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Serum noncholesterol sterols in Alzheimer's disease: the Helsinki Businessmen Study



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Cerebral cholesterol metabolism is perturbed in late-onset Alzheimer's disease (AD), but whether also the extracerebral cholesterol metabolism is perturbed is not known. Thus, we studied whole-body cholesterol synthesis and absorption with serum noncholesterol sterols in men without AD ($n = 114$) or with ($n = 18$) "pure" AD (no concomitant atherosclerotic cardiovascular disease) in a long-term cohort (the Helsinki Businessmen Study) of home-dwelling older men without lipid-lowering drugs and on their habitual home diet. Serum lipids did not differ between AD and controls, but age was higher (78 ± 1 vs 74 ± 0.3 years, mean \pm standard error, $P < 0.001$), age-adjusted plasma glucose concentration was lower (4.8 ± 0.3 vs 5.7 ± 0.1 mmol/L, $P = 0.011$), and APOE $\epsilon 4$ allele and frailty were more frequent in AD than in controls. Of the age and frailty-adjusted serum noncholesterol sterols desmosterol and lathosterol ratios to cholesterol reflecting cholesterol synthesis were lower in AD than in controls (eg, lathosterol 114 ± 12 vs 137 ± 5 $10^2 \mu\text{mol}/\text{mmol}$ cholesterol, $P = 0.004$). Cholesterol ratio to cholesterol was higher in AD than in controls suggesting increased cholesterol absorption. lathosterol/sitosterol ratio reflecting cholesterol metabolism was lower in AD than in controls (0.95 ± 0.28 vs 1.52 ± 0.11 $10^2 \mu\text{mol}/\text{mmol}$ cholesterol, $P = 0.027$). In AD, plasma glucose correlated negatively with cholesterol synthesis, whereas in controls the correlation was positive. In conclusion, extracerebral cholesterol metabolism was altered in AD. This finding along with the low plasma glucose concentration and its paradoxical interaction with cholesterol synthesis opens new perspectives in the regulation of cholesterol metabolism and glucose homeostasis in AD. (Translational Research 2018; 202:120–128)

Abbreviations: AD = Alzheimer's disease; A β = amyloid beta; APOE = apoprotein E; APP = amyloid precursor protein; BMI = body mass index; CVD = cardiovascular disease; FID = flame ionization detection; GLC = gas-liquid chromatography; GT = glutamyltransferase; HBS = Helsinki Businessmen Study; hs-CRP = high sensitive C-reactive protein; MMSE = Mini-mental state examination; SREBP 2 = sterol regulatory element-binding protein 2

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At A Glance Commentary

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Background

In vitro and animal studies suggest that the culprit of Alzheimer's disease (AD) amyloid beta is able to interfere with cholesterol metabolism. Thus, we studied extracerebral cholesterol metabolism in subjects with late-onset AD.

Translational Significance

Cholesterol synthesis and cholesterol metabolism were downregulated and cholesterol absorption was increased in AD. Plasma glucose was low in AD, and its association with cholesterol synthesis was paradoxical to controls. These findings open new perspectives to basic research to reveal the regulation of cholesterol metabolism and glucose homeostasis in AD thus helping to understand the development of AD.

INTRODUCTION

Late-onset Alzheimer's disease (AD) is a common type of dementia in advanced age. The main changes in AD are extensive deposition of amyloid beta ($A\beta$) peptide, a cleavage product of amyloid precursor protein (APP). The deposition of $A\beta$ triggers numerous processes leading to neuronal loss and impairment of memory and cognition.¹ In old age, the development of AD is a multifactorial process where aging and the presence of apoprotein E (APOE) $\epsilon 4$ allele are well-known risk factors.¹ Brain tissue is rich in cholesterol, which is completely derived from in situ biosynthesis.² During the past decades there is increasing evidence that cholesterol metabolism in brain is involved in the development of AD.³⁻⁵ For example, APOE has a strong isoform-related effect on cholesterol homeostasis in the brain as well as on the metabolism of $A\beta$. Cerebral cholesterol synthesis has been assessed with biomarkers, and in one of the three studies available cholesterol synthesis was unaffected,⁶ but in 2 studies it was lower in AD than in the control subjects.^{7,8} In 2 of these studies, 24S-hydroxycholesterol was lower in AD than in controls suggesting that also cholesterol elimination from brain was decreased in AD.^{6,7} Accordingly, cerebral cholesterol metabolism is conceivably perturbed in AD.

