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Plasma amyloid beta levels and platelet mitochondrial respiration in patients with Alzheimer's disease

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ABSTRACT

Objectives: Altered amyloid metabolism and mitochondrial dysfunction play key roles in the development of Alzheimer's disease (AD). We asked whether an association exists between disturbed platelet mitochondrial respiration and the plasma concentrations of $A\beta_{40}$ and $A\beta_{42}$ in patients with AD.

Design and methods: Plasma $A\beta_{40}$ and $A\beta_{42}$ concentrations and mitochondrial respiration in intact and permeabilized platelets were measured in 50 patients with AD, 15 patients with vascular dementia and 25 control subjects. A pilot longitudinal study was performed to monitor the progression of AD in a subgroup 11 patients with AD.

Results: The mean $A\beta_{40}$, $A\beta_{42}$ and $A\beta_{42}/A\beta_{40}$ levels were not significantly altered in patients with AD compared with controls. The mitochondrial respiratory rate in intact platelets was significantly reduced in patients with AD compared to controls, particularly the basal respiratory rate, maximum respiratory capacity, and respiratory reserve; however, the flux control ratio for basal respiration was increased. A correlation between the plasma $A\beta_{42}$ concentration and mitochondrial respiration in both intact and permeabilized platelets differs in controls and patients with AD.

Conclusions: Based on our data, (1) mitochondrial respiration in intact platelets, but not the Aβ level itself, may be included in a panel of biomarkers for AD; (2) dysfunctional mitochondrial respiration in platelets is not explained by changes in plasma Aβ concentrations; and (3) the association between mitochondrial respiration in platelets and plasma Aβ levels differs in patients with AD and controls. The results supported the hypothesis that mitochondrial dysfunction is the primary factor contributing to the development of AD.

1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease that causes dementia due to neuronal death and a loss of synapses in certain areas of the brain. Neuropathologically, AD is characterized by neuronal loss, increased formation of extracellular amyloid beta (Aβ) plaques and intracellular neurofibrillary tangles (NFT) formed by the hyperphosphorylated tau protein.

Critical roles have been assigned to Aβ metabolism [1–3], mitochondrial dysfunction [4–6], and oxidative stress [7,8], among other

Abbreviations: Aβ, amyloid beta; AD, Alzheimer's disease; ApoE, apolipoprotein E; APP, amyloid precursor protein; BAS, basal respiration; BMI, body mass index; C_I, complex I-linked respiration; C_I_II, respiration stimulated by substrates of both complex I and complex II; CSF, cerebrospinal fluid; CypD, cyclophilin D; DMP, oxygen flow after permeabilization of platelets with digitonin followed by addition of malate and pyruvate; GDS, Geriatric Depression Score; ETS, electron transport system; ETSC, maximal capacity of ETS at an optimum FCCP concentration; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; FCR, flux control ratio; HSD10, 17β-hydroxysteroid dehydrogenase type 10; LEAK, respiration caused by apparent proton leak after inhibition of the phosphorylation system; MMSE, Mini Mental State Examination; mPTP, mitochondrial permeability transition pore; NFT, neurofibrillary tangles; OXPHOS, oxidative phosphorylation; RES, respiration reserve capacity; ROS, reactive oxygen species; ROT, oxygen flow after inhibition of complex I by rotenone; ROX, residual oxygen flow after the addition of antimycin A; VD, vascular dementia

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pathways, in AD pathogenesis. The aim of the present study is to contribute to identify which processes are primary factors contributing to the development of AD, their feedback effects and interactions with intracellular signaling pathways involved in AD pathophysiology.

1.1. Amyloid metabolism in Alzheimer's disease

Abnormal amyloid precursor protein (APP) metabolism is a key process contributing to the pathogenesis of AD, since mutations responsible for the familial form of AD (early-onset AD) were identified in genes encoding APP or APP metabolizing enzymes (presenilins). However, mutations in these genes have little effect on the susceptibility to sporadic AD (late-onset AD), a multifactorial disorder resulting from the combination of both susceptible genes and environmental factors [9]. Until recently, the presence of the epsilon 4 allele of the gene encoding apolipoprotein E (ApoE) has been established as the strongest genetic risk factor for sporadic AD [10–13]. Several novel risk genes are being investigated. The cumulative effect of these genes seems to be manifested through the amyloid pathway and tau cascade [14,15].

APP is initially cleaved by β-secretase and the remaining membrane-bound fragment is further cleaved by γ-secretase to produce Aβ peptides, including the major $A\beta_{40}$ peptide consisting of 40 amino acids and the minor $A\beta_{42}$ peptide consisting of 42 amino acids. $A\beta_{42}$ is more likely to aggregate in the brain than $A\beta_{40}$ [16,17] and oligomers of $A\beta_{42}$ may initiate neurodegeneration [18].

1.2. Amyloid and mitochondrial hypotheses of Alzheimer's disease

A number of AD hypotheses have been formulated [19], of which the amyloid and mitochondrial hypothesis are the most popular. The amyloid cascade hypothesis postulates that the extracellular Aβ deposits are the fundamental cause of the disease and the pathological cascade consists of extracellular Aβ deposition, tau hyperphosphorylation and intracellular NFT formation, followed by neuronal death [18,20–23]. An important refinement of the amyloid hypothesis consisted of the assumption that soluble low molecular weight Aβ oligomers are responsible for Aβ neurotoxicity, as manifested by impairments in calcium homeostasis and synaptic functions, increased neuroinflammation and oxidative stress, and the formation of NFT at specific brain regions [24]. Both early-onset and late-onset AD lead to the increased production of $A\beta_{42}$, which tends to aggregate and/or to trigger a cascade of events leading to symptoms of AD. According to the amyloid hypothesis, the formation of NFTs, mitochondrial dysfunction, neuroinflammation, synaptotoxicity, and neuronal loss are proposed to result from an imbalance between Aβ production and Aβ clearance, leading to Aβ accumulation in the brain [25].

The amyloid cascade hypothesis can be explained by Aβ-induced mitochondrial dysfunction. However, the primary cause of the disease may be mitochondrial dysfunction [26] and/or changes in the functions of other upstream factors, such as ApoE4 and glycogen synthase kinase 3, inducing both Aβ and tau pathology [27,28]. Mitochondria and bioenergetics play major roles in APP processing and cellular trafficking [29]. Recently, mitochondria were shown to initiate, mediate, or contribute to AD pathophysiology. Functional deficits were observed in the mitochondria of patients with AD [30,31] and mitochondrial functions are the targets of new drugs for AD [32–34]. Direct interactions of Aβ with mitochondrial proteins are proposed to be related to the development of AD [35].

