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Abstract

The processing of the Amyloid Precursor Protein (*APP*) is a critical event in the formation of amyloid plaques which are composed of the 4kDa amyloid β-peptide (Aβ). Processing of APP occurs through a non-amyloidogenic pathway, mediated by initial α-secretase cleavage or through an amyloidogenic pathway via sequential cleavage by β- and γ-secretase enzymes, which produces Aβ peptides. Currently, the diagnosis of probable or possible Alzheimer's disease (AD) is primarily based on neuropsychological and neuroradiological assessment. Recent reports indicate that platelet β-secretase activity is moderately increased in patients with AD and mild cognitive impairment (MCI). To our knowledge platelet α-secretase activity has not yet been explored in this context and estimation of the ratio of the activities of α- and β-secretase in platelets may represent a useful surrogate marker of the balance between the two pathways of APP metabolism and be of importance for the diagnosis of AD. We therefore considered it of interest to develop assays of platelet α- and β-secretase activities suitable for such clinical investigations. Application of these assays to a Swedish population failed to uncover an effect of AD or MCI on individual platelet secretase activities or the secretase ratio. However, we did observe an inverse correlation between plasma triacylglycerol levels and the secretase ratio. The results are discussed in the context of the clinical usefulness of the secretase ratio as a biochemical adjunct to the diagnosis of AD.

Key words; alpha secretase, beta-secretase, Alzheimer's disease, biomarkers

INTRODUCTION

Alzheimer's disease (AD) is the most common form of late life dementia and is one of the leading causes of death in developed countries [1][2]. The clinical symptoms of the disease include a progressive decline in memory, judgement and cognitive function. Neurodegeneration, neurofibrillary tangles (NFTs) and the presence of senile or neuritic plaques in the brains of sufferers of the disease are the major pathological changes associated with AD [3]. The characteristic neuritic plaques of AD are composed of the 4kDa amyloid β- peptide (Aβ) [4][5] which is formed following the sequential cleavage of the Amyloid Precursor Protein (APP) by β- and γ-secretase, in what is known as the amyloidogenic pathway of APP processing. APP is a membrane spanning glycoprotein which under normal circumstances is predominantly cleaved by α-secretase, an ADAM (A Disintegrin And Metalloprotease) protein, to fragments other than Aβ [6][7]. The processing of APP by α-secretase precludes the accumulation of Aβ peptide and is named the non-amyloidogenic pathway. The cleavage results in the release of a soluble APP fragment (sAPPα) which has reported neuroprotective functions [8]. The remaining C-terminal stub is subject to cleavage by the γ-secretase complex, which releases a small peptide known as p3 that is considered non amyloidogenic.

Several members of the ADAM family, including ADAM9, ADAM10, ADAM17 (TACE), ADAM19 and ADAM8, have demonstrable α-secretase activity [9][10]. In contrast, the aspartyl protease beta-amyloid cleavage enzyme-1 (BACE 1) is the major source of β-secretase activity [11][12][13]. A related protease, BACE 2 also possesses β-secretase activity [14], though this protease cleaves APP more efficiently at an alternative

site located within the Aβ region and may play a greater role in the prevention of formation of Aβ peptide in cells that co-express BACE 1 and BACE 2 [15][16]. Greatest BACE 1 activity is present in the brain, particularly in the neurons [12][13]. Increases in both BACE 1 protein level and β-secretase activity have been reported in AD brains relative to healthy controls [17][18][19][20]. Conversely, the level of α-secretase activity has been reported to be lower in AD brains relative to healthy controls [19].