It is generally considered that plasma cholesterol concentration does not interact with the cerebral cholesterol if the blood-brain barrier is intact.⁹ However, APOE isoforms affect cholesterol metabolism also

extracerebrally, so that subjects with APOE $\epsilon 4$ allele have lower cholesterol synthesis and higher cholesterol absorption efficiency than subjects having the APOE $\epsilon 3$ or APOE $\epsilon 2$ alleles.¹⁰ Cholesterol synthesis alone without information on cholesterol absorption or cholesterol metabolism has been evaluated in AD in four studies with controversial results.¹¹⁻¹⁴ The interpretations are, however, complicated by the fact that in general the type of dementia is usually not well defined and diagnosis of AD in an older individual may be mixed with varying degrees of vascular disease. We aimed to control this in the long-term follow-up of the Helsinki Businessmen Study (HBS).^{15,16} In this cohort the cardiovascular risk factors, cognition, morbidity, and mortality has been followed up from midlife to old age.^{15,16} In Finland the diagnosis of AD is generally based on the criteria presented in Diagnostic and Statistical Manual of Mental Disorders, fourth edition, and by the National Institute of Neurological Disorders and Stroke-Alzheimer Disease and Related Disorders working group.^{17,18} The diagnosis is clinical, routinely associated with brain imaging, and made by a clinical specialist, a neurologist or a geriatrician.¹⁶ In this cohort we pragmatically separated men with AD diagnosis into "pure" AD—without any diagnosis of concomitant atherosclerotic cardiovascular disease (CVD) (coronary heart disease, cerebrovascular disease, or peripheral vascular disease)—and into a mixed dementia with a concomitant diagnosis of any atherosclerotic cardiovascular disease.¹⁶ As a further indication that "pure" AD defined accordingly indeed was different from dementia associated with CVD we noticed that midlife serum cholesterol concentration predicted mixed dementia, not "pure" AD, which paradoxically tended to be associated with lower midlife glucose.¹⁶ To this end, the aim of this study was to evaluate whether extracerebral cholesterol metabolism differs in men without AD or with late-onset "pure" AD in a long-term cohort of home-dwelling older men of the HBS study.

MATERIALS AND METHODS

Study population. The HBS cohort has been described earlier in detail.^{15,16} In short, initially a healthy cohort of 3490 executives and businessmen born in 1919–1934 participated in health check-ups during the 1960s and early 1970s at the Institute of Occupational Health in Helsinki. They have been followed up since then with questionnaires and clinical and laboratory examinations at the University of Helsinki as a clinical-epidemiologic longitudinal cohort. In 2003, a random sample of home-dwelling men of the cohort

(n = 651) participated in a health check-up including medical examination with weight, height, and waist circumference measurements, body mass index (BMI) calculation, Mini-mental state examination (MMSE) to evaluate cognition (maximum 30 points, <24 taken to denote cognitive impairment),¹⁹ frailty was evaluated using modified frailty phenotype,²⁰ and blood samples were drawn and analyzed to assess overall health and to examine serum and lipoprotein lipids and serum noncholesterol sterols.

The men were followed up to January 2014 with questionnaires, national registries, and death certificates for diagnosis of dementia.¹⁶ During the follow-up, 20 men, 3% of the 2003 cohort, had received a clinical diagnosis of "pure" AD. The classification of "pure" AD was performed by a neurologist and a geriatrician from our research team based on the information from questionnaires, health check-ups, national registers, and the information and narratives from clinical records and death certificates. If the available information included no hints of cardiovascular disease, after mutual consensus the diagnosis of "pure" AD was settled.¹⁶ Those subjects with dementia combined with signs of CVD (mixed dementia) were not included in this study. As a control group we included 153 men who were home-dwelling in 2014 and did not have any signs of cognitive disorders at an average age of 85 years. Two AD and 39 control men were taking lipid-lowering drugs in 2003, so that the final study population consisted of 18 AD and 114 control men.

