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Alzheimer disease and blood plasma lipids

(Big cohort study)

Dissertation

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Contents

Figures

Tables

1 Introduction

Alzheimer disease: epidemiology, etiology and pathogenesis

Alzheimer disease (AD) is most common neuro-degenerative disease, affecting millions people worldwide. It was first described by Alois Alzheimer, German psychiatrist and pathologist in 1906, but the exact molecular pathology is still not understood (Alzheimer A, 1907; Blennow K, 2006).
AD starts slowly and gets more symptoms over the time - it begins with short-term memory loss, prolonged with progressive decline in memory and other cognitive abilities such as language impairments, apraxia, worsening of semantic memory (most typically word-finding difficulties, a shrinking vocabulary, and poor understanding of complex words and concepts). Delusions and hallucinations occur in up to 50% of patients in the late stage of disease (Bacanu S, et al. 2005).

Age is the most important overall risk factor for AD. At the age of 65, nearly 1% of the population suffer from senile dementia, 10% at the age of 75, and 25–30% at the age of 85 (Knapp M, 2007). A positive family history is a second risk factor, although autosomal dominant presentations account for only 1% of the cases. Many epidemiological studies have established that women are at increased risk for AD (Vina J and Lloret AJ, 2010).

The rapid increase in the number of patients with dementia caused by AD will result in serious consequence for our society and economy. The group of Brookmeyer indicated that in 2006 the worldwide total number of patients with AD was 26.6 million, and by 2050 the number will quadruple (Brookmeyer R, 2007). It was estimated that about 43 % of AD patients require a high level of care such as nursing home and institutions. At the individual level, AD significantly shortens life expectancy and is one of the principal causes of physical disability, institutionalization, and decreased quality of life among the elderly. It is estimated that among individuals over 60 years of age dementia contributes 11.2 % of the years lived with disability, compared with 9.5 % for stroke, 8.9 % for musculoskeletal disorders, and 5.0 % for cardiovascular disease (Mathers C, 2009). Epidemiologic studies have confirmed the malignant nature of AD that could confers an excess risk of death for older people, in a similar extent to that of malignant tumours (Katzman R, et al., 1994).

The macroscopic features of Alzheimer's disease are cortical atrophy, particularly involving the medial temporal lobe and parietotemporal association areas with a relative sparing of the primary sensory, motor and visual cortices (Figure 1).

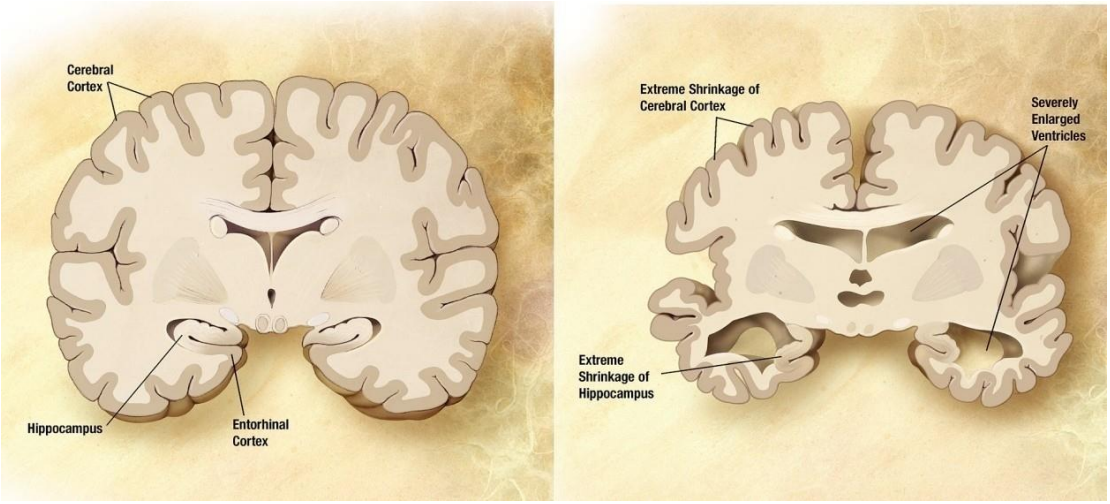

Figure 1. Comparison of a normal aged brain (left) and the brain of a person with Alzheimer's (right). Characteristics that separate the two are pointed out (Ames D, 2010)

Histopathologically AD is characterized by neuritic amyloid plaques, neurofibrillary tangles and a massive neuronal degeneration (Figure 2) (Snider BJ, et al., 2009). AD plaques consist of insoluble filaments, build by beta-Amyloid (Aβ). Those plaques are localized in the extracellular space of the cerebral cortex and vascular walls. The Aβ peptides are proteolytically derived from a larger transmembrane molecule, the amyloid precursor protein (APP) (Wharle S and Das P, 2002; Lichtenthaler SF, 2012).

The amyloid precursor protein (APP) is a transmembrane protein that plays major role in the regulation of several important cellular functions, especially in the nervous system, where it is involved in synaptogenesis and synaptic plasticity (Priller C and Bauer T, 2006). The secreted extracellular domain of APP, sAPP-alpha, acts as a growth factor for many types of cells and promotes neuritogenesis in post-mitotic neurons. Alternative proteolytic processing of APP releases potentially neurotoxic species, including the amyloid-beta (A-beta) peptide that is centrally implicated in the pathogenesis of Alzheimer's disease (Gralle M and Ferreira ST, 2007).

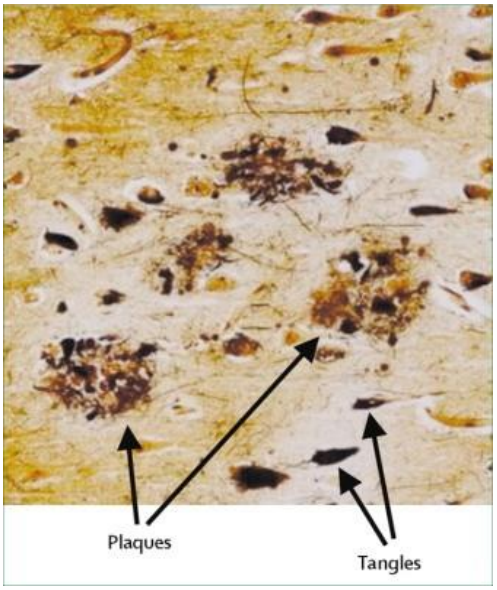

Figure 2. Microscopic alterations of brain tissue affected by Alzheimer disease (Blennow K, et al., 2006)

The second hallmark of AD is neurofibrillary tangles. Tangles are abnormal collections of twisted protein threads found inside nerve cells (Figure 2). The major component of tangles is the protein Tau. This microtubule associated protein, largely expressed in neurons, is predominantly found in axons (Popescu A, et al., 2004). Tau, in its hyperphosphorylated form, is the major component of PHF (paired helical filaments), observed in brains from patients with AD (Sergeant N and David J, 1997; Binder L, 2005).

The membrane protein APP is proteolytically cleaved in two pathways (Figure 3): in the non-amyloidogenic pathway, APP is first cleaved by the metalloprotease α-secretase. This cleavage yields the soluble APP ectodomain APPsα and a C-terminal fragment (C83), which is further processed by γ-secretase, leading to the secreted p3-peptide and AICD (Lichtenthaler SF, 2011). APP cleavage by β- and γ-secretase generates Aβ referred to the amyloidogenic pathway. β-Secretase cleavage occurs within the ectodomain of APP close to the transmembrane domain, resulting in the shedding of the soluble APP ectodomain (APPsβ) and the formation of the membrane-bound C-terminal fragment C99 (C-terminal 99 amino acids of APP). γ-Secretase cleavage of C99 leads to Aβ secretion and the formation of the APP intracellular domain (AICD).

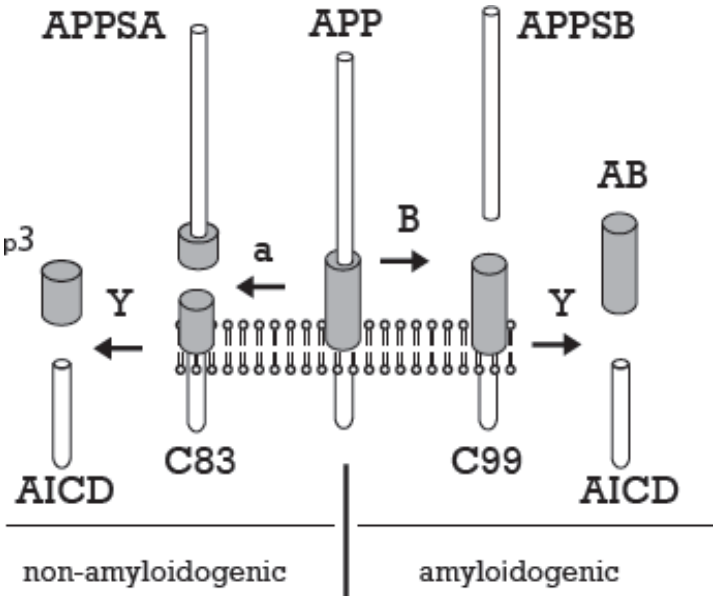

Figure 3. Alternative proteolytic processing of APP by α-, β- and γ-secretase (Lichtenthaler SF, 2011)

Although the precise pathophysiologic mechanism for Alzheimer's is unknown, the deposition of β-amyloid and the hyperphosphorylated form of tau-protein in CNS appears to be the central in the degeneration process (Kaplan and Sadock 2000; Mondragón-Rodríguez S, 2012). It was supported by studies in APP-transgenic mice model by Roberson et al. (Roberson ED, et al., 2011).

Lipoproteins: structure, properties, function

Lipoproteins are large, mostly spherical particles that transport lipids (primarily triglycerides, cholesteryl esters, and fat-soluble vitamins) in all body fluids (plasma, interstitial fluid and lymph). Cholesterol and triglycerides, being nonpolar lipid substances (insoluble in water), need to be transported in the plasma associated with various lipoprotein particles (Clinical Methods, 1990).

Lipoproteins are amphiphilic: the core of lipoproteins is hydrophobic, consisting of non-polar lipids (triglycerides and cholesterol esters); the outer layer consists of a “detergent” coat made up of a phospholipid monolayer and free (non-esterified) cholesterol which together with apolipoproteins facilitate solubility in aqueous body fluids (Figure 4).

Apolipoproteins are required for the assembly and structural stability of lipoproteins. They also serve to activate enzymes involved in lipoprotein metabolism and to mediate the binding of the lipoproteins to corresponding cell-surface receptors.

Lipoproteins play an essential role in the transport of dietary lipids to the liver, from the liver to peripheral tissues as well as in the transport of cholesterol from peripheral tissues back to the liver (Harrison 2001). Lipoproteins are classified by their density dependent on lipid/protein ratio. Major lipoproteins are: chylomicrons, very low density lipoproteins (VLDL), low density lipoproteins (LDL), high density lipoproteins (HDL) and intermediate density lipoproteins (IDL). The main characteristics of the different lipoprotein classes are summarized in Table 1.