The interconnection of the amyloid and mitochondria cascade hypotheses is evidenced by Aβ-induced impairment of mitochondrial energy metabolism [36–39], particularly the activity of the respiratory chain [39]. Aβ is imported into the mitochondria [40] and the oxidative phosphorylation (OXPHOS) pathway in the inner mitochondrial membrane may be disrupted upon an interaction with Aβ, leading to reduced ATP assembly and increased reactive oxygen species (ROS) production [41]. Other mitochondrial targets of Aβ include the enzymes 17β-hydroxysteroid dehydrogenase type 10 (HSD10, 17 β-HSD10, also known amyloid beta binding alcohol dehydrogenase, ABAD) and cyclophilin D (CypD). Inhibition of the Aβ-HSD10 interaction mitigates ROS production and apoptosis [42]. The Aβ-CypD interaction promotes the opening of the mitochondrial permeability transition pore (mPTP) and neuronal injury [43].

Mitochondrial function may also be affected by Aβ-induced changes in cytosolic free calcium concentrations [44,45]. Elevated cytosolic calcium levels may result in calcium overload in the mitochondria, leading to calcium-induced mPTP opening, inhibition of ATP production, increased ROS generation, dysregulated neuronal calcium signaling, and ultimately, an energy deficit, the release of proapoptotic factors, and neuronal injury. According to the calcium hypothesis [46], Aβ-induced changes in intracellular calcium homeostasis and the resulting changes in mitochondrial function initiate AD pathophysiology.

Currently, researchers are debating, whether the mitochondrial cascade occurs as a primary or secondary change in response to Aβ pathology [47]. The primary mitochondrial cascade hypothesis postulates that impaired mitochondrial bioenergetics alter amyloid metabolism and Aβ accumulation, which may or may not contribute to the development of AD pathology; i.e., researchers have hypothesized that mitochondrial dysfunction supersedes Aβ pathology and initiates pathological cascades during AD development [26,47,48]. Another approach is that Aβ induces mitochondrial dysfunction and triggers a cascade of pathological processes in patients with AD.

Therefore, the processes in which biological markers of the sporadic form of AD and targets of novel AD drugs are sought include Aβ metabolism, mitochondrial bioenergetics, and their interactions.

1.3. Blood biomarkers of Alzheimer's disease

The only valid method for identifying Aβ deposits in the living brain is Aβ-PET or Aβ measurements in cerebrospinal fluid (CSF) (a reduced $A\beta_{42}$ level or $A\beta_{42}/A\beta_{40}$ ratio). Disturbed neuronal metabolism of the tau protein in patients with AD is measured as increased concentrations of both total tau and phosphorylated tau proteins in the CSF [49]. Noninvasive cheap blood biomarkers that are suitable for AD diagnosis are currently being sought, because screening in preclinical stages is crucial for future therapy. The search is based on the assumption that changes in the levels of biochemical parameters measured in peripheral blood components reflect specific pathological processes in the brain or that these changes are systemic. Common changes in processes in the brain and periphery have been reported to be caused by oxidative stress [50].

A systematic review and meta-analysis confirmed that the levels of CSF biomarkers (total tau, phosphorylated tau, $A\beta_{42}$, and neurofilament light chain) are associated with AD, whereas plasma $A\beta_{42}$ and $A\beta_{40}$ levels are not [51]. Some results do not sufficiently support the hypothesis that plasma tau levels represent an AD biomarker [52]. However, recent highly sensitive and specific assays indicate reduced plasma $A\beta_{42}$ and $A\beta_{40}$ levels in patients with AD that correlate with both CSF concentrations and Aβ-positivity assessed using amyloid PET imaging [53]. Moreover, changes in the secondary structure of Aβ in human blood plasma may be a biomarker of prodromal AD [54]. Ratios of plasma concentrations of APP669–711/$A\beta_{42}$ and $A\beta_{42}/A\beta_{40}$ and their combinations are able to predict the individual Aβ-positive or Aβ-negative state in the brain (using PIB-PET as a standard), confirming the potential clinical utility of plasma biomarkers in predicting the brain Aβ load at an individual level [55,56]. In conclusion, the plasma $A\beta_{42}/A\beta_{40}$ ratio may reflect amyloid pathology in the brain [57].

Potential early AD biomarkers have also been identified in peripheral blood lymphocytes and platelets [58]. Mitochondrial health in platelets and monocytes determined based on bioenergetic profiles in blood sample has been shown to be related to brain bioenergetics and metabolism [59]. Platelets are recognized as a proper biological model

for the detection of Aβ-induced mitochondrial dysfunction [60], and platelets might serve as a biomarker for the detection of mitochondrial dysfunction during aging and AD [30,61,62]. The strong support for a role of deficits in complex I and IV activity in peripheral blood in the pathophysiology of AD has been confirmed by a meta-analysis [63].

Mitochondrial dysfunction is defined as a deviation in the oxygen consumption rate in various states of respiration [64–66] compared to the control rate. An assessment of mitochondrial bioenergetic functions in isolated mitochondria or intact or permeabilized cells has been enabled by the development of Oroboros and Seahorse extracellular oxygen flux technologies and the implementation of experimental protocols [67–70].

1.4. Aim

Based on our earlier findings that mitochondrial respiration in platelets is impaired in patients with AD [30], we measured plasma $A\beta_{40}$ and $A\beta_{42}$ concentrations and mitochondrial platelet respiration in patients with AD and healthy controls and tested the hypothesis that mitochondrial dysfunction in platelets from patients with AD is associated with changes in plasma Aβ concentrations. Moreover, we performed a pilot study (i) to evaluate plasma Aβ concentrations and platelet mitochondrial respiration during disease progression in patients with AD and (ii) to compare patients with AD with patients with vascular dementia (VD).

2. Materials and methods

2.1. Chemicals and solutions

Buffers and stock solutions used in assays of mitochondrial respiration were prepared as described in a previous study [30]. Briefly, inorganic Krebs and Henseleit isotonic medium (KH medium) containing 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4{\cdot}7H_2O$, 25 mM $NaHCO_3$, and 11.1 mM glucose (pH 7.4) was used to dilute intact platelets. Mitochondrial respiration medium (MiR05) containing 110 mM sucrose, 60 mM K-lactobionate, 20 mM taurine, 3 mM $MgCl_2{\cdot}6H_2O$, 10 mM KH_2PO_4, 0.5 mM EGTA, 1 g/L BSA, and 20 mM HEPES (pH 7.1) was used for assays of mitochondrial respiration in permeabilized platelets. Stock solutions of digitonin, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP, uncoupler), substrates (malate, pyruvate, glutamate, succinate, and ADP), and inhibitors (oligomycin, rotenone, and antimycin A) were stored at −20 °C or freshly prepared. All chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

2.2. Subjects

Patients with a probable diagnosis of Alzheimer's disease (AD) or vascular dementia (VD) aged greater than 55 years were recruited from the Department of Psychiatry of the First Faculty of Medicine, Charles University and General University Hospital in Prague, Czech Republic.