All subjects gave their written informed consent. The study was performed according to the principles of the Declaration of Helsinki. The Ethics Committee of the Department of Medicine, University of Helsinki (Revised protocol Dnro 246/E5/2002) has approved the study follow-up of the HBS cohort.

Methods and measurements. Plasma glucose, serum uric acid, high sensitive C-reactive protein (hs-CRP), and serum glutamyltransferase (GT) concentrations were analyzed with standardized methods at the Central Laboratory of Helsinki University Hospital. Serum total, low density lipoprotein and high density lipoprotein cholesterol and serum triglyceride concentrations were analyzed enzymatically using automated analyzers of our hospital laboratory. APOE genotypes were determined with the polymerase chain reaction method.

Serum cholesterol and noncholesterol sterols Δ^8 -cholestenol (5 α -cholest-8-en-3 β -ol), desmosterol, lathosterol, campesterol, sitosterol, and cholestanol were analyzed using gas-liquid chromatography (GLC) with flame ionization detection (FID) and a 50-m capillary column (Ultra 2, Agilent Technologies, Wilmington, DE) with 5 α -cholestane as internal standard.²¹ The samples were extracted with chloroform-methanol, saponified with potassium hydroxide in ethanol and

silylated with trichloromethylsilane prior to GLC analysis. In GLC the mobile phase is gas (pure helium), and a liquid stationary phase is deposited on the inner wall of a long capillary column. The separation of cholesterol and noncholesterol sterols in serum depends on their retention time. When this analysis was developed in our research laboratory the quantification and characterization of the sterols were checked with mass spectrometry and using commercial pure sterol standards. It turned out as it is also generally considered today that GLC with FID detection is reliable and it has a good peak resolution when analyzing serum cholesterol and the 6 noncholesterol sterols described above.²¹ However, the detection is not sufficient enough to analyze the serum oxysterols 24S-hydroxysterol and 27-hydroxysterol. Serum noncholesterol sterols were expressed as ratios to cholesterol by adjusting the concentrations with the cholesterol value of the same GLC run. Ratios to cholesterol of the serum cholesterol precursors (Δ^8 -cholestenol, desmosterol, and lathosterol) reflect cholesterol synthesis, while those of plant sterols (campesterol and sitosterol) and cholestanol reflect cholesterol absorption efficiency.²²⁻²⁴ We also calculated the lathosterol/sitosterol ratio, which reflects whole-body cholesterol metabolism,^{23,24} and the lathosterol/desmosterol ratio reflecting the activity of the 2 different pathways of cholesterol synthesis.

Statistical analysis. Statistical analyses were conducted using SPSS version 22. According to power analysis based on previous data¹¹ to detect a significant difference in serum lathosterol to cholesterol ratio between AD vs controls with an α level of 0.05 (two sided) and statistical power of 0.80, the required minimal study population should be 46. Normality and homogeneity of variance assumptions were checked before further analyses. Univariate analysis of covariance was used to compare the values between the groups. Variables not normally distributed even after logarithmic transformation, nonhomogenous in variance, or noncontinuous were tested with Mann-Whitney *U* test or Fisher's exact test. Spearman correlation coefficients were calculated. A *P* value of <0.05 was considered statistically significant. The results are given as mean \pm standard error.