Table 1. Composition of lipoproteins

Lipoproteins	Source	Diameter, (nm)	Density, (g/cm³)	Proteins, (%)	Lipids, (%)			
					TG	PL	CE	Chol
Chylomicrons	Intestine	100 – 1000	0.95	1 – 2	86	8	3	2
VLDL	Liver	30 – 80	0.95 – 1.006	6 - 10	55	18	13	7
IDL	VLDL	25 – 30	1.006 – 1.019	15 - 20	25	21	28	9
LDL	VLDL, IDL	20 – 25	1.019 – 1.063	22 - 30	9	20	40	8
HDL2	Liver, Intestine	9 – 12	1.063 – 1.125	35 -45	5	33	17	5
HDL3		5 - 9	1.125 – 1.21	50 -55	3	28	12	3

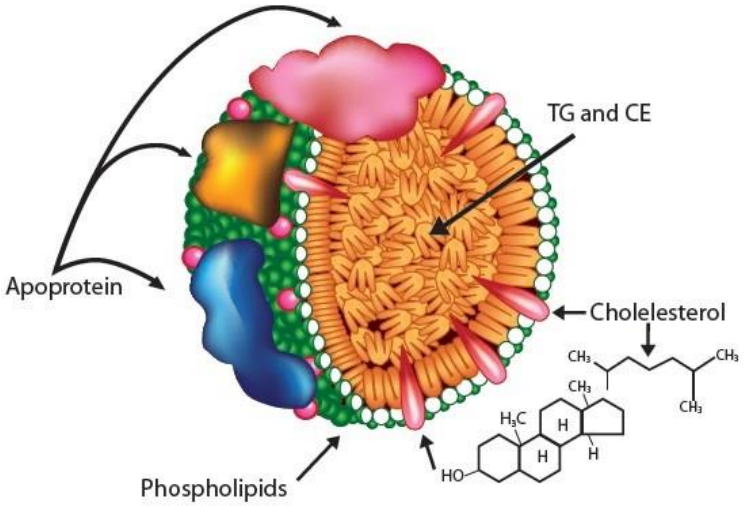

Figure 4. Lipoprotein particle (addapted from http://www.bioguo.org)

Chylomicrons

Dietary lipids absorbed by the intestine are packaged into large triacylglycerol-rich particles called chylomicrons. They pass into the lymphatic system and ultimately into the circulation. ApoB48 is required for the assembly and export of chylomicrons that are synthesized by enterocytes. In the plasma apolipoproteins CII and E are transferred from HDL to nascent CM, creating the mature particles (Tomkin GH, 2012). Triglycerides in chylomicrons undergo lipolysis in a reaction catalysed by lipoprotein lipase (LPL), an enzyme found on the endothelial surfaces of capillaries, especially in the adipose tissue, the heart and the skeletal muscle. The resulted fatty acids are released for storage in adipose tissue and as energy source in muscle and heart (Figure 5).

LPL is the key enzyme in the intravascular hydrolysis of TG-rich lipoproteins (TRL), a reaction that is dependent on the essential cofactor ApoCII. Furthermore, it has been shown that LPL can mediate cellular binding and uptake of TRL *in vivo*. During intravascular lipolysis, chylomicrons decrease its core TG content and are reduced to cholesterol ester-enriched chylomicron remnants, which are taken up by the liver mediated by lipoprotein receptor binding protein (Laatsch A, et al., 2012). In this way chylomicrons function to deliver fatty acids from dietary triglycerides to muscles and adipose tissue and dietary cholesterol with fat soluble vitamins to the liver. This

process is called the exogenous pathway of triglycerides and cholesterol transport (Figure 5).

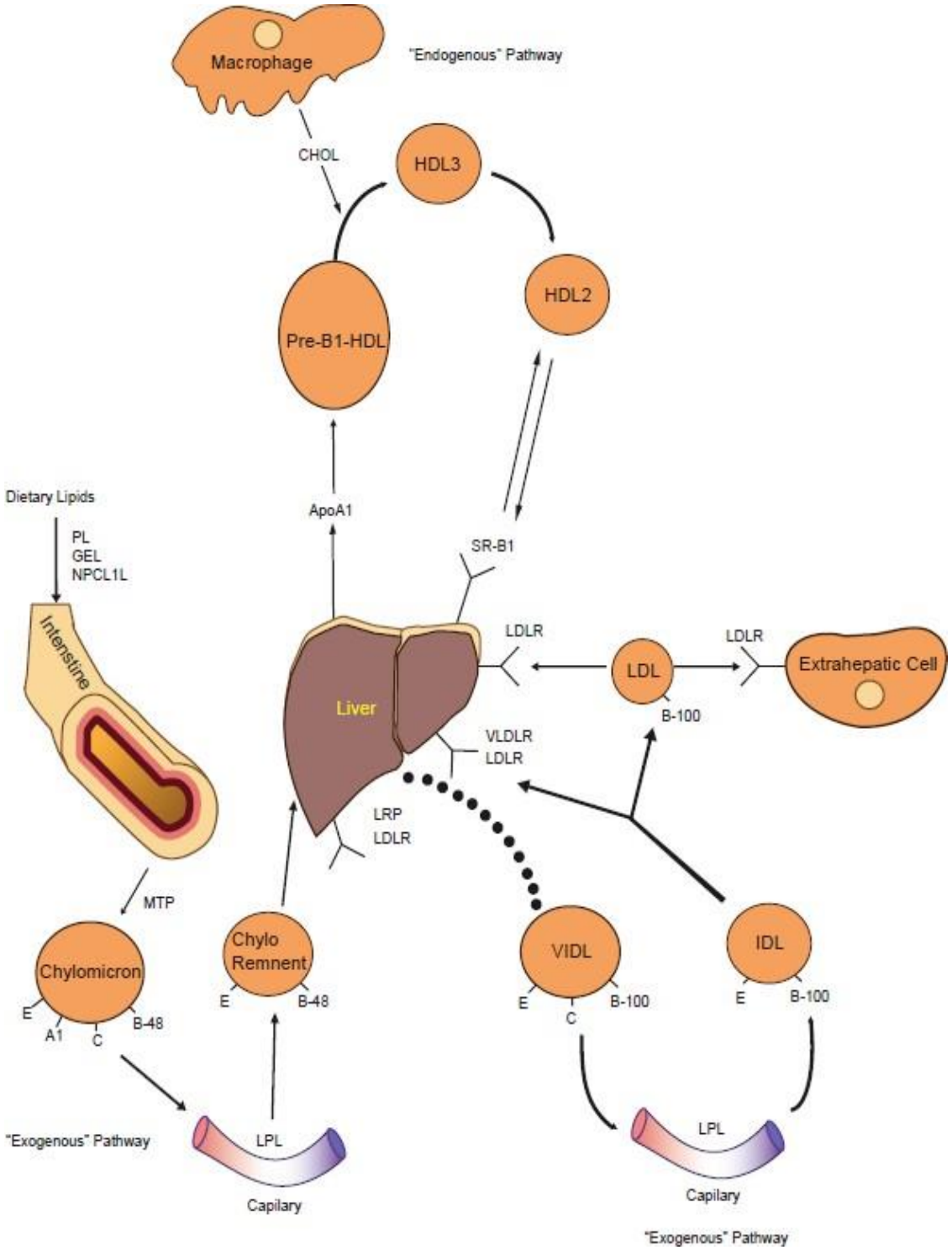

Figure 5. Schematic model of lipid metabolism. Capillary in the adipose tissue, heart, and skeletal muscle

VLDL

The other triglyceride-rich lipoprotein, VLDL, is produced by the liver. VLDL secreted into the circulation contains ApoB100 that has a structural function, similar to B48 in chylomicrons, and it serves as ligand for the LDL receptor (see below). The major role of this lipoprotein is to transport endogenously synthesized triglycerides from the liver to extrahepatic tissues. The second function of VLDL is to carry liver-generated cholesterol esters to peripheral cells after its conversion to LDL. Like in chylomicrons, triglycerides in VLDL undergo intravascular lipolysis and the fatty acids are stored in adipose tissue or used by muscle. The VLDL metabolism to IDL and LDL, is known as the endogenous lipoprotein pathway (Figure 5).

IDL and LDL

As triglycerides are hydrolysed, the VLDL particle becomes smaller and ApoC-II as well as ApoE are transferred back to HDL. This results first in the formation of an IDL (intermediate density lipoprotein). Further hydrolysis of triglycerides in IDL by lipoprotein lipase results in the formation of LDL that only contains ApoB100. Cholesterol ester in LDL may originate from cholesterol directly synthesized by the liver (endogenous pathway), or indirectly from the diet via the clearance of chylomicron remnants by the liver (exogenous pathway). The primary function of LDL is to provide cholesterol to peripheral tissues via binding to LDLR (Brown MS and Goldstein JL, 1986).

HDL

HDL is able to extract and transport cholesterol from peripheral tissues back to the liver. Mature HDL contains the crucial structural protein ApoAI, cholesterol ester, and other apolipoproteins, such as CII, CIII and E. Nascent HDL is synthesized and secreted as an ApoAI containing lipid-poor discoidal particle by the liver (Figure 5). Circulating nascent HDL acquires cholesterol by using Lecithin-Cholesterin-Acyltransferase (LCAT) to extract cholesterol from plasma membranes of peripheral cells (Santos-Gallego CG and Badimon BI, 2008).

ApoAI activates LCAT in the nascent HDL, and also functions as a ligand for a cell surface receptor that exists on peripheral cells. Cholesterol esters, the product of

LCAT catalysis, move to the core of nascent HDL. The whole process of LCAT extraction of cell cholesterol and its incorporation into HDL for liver clearance is called "reverse cholesterol transport" (Figure 5) (Cimmino G, 2015). HDL therefore functions as a cholesterol scavenger, facilitating the transport of excess cholesterol to the liver for conversion to bile acids and eventual elimination. It is this cholesterol-removing property that renders HDL as the "good" cholesterol carrier. Another major function of HDL is to serve as a repository for apolipoproteins A1, CII and E. Transfer of ApoCII is required for the metabolism of chylomicrons and VLDL, and ApoE is crucial for clearance of chylomicron remnants, IDLs and HDLs. Thus, HDL contributes to both the exogenous and endogenous pathways of lipid transport.

Apolipoproteins: structure, properties and function

Apolipoproteins are specific lipid-binding proteins that are integrated to the surface of the lipoprotein. Each type of lipoprotein has a characteristic apolipoprotein composition. Beyond the physical surface stabilization of lipoprotein particles, apolipoproteins have 3 main functions: intracellular recognition and traffic of the nascent particle after synthesis; activation of lipid-processing enzymes in the bloodstream; and binding to cell surface receptors for endocytosis and clearance (Alaupovic P, 1991).

ApoB is the major protein of chylomicrons, VLDL, IDL, and LDL particles. The larger ApoB100 is associated with VLDL and LDL of hepatic origin, while having a lower-molecular-weight. ApoB48 is found in intestinally derived chylomicrons only (Lim JS, 2011) (Table 2).

ApoE is synthesized in the liver and is present on all forms of lipoproteins, except LDL. ApoE binds both heparin-like molecules (which are present on all cells) and the LDL receptor. ApoE displays genetic polymorphism; three alleles of the apo-ε gene express six possible phenotypes, which differ in their ability to bind the LDL receptor (Heeren J, 2003).

Most HDL particles contain apoproteins AI, AII, AIV, and C. ApoAI and ApoAIV are believed to be activators of lecithin:cholesterol acyltransferase (LCAT), an enzyme that esterifies cholesterol in the plasma.

ApoAI is a crucial structural protein for HDL and it is one of the Aβ-binding proteins, the major component of senile plaques in the brain of AD patients (Merched A and

Xia Y, 2000). This link provides a possible connection between lipoprotein metabolism and Alzheimer disease.

Table 2.Apolipoproteins in human plasma

Apolipo-Proteins	Molecular weight, (Da)	Lipoproteins	Function
AI AII	29.000 17.000	HDL, Chylomicrons	Structure protein of HDL, ApoAI activates LCAT
B-48	241.000	Chylomicrons	Structure proteins of Chylomicrons
B-100	513.000	VLDL, LDL	Structure proteins of VLDL, IDL and LDL. Unic protein for LDL. Internalisation of LDL
C-I C-II C-III	6.6000 8.900 8.800	Majority of lipoproteins	Easy transferred between different LP classes. CII – cofactorfor LPL
D	19.000	HDL	Function unknown
E	34.000	VLDL, IDL, chylomicrons	Internalization of chylomicrons and IDL by liver

ApoE

ApoE is a 34 kDa amphipathic protein that associates with serum triglyceride-rich and high density lipoproteins and is involved in the transport of cholesterol between tissues (Mahley R, et al., 2009).

ApoE exists in human beings as three major genetically determined isoforms that can be separated with isoelectric focussing on the basis of charge differences resulting from a single amino-acid substitution. These ApoE isoforms are encoded by three different alleles at the ApoE gene locus (ε2, ε3, ε4), resulting in three homozygous (ε4/4, ε3/3, ε2/2) and three heterozygous (ε4/3, ε4/2, ε3/2) genotypes (Hopkins P and Huang Y, 2002). ApoE3 is the wild-type protein, with arginine at residue 112 and cysteine at residue 158. ApoE4 has an arginine residue at 112 position and ApoE2 a cysteine residue at 158 position. ApoE consists of two domains: the aminoterminal domain – this is the receptor binding region, and the carboxyterminal domain – the major lipid binding region. ApoE4 is mainly found in VLDL; ApoE4 is less recycled and forms less HDL_E (Heeren J, et al., 2003) compared to ApoE3.