Several peripheral blood cells contain the required components for the processing of APP but platelets are the major source of Aβ in the peripheral blood [21]. Studies have indicated that platelet APP processing pathways are altered in AD [22][23][24], with a shift towards the amyloidogenic pathway which appears to reflect the overall disease process. These results suggest that alterations in the levels and activities of the enzymes involved in platelet APP processing could potentially be useful in aiding diagnosis of AD. Given that the amyloidogenic and non-amyloidogenic pathways represent two alternative and competing pathways for APP processing [25], it is probable that the ratio of their activities would be more informative than the respective α- or β- secretase activity alone. From a diagnostic point of view, the secretase ratio would be a more powerful tool than either α- or β-secretase activity alone since it gives an integrative overview of both the amyloidogenic and non amyloidogenic pathways.

Up to now, the diagnosis of probable or possible AD is primarily based on neuropsychological and neuroradiological assessment. In some countries, biomarkers in the cerebrospinal fluid (CSF) such as Aβ42 and total or phosphorylated tau are also used. A potential biomarker such as the platelet α/β-secretase activity ratio, which is readily accessible and relatively easily assayed, would significantly enhance and simplify the

diagnosis of AD. The possibility to use a biomarker supporting the clinical diagnosis is of great relevance for early detection of the disease and also for the evaluation and monitoring of potential therapies.

A recent study by Johnston et al has demonstrated a slightly increased β-secretase activity in platelet membrane from AD patients relative to healthy controls [22]. Platelet ADAM 10 expression has been shown to be reduced in AD [23][24]. To the best of our knowledge, platelet α-secretase activity has not yet been explored in AD patients or healthy controls.

The aim of the present study was to investigate the levels of platelet α- and β-secretase activity, and the ratio between those activities in healthy controls, mild cognitive impairment (MCI) and AD.

MATERIALS AND METHODS

The platelet α- and β-secretase activity was analysed in 30 healthy control subjects, 6 MCI patients and 20 patients with probable AD. The diagnosis of probable AD and MCI was made on the basis of the case history, clinical evaluation, magnetic resonance imaging studies, mini-mental state examination (MMSE) and neuropsychological assessment. The study was approved by the local ethics committee at Karolinska Institutet, Stockholm. Informed consent was obtained from all controls and patients, and the study was carried out in accordance with the Helsinki Declaration.

Blood was taken into a 5ml EDTA collection tube. Within 30 minutes of collection, the blood was centrifuged at 1300 g for 15 minutes at 4°C. The uppermost 1.2ml of platelet rich plasma (PRP) was removed to a separate centrifuge tube. Two 400µl aliquots of this PRP were transferred to 10ml centrifuge tubes for the analysis of platelet α- and β-secretase activity. A platelet count was performed on the remaining 400µl of PRP using a Sysmex XE-5000 Automated Haematology Analyser. The aliquots of PRP were centrifuged at 4700 g for 15 minutes at 4°C to collect the platelets. The supernatants were removed completely and discarded. The samples were analysed using slight modifications to the protocols included in commercially available kits which are intended for the measurement of cellular α- and β-secretase activity. The substrate for either assay is a short sequence of amino acids which includes the cleavage sites for α- or β-secretase and utilises the EDANS/DABCYL fluorometric reporter system. The rate of change in fluorescence is correlated to the enzymatic activity in the sample.