Specific criteria were used to diagnose AD and VD, including the International Classification of Diseases, Tenth Edition (ICD-10) criteria, NINDS-AIREN vascular dementia criteria [71], the NINCDS-ADRDA Alzheimer's criteria [72,73], and the Hachinski Ischemic Score [74]. Patients with AD met previously described inclusion criteria [75], including the presence of cortico-subcortical atrophy (confirmed by magnetic resonance imaging of the brain). Exclusion criteria for both AD and VD included: patients with a diagnosis of Parkinson's disease, other neurodegenerative or mental illnesses, including secondary dementia, decompensated somatic disease, cancer, type 1 diabetes mellitus, uncompensated type 2 diabetes mellitus, autoimmune diseases, an addiction to addictive substances, alcohol and other substances; long-term use of central anticholinergics; or missing data. The severity of the illness was quantified using the Addenbrooke's Cognitive Examination-Revised (ACE-R) [76] including the Mini-Mental State Examination (MMSE) questionnaire and by the short version of the Geriatric Depression Scale (GDS) [77]. The control group included healthy volunteers aged greater than 55 years without dementia, mental disorders, or organic brain damage. Alzheimer's disease progression was observed in the subgroup of patients with AD after approximately 1 year.

The study was conducted according to the principles of the Declaration of Helsinki, and the study protocol was approved by the Ethical Review Board of the First Faculty of Medicine, Charles University and General University Hospital in Prague. Written informed consent was obtained from participants.

2.3. Blood samples

Fasting blood samples were collected by venipuncture between 7:00 and 8:00 am, prior to the administration of morning medications. BD Vacutainer® blood collection tubes (Becton, Dickinson and Company, Franklin Lakes, USA) were used, with EDTA as an anticoagulant. Platelet-rich plasma (PRP) was separated by centrifugation at 150 ×*g* for 20 min at 25 °C and platelets were counted. Aliquot of PRP was diluted 1:1 with KH medium and used to analyze mitochondrial respiration in intact platelets. An aliquot of PRP was centrifuged at 1500 ×*g* for 10 min at 25 °C. The plasma (supernatant) was then collected and stored at −70 °C until $A\beta_{40}$ and $A\beta_{42}$ assays. The pellet was resuspended in two volumes of respiration medium MiR05, treated with digitonin and used to measure mitochondrial respiration in permeabilized platelets.

2.4. Plasma Aβ assays

Plasma $A\beta_{40}$ concentrations were measured using sandwich enzyme immunoassay with Amyloid beta 40 ELISA Kit ABIN1118191 (antibodies-online GmbH, Aachen, Germany) for an *in vitro* quantitative measurement of $A\beta_{40}$ levels in human plasma (detection range 15.625–1000 pg/mL, minimum detection limit 15.625 pg/mL, and sensitivity 9.375 pg/mL). Plasma $A\beta_{42}$ concentrations were measured with a competitive ELISA using the highly sensitive kit ABIN4947345 (antibodies-online GmbH, Aachen, Germany) for an *in vitro* quantitative measurement (detection range 3.7–300 pg/mL, minimum detection limit 3.7 pg/mL, and sensitivity 1.31 pg/mL). Both Aβ assays were performed using the analyzer SLT Spectra (SLT Labinstruments, Austria) and KIMW software (Daniel Kittrich, Prague, Czech Republic).

2.5. Mitochondrial respiration

Changes in the platelet mitochondrial oxygen consumption rate and derived respiratory parameters characterizing changes in activities of various components of the OXPHOS system were determined using high-resolution respirometry in intact or permeabilized platelets as described in previous studies [30,78]. Oxygen concentrations were measured polarographically with Clark oxygen electrodes at 37 °C using the O2k-Respirometer (Oroboros Instruments Corp, Innsbruck, Austria).

The following oxygen consumption rates were determined in intact platelets: BAS – physiologically relevant endogenous basal respiration in diluted plasma before the addition of any substances; LEAK – respiration caused by apparent proton leakage after the inhibition of the phosphorylation system by 2 μg/mL oligomycin; ETSC – maximal capacity of ETS (maximal respiratory rate in a common reference state) after titration with the protonophore carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), i.e. uncoupled respiration at an optimum FCCP concentration; ROT – oxygen flow after the inhibition of complex I by 2.5 μM rotenone; and ROX – residual oxygen flow after the addition of 2.5 μg/mL antimycin A.

When studying permeabilized platelets, substrate-uncoupler-inhibitor-titration protocols were used to establish the values of the following parameters: DMP – oxygen flow after the permeabilization of

platelets with 50 μg/mL digitonin followed by the addition of 5 mM malate and 5 mM pyruvate; C_I – complex I-linked respiration after the addition 1 mM ADP and 5 mM glutamate; C_I_II – respiration at saturating ADP concentrations that was supported by substrates of both complex I and complex II after the addition of 10 mM succinate (OXPHOS capacity); LEAK – respiration caused by apparent proton leakage after the inhibition of the phosphorylation system by 2 μg/mL oligomycin; ETSC – maximal capacity of ETS after titration with FCCP (maximal noncoupled respiration); ROT – oxygen flow after the inhibition of complex I by 0.5 μM rotenone; and ROX – residual oxygen flow after the final addition of 1.25 μg/mL antimycin A.

Oxygen consumption rates were normalized to the platelet concentration (pmol O_2 per *sec* per 10^6 platelets) and ROX was subtracted from all other respiratory parameters. Respiration reserve capacity (RES) was calculated as ETSC-BAS in intact platelets and as ETSC-C_I_II in permeabilized platelets. Flux control ratios (FCRs) were calculated as ratios of oxygen flow in different respiratory control states to ETSC. FCRs, which represent the efficiency of respiration during several states of respiration and are independent of the mitochondrial content and cell size, were used to determine the relative contributions of the different respiratory states of mitochondria: (i) (BAS-ROX)/(ETSC-ROX), (LEAK-ROX)/(ETSC-ROX), (BAS–LEAK)/(ETSC-ROX), (ROT-ROX)/(ETSC-ROX), ROX/ETSC, and RES/(ETSC-ROX) in intact platelets; and (ii) (C_I-ROX)/(ETSC-ROX), (C_I_II-ROX)/(ETSC-ROX), (LEAK-ROX)/(ETSC-ROX), (ROT-ROX)/(ETSC-ROX), ROX/ETSC, and RES/(ETSC-ROX) in permeabilized platelets.