ApoE plays a major role in lipid metabolism by mediating cellular uptake of lipoprotein particles via ApoE- and ApoB/E-specific receptors in the liver and other tissues and it contributes to the reverse cholesterol transport (Heeren J, et al., 2004). In numerous studies (Kobayashi J and Saito Y, 2001; Zhijian Y and Tiebing Z, 2001) E4/3 subjects exhibited higher fasting levels of both cholesterol and triglyceride than E3/3 individuals. ApoE3 and ApoE4 isoforms of ApoE have differential effects on lipoprotein metabolism. For example, plasma clearance of VLDL in ApoE4 is higher compared with ApoE3 mice (Knouff C and Hindsdale M, 1999).

After receptor-mediated endocytosis of TRL into the liver, TRL particles are immediately disintegrated in peripheral endosomal compartments. Whereas core lipids and ApoB are delivered for degradation into lysosomes, TRL-derived ApoE is efficiently recycled back to the plasma membrane. This is followed by ApoE re-secretion and association of ApoE with HDL (Heeren J, et al., 2003).

Ikewaki and his group compared the catabolism of ApoE2 and ApoE4 in heterozygous E2/4 subjects in vivo (Ikewaki K, 2002) and demonstrated that ApoE4 was catabolized three times faster than ApoE2, indicated that these two ApoE isoforms have distinct metabolic pathways. The production rate of ApoE4 was slightly higher than that of ApoE2 but was not significantly different. Most radiolabelled ApoE2 was present on HDL, whereas radiolabelled ApoE4 was present in both VLDL and HDL.

The absence of free SH-groups at positions 112 and 158 of the mature ApoE4 isoform may affect its distribution between high-density lipoproteins and triglyceride-rich lipoproteins. It was proposed that association of ApoE4 with chylomicrons underlie the faster removal of alimentary lipoproteins from the circulation which results in down-regulation of hepatic LDL receptors and its attendant elevations of LDL cholesterol (Hopkins P and Huang Y, 2002).

Role of the apolipoproteins and lipids in AD

In 1993, a study in families with AD first implicated the ε4 allele in late-onset AD (Saunders A and Strittmatter W, 1993). In these families, the risk of AD increased with increasing gene dose (up to a 4.5-fold higher risk in those homozygous for ε4) while age at onset AD decreased from an average of 84 to 68 years. Since then, a

large number of population-based epidemiological studies have confirmed this association, and ApoE4 is one of the best-established risk factors for AD (Mahley RW, Weisgraber KH, Huang Y, 2006).

The ε4 allele of ApoE, as well as recently discovered TREM2 (transmembrane glycoprotein) as a risk factor for Alzheimer's disease (Jonsson J, 2013), remains by far the most important sequence variant affecting the risk of late-onset Alzheimer's disease because of its prevalence and the size of its effect on risk.

Individuals with AD carrying the ApoE (ε4) allele have more profuse deposits of Aβ in the cerebral cortex and vascular walls than AD patients carrying other ε alleles (Saunders A and Strittmatter W, 1993; Premkumar D and Cohen D, 1996; Wilhelmus M, et al., 2005). The underlying pathophysiology that explains the relationship between ApoE4 and AD remains speculative, but may involve an ApoE4-mediated increase in serum cholesterol levels. E4/3 subjects exhibited higher fasting levels of both cholesterol and triglyceride than E3/3 individuals (Boerwinkle E and Brown S, 1994; Egert S, et al., 2010). Increased association of ApoE4 with chylomicrons was thought to underlie the faster removal of alimentary lipoproteins from the circulation which results in down-regulation of hepatic LDL receptors and its attendant elevations of LDL cholesterol (Weintraub M and Eisenberg S, 1987).

Cholesterol requires the presence of ApoE to influence Aβ. ApoE knockout APP (amyloid precusor protein) mice fed high-cholesterol diets have unaltered Aβ levels (Puglielli L and Tanzi R, 2003). A transgenic-mouse model for AD amyloidosis was used to test the hypothesis whether Aβ deposition can be modulated by diet-induced hypercholesterolemia (Refolo M and Pappolla M, 2000). It was shown that diet-induced hypercholesterolemia resulted in significantly increased levels of Aβ peptides in the CNS. Furthermore, the levels of total Aβ were strongly correlated with the levels of both plasma and CNS total cholesterol. High cholesterol levels increase both ApoE expression and Aβ levels in rabbit brain (Wu C and Liao P, 2003). Additionally, ApoE4 is involved in senile plaque formation by its affinity for β-amyloid peptide leading to insoluble complexes and in turn amyloidogenesis (Weisgraber K and Mahley R, 1996).

Several lines of evidence suggest that cholesterol metabolism may play an important role in the development of AD pathology. Cholesterol is transported out of cells by high-density lipoproteins (HDL), which also include ApoE. A strong linear association was found between increasing levels of plasma high density lipoprotein cholesterol (HDL-C) during late life and AD pathology in a large post mortem study (Kalman J and Kudchodkar B, 1999; Launer L and White L, 2001). In a case-control study provided by the group of Renato Fellin an association between low HDL-C levels and vascular dementia, but not late onset of AD, was shown (Zuliani G and Ble A, 2001). Further support for the link between cholesterol and AD comes from the group of Refolo and Pappolla (Refolo M and Pappolla M, 2000). The authors reviewed autopsy cases of patients at the age 40 years or older and found a correlation between cholesterolaemia and presence or absence of amyloid deposition; cholesterolaemia and amyloid load. They conclude, that serum hypercholesterolaemia may be an early risk factor for the development of AD amyloid pathology (Pappolla M and Bryant-Thomas T, 2003). The Aulikki Nissinen group from the University of Helsinki showed an association between ApoE4 and AD and also with elevated serum total cholesterol and LDL levels. They conclude that high serum total cholesterol may be an independent risk factor for AD and some of the effect of the ApoE4 on risk of AD might be mediated through high serum cholesterol (Notkola I and Sulkava R, 1998; Reed B, et al., 2014).

In the study provided by Wolf et al. the impact of baseline and lifetime plasma total cholesterol levels averaged across many years on the risk for AD was examined in a large, population-based cohort (the Framingham Study). The study sample consisted of 1026 subjects from this cohort. The authors conclude that long-term average serum cholesterol levels were not associated with the risk for incident AD (Zaldy S and Seshardi S, 2003).

Cholesterol may also be involved in the cleavage of the amyloid precursor protein (APP) (Howlands D and Trusko S, 1998; Sjögren M, Blennow K, 2005). The effect of "Statins", which reduce the level of plasma cholesterol, on the prevalence of AD was investigated (Wolozin B and Kellman W, 2000). It was reported that patients who have taken lovastatin or pravastatin (inhibitors of the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase) have a lower prevalence of probable AD. Several reports, however, have provided contradictory information (Sparks D, 1997; Roher A and Kuo Y, 1999). Also mutations in the LPL gene have

been linked to AD, the risk was increased in Asn291Ser carriers (Baum L and Chen E, 1999). It has been suggested that increased plasma LDL levels, besides their role in the development of atherosclerosis (Steinberg D, 1997), might be involved in the pathogenesis of AD (Kuo Y and Emmerling M, 1998). Additional findings indicate that LPL is a novel Aβ-binding protein promoting cellular uptake and subsequent degradation of Aβ (Nishitsuji K, et al., 2011).

No significant difference in ApoAI brain expression (Harr S and Uint L, 1996) or in CSF concentration was found between AD patients and non-AD's (Song H and Saito K, 1997). However, as shown in the studies (Kawano M and Kawakami M, 1995; Merched A and Xia Y, 2000) plasma ApoAI was reduced in AD and dementia patients. Variation in the ApoAI gene might influence the function of the protein and, thus, brain cholesterol metabolism, leading to an increased risk for AD (Smach A M, et al., 2012).

Extracellular amyloid plaques, intracellular neurofibrillary tangles, and loss of basal forebrain cholinergic neurons in the brains of AD patients may be the end result of abnormalities in lipid metabolism.

1.4 Aim of the study

Modern data is still controversial regarding casual connections between lipid metabolism and Alzheimer disease. The actual study aims to investigate the interplay between gender, ApoE genotype and plasma lipids in respect to Alzheimer disease. For this purpose, data sets of ARG (existing database in the psychiatric clinic and the neurological clinic of University Medical Center Hamburg Eppendorf) should be statistically analysed to determine the interaction between total cholesterol, HDL, LDL, ApoAI, ApoE and the ApoE genotype with AD. Additionally, detailed analysis of FPLC fractionated plasma was attempted to find factors which influence the plasma HDL and ApoAI levels in AD patients.

2 Material and methods

Patients and sample preparation

Patients

At first, data from total 546 persons including AD patients (n=228) and controls (non-AD) (n=318) were selected for investigation from ARG database. The AD patients were all seen in the outpatient “memory clinic” and diagnosed as “clinically probable” AD according to the NINCDS-ADRDA and DSM-IV criteria for primary degenerative dementia, Alzheimer type (McKahn G and Drachman D, 1984). The non-AD subjects attended the neurological clinic and underwent lumbar puncture for diagnostic purpose. Basic information including age and gender were obtained in the psychiatric clinic and the neurological clinic of Hamburg University Hospital. Plasma lipid parameters, ApoE-genotype and apolipoproteins in plasma where determined routinely in the Institute of Biochemistry and Molecular biology II (headed at that time by Prof. Ulrike Beisiegel).

In the last part of this work, detailed analysis of lipoproteins was performed. For this purpose, frozen plasma from AD patients (n=10: ApoE3/3 – 6, ApoE 4/4 – 4) and non-AD (n=11: ApoE3/3 – 6, ApoE 4/4 – 5) were obtained in the psychiatric clinic of Hamburg University Hospital.

Sample preparation

Materials

- 9 ml EDTA container
- aprotinin
- tetrahydrolipstatin
- Centrifuge 2500 rpm
- 1,5 ml Eppendorf tubes

Method

The blood puncture was done after an overnight fasting period. First, 100 µl of 2,5 mg/ml aprotinin (diluted in 0,9% NaCl) and 10 µl of tetrahydrolipstatin (protease and lipase inhibitors, respectively) were added to a 9 ml EDTA container. From each patient, 9 ml of ethylendiaminetetraacetic acid (EDTA)-anticoagulated blood were sampled on the same visit and immediately placed on ice. Blood was centrifuged at 4°C for 10 min at 2500 rpm to obtain plasma. Plasma was freshly frozen at – 80°C in 1,5 ml Eppendorf tubes (aliquots corresponds to the amount required for a FPLC run), no later than 30 min after puncture. The samples were thawed at room temperature immediately before analysis.

Isolation of lipoproteins from plasma using FPLC

FPLC was performed for the separation of different lipoproteins from plasma samples and exact examination of their composition.

Materials

- FPLC machine Pharmacia LKB Controller LCC-500 Plus

- HiLoad TM 16/60, Superdex TM 200, prep grade column

- FPLC buffer as mobile phase 10 mM TRIS-HCl (pH 8.0); 0.15 M NaCl; 10 mM EDTA; pH 8. This puffer has to be sterile filtered before using with filtration system (Nalgene, Nunc Products).

Method

One milliliter of fresh plasma, or thawed at room temperature plasma was loaded in 1ml loop. Separation took place at 4°C. After one FPLC run 50 FPLC-fractions (1ml volume in each fraction) was obtained and used for the different analytic procedures. According to the concentration of cholesterol 8 pools were collected: fractions with the largest values were grouped in the first pool - pool 1 – containing VLDL and LDL, pools 2, 3 – HDL2, pools 4, 5 – HDL3, and the smallest concentaratoins were in pools 6, 7, 8 – containing only proteins.

Measurement of cholesterol, triglycerides and phospholipids

In each fraction pool cholesterol, triglycerides and phospholipids were measured.

