In a cohort study in France including 19 PXE- families, there was only one patient showing the complete picture of organ involvement at the age of 51 years. In the case of our patient both detected genetic alterations were genomic deletions, the deletions correspond to regions with major importance in the function of ABCC6. The severe clinical picture, the very early onset and the systemic character of the disease seen in our patient, underscore the importance of these regions. Furthermore we provide the first evidence that compound heterozygous deletions can be causative for PXE. In our case the critical alterations of both alleles were associated with an early onset and severe clinical picture. All the involved organs were already affected in young adulthood. These findings strongly suggest a genotype-phenotype correlation considering cases described in the literature. Further studies are required to verify the genotype/phenotype correlation.

In future studies genetic analysis of PXE patients should include screening for genomic deletions, since exon by exon specific PCRs and sequencing will lead to misinterpretation of genetic alterations in individuals with deletions.

6. Summary

Common alterations, such as decreased antioxidant capacity, dyslipidaemia, increased serin protease activity and extracellular matrix alterations are present behind the pathomechanism of very different disease states that we studied.

In the two forms of dementias the decrease of PON1 activity relative to HDL was demonstrated. This is plausible in vascular dementia, where the basis of changes is atherosclerosis. The decrease in PON1 activity and its connection with the progression of atherosclerosis is well known.

There are several reports on the role of increased oxidative stress and lipid abnormalities in Alzheimer's disease. Besides such alterations, the etiologic role of decreased capacity of the antioxidant systems can be suggested based on our studies, because decreased activity of PON1 was detected. The decrease in PON1 activity is rather the consequence of microenvironmental changes, than the result of genetically determined polymorphisms.

Pseudoxanthoma elasticum is caused by defective ABCC6 transport protein. The natural substrate of ABCC6 is unknown and the connection between the function of this transport protein and the rupture of elastic fibers is also unclear. Two additional mutations were detected in our PXE patients.

The functional studies on fibroblasts from PXE patients revealed increased elastase activity, similar to atherosclerosis. The cells were less sensitive to stimulation by IL-1 β , which raises the possibility that this inflammatory cytokine might have a role in the development of symptoms. Further studies are under way to examine the alterations of IL-1 β regulation and the derangement of extracellular matrix that might add new information on the role of ABCC6 transport protein.

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