2.6. Data analysis

The Shapiro-Wilk W test was used to assess the normal distribution of the measured plasma and mitochondrial parameters. The relations between all measured parameters and age were determined by calculating Pearson product-moment correlation coefficients (r). Demographic factors were compared using the Mann-Whitney U test. Scheffé's method was used to adjust significance levels in an analysis of variance to account for multiple comparisons between patients with AD, patients with VD and controls. Multiple comparisons of patients with AD and VD with the control group were performed using Dunnett's test. A comparison was performed using a paired difference test of the parameters measured at the time of inclusion of patients with AD into the study and after approximately 1 year. The association between Aβ levels and mitochondrial parameters was analyzed by calculating partial correlation coefficients (ρ). All multiple comparisons and partial correlations were controlled for age, years of education, and body mass index (BMI).

Thea Statistica data analysis software, version 13 (TIBCO Software Inc., Palo Alto, CA, USA) was used for statistical analyses. DatLab software version 7 (Oroboros Instruments Corp, Innsbruck, Austria) was used to acquire and analyze respirometry data.

3. Results

Plasma $A\beta_{40}$ and $A\beta_{42}$ concentrations and mitochondrial respiration parameters in platelets were measured in 50 subjects with AD, 15 subjects with VD, subgroups of 11 patients with AD after 487 ± 103 (mean ± SD) days from the first examination (AD2), and 25 controls. Selected clinical and demographic data are summarized in Table 1.

In the control group, we did not observe significant differences in the measured plasma and mitochondrial parameters between women and men. Therefore, in the analysis of the data obtained from patients with AD, patients with VD and controls, women and men were evaluated together.

In the control group, a significant negative correlation was observed between age and the $A\beta_{42}/A\beta_{40}$ ratio ($r = -0.470$, $p = .018$) and between BMI and RES in permeabilized platelets ($r = -0.411$, $p = .041$). Statistical analyses of plasma and mitochondrial parameters (comparison of mean values and partial correlations) were therefore performed by controlling for age, BMI, and education level.

3.1. Mean values

We did not observe significant differences between mean plasma $A\beta_{40}$ and $A\beta_{42}$ concentrations, nor between $A\beta_{42}/A\beta_{40}$ in patients with AD, patients with VD, patients with AD2, and controls (Table 1).

In intact platelets from patients with AD, the mitochondrial respiratory parameters BAS ($p = .014$), ETSC ($p = .0002$), and RES ($p = .0004$), as well as the flux control ratio RES/ETSC ($p = .0036$), were significantly reduced compared to controls; the flux control ratios BAS/ETSC ($p = .015$) and (BAS-LEAK)/ETSC ($p = .045$) were increased. In permeabilized platelets, respiratory parameters were not significantly altered in patients with AD compared with controls (Table 1).

Pilot data only showed significant reductions in ETSC ($p = .039$) in intact platelets from patients with VD compared to controls. A significant difference was not observed between patients with AD and VD.

A comparison of respiratory parameters measured in the subgroup of patients with AD at the time of study entry and after 487 ± 103 days was performed using a paired difference test. We detected a significant increase in ROX ($p = .0023$) and the ROX/ETSC ratio ($p = .0007$) in intact platelets and a significant increase in DMP ($p = .029$), LEAK ($p = .049$), DMP/ETSC ($p = .020$), and LEAK/ETSC ($p = .049$) in permeabilized platelets in the AD2 group. The RES parameter decreased to zero (Table 1).

3.2. Partial correlations

All partial correlation coefficients were controlled for age, years of education, and BMI. A significant partial correlation between plasma $A\beta_{42}$ and $A\beta_{40}$ levels was observed in controls ($\rho = 0.494$, $p = .032$), but not in patients with AD ($\rho = -0.232$, $p = .124$) or VD ($\rho = -0.321$, $p = .366$) (Fig. 1).

A significant partial correlation was observed between the MMSE score and BAS in intact platelets from patients with AD ($\rho = 0.428$, $p = .003$) (Fig. 2). In intact platelets, significant negative correlations were observed between $A\beta_{42}/A\beta_{40}$ and ROX and the ROX/ETSC ratio in both controls and patients with AD (Table 2 and Fig. 3). Significant positive correlations between $A\beta_{42}$ and BAS/ETSC and (BAS-LEAK)/ETSC were observed in patients with AD but not in controls (Fig. 4).

In permeabilized platelets, a significant negative correlation between the $A\beta_{42}/A\beta_{40}$ ratio and the C-I mitochondrial parameter was observed in controls, but not in patients with AD (Table 3). In contrast, in patients with AD, but not in controls, a significant positive correlation was identified between the $A\beta_{42}$ concentration and C_I_II/ETSC (Fig. 5).

4. Discussion

Mitochondrial dysfunction is a common feature of various neurodegenerative disorders and is probably involved in the initiation and/or amplification of oxidative stress during the onset and progression of AD. In our study, we tested if mitochondrial dysfunction observed in peripheral blood components is associated with plasma $A\beta_{42}$ levels, which would potentially indicate that of mitochondrial dysfunction is secondary to the effects of Aβ.

We did not observe significant differences in the mean plasma concentrations of $A\beta_{40}$ and $A\beta_{42}$ or the $A\beta_{42}/A\beta_{40}$ ratio in patients with AD, patients with VD and controls, which was due to the large range of these values observed in all groups. Namely, the range of the $A\beta_{42}/A\beta_{40}$ ratio in the controls was 0.008–0.268, 0.005–0.407 in the patients with AD and 0.051–0.417 in patients with VD. Thus, low or high plasma $A\beta_{42}$ subgroups were present, regardless of the neurodegenerative disease. According to our data, the difference in plasma Aβ

Table 1
Demographic, clinical and biochemical data from participants with Alzheimer's disease (AD and AD2), vascular dementia (VD), and controls.