Materials

- ELISA-reader, Dynatech Laboratories, MRX version 1.12
- Cholesterol, triglyceride and phospholipide reagent (Roche, Germany)
- Precipath L, control plasma (Roche)
- 96-well micro-titre plate (Nalge Nunc International, Denmark)

Method

Cholesterol, triglyceride and phospholipids concentration were determined in FPLC plasma fractions using colour test photometric reactions. In 96-well (micro-titre plate) were mixed 100 µL of the FPLC probe with 200 µL of the reagent. Reaction developed at 37°C for 10 min. Then optical density was measured at 550 nm using a micro-titre plate reader. To determine the concentration of lipids in absolute values a standard curve was generated using Precipath L at a different dilution (1:2, 1:4 etc.) in FPLC buffer. As reference point the FPLC buffer was taken. Since Ca^{2+} ions are essential for phospholipids test, the method was modified (Dr. S. Ragozin, personal communication). To overcome 10 mM EDTA in the FPLC buffer, reaction was supplied with 5 µL of 2 M CaCl2 – ten times molar excess Ca^{2+} on EDTA.

Determination of protein concentration

Protein levels were detected in each of 8 pools.

Materials

- ELISA-reader, Dynatech Laboratories, MRX version 1.12
- 96-well micro-titre plate (Nalge Nunc International, Denmark)

- Lowry A	2% Na2CO3 in 0.1 MNaOH
- Lowry B	1% CuSO4 in diH2O
- Folin's Reagent	Phenol reagent - 2N (Folin-Ciocalteau reagent)
- Standard	Bovine serum albumin (BSA), fraction V (Pierce, USA)

Method

Add 100 µl of sample (20µl sample + 80 µl buffer = 100 µl) per tube. Then add 1 ml of Lowry stock reagent (49 ml Lowry A + 1 ml Lowry B) to each tube. Incubate 10 min at room temperature. Add 100 ml of Folin's reagent to each tube (Dilute 1:1 in diH2O before use). Afterwards incubate for 30 min at room temperature in darkness and transfer 300 µl from each tube on the 96-well micro-titre plate. Read in a spectrophotometer at 760 nm.

A protein-free sample of the reagent served as a reference for the computation of the optical density values

Under alkaline conditions, copper complexes with protein. When Folin-phenol reagent (phosphomolybdate-phosphotungsttate reagent) is added, the Folin-phenol reagent binds to the protein. The bound reagent is slowly reduced and changes colour from yellow to blue.

Enzyme-linked Immunosorbent Assay

ApoE ELISA

We performed ELISA for the examination of ApoE in each of 8 pools.

Materials

- Incubator (“Binder”, 37°C)
- ELISA-reader, Dynatech Laboratories, MRX vers. 1.12
- Microtiter stripes (Immuno-modules, Maxisorp, Nunc)
- Wash buffer 0.5% NaCl
  0.02% Tween 20
  5 l Aqua dest.
- Blocking buffer 0.1 M NaH2PO4/Na2HPO4 (p.A.)
  0.15 M NaCl
  1.0% BSA (Merck 112018)
  1.0 mM EDTA (Titripoex III, Merck 1.08418)
  0.055 Tween 20 (Bio-Rad 170-6531)

0.01% Thimerosal (FLUKA 71230)

5 l Aqua dest., pH 7.0

- Serum (Behring) as a control
- Polyclonal ApoE primary antibody (DAKO)
- Secondary antibody (GARPO)
- Capture EE7 antibody
- HRP-substrate buffer 1.3 M Malonic acid (Fluca 63290)

  0.6 M Na-Acetat (Merck 1.006268)

  NaOH 1.11 M (Merck 1.06495)

  0.2% H2O2.

  0.3% Tetramethylbencidine (TMB)

  2M H2SO4

Method

We coated the Microtiter stripes with EE7 antibody (374 µl of monoclonal EE7 antibody in 5.126 ml NaHCO3 buffer). The antibody was bound to the bottom of each well by adding approximately 100 µl of antibody solution to each well. Afterwards the plate was incubated overnight at 4°C to allow complete binding. The wells were washed twice with wash buffer (300 ml pro well). The remaining sites for protein binding on the microtiter plate were saturated by incubating with blocking buffer (300 µl pro well). The plate was incubated for 90 minutes at 37°C. Afterwards the plate was decanted and each microwell was washed three times with 300 µl wash buffer. Then 100 µl of the 1:6000 diluted plasma was added to the wells. Probes were incubated for 2 hours at 37°C. Afterwards each microwell was decanted and washed three times with 300 µl wash buffer. 100 µl/well primary antibody anti-ApoE (DAKO) was added. Probes were incubated at 37°C for 2 hours. Afterwards each microwell was decanted and washed three times with 300 µl wash buffer, then incubated with 1:60000 diluted GARPO for one hour at 37°C. Afterwards each microwell was decanted and washed three times with 300 µl wash buffer. Substrate was added as indicated by the manufacturer (250 µl of mix from 1ml of substrate buffer, 1 ml of H_2O_2 and 1 ml of TMB + 25 ml of distilled water). After the

suggested incubation time had elapsed (20 minutes for changing colour), the reaction was stopped with 50 µl of H_2SO_4. Optical density was measured at 450 nm on an ELISA reader.

ApoAI ELISA

The same method was used for quantitative ApoAI detection.

Materials

- 37°C incubator
- ELISA-reader, Dynatech Laboratories, MRX vers. 1.12
- Rabbit Anti-Human ApoAI coated microwell strips 12 x 8 with plastic frame
- HRP conjugated affinity purified goat anti-ApoAI – 12 ml
- ApoAI standard (pre-diluted 1:10000), 335 mg/dl
- TMB/peroxide substrate colour developer – 12 ml
- ApoAI specimen diluent – 2 x 60 ml
- Sulfuric acid termination reagent (0.5 N) – 12ml
- 15 x washing buffer concentrate – 60 ml

Method

100 µl of diluted specimen and standard were added to each microwell. Probes were incubated at room temperature for 2 hours. Afterwards each microwell was decanted and washed three times with 300 µl wash buffer. 100 µl of HRP conjugated goat anti-ApoAI was added to each well. Probes were incubated for 2 hours at room temperature. Afterwards each microwell was decanted and washed three times (as in step c). 100 µl of TMB/peroxide substrate was added and incubated at room temperature for 30 minutes. The reaction was stopped with 100 µl of 0,05 N sulfuric acid, diluted 1:10. The optical density (O.D.) of the remaining wells was determinated. A standard curve was constructed using the O.D. values obtained for each of the standards. The unknowns (samples values) were interpolated from the standard curve (Excel).

SDS-PAGE

Separation of the proteins in polyacrylamid gel was performed for the further specific determination using Western-blotting procedure.

Materials

- Protein standard	Rainbow Marker RPN 800
	(Amersham Life Science)
- Standard	Bovine serum albumin (BSA)
	fraction V (Pierce, USA)
- Lower buffer	TRIS base - 258.5 g
	5 l Aqua dest., pH 9.5
- Upper buffer	TRIS base - 24.8 g
	Boric acid - 12.4 g
	SDS -5 g
	5 l Aqua dest., pH 8.6

- Minigel-electrophoresis apparatus and accessories (BioRad)
- Power Pac 300 (BioRad)
- Thermomixer compact (Eppendorf)
- Centrifuge, Sigma 1-15 K
- 1,5mm thick combs and glass plates (BioRad)

- Separating gel (10%)	2 ml Acrylamid/Bis (40%N,N'Methylenbisacrylamid)
	2 ml lower buffer - 1.7 M Tris-HCl, pH 9.18
	10 µl Temed (Tetramethylethylendiamin)
	40 µl APS (10% Ammoniumpersulfat in A. dest., fresh!)
	Aqua dest., 4 ml
- Stacking gel (3%)	385 µl Acrylamid
	(40% N,N'-Methylenbisacrylamid) - BioRad

Method

After SDS-PAGE gel was washed for 5 minutes in PBS, it was covered with a staining solution, sealed in a plastic box and left overnight or for 2 to 3 hours on a shaker (RT) at 37°C. Gel was then washed until the band pattern became well visible. Afterwards gel was kept overnight between two gel drying films at 4°C.

A reuse of the staining solution is not recommended to prevent cross contamination between gels.

Western Blot

Proteins separated in SDS-PAGE were transferred onto a nitrocellulose membrane and detected with antibodies and chemo-fluorescent visualisation.

Materials

- Blotting cassettes for mini-gel and accessories
- Power Supply LKB (Bromma)
- Film Developing machine Kodak X-Omat 1000 Processor
- Gel-Blotting Paper (Schleicher and Schuell)
- Nitrocellulose Transfer Membrane pore size 0.45 µm (Protran)
- Ponceau S solution 0.2% in 3% TCA (Serva)
- Kodak Biomax MR scientific imaging film
- Photokassette (Rego, Germany)
- Sterile filter 0.2 µm (Schleicher and Schuell)
- Blotting buffer 20 mM Tris
  150 mM Glycin
  20% Methanol
  5 l Aqua dest.
- Wash buffer A 10mMTris pH 7.4
  154 mM NaCl
  0.1% Tween
  5 l Aqua dest.

- Wash buffer B	10mMTris pH 7.4
	154 mM NaCl
	0.1% Tween
	1% SDS
	0.25 M Natriumdesoycholat
- Blocking buffer	10% milk-powder in wash buffer A

- 5% dilution of BSA (Bovine serum albumin)

- Western blotting detection reagents 1 + 2 (Amersham Biosciences) for the ECL reaction

Method

Filter paper (2 sheets per gel) and nitrocellulose membrane (1 sheet per gel) were cut. Once the gel electrophoresis was completed, some transfer buffer was placed in a plastic container and 2 sheets of scotch bright, 2 sheets of filter paper and 1 sheet of nitrocellulose paper were prepared. Afterwards one holder, 1 sheet of scotch brite®, 1 sheet of filter paper, and the piece of nitrocellulose were placed in the plastic container. The gel from the electrophoresis chamber was removed and one glass plate from the gel was carefully removed, leaving the stacking gel intact. The gel was placed onto the filter paper and the remaining glass plate was removed. The second piece of filter paper was placed on the gel and any air bubbles were pressed out. A sponge was placed on top of the filter paper. Afterwards air bubbles were pressed out; the holder was closed. This sandwich was placed in the transfer chamber, filled with blotting buffer, so that the nitrocellulose faced the red electrode (positively charged). The transfer ran for 2 hours at a constant 250 milliampere. Once the transfer was completed, the cassette was removed and opened so that the gel was exposed. Afterwards the wells locations on the cellulose were marked with a pen and the gel was removed from the nitrocellulose. The nitrocellulose was placed on a glass plate.

Ponceau staining of proteins

The nitrocellulose membrane was stained with Ponceau Red solution for 15 minutes to visualize protein bands. The membrane was rinsed in PBS until protein bands were distinct and the position of the molecular weight markers were marked with a ball point pen. The Ponceau Red stain was washed off the membrane during the blocking step. The membrane was scanned and saved for analysis.

Protein detection

The nitrocellulose strips were placed in blocking solution for 1 hour to avoid nonspecific bounding of antibodies (wash buffer A with 10% of milk powder + 5% bovine serum albumin). Strips were washed in wash buffer A on a rocker for 15 minutes. The strips were incubated at 4°C overnight in the primary antibody-containing fluids. Afterwards the strips were washed again in wash buffer A on a rocker for 5 minutes, then in wash buffer B for 10 minutes (2 times) and in the end in PBS for 1 minute. Afterwards they were incubated with the secondary antibody on a rocker at 37°C for two hours. The strips were washed the same way like after incubation with the primary antibody. Then blot was incubated for 1 min with ECL solution and exposed to the black-&-white negative photo material. Membranes were placed in plastic envelope and stored at 4°C.

Statistical analysis

Between-group differences in continuous variables were analysed by ANOVA with or without co-variables. The non-parametrical Spearman correlation was used to evaluate relationships between multinomial (e.g. number of ApoE4 alleles) and continuous variables. All results are expressed as means (standard deviations) if not otherwise indicated. Statistical analysis was performed using STATISTICA 5.5 software (Stat Soft, Inc., Tulsa, OK, USA).