The platelet pellet for the α-secretase assay was resuspended in 500µl of a 25mM Tris-HCL buffer pH 8. The pellet for the analysis of β-secretase activity was resuspended in 5mls of wash buffer (450mM Tris pH 7.4, 12mM NaCl, 1.5mM EDTA) and the platelets were recovered following centrifugation at 4700 g for 15 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in 250µl of cell extraction buffer (R&D Systems UK, Component of kit FP002). An aliquot of this suspension was diluted 1/40 in the same buffer prior to assay. These dilutions were sufficient to ensure that the enzymatic activity would fall within the linear range for each assay which was previously established through analysis of α- and β-secretase activity in serial dilution of platelets from a 23 year old control subject. Platelet α- and β-secretase activity was measured using commercially available kits (FP001, FP002) which were obtained from R&D systems UK. 50µl of diluted platelet suspension was transferred in triplicate to a 96 well micro-plate (Nunc, Roskilde, Denmark). The addition of 50µl of 2X Reaction buffer and 5µl of the appropriate substrate (YEVHHQKLV – α-secretase, REEVNLDAEFKR – β-secretase) to each well brought the final volume per well to 105µl. Each plate also included (in triplicate) a 2µg/ml Recombinant Human TACE Positive control and a 2.5µg/ml Recombinant Human BACE Positive control, which were both sourced from R&D Systems UK. Each plate contained three blanks. The blank well for the platelet α-secretase, and TACE Positive control contained 50µl of 25mM Tris-HCL buffer pH 8, 50µl 2X Reaction buffer and 5µl of α-secretase substrate. The blank wells for the platelet β-secretase and BACE Positive control contained 50µl of Cell extraction buffer (Platelets) or 50µl of a 50mM Tris-HCL, 150mM NaCl pH 7.5 buffer, (BACE Positive control) as well as 50µl 2X Reaction Buffer and 5µl β-secretase substrate. The plate was

tapped gently to mix and the increase in fluorescence over a 95 minute time period at 37°C was followed using a Safire II Microplate reader with Magellan PC software (v6.3; Tecan Austria Gmbh, Austria). At each time-point, the fluorescence reading for the relevant blank (average of triplicates) was subtracted from the average fluorescence of the triplicate sample/control wells. The resultant values were plotted in a scatter diagram, with relative fluorescence units (RFU) on the Y-axis and time in minutes on the X-axis. For the α-secretase assay, the initial rates during the 5-25 minute linear phase were calculated by least squares approximation and were expressed as $\Delta RFU/h/10^6$ platelets. The linear phase for the β-secretase assay began at 25 minutes and continued for the duration of the assay. The rate of change in RFU per minute over the 25-95 minute time interval was calculated by least squares approximation and expressed as $\Delta RFU/h/10^6$ platelets.

The method was linear up to 39×10^6 platelets per well for the α-secretase activity (Fig.1A) assay and 1.28×10^6 platelets per well for the β-secretase activity assay (Fig.1B). The average intra-assay coefficient of variation of triplicates was 4.7% and 3.3% respectively for the platelet α- and β-secretase activity assays. The interassay coefficient of variation for the platelet α- and β-secretase activity assays was 12.2% and 5.2% respectively.

All platelet secretase activity measurements were carried out on fresh samples and were fully completed within 5 hours of blood collection.

Serum lipid profiles were obtained for 16 of the patients and CSF Aβ levels were obtained for 18 of the patients. Lipid and CSF Aβ levels measurements were carried out

in the Clinical Chemistry laboratory at Huddinge University Hospital according to routine procedures.

Student's t-Test was used to test for statistically significant differences in results between the control and patient groups at the $p \leq 0.05$ level of significance. The Pearson correlation test was used investigate possible relationships between the platelet secretase activities and age, MMSE score, CSF Aβ levels, Total cholesterol, HDL cholesterol, LDL cholesterol and triglyceride levels.

RESULTS

Patients in the demented group were significantly older than those in the control group (P < 0.001), while there was no significant difference between the ages of the MCI and control group, or the MCI and dementia groups. There was a higher proportion of female subjects in the healthy control group than the MCI or AD groups (Table 1) but there was no significant effect of gender on the platelet secretase activity in any of the groups when tested using Students two tailed t-Test. The patients sampled in this study had a range of MMSE score (15-30), indicating relative heterogeneity in terms of disease progression (Table 1).