Characteristic	Control	AD	VD	AD2
Demographic and clinical data				
Age (years)	67.0 ± 6.4	***76.2 ± 7.3	*72.2 ± 6.1	74.9 ± 7.3
Education (years)	14.4 ± 2.9	14.4 ± 2.7	12.7 ± 1.8	14.5 ± 2.7
BMI	27.5 ± 5.2	*24.6 ± 3.3	25.8 ± 3.9	26.4 ± 3.8
GDS	3.6 ± 2.0	4.4 ± 2.0	5.3 ± 2.5	5.0 ± 2.8
MMSE	29.0 ± 1.5	***21.7 ± 5.7	24.7 ± 3.0	#***20.2 ± 7.1
Plasma concentration				
$A\beta_{40}$ (pg/mL)	262 ± 92	293 ± 108	316 ± 144	282 ± 48
$A\beta_{42}$ (pg/mL)	40 ± 23	49 ± 22	52 ± 20	57 ± 21
$A\beta_{42}/A\beta_{40}$	0.161 ± 0.084	0.190 ± 0.089	0.198 ± 0.107	0.210 ± 0.081
Respiration of intact platelets				
ROX	0.018 ± 0.007	0.016 ± 0.005	0.015 ± 0.004	##**0.020** ± 0.004
BAS-ROX	0.103 ± 0.017	***0.091** ± 0.015	0.093 ± 0.014	0.092 ± 0.018
LEAK-ROX	0.005 ± 0.004	0.005 ± 0.005	0.008 ± 0.006	0.007 ± 0.005
ETSC-ROX	0.127 ± 0.028	***0.100** ± 0.021	*0.104 ± 0.022	**0.092 ± 0.023
ROT-ROX	−0.002 ± 0.005	0.000 ± 0.003	0.000 ± 0.006	−0.002 ± 0.001
RES	0.024 ± 0.017	***0.009 ± 0.011	0.011 ± 0.012	***0.000 ± 0.016
(BAS-ROX)/(ETSC-ROX)	0.827 ± 0.103	*0.917 ± 0.103	0.904 ± 0.085	***1.016 ± 0.157
(LEAK-ROX)/(ETSC-ROX)	0.040 ± 0.031	0.055 ± 0.049	0.078 ± 0.060	0.077 ± 0.054
(BAS-LEAK)/(ETSC-ROX)	0.787 ± 0.102	*0.862 ± 0.082	0.847 ± 0.130	**0.939 ± 0.149
(ROT-ROX)/(ETSC-ROX)	−0.013 ± 0.034	−0.006 ± 0.033	−0.008 ± 0.055	−0.019 ± 0.015
ROX/ETSC	0.126 ± 0.037	0.139 ± 0.041	0.130 ± 0.022	###**0.181 ± 0.040
RES/(ETSC-ROX)	0.173 ± 0.103	**0.072 ± 0.094	0.096 ± 0.085	***−0.016 ± 0.157
Respiration of permeabilized platelets				
ROX	0.009 ± 0.004	0.008 ± 0.006	0.011 ± 0.014	0.007 ± 0.006
DMP-ROX	0.040 ± 0.013	0.035 ± 0.013	0.039 ± 0.021	#**0.046** ± 0.016
C_I-ROX	0.117 ± 0.030	0.109 ± 0.030	0.114 ± 0.026	0.111 ± 0.024
C_I_II-ROX	0.173 ± 0.037	0.171 ± 0.034	0.180 ± 0.036	0.166 ± 0.036
LEAK-ROX	0.033 ± 0.009	0.030 ± 0.010	0.037 ± 0.015	#**0.036** ± 0.013
ETSC-ROX	0.184 ± 0.038	0.175 ± 0.036	0.194 ± 0.045	0.175 ± 0.052
ROT-ROX	0.071 ± 0.022	0.074 ± 0.018	0.080 ± 0.018	0.073 ± 0.021
RES	0.011 ± 0.016	0.011 ± 0.044	0.040 ± 0.086	0.009 ± 0.020
(DMP-ROX)/(ETSC-ROX)	0.220 ± 0.065	0.205 ± 0.064	0.197 ± 0.077	#**0.270** ± 0.070
(C_I-ROX)/(ETSC-ROX)	0.640 ± 0.115	0.624 ± 0.095	0.615 ± 0.075	0.656 ± 0.114
(C_I_II-ROX)/(ETSC-ROX)	0.945 ± 0.091	0.987 ± 0.101	0.971 ± 0.091	0.981 ± 0.135
(LEAK-ROX)/(ETSC-ROX)	0.183 ± 0.049	0.170 ± 0.045	0.190 ± 0.052	#**0.207** ± 0.046
(ROT-ROX)/(ETSC-ROX)	0.385 ± 0.093	0.431 ± 0.088	0.423 ± 0.068	0.424 ± 0.070
ROX/ETSC	0.047 ± 0.022	0.042 ± 0.031	0.042 ± 0.020	0.039 ± 0.027
RES/(ETSC-ROX)	0.043 ± 0.088	0.055 ± 0.229	0.170 ± 0.367	0.019 ± 0.135
N (women/men)	25 (15/10)	50 (25/25)	15 (12/3)	11 (6/5)

Plasma amyloid beta (Aβ) levels were measured using sandwich enzyme immunoassay ($A\beta_{40}$) kit or competitive enzyme immunoassay ($A\beta_{42}$) kit. Respirometry parameters were measured in intact and permeabilized platelets using protocols for the O2k-Respirometer (Oroboros). Demographic factors were compared using the Mann-Whitney test. Plasma Aβ levels and platelet mitochondrial respiration were analyzed with general linear models controlling for age, education level, and BMI; statistical significance was determined using ANOVA and the Scheffé post hoc test. Statistically significant differences compared with controls is presented as $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$. In the subgroup of patients with AD, parameters measured upon patient admission to the study and after 487 ± 103 days (AD2) were compared using the Wilcoxon matched pairs test. Statistically significant differences between AD and AD2 groups are presented as $^{\#}p < .05$, $^{\#\#}p < .01$, and $^{\#\#\#}p < .001$.

AD, Alzheimer's disease; VD, vascular dementia; MMSE, Mini Mental State Examination; GDS, Geriatric Depression Score; BMI, body mass index; Aβ, amyloid beta; BAS, physiologically relevant endogenous basal respiration in diluted plasma before the addition of any substances; LEAK, respiration caused by apparent proton leakage after the inhibition of the OXPHOS system by 2 μg/mL oligomycin; ETSC, maximal capacity of the electron transport system (ETS) at an optimum carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) concentration; ROT, oxygen flow after the inhibition of complex I by 2.5 or 0.5 μM rotenone; ROX, residual oxygen flow after the addition of 2.5 or 1.25 μg/mL antimycin A; DMP, oxygen flow after the permeabilization of platelets with 50 μg/mL digitonin followed by the addition of 5 mM malate and 5 mM pyruvate; C_I, complex I-linked respiration after the addition of 1 mM ADP and 5 mM glutamate; C_I_II, respiration stimulated by substrates of both complex I and complex II after the addition of 10 mM succinate. Respiration reserve capacity (RES) was calculated as ETSC-BAS in intact platelets and as ETSC-C_I_II in permeabilized platelets.

The respiratory parameters ROX, BAS, LEAK, ETSC, ROT, RES, DMP, C_I, and C_I_II are expressed in units pmol/(*sec*·10^6 platelets).

Statistically significant values are diplayed in bold.

concentrations between controls and patients with AD is merely due to the difference in the correlation between $A\beta_{40}$ and $A\beta_{42}$, which is significantly positive for controls, whereas it is insignificantly negative in patients with AD (Fig. 1). This may reflect different amyloid metabolism in patients with AD compared with controls.