3 Results

Study Cohort Composition

Gender, Age and Diagnosis

Alzheimer Research Group database (ARG) was used in this study, where 228 patients were diagnosed with AD and 318 cognitively-normal control individuals with no dementia. Altogether 366 men and 180 women were included in the study.

Due to significantly older patients of the AD group, analysis was performed including only individuals older than 55 years (age-adjusted) to eliminate a age-related influence as good as possible (due to ethical restrictions it was difficult to get enough old controls) (Table 3).

Table 3. Age, gender and frequency of the E4-allele of the ARG database (patients > 55 years). Age values indicates mean ± standard deviation. Total group n=350

	Control, n=127	AD, n=223
Age	57.8 ± 1.4	72 ± 8.5
Gender F/M, age > 55 years	35/92	143/80
frequency of the ε4 allele of ApoE	0.11	0.36

In age-adjusted group out of total 350 participants Alzheimer disease was diagnosed in 223 (64%), among them80 (23%) were male, and 143 (40.7%) female. In control group of 127 (36%) persons, there were 92 (26%) males and 35 (10%) females. AD was diagnosed twice more common in females.

ApoE4 frequency and Alzheimer disease

All 350 persons were genotyped. Among them - 153 participants were ApoE4 positive (47%). Cross table helps to uncover a distribution of ApoE4 allele in the groups depending on gender and diagnosis.

In AD group 132 (60%) of 223 patients were ApoE4-carriers. In the control group only 30 (23.5%) of 127 have this allele. This is line with the literature that the ε4 isoform correlates with a higher risk of the Alzheimer disease.

The analysis was conducted on 173 men and 177 women. There were in total 92 (53%) ApoE4 positive females and 53 (30%) males. This shows that ε4 is more frequent in women. Among 145 AD females, 88 (60%) were ApoE4 positive. AD males in 42 (52.5%) cases were ApoE4-carriers. Therefore it is possible to speculate that ApoE4 interacts with female gender, further increasing risk to develop AD for women.

In the control group four of 35 (11.4%) females and eleven of 92 (12%) males were ApoE4-carriers. Therefore, ApoE4 genotype was in both females (60% vs 11.4%) and males (52.5% vs 12%) higher associated with AD than in the control group (Tab. 4).

Table 4. ApoE4 distribution depending on gender and diagnosis

		WithApoE4	Without ApoE4
AD	Female	88 (60%)	54 (40%)
	Male	42 (52.5%)	39 (47.5%)
Control	Female	4 (11.4%)	31 (88.6%)
	Male	11 (12%)	81 (88%)

Plasma lipid parameters and Alzheimer disease

Plasma concentrations of HDL, LDL, ApoAI, and ApoB and cholesterol of 223 AD patient and 127 control persons were examined and statistically evaluated as function of AD diagnosis, ApoE4 genotype and gender. Those correlations are shown below.

HDL

Association of plasma HDL levels and AD is still a controversial issue: some examinations reported associations between decreased levels of HDL and AD and low levels of cholesterol and risk of AD; some other - no association of HDL, TG, or LDL levels with AD (Reitz Ch, 2012).

The mean value of plasma HDL level in this study in the AD group was higher as compared to controls (54 ±16 mg/dl vs 46 ±13 mg/dl respectively). Same effect was observed in females as compared to males (58 ±16 mg/dl vs 45 ±12 mg/dl). Those two observations are significant as t-test resulted in p-value smaller then 0.1%.

There was no significant difference of HDL concentration among ApoE4 positive and negative probands (Figure 6).

Higher HDL levels might be a marker of prevalent AD, especially in females, but were not associated with risk of future dementia.

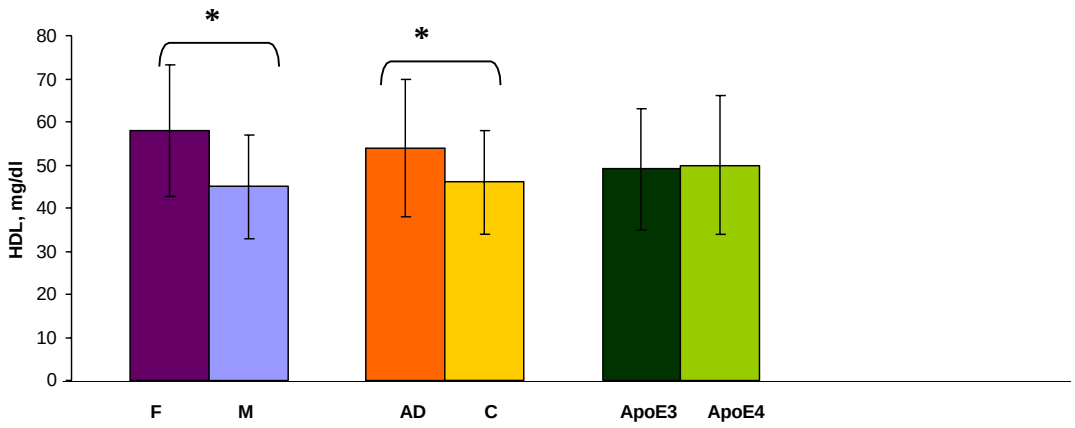

Figure 6. Plasma HDL concentration depending to gender, diagnosis and ApoE4 genotype in whole cohort. Error bars represent SD. * - p-value <0.001; ** p-value <0.002; *** p-value <0.005

LDL

Controversial literature data concerning levels of LDL and HDL in AD patients independently of ApoE genotype has been discussed (Kim J, 2009) and needs to be proven in further experiments to collect more evidence of a direct relationship between cholesterol distribution among plasma lipids and AD.

Analysis of the ARG database shows significant higher plasma LDL concentration in AD group compared to controls (141 ±35 mg/dl vs 129 ±30 mg/dl respectively). Same effect on plasma LDL levels could be demonstrated in females 140 ±37 mg/dl as compared to males with 131 ±30 mg/dl and in ApoE4 carriers 140 ±31 mg/dl as compared to non ApoE4 130 ±33 mg/dl, see figure 7. Numerically the values do not differ much. However, statistical analysis using t-test reveals very low probability to observe such differences in studied groups by chance, with p-value smaller 0.5%.

The epidemiological evidence on serum LDL and AD is, however, complex. Higher plasma LDL has been associated with elevated risk of AD in this study. Thus, if a high LDL level has a causal effect on risk of Alzheimer's disease, LDL lowering drugs or some genetic variants that lower LDL levels lifelong would be expected to also decrease the risk of disease.

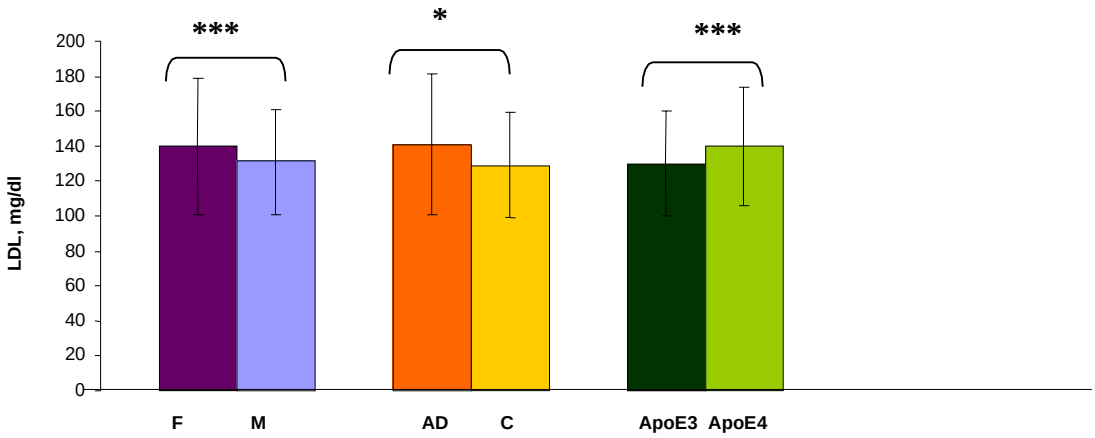

Figure 7. Plasma LDL concentration depending to gender, diagnosis and ApoE4 genotype in whole cohort

ApoAI

ApoAI is the major component of high-density lipoprotein in the blood, and is under investigation by cardiovascular researchers for its ability to protect against heart disease (Brewer H, 2004). Some evidence suggests that it might also help to defend the brain against the cognitive deficits associated with Alzheimer's pathology (Lewis T, 2010).

Similar correlation between plasma ApoAIlevels in investigated groups, as we observed in HDL concentration before - significantly higher plasma ApoAI levels in AD group compared to controls (147 ±25 mg/dl vs 135 ±17 mg/dl respectively). Females showed similar correlation (153 ±23 mg/dl as compared to males with 133 ±18 mg/dl). ApoE genotype didn't influence the ApoAI plasma concentration (Figure 8).

This finding adds to the evidence that higher ApoA-I levels have been associated with prevalent AD.

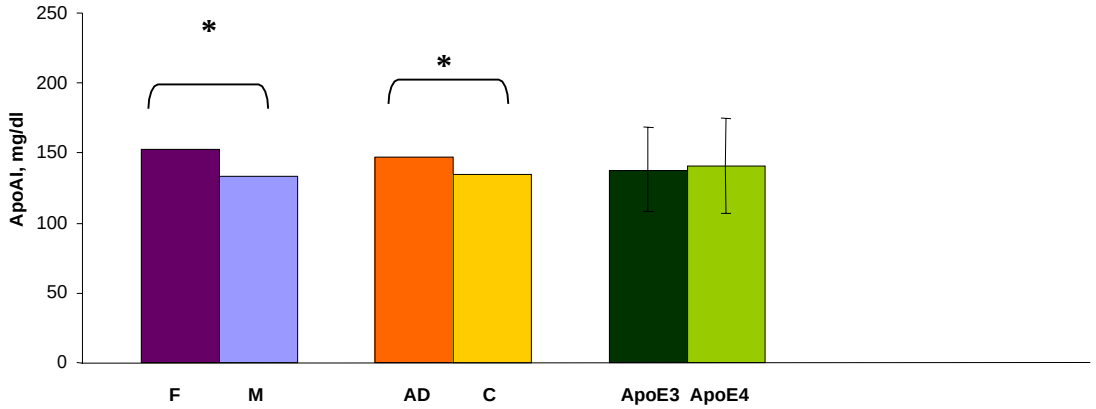

Figure 8. Plasma ApoAI concentration depending to gender, diagnosis and ApoE4 genotype in whole cohort

ApoB

ApoB is the main apolipoprotein of chylomicrons and low-density lipoproteins regulate lipoprotein metabolism, binds and transports LDL, react with tissue receptors. Multiple studies revealed associations of abnormal plasma ApoB level and AD (Caramelli P, 1999).

AD patients in our study had significantly higher plasma concentration of ApoB as compared to controls (108 ±26 mg/dl vs 98 ±20 mg/dl). ApoE4 carriers demonstrate same effect on ApoB plasma level as compared to ApoE3 genotype (109 ±25 mg/dl vs 97 ±22 mg/dl). The ApoB level was not significantly different among females and males (105 ±28 mg/dl vs 100 ±21 mg/dl), see figure 9.

Presented data prove, thatAD may be indeed associated with high plasma concentration of ApoB. This finding suggests that HDL, ApoAI or ApoE may not be the single factors in lipid metabolism to play a role in AD pathogenesis.