RESULTS

Table 1 summarizes the key characteristics of the men with "pure" AD and the controls without any signs of cognitive decline. The mean age was 77.7 years (range 70-84 years) in the AD and 74.1 years (range 69-84 years) in the control group (*P*<0.001), so that all further analyses were performed with age as a covariate. Frailty was more prevalent in the AD than in the control group, so that frailty was also taken as a covariate in all analyses. The range of BMI varied from 19 to 31 kg/m² in both groups, and the mean values of BMI and waist

Table 1. Clinical characteristics of the study population in 2003

Variables	Control group n = 114	AD group n = 18	P (adjusted for age)	P (adjusted for age and frailty)
Age, y	74.1 ± 0.3	77.7 ± 1.0	<0.001 (not adjusted)	—
BMI, kg/m ²	25.7 ± 0.3	24.3 ± 0.9	0.185	0.052
Waist circumference, cm	100 ± 1.0	102 ± 2.5	0.355	0.936
Plasma glucose, mmol/L	5.71 ± 0.12	4.84 ± 0.31	0.011	0.284
Uric acid, μmol/L	365 ± 8	363 ± 20	0.934	0.280
hs-CRP, mg/L	2.77 ± 0.62	1.40 ± 1.71	0.795	0.796
GT, IU/L	31.6 ± 6.0	78.6 ± 16.4	0.091	0.093
APOE ε4 allele, n (%)	27 (25.2)	9 (50.0)	0.047	0.034
MMSE, median (interquartile range)	29 (28–30)	26 (25–28)	<0.001	<0.001
Frailty, n (%)	30 (39.0)	10 (88.3)	0.005	—
Alcohol consumption, g/wk	104 ± 11	121 ± 29	0.617	0.748
Smokers, %	5.3	5.6	0.960	0.850
Hypertension, %	39.1	52.0	0.240	0.190
Major CVD, %	14.0	0	0.090	0.100
Type 2 diabetes, %	7.0	0	0.250	0.300
History of cancer, %	15.8	16.7	0.920	0.840
Statin treatment, %	0	0	—	—

Abbreviations: AD, Alzheimer's disease; APOE, apolipoprotein E; BMI, body mass index; CVD, cardiovascular disease; hs-CRP, high sensitive C-reactive protein; GT, glutamyltransferase; MMSE, Mini-mental state examination.

Continuous data are mean ± standard error.

circumference were similar between the groups. Mean plasma glucose was significantly lower in the AD compared with the control group, but serum uric acid, hs-CRP, and GT concentrations did not differ between the groups and were within the reference values. Half of the AD men had at least one APOE ε4 allele compared to 25% in the control men (P<0.05). MMSE was different between the groups, but also in the AD group 85% had at least 24 points, which is the conventional cutoff of cognitive decline. This indicates that clinical AD was mainly developed after the health check-up in 2003; in fact the clinical diagnosis of AD had been set during the following 3 to 10 years (mean 7.8 years) after 2003. Alcohol consumption and smoking did not differ between the groups.

Hypertension had been diagnosed in 52% of the men with AD and in 39% of the controls, and the difference was not significant between the groups (Table 1). None of the men with AD had a history of major CVD (coronary heart disease, cerebrovascular disease, or peripheral vascular disease) or type 2 diabetes, whereas in the control group 14% had a positive history of atherosclerotic vascular disease and 7% had type 2 diabetes (Table 1). The prevalence of these diseases was, however, not significant between the groups, neither was the prevalence of the history of cancer, which had been treated and was in remission.

Serum and lipoprotein cholesterol and serum triglyceride concentrations did not differ between the groups (Table 2). The range of serum cholesterol varied from 3.6 to 6.9 mmol/l in the AD and from 3 to 7.5 mmol/l in the control group.

After adjustment for age and frailty, serum desmosterol and lathosterol ratios to cholesterol were significantly lower in the AD than in the control group (Table 2). Lathosterol/desmosterol ratio did not differ between the groups. Regarding cholesterol absorption markers, serum cholestanol ratio to cholesterol was higher in the AD than in the control group. Lathosterol/sitosterol ratio, marker of cholesterol metabolism, was lower in the AD than in the control group. Serum plant sterols did not significantly differ between the groups.