Platelet membrane α-secretase activity and β-secretase activity were measured in fresh samples from healthy controls and subjects with MCI and probable AD as described in methods. Freezing and storage of platelet pellets at -70°C led to great variation in the levels of α- and β-secretase activity measured, when compared to the activities detected in a corresponding fresh sample. It is likely that this may be due to alterations in the physical properties of the platelet membrane as a result of the freeze-thawing process, as the platelet membrane integrity is known to be sensitive to temperature. Plasma was found to be a potent inhibitor of platelet α-secretase activity, most likely due to the presence of endogenous protease inhibitors. There was a complete inhibition of α-secretase activity in platelet rich plasma. As a result of this, the accurate measurement of α-secretase activity in the purified platelet pellet depended greatly on the complete removal of the platelet poor plasma prior to resuspension in the final assay buffer.

The composition of the centrifuge tubes used in the platelet preparation steps was also found to have a significant effect on the levels of detected α-secretase activity, most

Comment [SM1]: Maybe it would be worth adding in a comment on immunodepletion strategies for antu-proteases?

This also raises the question of whether there is a relationship between secretase activities, product levels and the genotype of AAT

likely because of small differences in the amount of residual platelet poor plasma left prior to resuspension in the assay buffer. During the course of this study, two different types of centrifuge tubes were used. One specific type of centrifuge tube was used in the preparation of the first 28 samples and a different type of centrifuge tube was used in the preparation of the remaining samples. The comparison of α-secretase activity levels in 8 samples that were prepared using both the old and new centrifuge tubes led to the finding that the detected α-secretase activity was, on average, 1.45 times higher in the second type of centrifuge tubes. The calculated value (1.45) was used to normalise the data and allow for the comparison of levels of α-secretase activity between the two different types of centrifuge tubes. The effect of centrifuge tube on platelet β-secretase activity was minimal.

There was no significant difference in the yield or the purity of the isolated platelets between the control and dementia groups or the control and MCI groups (Table.1). No significant effect of age or MMSE score on the α-secretase activity, β-secretase activity or the α-/β-secretase activity ratio in either patient or control groups was observed (Table.2) There was no statistically significant difference between the levels of α-secretase activity, β-secretase activity or the derived secretase activity ratio in the MCI or dementia groups when compared to the healthy control group (Fig.2).

There was no significant correlation observed between CSF Aβ levels, total, HDL or LDL cholesterol and any secretase activity in the patients for whom these values were available (Table 2). However, a statistically significant correlation was observed between triglyceride levels and the α-/β-secretase activity ratio (Fig.3)

DISCUSSION

The general consensus is that the levels of α- and β-secretase protein and activity are altered in AD brains compared to healthy controls [17][18][19][20] but such tissue is only available at post mortem. Since CSF comes into direct contact with the extracellular spaces of the brain, it is attractive to use this as a source of biomarkers for neurological diseases. One recent investigation failed to demonstrate a significant difference between AD-patients and controls with respect to β-secretase activity in CSF [26]. However the same study did report the finding of a slightly but significantly higher CSF β-secretase activity in MCI than in AD or control group. One of the major drawbacks to analysis of CSF is the difficulty in obtaining a sample and the discomfort and stress caused to the patient by the sampling procedure. In contrast to this, a whole blood sample can be acquired with minimal invasiveness. Given that platelets express both α- and β-secretase they would appear to be a viable source of biomarkers for AD. To this end, the primary objective of this study was to establish assays for platelet α- and β-secretase activity and to test the hypothesis that these activities are affected by dementia and AD.

The levels of the α-secretase ADAM 10 have been shown to be lower in platelets from AD patients than in healthy controls [23][24]. To the best of our knowledge platelet α-secretase activity has not previously been investigated in healthy controls or AD patients but it seems likely that expression levels would be correlated to activity. One group has recently reported elevated platelet β-secretase activity in AD and MCI [22][27]. However, within our population we failed to demonstrate a significant effect of AD or MCI on either of the two activities or the ratio between the two activities. Potential

explanations for the lack of agreement between the studies could be the different platelet preparation methodology used in the studies or differences in the disease states and demographics of the sample populations. The fact that our control group was significantly younger than our patient group could not explain our failure to demonstrate a difference between the groups since there was no significant effect of age on the platelet secretase activities in any of the groups (Table 2). However, our finding that plasma TAG levels are inversely correlated to the secretase activity ratio (cf below) may provide some explanation for this.