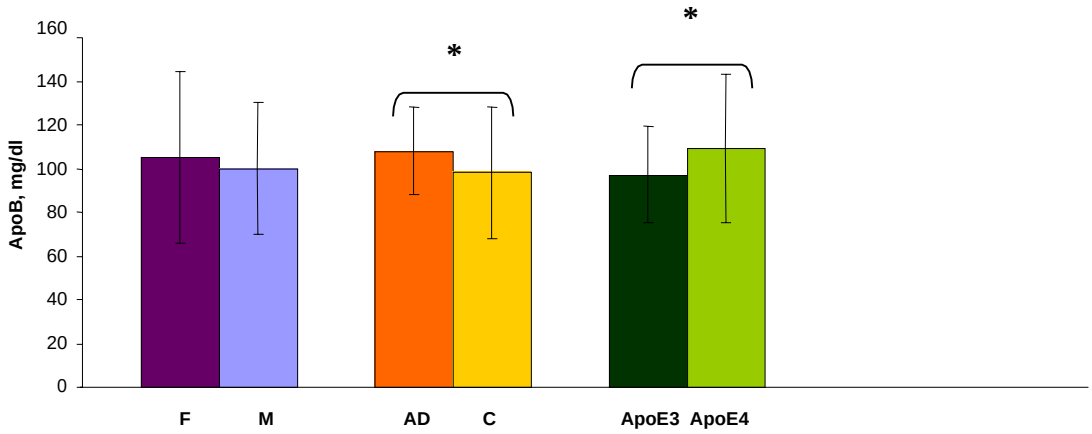

Figure 9. Plasma ApoB concentration depending to gender, diagnosis and ApoE4 genotype in whole cohort

Cholesterol

In many studies a strong correlation between high plasma cholesterol concentration and AD pathology was described (Sjögren M and Blennow K, 2005). High cholesterol in plasma was shown to be an independent risk factor for AD. However, cholesterol is a major constituent of the myelin encircling neurons in the brain, and the risk of Alzheimer's disease have been reported in people treated with traditional cholesterol lowering drugs, in particular statins, although results have been conflicting.

The mean value of plasma cholesterol level in the AD group was significantly higher as compared to controls (221 ±38 mg/dl vs 205 ±33 mg/dl), see figure 10. The same significant effect on cholesterol levels in plasma was seen in females compared to males (224 ±39 mg/dl vs 206 ±32 mg/dl) and in probands with ApoE4 genotype compared to ApoE3 genotype (219 ±35 mg/dl vs 207 ±35 mg/dl).

It is probable that high plasma cholesterol will further aggravate the AD prognosis in females with ApoE4 genotype.

We compared serum lipid levels of AD and non-AD subgroups to demonstrate a correlation between AD and plasma lipids, independent of gender or ApoE phenotype. All lipid parameters are summarised in Table 5. Student's t-test was used to calculate statistical significance.

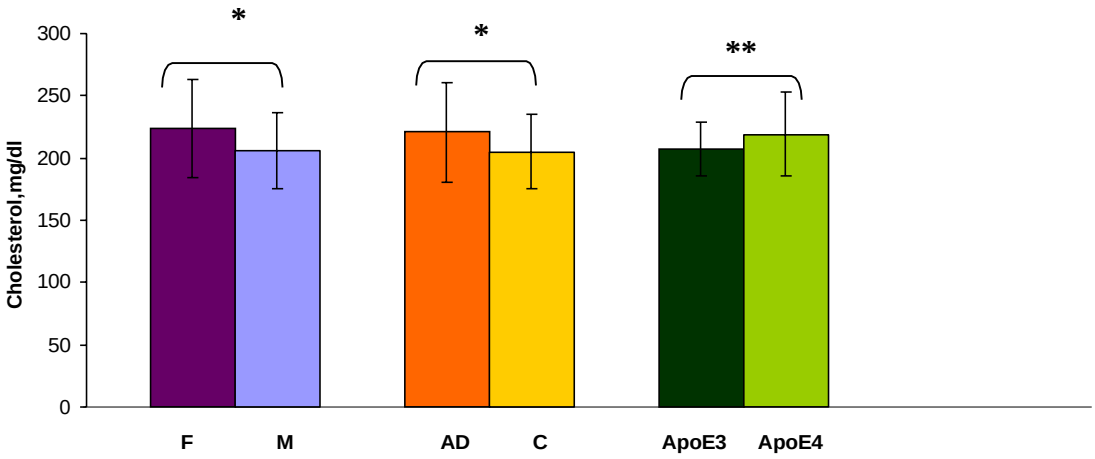

Figure 10. Plasma Cholesterol concentration depending to gender, diagnosis and ApoE4 genotype in whole cohort

Table 5. Lipoprotein profile from350 patients from the database of Alzheimer Research Group (age-matched subgroup). Data are given as means (mg/dl) ± SD

	Diagnosis		ApoE genotype		Gender	
	AD	Non-AD	ApoE4	ApoE3	Female	Male
N	223	127	145	205	177	173
Chol	221 (±38)	205 (±33)	223 (±35)	210 (±41)	225 (±39)	206 (±35)
LDL	141 (±35)	129 (±30)	143 (±31)	133 (±38)	141 (±37)	133 (±33)
HDL	54 (±16)	48 (±16)	50,2 (±16)	49 (±14)	59 (±16)	45 (±14)
ApoAI	147 (±26)	136 (±21)	141 (±25)	138 (±19)	154 (±23)	132 (±21)
ApoB	108 (±26)	98 (±20)	111 (±25)	99 (±24)	105 (±28)	103 (±21)

The important result of this study is the finding of highly significantly increased levels of plasma HDL and ApoAI concentration in AD group as compared to Non-AD (HDL: 54 ±16 mg/dl in AD and 48 ±16 mg/dl in control group, $p<0.05$; ApoAI: 147 ±26 mg/dl in AD and 136 ±21 mg/dl in Non-AD group, $p<0.05$) see table 5. After adjustment on a gender and ApoE allele this effect was seen only in males with ApoE4. In other subgroups no significant effect was observed (Table 6).

Comparing the lipid parameters stratified to gender

In previous part of this study, we demonstrated significantly higher plasma concentrations of different lipids and apolipoproteins in AD patients compared to control group. Our results are well in line with literature that female gender is a risk factor on late onset of AD (Payami H, 1994). The ε4 allele of the ApoE gene (ApoE4) is a potent genetic risk factor for sporadic and late-onset of AD (Chartier-Harlin MC, 1994). Our data also supports this statement. While estimates vary across studies and ethnic backgrounds, the ApoE4 allele is typically present in more than 50% of AD patients, whereas it is found only in about 15% in healthy elderly. To investigate simultaneous influence of gender and ApoE genotype on plasma lipids and apolipoproteins, we stratified AD group and controls according to gender. Stratification in this case means that analysis is based on gender specific variations. Furthermore, results are gathered in four groups whatever AD or control as well as ApoE4 positive or negative.

AD with ApoE4

As mentioned before, ApoE4 genotype and female gender are important risk factors for a late onset AD (Neu SC, 2017). How this setting influences plasma lipids and apolipoproteins of the investigated cohort from ARG database is demonstrated in figure 11.

Cholesterol (234 ±34 mg/dl vs 212 ±27 mg/dl), HDL (57 ±16 mg/dl vs 46 ±13 mg/dl) and ApoAI (151 ±25 mg/dl vs 136 ±26 mg/dl) were significantly higher in females as compared to males.

The influence of high lipids plasma concentration on AD mainly seen in females. Initially, this finding was viewed as controversial, with some studies attributing the increased prevalence of ApoE4 alleles in AD females to the longer survival of AD female carriers (Corder EH, 1995). This substantial trend, found by us, suggests a greater risk to became AD in female ApoE4 carriers.

AD without ApoE4

To prove if only ApoE4 genotype has such effect on plasma lipids and apolipoproteins in females compared to males, ApoE3 AD carriers was compared.

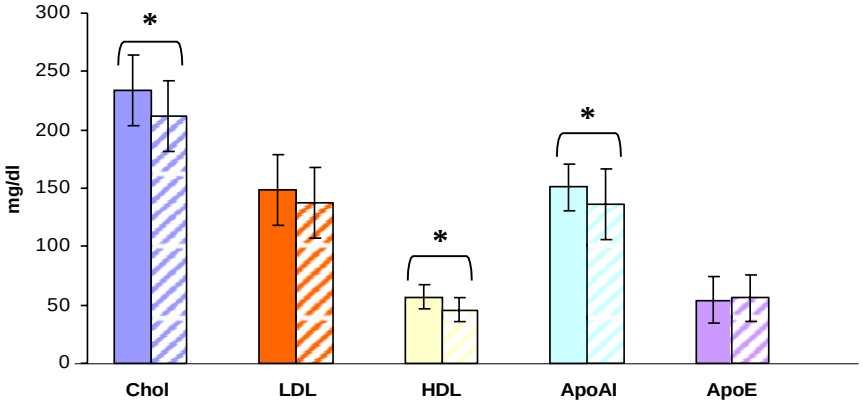

Figure 11. AD group with ApoE4. Shaded – female, hatched – male. Error bars represent SD.* - p-value <0.001; ** p-value <0.002; *** p-value <0.005

Alzheimer patients without ApoE4 demonstrated the same significant higher plasma concentration of Cholesterol (226 ±39 mg/dl vs 199 ±37 mg/dl), LDL (145 ±41 mg/dl vs 124 ±32 mg/dl), HDL (58 ±16 mg/dl, vs 46 ±14 mg/dl) and ApoAI (154 ±24 mg/dl vs 127 ±20 mg/dl) in females vs males as ApoE4 carriers (Figure 12).

Independent of the ApoE genotype, AD females demonstrated higher levels of investigated plasma lipids and apolipoproteins compared to males.

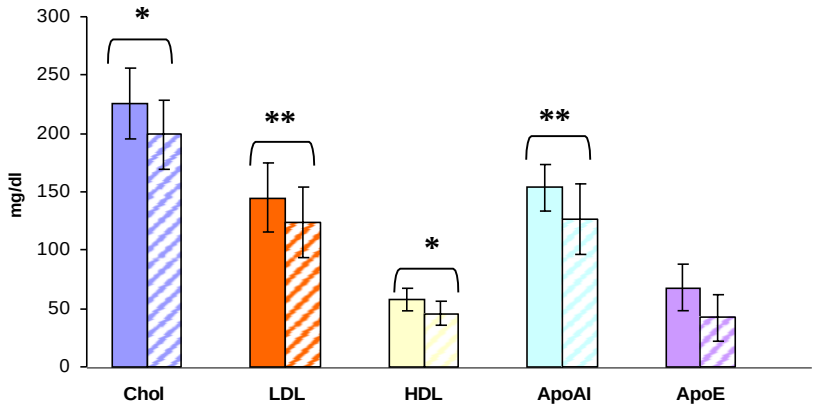

Figure 12. AD group without ApoE4

3.3.3 Non-AD with ApoE4

Plasma lipid parameters in control group was analysed to compare same settings in participants without cognitive disturbances.

Plasma HDL levels (72 ±27 mg/d vs 36 ±8 mg/dl) as well as ApoAI levels (170 ±32 mg/dl vs 124 ±14 mg/dl) were significantly higher among ApoE4-carrying non-AD females and males (Figure 13).

High levels of HDL and ApoAI in plasma remain still significantly higher in females. The exact clinical outcome of ApoE genotype on AD prognosis depends predominantly on patient's gender.

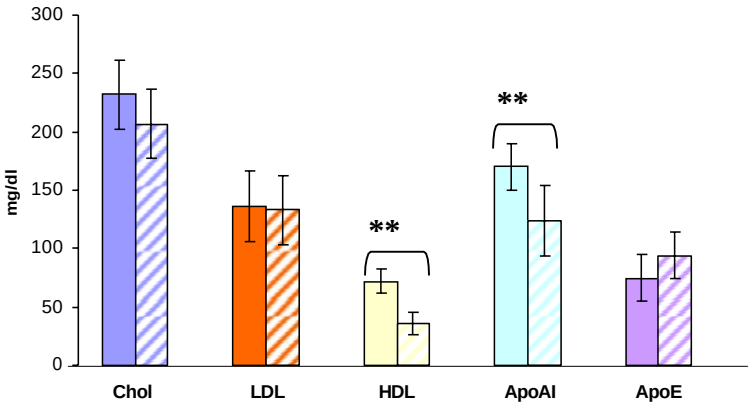

Figure 13. Non-AD group with ApoE4

3.3.4 Non-AD without ApoE4

Lower plasma lipid levels in this group, compare to other settings, was observed, with still significant gender-specific differences inside the group.

Comparing non-AD females and males without ApoE4 the same differences in plasma HDL and ApoAI levels were seen: a significantly higher plasma concentration in females vs males (HDL: 59 ±13 mg/dl vs 45 ±13 mg/dl; ApoAI: 149 ±18 mg/dl vs 124 ±1 g/dl). See figure 14.

Higher levels of HDL and ApoAI in females appear to be independent of cognitive disorder status.