In both groups, the cholesterol synthesis markers were interrelated depicted for serum lathosterol and desmosterol in Fig 1, panel A. Likewise, cholesterol absorption markers were interrelated shown for serum cholestanol and sitosterol in Fig 1, panel B. In the AD and control groups, cholesterol homeostasis was intact shown for the inverse interrelation between serum lathosterol and sitosterol in Fig 2. Plasma glucose correlated positively with serum Δ⁸-cholestanol in the control group but negatively in the AD group (Fig 3). Frailty was not associated with age, BMI, MMSE, plasma glucose, hs-CRP, APOE isoforms, serum or lipoprotein lipids or serum noncholesterol sterols in either of the groups.

DISCUSSION

The new observations in the present study were that in men with late-onset "pure" AD—dementia diagnosis without signs of concomitant clinical atherosclerosis—the extracerebral cholesterol synthesis and cholesterol metabolism were downregulated compared with the control men. The synthesis markers, serum desmosterol and lathosterol ratios to cholesterol, were 5%–18% lower and the lathosterol/sitosterol ratio was 37% lower in the AD compared with the control group. Cholesterol synthesis was downregulated within its both pathways, that is, in the Bloch unsaturated and the Kandutsch-Russell saturated side chain pathway. One of the surrogate markers of cholesterol absorption

Table 2. Serum and lipoprotein lipids and serum noncholesterol sterols in the study population in 2003

Variables	Control group n = 114	AD group n = 18	P (adjusted for age)	P (adjusted for age and frailty)
Serum cholesterol, mmol/L	5.36 ± 0.09	5.66 ± 0.22	0.256	0.658
LDL cholesterol, mmol/L	3.27 ± 0.09	3.12 ± 0.22	0.580	0.305
HDL cholesterol, mmol/L	1.44 ± 0.05	1.63 ± 0.13	0.007	0.090
Serum triglycerides, mmol/L	1.39 ± 0.06	1.24 ± 0.15	0.257	0.065
Δ ⁸ -Cholestanol*	24.4 ± 0.8	20.1 ± 1.9	0.061	0.075
Desmosterol*	109 ± 2	104 ± 5	0.424	0.006
Lathosterol*	137 ± 5	114 ± 12	0.101	0.004
Campesterol*	264 ± 15	251 ± 37	0.773	0.734
Sitosterol*	130 ± 6	119 ± 16	0.573	0.537
Cholestanol*	145 ± 3	153 ± 7	0.286	0.040
Lathosterol/desmosterol*	1.24 ± 0.03	1.10 ± 0.08	0.156	0.092
Lathosterol/sitosterol*	1.52 ± 0.11	0.95 ± 0.28	0.079	0.027

Mean ± standard error.

*10² × μmol/mmol of cholesterol. AD, Alzheimer's disease; HDL, high density lipoprotein; LDL, low density lipoprotein.

efficiency, cholestanol to cholesterol ratio, was increased by 6% suggesting that cholesterol absorption efficiency was upregulated. The serum noncholesterol sterol to cholesterol ratios, validated biomarkers in several different non-AD populations²²⁻³⁰ proved to reflect cholesterol metabolism also in AD.

We have demonstrated earlier in a large cohort of the HBS population that lower midlife glucose was associated with "pure" AD.¹⁶ Also in this study plasma glucose concentration was lower in the AD than in the control group. Although diabetes is usually considered an AD risk factor, this may not be the case if AD pathology is verified and vascular contribution excluded.³¹⁻³³ Another unexpected finding related to plasma glucose was that in AD plasma glucose concentration correlated negatively with cholesterol synthesis and not positively as expected.³⁴ This paradoxical finding opens new perspectives to find out whether the regulation between cholesterol metabolism and glucose homeostasis is perturbed in AD.

AD was developed during the following 3–10 years after the present health check-up in 2003. As expected, higher age and the presence of APOE ε4 allele characterized the development of AD. Frailty was also more frequent in the AD than in the control group already in 2003. On the contrary, the frequency of the most prevalent diseases, smoking habits, alcohol consumption, or the rest of the clinical variables including hs-CRP, GT, and uric acid did not seem to interfere with the development of AD. The mean BMI value was normal in both groups, and none of the subjects in either of the groups had below normal BMI.