Numerous studies have reported that cholesterol exerts a regulatory influence on APP processing and Aβ formation. One recent study has demonstrated that reducing cellular cholesterol levels has an inhibitory effect on β- and γ- secretase activity [28]. Another study reports an increased α- secretase activity after cholesterol depletion in several different cell lines [29]. Very recently Liu et al have reported that the absolute cholesterol levels may be important for the platelet BACE activity [30]. In the present study we could not detect any significant correlations between circulating cholesterol levels and platelet α- or β-secretase activity. However, despite the small sample size (n=16) we did find a statistically significant correlation between the levels of TAG and the α-/β-secretase activity ratio. It should be noted in this context that the correlation is between plasma lipid levels and activity, rather than platelet lipid level and activity.

The presence of a statistically significant correlation between TAG levels and platelet β-secretase activity but not α-secretase activity indicates that the relationship between TG and the α-/β-secretase activity ratio is mainly due to an effect on β-secretase activity. In contrast to our results which suggest the existence of a relationship between

TAG and β-secretase activity, a recent cell-based study has tested the effect of several different TAGs on β-secretase activity in HEK cells stably expressing BACE-1 and found no significant effect [31]. The results of this study may however be confounded by the high level expression strategy dependent on an exogenous promoter which may circumvent normal homeostatic mechanisms.

It should be pointed out that the α-secretase assay measures overall activity and does not distinguish between various types of α-secretase which may be present in platelets. Thus, we cannot exclude the possibility that the activity of one specific protein with α-secretase activity could be decreased without a significant effect on the total platelet α-secretase activity.

Interestingly, several recent studies have implicated interleukins, including IL-1, with a role in upregulating the non-amyloidogenic pathway of APP processing, via upregulation of ADAM 10 and TACE [32][33]. One study has identified an increased plasma level of IL-1 in AD [34], which could potentially lead to a shift towards the non-amyloidogenic pathway in platelets.

In conclusion, we have demonstrated that it is possible to kinetically assay both α- and β-secretase activity in human platelets. To the best of our knowledge, this is the first study to report the measurement of α-secretase activity in human platelets. We have identified an apparent relationship between the level of TAG and platelet β-secretase activity and hence the α-/β-secretase activity ratio which will require further investigation. If the existence of such a relationship is confirmed, it is possible that it may be necessary to normalise the β-secretase activity with respect to TAG levels before testing for differences in secretase activities between patient and control groups. It would

also be of interest to directly investigate the effect of specific species of TAG on platelet β-secretase activity.

In the present study we were unable to uncover any significant differences in the levels of the secretase activities in health, MCI or dementia. Given the relatively small number of patient and control samples in our study, and the positive studies previously published, we can not completely exclude an effect of neurodegenerative diseases on the platelet α- and/or β-secretase activities. If such an effect exists, however, it is likely to be small and at this stage it is difficult to assess its usefulness for routine diagnostic purposes.

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Figure Legends

**Fig.1.** Linearity studies for platelet α- and β-secretase activity.

(**A**) Platelet α-secretase activity increased linearly up to $39*10^6$ platelets per well ($R^2 =$ 0.9943). (**B**) Platelet β-secretase activity increased linearly up to $1.28*10^6$ platelets per well ($R^2 = 0.9908$).

Fig.2. Platelet secretase activity stratified by diagnosis. HC = healthy controls, MCI = mild cognitive impairment and AD = Alzheimer disease. Secretase activities are expressed as $\Delta RFU/h/10^6$ platelets.