We confirmed previously observed [30] significant reductions in the basal respiratory rate, maximal electron transfer system capacity, and respiration reserve capacity in intact platelets from patients with AD compared with controls (Table 1). These data confirmed that mitochondrial dysfunction and perturbed bioenergetics in patients with AD are not restricted to brain [79–81]. In addition, the significant positive correlation between MMSE and BAS observed in patients with AD (Fig. 2) indicates the possibility that the basal respiratory rate of the mitochondria in intact platelets might reflect the degree of cognitive

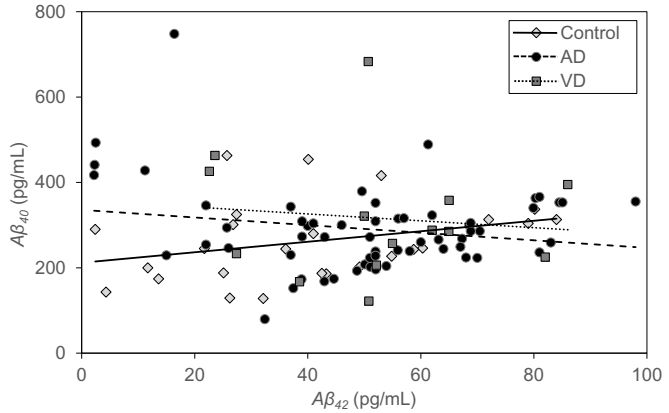

Fig. 1. Correlation between plasma $A\beta_{40}$ and $A\beta_{42}$ levels in controls and patients with Alzheimer's disease (AD) or vascular dementia (VD). The partial correlation coefficient (ρ) adjusted for age, education and body mass index was significant in controls ($\rho = 0.494$, $p = .032$) but not in patients with AD ($\rho = -0.232$, $p = .124$) or VD ($\rho = -0.321$, $p = .366$).

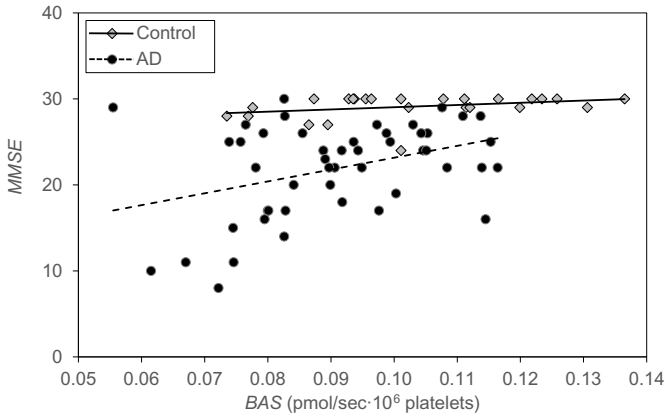

Fig. 2. Correlation between the Mini-Mental State Examination score (MMSE) and basal respiration in intact platelets (BAS) from patients with Alzheimer's disease (AD) and controls. The partial correlation coefficient (ρ) adjusted for age, education and body mass index was significant in patients with AD ($\rho = 0.428$, $p = .003$).

impairment in patients with AD. Decrease of the respiratory reserve in the intact platelets could be used as a biomarker of incipient AD.

The hypothesis that direct interactions of Aβ with mitochondrial enzymes are responsible for reducing the respiratory rate of mitochondria in platelets from people with AD was not confirmed, as a significantly reduced respiratory rate in intact platelets was observed with unchanged mean plasma concentrations of $A\beta_{42}$, $A\beta_{40}$, or $A\beta_{42}/A\beta_{40}$. Therefore, plasma Aβ levels do not appear to be a proper biomarker of AD. Our results supported the hypothesis that mitochondrial dysfunction in patients with AD exists independent of Aβ pathology and therefore the mitochondria may initiate pathophysiological cascades associated with AD [47].

According to the pilot data, differences in the measured parameters do not exist between patients with AD and VD, potentially indicating that the disturbance in mitochondrial respiration is not specific to patients with AD, but generally occurs in patients with neurodegenerative diseases. However, this finding must be confirmed with a larger data set. Mitochondria undoubtedly contribute to processes such as oxidative stress, calcium homeostasis and apoptosis, which are involved in neurodegeneration. Common mitochondrial dysfunction may be the basis for subsequent specific pathological changes in patients with a particular neurodegenerative disease, e.g., amyloid metabolism in patients with AD.

As shown in a pilot longitudinal study, the subgroup of patients with AD who were clinically examined and measured at baseline and after more than 1 year displayed increased residual ROX respiration in intact platelets and an increase in the nonphosphorylated LEAK respiratory rate in both intact and permeabilized platelets. Thus, an increased disruption of the intrinsic mitochondrial membrane permeability to protons may occur with AD progression.

Although we did not observe a direct association between mean plasma Aβ concentrations and mitochondrial dysfunction in platelets, we identified significant partial correlations between Aβ and mitochondrial respiration parameters (Tables 2 and 3). The significant negative correlations between $A\beta_{42}/A\beta_{40}$ and ROX (Fig. 3) and ROX/ETSC indicates the different effects of the $A\beta_{40}$ and $A\beta_{42}$ peptides on the oxidative side reactions remaining after the inhibition of ETS in intact platelets. In people with AD, but not in controls, a positive correlation was observed between $A\beta_{42}$ and (i) the basal respiration normalized for maximal capacity of ETS (BAS/ETSC), (ii) the normalized phosphorylation-related respiration corrected for LEAK respiration ((BAS-LEAK)/ETSC) in intact platelets (Fig. 4), and (iii) the normalized respiration stimulated by substrates of both complex I and complex II (C_I_II/ETSC) in permeabilized platelets (Fig. 5). These findings suggest a possible role for $A\beta_{42}$ in the mechanism regulating mitochondrial respiration in platelets that is specific for patients with AD. We have not yet determined what factors are responsible for the potential differences in the sensitivity of mitochondrial respiration to the effects of $A\beta_{42}$ in patients with AD, but changes in the lipid composition of membranes and interactions of $A\beta_{42}$ with the lipid part of membranes are expected to contribute to the toxicity of Aβ in patients with AD [82].

A negative correlation between $A\beta_{42}/A\beta_{40}$ and mitochondrial respiration in permeabilized platelets supported by complex I substrates (C_I) was observed in controls, but not in patients with AD (Table 3), which confirms that the response to the $A\beta_{42}$-mitochondria interaction may be different in patients with AD than in controls.

Because a direct interaction of $A\beta_{42}$ with the mitochondria causes dysfunction, including the inhibition of respiratory rate [83–85], the positive correlation between plasma $A\beta_{42}$ levels and flux control ratios in platelets (Tables 2 and 3), as well as increase of some flux control ratios in intact platelets (Table 1) in the present study suggests the existence of adaptive changes in the respiratory chain that partially compensate for mitochondrial dysfunction in patients with AD. This compensation may consist, for example, of an increase in complex I activity observed in platelets from patients with AD [30]. The observation that this compensation is incomplete reveals a reduction in both basal and maximum respiratory rates in intact platelets and a decrease in the respiratory reserve to zero (Table 1).