In older subjects with AD, extracerebral cholesterol synthesis has earlier been evaluated in four studies.¹¹⁻¹⁴ In three of these studies, the possibility of vascular dementia had been screened by brain imaging.^{11,12,14} In three studies serum lathosterol or desmosterol ratio to cholesterol was lower in the AD than in the control group,¹¹⁻¹³ but in one study no difference could be observed between the groups.¹⁴ Thus, the present study confirmed the downregulated cholesterol synthesis in AD but also demonstrated reduced whole-body cholesterol metabolism and a slight increase in cholesterol absorption efficiency. We have demonstrated earlier that old age without AD interferes with cholesterol metabolism, so that in 75-year-old men cholesterol absorption efficiency, bile acid synthesis, and biliary cholesterol excretion were diminished compared with 50-year-old men, but cholesterol synthesis was preserved at the same level in old age as in middle-age.³⁵ Accordingly, AD seems to have a different impact on cholesterol metabolism than aging alone.

What are the possible mechanisms interfering with cholesterol metabolism in AD? APOE ε4 isoform

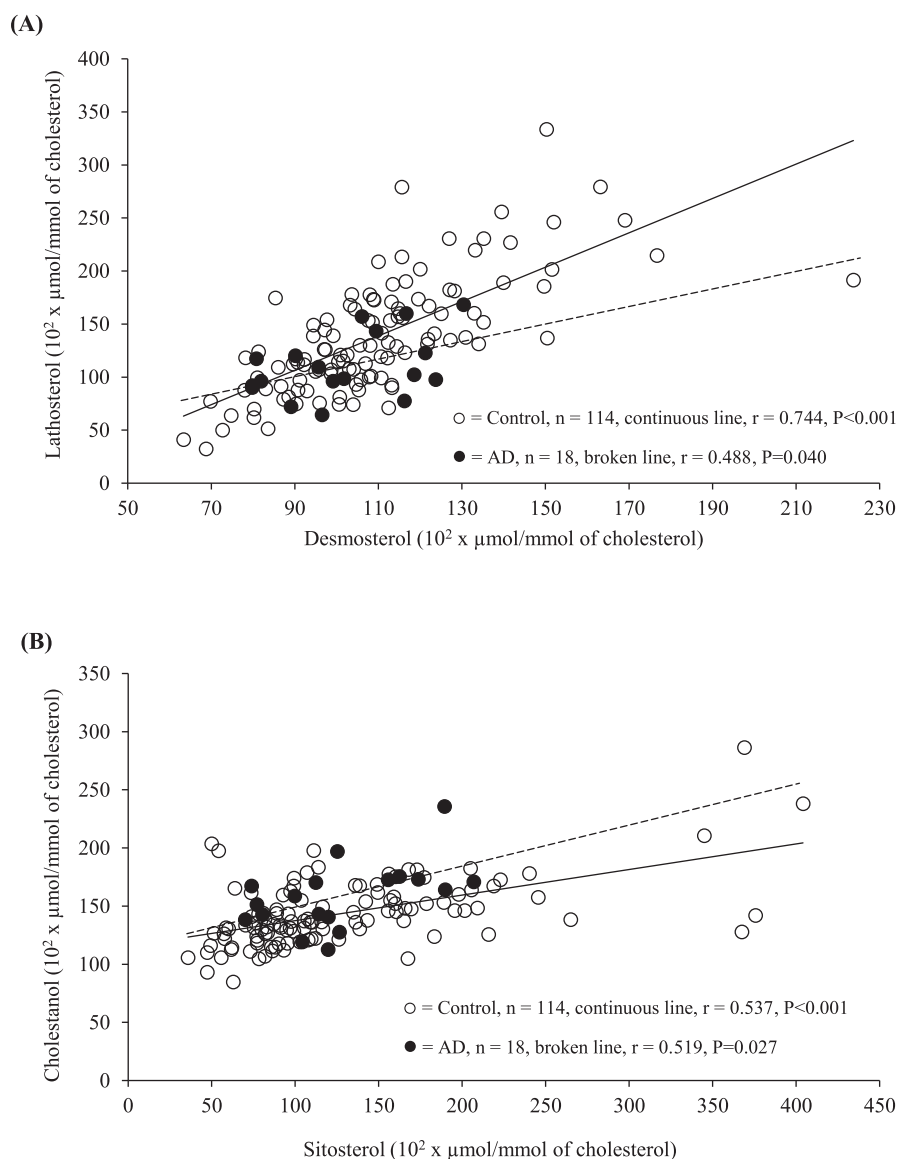


Fig 1. Correlation of (A) serum lathosterol to desmosterol and (B) cholestanol to sitosterol in the control men and in men with Alzheimer's disease (AD).

decreases cholesterol synthesis and serum lathosterol to cholesterol ratio¹⁰ similarly as observed in the present study making it a possible factor. Frailty, the other variable frequent in AD is characterized by reduced physiological reserves and