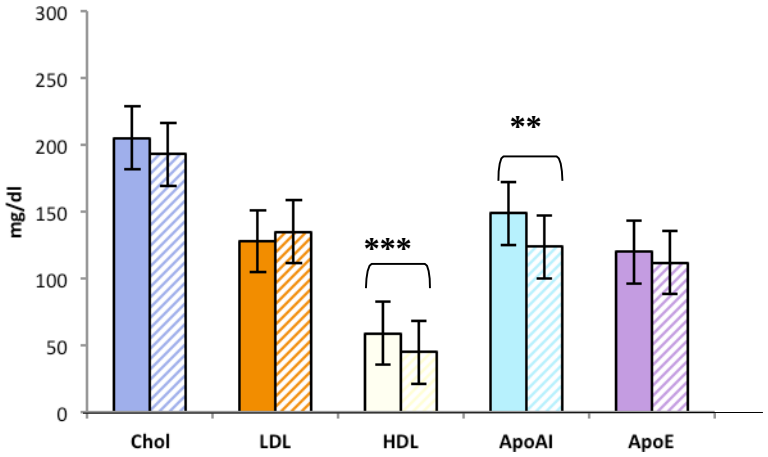

Figure 14. Non-AD group without ApoE4

In summary, cholesterol plasma concentration, LDL and its main protein ApoB were significantly higher in ApoE4-carriers vs ApoE3 - cholesterol (223 ±35 mg/dl vs 210 ±41 mg/dl), LDL (143 ±31 mg/dl vs 133 ±38 mg/dl), ApoB (111 ±25 mg/dl vs 99 ± 24 mg/d) see table 5. ApoE-genotype alone does not influence plasma ApoAI and HDL levels. After adjustment on AD and gender significant lower HDL level was observed in ApoE4 males but not in females (36 ±8 mg/dl vs 45 ±13 mg/dl) see table 6. The same effect of ApoE4 on HDL levels was established in the study of Robitaille (Robiteille N, et al., 1996) with 435 participants. Other authors do not observe any effect of ApoE polymorphism on HDL lipid level. It is possible that this effect is weakly or secondarily influenced by other factors.

Table 6. Lipoprotein profile (age-matched, adjusted groups). Data are given as means (mg/dl) ± SD

	Alzheimer				Non-AD			
	Females		Males		Females		Males	
	ApoE3	ApoE4	ApoE3	ApoE4	ApoE3	ApoE4	ApoE3	ApoE4
Age	75(±8)	71(±7)	70(±9)	72(±6)	58(±1)	59(±1)	58(±1)	58(±1)
N	59	83	39	42	31	4	81	11
Chol	226(±43)	234(±34)	199(±37)	212(±27)	190(±33)	232(±34)	207(±40)	211(±35)
TG	118(±52)	138(±70)	140(±70)	136(±64)	115(±60)	114(±32)	145(±74)	179(±88)
LDL	145(±41)	149(±33)	124(±32)	138(±26)	124(±27)	136(±50)	133(±39)	139(±26)
HDL	58(±16)	57(±16)	46(±14)	46(±13)	59(±13)	72(±27)	45(±13)	36(±8)
VLDL	24(±10)	27(±14)	28(±14)	27(±13)	23(±12)	23(±6)	29(±15)	36(±18)
ApoAI	154(±24)	151(±25)	127(±20)	136(±26)	149(±18)	170(±32)	132(±18)	124(±14)
ApoE	68(±53)	54(±38)	42(±27)	56(±61)	114(±34)	75(±13)	106(±45)	94(±40)

3.4 Detailed analysis of plasma lipoproteins (selected group)

There is ongoing discussion regarding the role of subtle differences in HDL composition played in developing of AD (Ohtani R, et al., 2018). Here methodology was established to investigate specific plasma components based on fractionation in light of AD. For the fractionation and the subsequent examination of plasma lipids composition, we performed size exclusion Fast Performance Liquid Chromatography (Figure 15). Fraction determination was based on time individual fraction leaves the column. Neighbouring fractions of similar nature were pooled and numbered. The first pool is represented by the mixture of VLDL and LDL particles. In the group of high density lipoproteins (pools 2-5), two major HDL subfractions - HDL2 (the larger-size cholesterolester-enriched HDL subfraction) and HDL3 (which constitute the predominant HDL subfraction) were separated. Consequently cholesterol was measured in all fractions and, ApoAI and ApoE abundance was estimated with the help of Western Blot in pools. Most of them contain ApoAI as the quantitatively most important protein constituent.

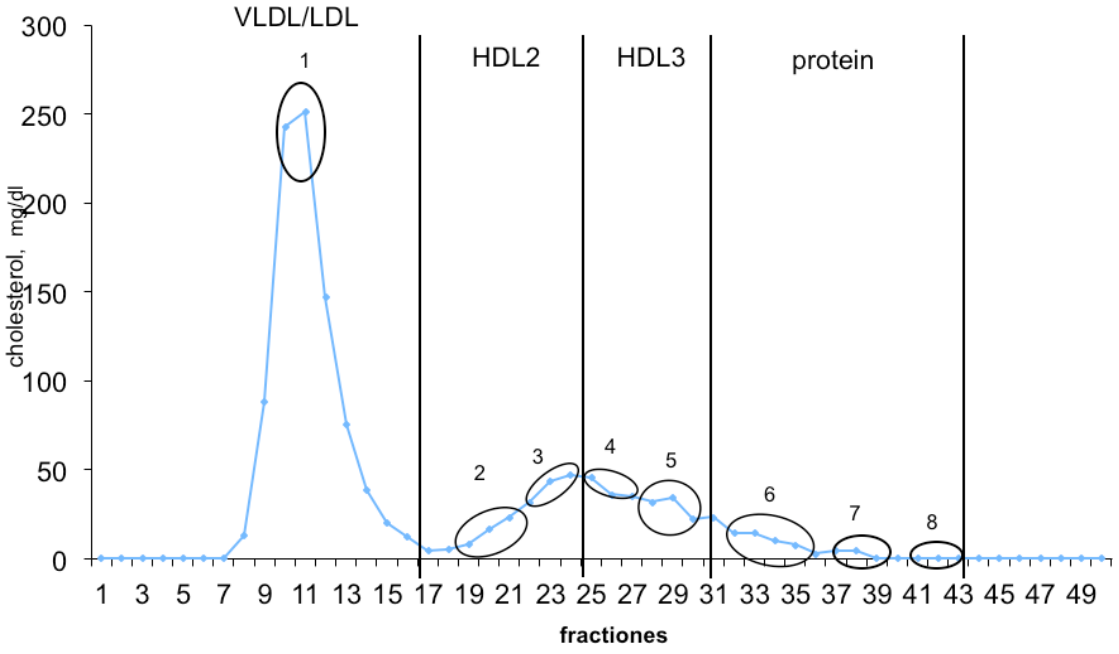

Figure 15. Separation of plasma with FPLC. Fractions are pooled as indicated by the numbers

After gel filtration of one millilitre human plasma, each of the 8 pools were examined and characterized. Western Blot procedure, ApoE-, and ApoAI ELISA were performed for each pool.

The Western Blot (Figure 16) showed a qualitative difference of ApoE and ApoAI concentrations. Maximum of ApoE levels were detected in pool 1 – VLDL and LDL. Non significant levels of ApoAI in VLDL and LDL (pool1) fractions were detected – almost all ApoAI was detected in HDL fractions (pools 2-5). In protein fractions some trace amounts of both ApoE and ApoAI were also detected (pool 6).

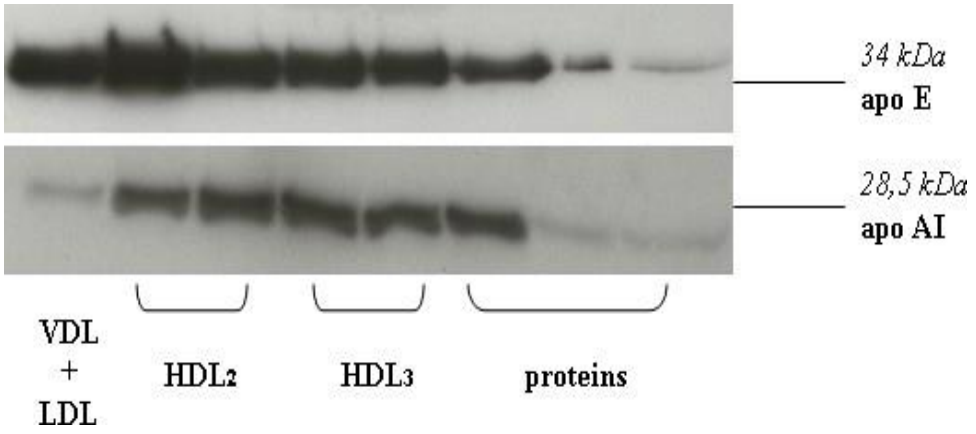

Figure 16. Qualitative differences of ApoE and ApoAI protein abundance in different plasma fractions. Pool numbers correspond to Figure 15.

ELISA detected ApoE in all lipoprotein fractions (pools 1-5). Vast majority of ApoE was detected in pool 1 (VLDL/LDL fraction). Interestingly, most dramatic difference of ApoE concentration was observed in the HDL2 fraction (pools 2, 3) if compared between ApoE genotypes. ApoE4-carring AD patients demonstrated 2-times lower ApoE concentration in HDL2 as compared to AD ApoE3-carriers (Figure 17).

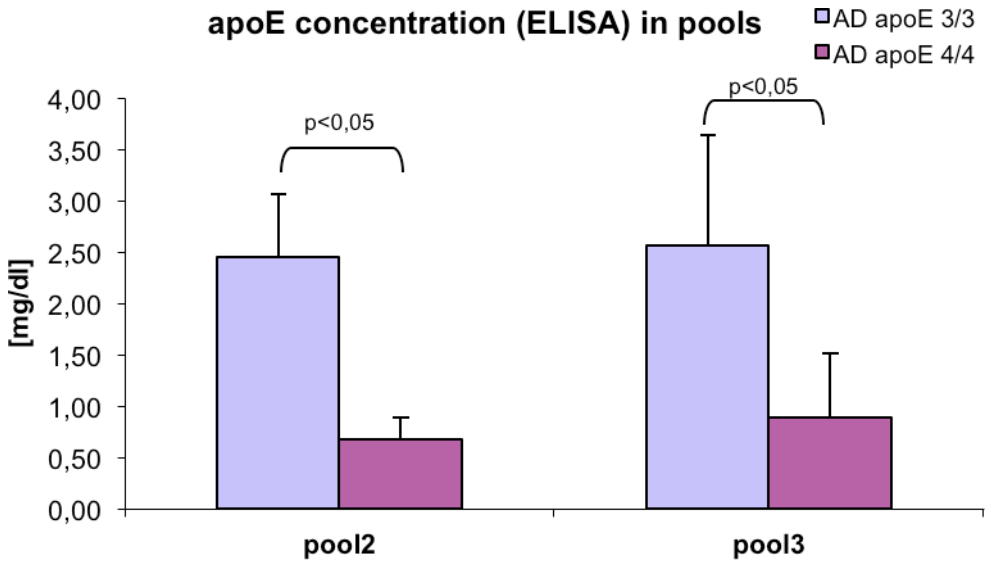

Figure 17. Concentration of ApoE in AD ApoE3 vs AD ApoE4. ELISA test showed quantitative differences of Apo E concentrations in HDL2 pools of AD patients with different ApoE phenotypes: ApoE3 (n=6) and ApoE4 (n=5) (selected group)

FPLC coupled to Western Blot or ELISA might help to shed some light on complex lipid interaction observed in AD patients. On the other side, this strategy is highly laborious and expensive, therefore it is difficult to recommend as first screening methods of big number of cases.

4 Discussion

There is much evidence suggesting that there is a strong relationship between the deterioration of brain lipid homeostasis, vascular changes and the pathogenesis of Alzheimer disease (Poirer J, 2003).

These associations include: (1) recognition that a key cholesterol transporter, ApoE4, acts a major genetic risk factor for both familial and sporadic AD; (2) epidemiological studies linking cardiovascular risk factors, such as hypertension and high plasma cholesterol, to dementia; (3) the discovery that small strokes can precipitate clinical dementia in cognitively normal elderly subjects; and (4) the beneficial effect of cholesterol-lowering drugs, such as Probucol and statins, in AD (Poirier J, 2005).