Mitochondrial dysfunction has been documented in the peripheral blood, muscles, and other tissues of patients with AD and indicates either (1) a general direct effect of APP, Aβ, or other unrecognized peripheral AD-specific substances on cellular bioenergetics, or (2) the primary occurrence of mitochondrial dysfunction throughout the body, which may be associated with a disruption of amyloid metabolism in the brain. Since we have not observed an association between a disruption of mitochondrial respiration in platelets and mean plasma concentrations of $A\beta_{40}$ and $A\beta_{42}$ or the ratio $A\beta_{42}/A\beta_{40}$ in patients with AD, our results support the hypothesis that the mitochondrial dysfunction associated with AD occurs throughout the body, and mitochondrial dysfunction may primarily be responsible for the development of AD by disrupting amyloid metabolism in the brain [86]. We speculated that specific positive feedback mechanisms exist in the brains of patients with AD between the disruption of mitochondrial functions and production of pathological forms of Aβ. Different correlations between mitochondrial respiration parameters and plasma Aβ concentrations in patients with AD and controls support this conclusion.

The greater influence of mitochondrial dysfunction on amyloid metabolism in the brain than in peripheral tissues can be explained by an increase in ROS production due to OXPHOS disruption because ROS

Table 2
Correlation between plasma amyloid beta (Aβ) levels and mitochondrial respiration in intact platelets.

Partial correlation	Control			AD		
	$Aβ_{40}$	$Aβ_{42}$	$Aβ_{42}/Aβ_{40}$	$Aβ_{40}$	$Aβ_{42}$	$Aβ_{42}/Aβ_{40}$
ROX	0.387	−0.311	**−0.628**	**0.334**	−0.163	**−0.381**
	p = .101	p = .195	***p* = .004**	***p* = .027**	p = .291	***p* = .011**
BAS-ROX	−0.088	−0.251	−0.314	0.049	0.099	0.071
	p = .721	p = .300	p = .191	p = .752	p = .524	p = .645
LEAK-ROX	0.020	0.257	0.392	0.008	−0.002	0.007
	p = .934	p = .287	p = .097	p = .958	p = .990	p = .964
ETSC-ROX	−0.025	−0.100	−0.206	0.097	−0.110	−0.068
	p = .919	p = .683	p = .397	p = .530	p = .476	p = .660
ROT-ROX	0.241	0.319	0.148	−0.211	−0.152	−0.032
	p = .320	p = .184	p = .546	p = .169	p = .324	p = .839
RES	0.047	0.085	−0.028	0.165	−0.298	−0.156
	p = .849	p = .728	p = .910	p = .308	p = .062	p = .335
(BAS-ROX)/(ETSC-ROX)	−0.079	−0.182	−0.086	−0.091	**0.309**	0.193
	p = .747	p = .457	p = .725	p = .563	***p* = .044**	p = .216
(LEAK-ROX)/(ETSC-ROX)	0.055	0.245	0.396	−0.012	−0.031	−0.012
	p = .824	p = .312	p = .094	p = .939	p = .844	p = .938
(BAS-LEAK)/(ETSC-ROX)	−0.096	−0.255	−0.203	−0.110	**0.430**	0.261
	p = .696	p = .292	p = .406	p = .484	***p* = .004**	p = .091
(ROT-ROX)/(ETSC-ROX)	0.191	0.276	0.121	−0.216	−0.179	−0.043
	p = .433	p = .253	p = .622	p = .163	p = .251	p = .782
ROX/ETSC	**0.514**	−0.242	**−0.544**	0.287	−0.146	**−0.379**
	***p* = .024**	p = .318	***p* = .016**	p = .059	p = .345	***p* = .011**
RES/(ETSC-ROX)	0.079	0.182	0.086	0.116	−0.253	−0.094
	p = .747	p = .457	p = .725	p = .476	p = .116	p = .564

Partial correlation coefficients (ρ) adjusted for age, education level and body mass index are displayed.
AD, Alzheimer's disease; ROX, residual oxygen consumption after the addition of 2.5 μg/mL antimycin A; BAS, endogenous basal respiration in diluted plasma before the addition of any substances; LEAK, respiration caused by apparent proton leakage after the inhibition of the OXPHOS system by 2 μg/mL oligomycin; ETSC, maximal capacity of the electron transport system (ETS) at an optimum carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) concentration; ROT, oxygen flow after the inhibition of complex I by 2.5 μM rotenone; RES, respiration reserve capacity (RES = ETSC-BAS).
Statistically significant values are diplayed in bold.

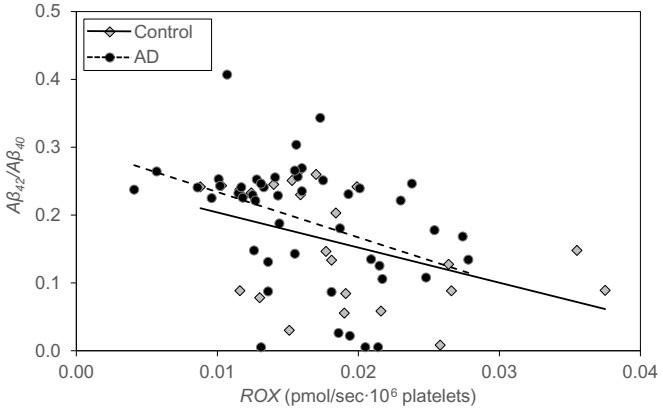

Fig. 3. Correlation between the $Aβ_{42}/Aβ_{40}$ ratio and residual oxygen flow (ROX) in intact platelets from patients with Alzheimer's disease (AD) and controls. Partial correlation coefficients (ρ) adjusted for age, education and body mass index were significant in both patients with AD (ρ = −0.381, p = .011) and controls (ρ = −0.628, p = .004).

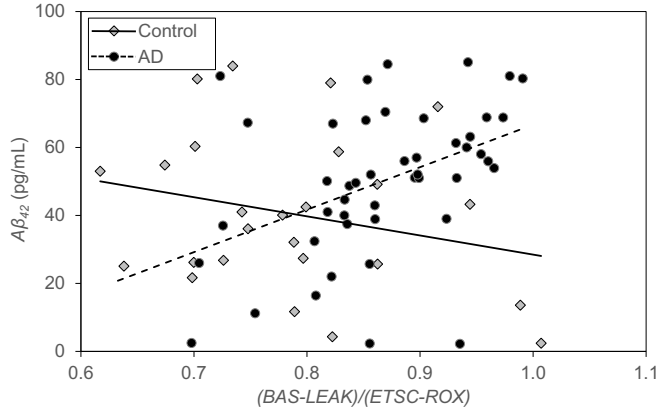

Fig. 4. Correlation between $Aβ_{42}$ levels and normalized net respiratory rate (BAS-LEAK)/(ETSC-ROX) in intact platelets from patients with Alzheimer's disease (AD) and controls. The partial correlation coefficient (ρ) adjusted for age, education and body mass index was significant in patients with AD (ρ = 0.430, p = .004), but not in controls (ρ = −0.255, p = .292).

increase Aβ production [87]. As a result, our results support the hypothesis that AD is potentially a consequence of neuropathological processes involving the actions of Aβ in the brain and tau pathology, whereas mechanisms triggering sporadic AD are age-related mitochondrial dysfunctions and oxidative stress that disrupt of amyloid metabolism in certain areas of the brain in susceptible individuals. Reduced antioxidant activity, increased neurotoxicity, and genetically determined mitochondrial dysfunction (e.g., mediated by the influence of ApoE4) may contribute to susceptibility to the development of sporadic AD. We postulate that dietary and/or pharmacological interventions targeting the mitochondrial regulatory mechanisms that inhibit Aβ-mitochondrial protein interactions, eliminate ROS, improve calcium homeostasis, or improve overall cell bioenergetics [88–90] would potentially prevent the development of the disease.