increased vulnerability, but the exact biological processes causing this syndrome are mainly unknown. In this study frailty did not correlate with any of the clinical or metabolic variables. According to these results, it seems unlikely that frailty has any impact on cholesterol metabolism in AD.

In vitro and in animal studies the cleavage products of APP are able to regulate cholesterol homeostasis.³⁶ The β - cleavage product of APP, $A\beta$, was able to downregulate the key regulator of cholesterol

synthesis, sterol regulatory element-binding protein 2 (SREBP 2) in astrocytes and hepatic cells resulting in decreased cholesterol synthesis and low density lipoprotein receptor content in these cells. Moreover, in human studies two subjects with an autosomal dominant form of early-onset familial AD caused by a duplication of the APP gene (APP^{Dp}) in combination with increased β -cleavage, serum precursors (squalene, lathosterol, and desmosterol ratios to cholesterol) were reduced suggesting that $A\beta$ conceivably regulates cholesterol synthesis also in human subjects.³⁶ The surrogates of cholesterol absorption efficiency remained within reference values. The ability of $A\beta$ peptide to downregulate SREBP 2 may also explain the novel

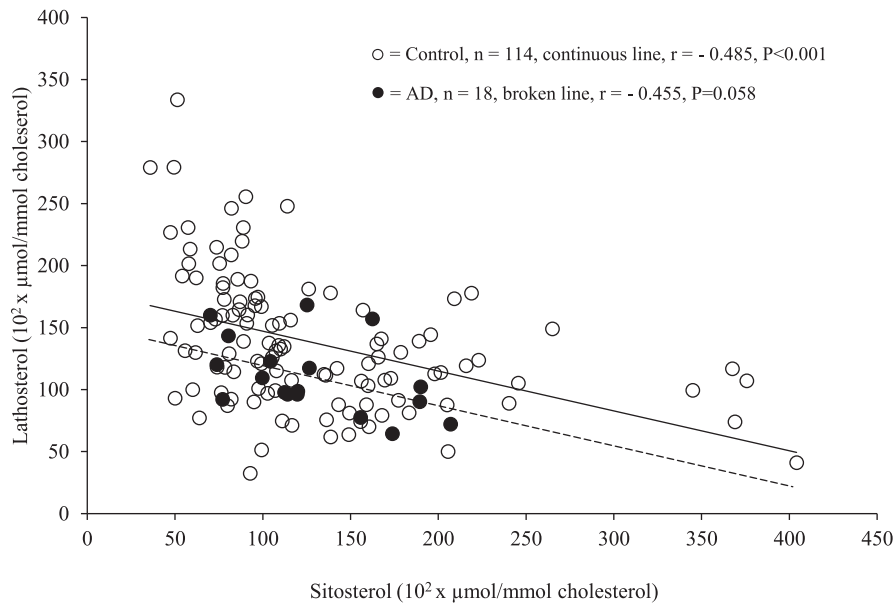


Fig 2. Correlation of serum lathosterol to sitosterol in the control men and in men with Alzheimer's disease (AD).

findings that the expected positive associations of plasma glucose concentration with cholesterol synthesis observed in non-AD populations³¹ was turned negative in the AD subjects.