Fig.3. Plasma triacylglycerol levels are inversely correlated with the platelet secretase activity ratio in Alzheimer disease patients. Open circles = male, filled circles = female.
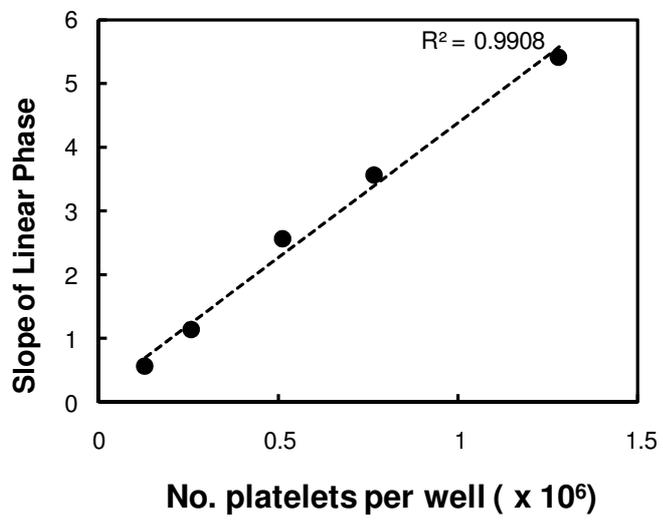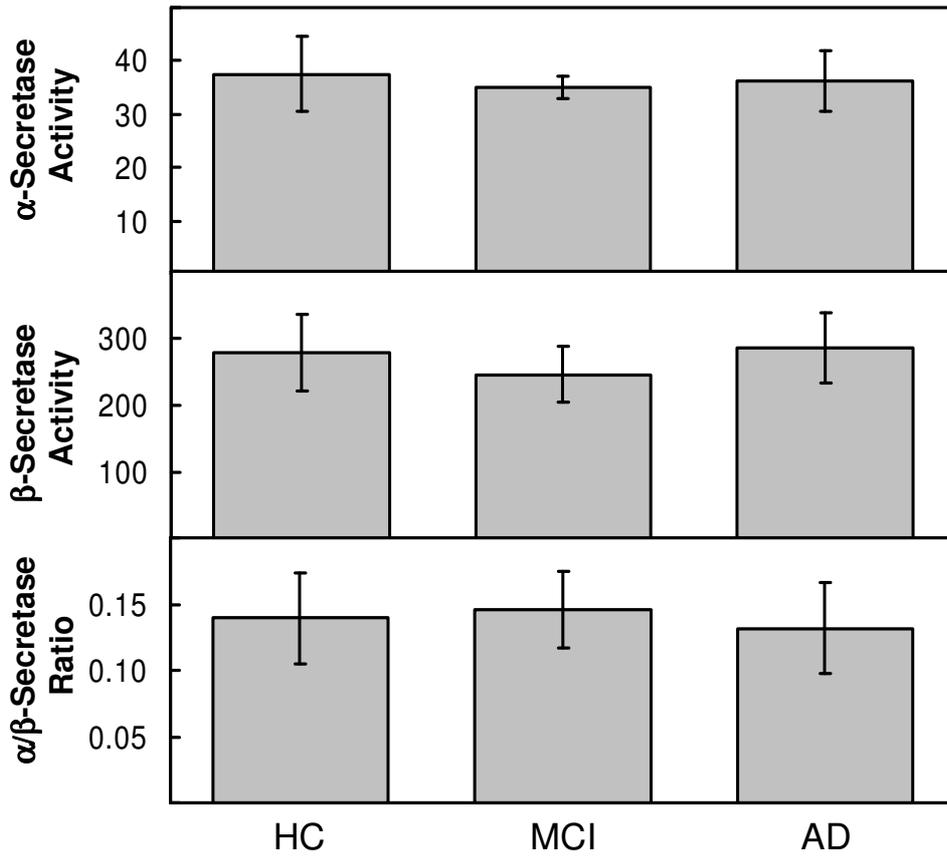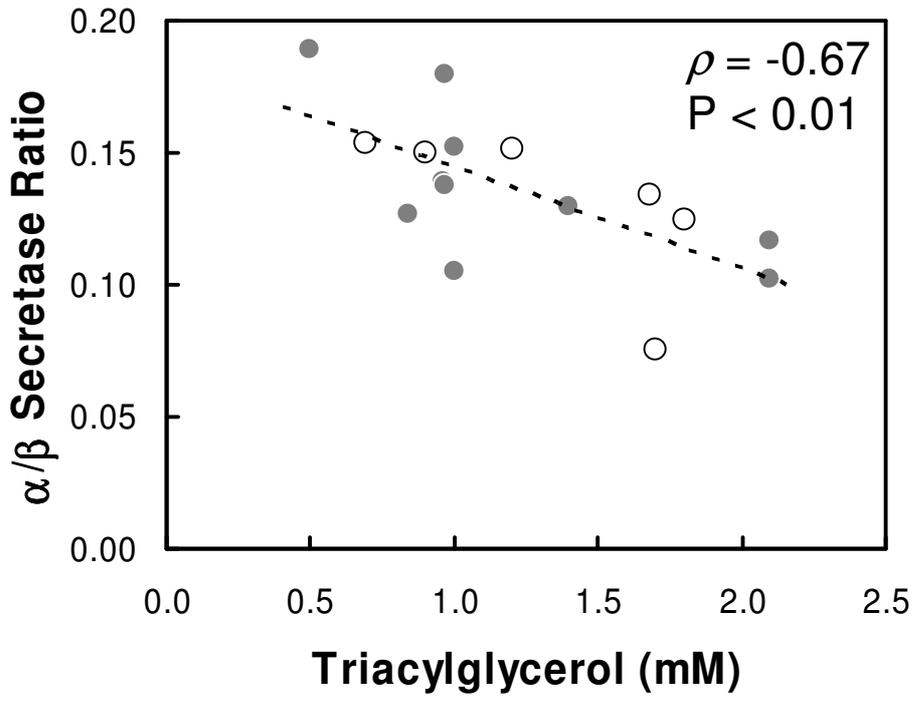
Fig.1