The discovery that the ApoE4 genotype is genetically linked to AD, raises the possibility that a dysfunction of the lipid transport system could seriously affect lipid homeostasis in the brain and become a trigger of AD onset.

That's why we examined the association between plasma lipid parameters and Alzheimer disease more closely with respect to gender and different ApoE genotypes. Firstly, we compared the association between AD and ApoE4 in the whole study population. Then, the influence of the ApoE polymorphism and gender on the distribution of plasma of total cholesterol, LDL, triglycerides, VLDL, HDL, and ApoAI, ApoE and ApoB and, finally, to detect a possible difference in HDL composition between AD patients with and without the ε4 allele with respect to gender to get a clearer picture of the relation between plasma lipid levels and Alzheimer disease.

AD and lipid parameters

Brain cell synthesis, efflux, and influx of cholesterol are tightly regulated (Shimano H, 2001). Neuronal synaptic requirements for cholesterol needed in membrane lipid raft composition, plasticity, and regeneration are met through the uptake of ApoE-cholesterol complexes produced by glia in addition to axonal transport of cholesterol produced in cell bodies. Disturbances in cholesterol homeostasis in either direction may promote AD pathology. Lack of cholesterol supply to neurons impairs neurotransmission and synaptic plasticity (Mauch D, et al., 2001), inducing neurodegeneration and tau pathology.

The ε4 allele of the ApoE is associated with Alzheimer's disease and also with elevated serum total cholesterol and LDL levels. The role of serum total cholesterol in the pathogenesis of AD in a population-based sample of 444 men, aged 70-89 years was examined (Notkola I and Sulkava R, 1998), and concluded that high serum total cholesterol may be an independent risk factor for AD and some of the effect of the ApoE4 allele on risk of AD might be mediated through high serum cholesterol.

Different results were found in the literature. Plasma HDL and ApoAI were either found to be unchanged (Tynkkynen J, et al., 2016; Hoshino T and Kamino K, 2002), increased (Kuo Y and Emmerling M, 1998; Carantoni M and Zuliani G, 2000; Merched A and Xia Y, 2000) or decreased (Reitz Ch, et al., 2010) in AD patients versus non-AD's. These differences might be due to the selection of patients and non-AD's, which may have a crucial influence on plasma lipoprotein levels (family and individual history, accompanying illnesses, environmental factors, etc.).

It has been suggested that HDL particles might play a role in the removal of excess cholesterol from the brain by interaction of ApoE and heparansulfate proteoglycans in the subendothelial space of cerebral microvessels (Matsuda Y, et al., 1998). It is also known that HDL particles favour endothelium dependent vasorelaxation by inhibiting the action of oxidized LDL particles (Matsuda Y, Hirata K, et al., 1993), and interfere with induction of endothelial cell adhesion molecules (Cockerill G, Rye K, et al., 1995); these mechanisms might be involved in the pathogenesis of small vessel disease.

Low levels of total cholesterol and HDL that have been observed in a *post mortem* study (Kuo Y and Emmerling M, 1998) might at least be partly related to malnutrition in the late stages of AD or other severe disease before death in AD patients and non-AD's. An increase of plasma HDL was found in in an age- and gender matched study population (Kalman J and Kudchodkar B, 1999). Interestingly, the CSF levels of ApoAI, which closely correlate to plasma ApoAI levels (Fagan A and Youkin L, 2000), and have also been found to be elevated in AD (Demester N and Castro G, 2000).

In general, the association of dyslipidemias with AD is still a controversial issue, due to: 1) the very small number of patients included in most of the studies, and 2) the exclusion from the analysis of some important factors that might induce significant

modifications in lipoprotein profiles. With regards to this aspect, several conditions are known to modify plasma lipids in the elderly including age itself, gender, dietary lipid intake, obesity, diabetes mellitus, drug therapy, ApoE phenotype, an inflammation state, etc.

Gender and lipid parameters

We measured and compared levels of plasma lipoproteins of the males and females in both AD and non-AD groups to uncover the effect of the gender on lipid differences of the groups.

Analysing the database of the psychiatric clinic and the neurological clinic of Hamburg University Hospital it was revealed that females on average showed a higher plasma LDL, HDL, ApoAI and cholesterol levels as males. In two big population studies the plasma lipid profile of 538 females and 446 males (Kotteke BA, 1991) and 163 females and 184 males (Mattila KS, et al., 1986) was examined. In both studies the same effect was seen – significantly higher HDL and ApoAI plasma concentration in females. Mattila KS et al., which examined Finnish cohorts was able to demonstrate higher LDL and ApoB plasma levels in association with female gender.

There was no significant gender related difference of the plasma ApoE and ApoB levels, but females are characterized by slightly increased ApoB levels (105 ±28 mg/dl vs 103 ±21 mg/dl).

Therefore the plasma lipid parameters measured in our cohort are similar to the results of other populations. Light divergences between the single random checks may lie in the choice of the participants. For example diuretics and ß-blocker may influence lipid metabolism. Age as well influenced lipid metabolism – plasma LDL and ApoB concentration in general lower with age.

ApoE4 and lipid parameters

ApoE is a protein that plays an essential role in lipid metabolism and distribution (Huang Y and Mahley RW, 2014). The ApoE gene is polymorphic, and its three alleles code for isoforms E2, E3, and E4, which differ by single-amino-acid substitutions (Eisenberg DT, 2010). The ApoE3 allele is the predominant isoform in all populations studied. The ApoE4 allele is associated with increased total serum

cholesterol and it constitutes a major risk factor for Alzheimer disease (Hendrie H, 2014). The ApoE2 allele seems to have a protective effect against Alzheimer disease and is associated with longevity (Kulminski A, 2016).

One of the objective of this work was to show an influence of ApoE4 on plasma lipoproteins and to compare this effect on AD. For this reason we compared plasma lipid parameters of recipients with one or two E4-allels with recipients without the E4-allele.

Data from the literature (Sing CF, Davignon J, Ehnholm J, et al.; Xu CF et al.; Robitaille N, et al.; Garces C, et al. and Lussier-Cacan S et al. with big cohorts between 122 and 1695 participants) are in accordance with data obtained from our study – positive influence of ApoE4 on cholesterol, LDL and ApoB, as well as no effect on ApoAI.

Differences in composition of HDL subclasses

Plasma of patients from total study population was separated by FPLC analysis for detailed characterization of HDL subtypes. For a further fractionation of HDL, 1 ml of plasma was applied to the gel filtration and the plasma lipoproteins HDL2 and HDL3 were isolated.

After detailed analysis of HDL subpopulations, it was revealed that HDL3 particles of AD ApoE4/4 group have a lower cholesterol concentration vs AD ApoE3/3 group, but they were loaded more with ApoAI. ApoAI concentration was 2-3-times higher in AD-carrying patients vs non-AD's. Our results are in agreement with the data of Merchedet al scientific group (Merched A and Xia Y, 2000), additionally they suggested that the AD patients with higher serum ApoAI concentrations have less cognitive impairment. Considering the anti-atherogenic properties of high density lipoprotein, it is possible to identify an analogy between biochemical markers of atherosclerosis (ApoE4, ApoAI, HDL, etc.) and markers of Alzheimer's disease. Interestingly, studies in the elderly have shown that high levels of serum ApoAI and HDL are associated with low total mortality and enhanced longevity (Luoma P, 1997).

It is not clear, which role elevated HDL levels might play in AD pathophysiology. Generally, it is believed that HDL removes cholesterol from peripheral organs and

tissues (Segrest J and Li L, 2000). We speculate that HDL3 particles could be involved in the transport of 24-OH-cholesterol, the main product of cholesterol degradation in the brain, at the blood brain barrier, in spite of this, they have lower cholesterol concentration (cholesterol can be substituted with 24-OH-cholesterol). It has been shown that 24-OH-cholesterol is increased in plasma and CSF of AD patients (Selkoe D and Schenk D, 2003). Increased ApoAI and HDL might be connected with increased cholesterol turnover in the brains of AD patients.

In spite of the small number of the participants (n=21), the ApoE phenotype distribution confirmed the increased ApoE4 allele frequency in the group of Alzheimer patients, compared to our non-AD controls (Table 5, 6).

Experimentally it was revealed that ApoE concentration in human plasma was higher by AD ApoE3/3 patients vs AD ApoE4/4. After detailed analyse of each pool (ELISA) significantly higher ($p<0,05$) ApoE concentration in pools 2 and 3 (HDL2) was observed. ApoE concentration was almost 5-time higher in the VLDL fraction than in HDL2 or HDL3 fractions of AD patients with ApoE4/4 genotype. In the non-AD group with ApoE4/4 genotype equally ApoE distribution between different lipoprotein classes was observed. It is not clear, which factor or mechanism influenced the distribution of ApoE between lipoprotein classes by patients with AD, but this factor might play a role in AD pathophysiology. In principle, lipoprotein lipase (LPL) could be involved in this process (Baum L, 2000). Bound to the vascular endothelium, LPL hydrolyses triglycerides of chylomicrons and very low density lipoproteins (VLDL). However, there is growing evidence that LPL is a multifunctional protein in lipoprotein metabolism. In addition to lipolysis, LPL mediates the uptake and degradation of lipoproteins by cells. Beisiegel, Weber, and Bengtsson-Olivecrona (Beisiegel U, et al., 1991) showed an LPL-induced increase in the binding of ApoE-rich lipoproteins to cells in vitro. LPL also promoted the binding of ApoE-containing micelles to the low density lipoprotein receptor-related protein (LRP). Direct binding of LPL to LRP1 has been demonstrated as well and this interaction apparently mediates lipoprotein degradation (Nykjaer A, et al., 1993). Based on these observations it was suggested that LPL stimulates the cellular catabolism of triglyceride-rich lipoproteins and modulates distribution of ApoE between lipoprotein classes.

5 Summary and Outlook

In this study plasma lipids were investigated in association with Alzheimer disease. Analysis of total 350 age matched probands shows that AD was twice more common in females as well as ApoE4 allele was more frequent in females as compared to males. Elevated plasma concentrations of LDL, HDL and ApoAI were clearly associated with AD diagnosis and female gender. These results suggest that changes in metabolic conditions, reflected by ApoAI and HDL levels, may be important in AD development and progression. Association of high LDL cholesterol with AD might indicate that cholesterol lowering drugs could help to decrease the risk of AD. Limitations of the current study were the relative small number of ApoE4 homozygotes and the limited sample number for HDL subtype analysis. In future, the quantitative analysis of HDL2 versus HDL3 may help to explain the differences in cholesterol levels observed in individuals without and with AD.

6 List of abbreviations

AD	–	Alzheimer Disease
Aβ	–	Amyloid beta
AICD	–	APP intracellular domain
ARG	–	Alzheimer Research Group
Apo	–	Apolipoprotein
APP	–	Amyloid Precursor Protein
Chol	–	Cholesterol
CM	–	Chylomicrones
CNS	–	Central Nervous System
DACO	-	Background-reducing antibody diluent
EDTA	–	Ethylendiaminetetraacetic Acid
ELISA	–	Enzyme-linked Immunosorbent Assay
FPLC	–	Fast Performance Liquid Chromatography
HDL	–	High Density Lipoproteins
HMG-CoA	–	3-Hydroxy-3-Methylglutaryl Coenzyme A
IDL	–	Intermediate Density Lipoproteins
LCAT	–	Lecitin:cholesterol Acyltransferase
LDL	–	Low Density Lipoproteins
LPL	–	Lipoprotein Lipase
LRP	–	Lipoprotein receptor-related protein
NFT	–	Neurofibrillary Tangles
O.D.	-	Optical Density
PHF	–	Paired Helical Filaments
SDS-PAGE	–	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
TG	–	Triglycerides
TRL	–	Triglyceride- rich Lipoprotein
VLDL	–	Very Low Density Lipoproteins
CSF	–	Cerebrospinal Fluid
WHO	–	World Health Organisation

7 Literature

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9 Curriculum Vitae

Lebenslauf entfällt aus datenschutzrechtlichen Gründen.

10 Eidesstattliche Versicherung

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