The study has both limitations and strengths. First, the study population consisted only of men of highest social strata with late-onset AD so that the results are

not extendable to other AD populations. The number of men developing AD in this cohort was also smaller than expected, only 20 out of 651 men (3%). However, the size of the study population exceeded the demand of the power calculation. Second, the serum noncholesterol sterols could not be validated with the absolute measurements of cholesterol metabolism, because they

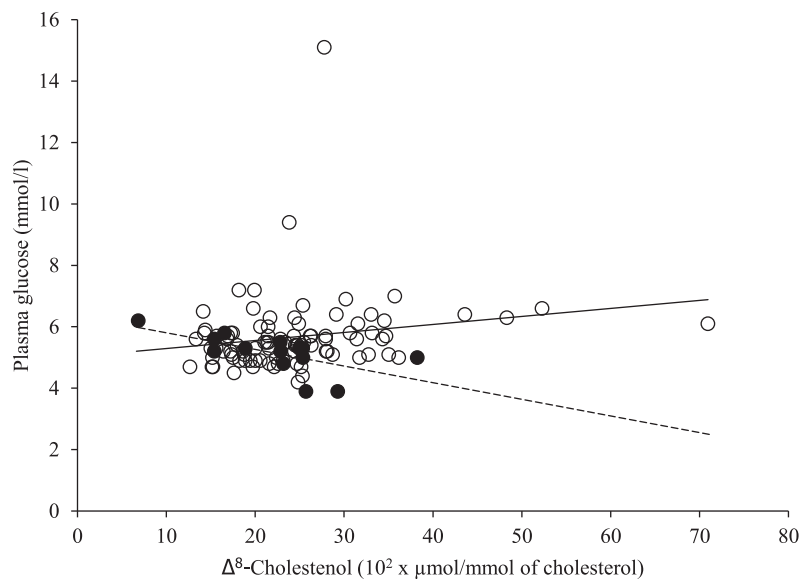


Fig 3. Correlation of plasma glucose to serum Δ^8 -cholestenol in the control men (open circles, continuous line, $r = 0.265$, $P = 0.011$) and in men with Alzheimer's disease (AD) (closed circles, broken line, $r = -0.757$, $P = 0.002$).

are too complex and demanding to be used in the AD population. The assessment of validity of the serum biomarkers had to be based on the information that cholesterol homeostasis was intact and that the synthesis markers as well as the absorption markers were interrelated. Today, the noncholesterol sterols are widely used in sterol research relying on the earlier results of validation in different non-AD populations.

Finally, the strengths of this study are the socio-economically homogenous population and the aim to "purify" the AD diagnosis from vascular components. For the latter, an obvious limitation is that diagnosis of AD was not made personally by the present study team. However, in Finland dementia diagnosis is generally made by specialists in a structured fashion including brain imaging, and for each man the study team checked carefully the clinical history from the HBS longitudinal data, patient records, drug reimbursement registers, and narratives and diagnoses of death certificates.¹⁶ None of the men with AD had a history of major atherosclerotic CVD (coronary heart disease, cerebrovascular disease, or peripheral vascular disease). In addition, hypertension was not more prevalent in AD compared with controls, and midlife cholesterol predicted mixed dementia but not AD.¹⁶ Although it is not guaranteed that our definition of "pure" AD is devoid of any vascular component, we believe that reducing the atherosclerotic burden from the AD diagnosis nevertheless can better reveal the real biologic processes involved in AD.

In conclusion, the novel findings were that cholesterol synthesis and cholesterol metabolism were lower whereas cholesterol absorption was higher in the AD compared with the control subjects. Plasma glucose concentration was low in AD, and its association with cholesterol synthesis was paradoxical to controls. These findings open new perspectives in the regulation of cholesterol metabolism and glucose homeostasis in AD.

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All authors have read the journal's policy on disclosure of potential conflicts of interest and have none to declare. All authors have read the journal's authorship agreement and the manuscript has been reviewed by and approved by all named authors (CS, PS, MJN, HG, and TES). There has been no outside editorial support for preparation of the manuscript.

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