Declarations of interest

The authors have no conflicts of interest, financial or otherwise, to declare.

Table 3
Correlation between plasma amyloid beta (Aβ) levels and mitochondrial respiration in permeabilized platelets.

Partial correlation	Control			AD		
	$Aβ_{40}$	$Aβ_{42}$	$Aβ_{42}/Aβ_{40}$	$Aβ_{40}$	$Aβ_{42}$	$Aβ_{42}/Aβ_{40}$
ROX	0.442	−0.040	−0.245	0.120	−0.075	−0.038
	$p = .058$	$p = .870$	$p = .313$	$p = .433$	$p = .626$	$p = .804$
DMP-ROX	0.021	0.165	−0.028	−0.020	−0.188	−0.122
	$p = .933$	$p = .500$	$p = .908$	$p = .898$	$p = .227$	$p = .437$
C_I-ROX	0.091	−0.395	**−0.488**	0.077	−0.263	−0.245
	$p = .710$	$p = .094$	$\mathbf{p = .034}$	$p = .623$	$p = .088$	$p = .113$
C_I_II-ROX	0.090	−0.243	−0.319	0.050	−0.124	−0.108
	$p = .715$	$p = .316$	$p = .183$	$p = .750$	$p = .429$	$p = .490$
LEAK-ROX	0.072	−0.254	−0.454	0.215	−0.243	−0.249
	$p = .771$	$p = .295$	$p = .051$	$p = .156$	$p = .108$	$p = .100$
ETSC-ROX	0.148	−0.180	−0.323	0.062	−0.264	−0.216
	$p = .547$	$p = .460$	$p = .177$	$p = .684$	$p = .079$	$p = .155$
ROT-ROX	0.004	−0.049	−0.047	0.041	−0.056	0.007
	$p = .986$	$p = .841$	$p = .849$	$p = .788$	$p = .717$	$p = .966$
RES	0.134	0.145	−0.013	0.059	0.020	−0.053
	$p = .584$	$p = .553$	$p = .959$	$p = .702$	$p = .898$	$p = .728$
(DMP-ROX)/(ETSC-ROX)	−0.120	0.254	0.158	−0.043	−0.065	−0.031
	$p = .625$	$p = .294$	$p = .519$	$p = .784$	$p = .677$	$p = .843$
(C_I-ROX)/(ETSC-ROX)	0.002	−0.355	−0.329	0.076	−0.058	−0.133
	$p = .994$	$p = .136$	$p = .169$	$p = .630$	$p = .712$	$p = .395$
(C_I_II-ROX)/(ETSC-ROX)	−0.045	−0.094	0.011	−0.031	**0.416**	0.276
	$p = .855$	$p = .703$	$p = .964$	$p = .843$	$\mathbf{p = .006}$	$p = .074$
(LEAK-ROX)/(ETSC-ROX)	0.001	−0.122	−0.247	0.223	−0.163	−0.196
	$p = .996$	$p = .620$	$p = .308$	$p = .140$	$p = .284$	$p = .198$
(ROT-ROX)/(ETSC-ROX)	−0.091	0.131	0.222	−0.039	0.206	0.223
	$p = .711$	$p = .593$	$p = .361$	$p = .801$	$p = .174$	$p = .141$
ROX/ETSC	0.361	−0.004	−0.123	0.109	−0.073	−0.046
	$p = .129$	$p = .989$	$p = .616$	$p = .476$	$p = .635$	$p = .763$
RES/(ETSC-ROX)	−0.135	−0.085	−0.065	0.062	−0.018	−0.074
	$p = .583$	$p = .728$	$p = .790$	$p = .686$	$p = .905$	$p = .629$

Partial correlation coefficients (ρ) adjusted for age, education level and body mass index are displayed.
AD – Alzheimer's disease; ROX – residual oxygen consumption after the addition of 1.25 μg/mL antimycin A; LEAK – respiration caused by apparent proton leakage after the inhibition of the OXPHOS system by 2 μg/mL oligomycin; ETSC – maximal capacity of the electron transport system (ETS) at an optimum carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) concentration; ROT – oxygen flow after the inhibition of complex I by 0.5 μM rotenone; DMP – oxygen flow after the permeabilization of platelets with 50 μg/mL digitonin followed by the addition of 5 mM malate and 5 mM pyruvate; C_I – complex I-linked respiration after the addition of 1 mM ADP and 5 mM glutamate; C_I_II – respiration stimulated by substrates of both complex I and complex II after the addition of 10 mM succinate; RES, respiration reserve capacity (RES = ETSC-C_I_II).
Statistically significant values are diplayed in bold.

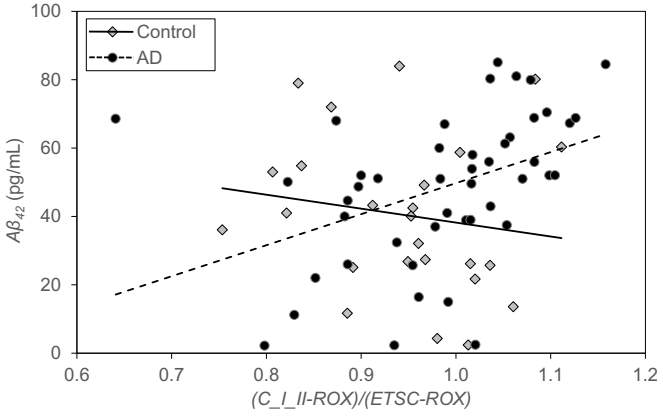

Fig. 5. Correlation between $Aβ_{42}$ levels and normalized respiration supported by substrates of both complex I and complex II (CI_II-ROX)/(ETSC-ROX) in permeabilized platelets from patients with Alzheimer's disease (AD) and controls. The partial correlation coefficient (ρ) adjusted for age, education and body mass index was significant in patients with AD ($ρ = 0.416$, $p = .006$), but not in controls ($ρ = −0.094$, $p = .703$).

Acknowledgements

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