(A)

(B)
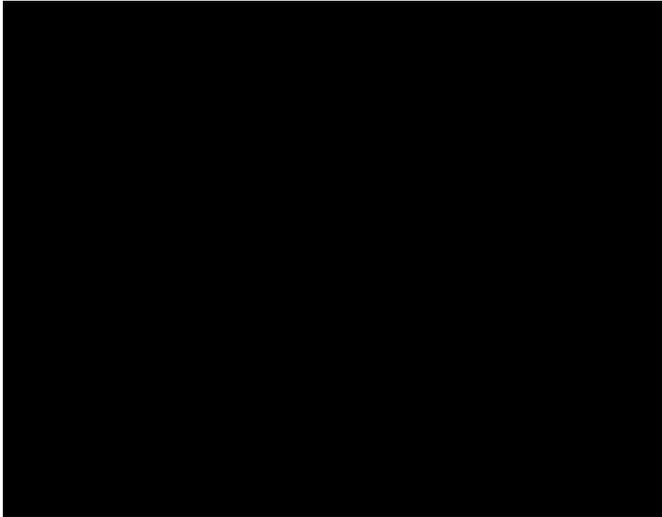
Fig.2.

Fig.3.

Fig.3.

Group	n	Age° (years)	Gender (F:M)	MMSE°	Platelet Yield° (*10^9/L)	Purity* (%)	α-secretase activity° (ΔRFU/h/10⁶ platelets)	β-secretase activity° (ΔRFU/h/10⁶ platelets)	α-/β-secretase activity ratio°
Controls	30	61.7 ± 7.5	23 : 7	NA	269 ± 65	93.8 ± 5.6	37.6 ± 6.9	279 ± 57	0.1396 ± 0.0343
AD	20	71.6 ± 9.8	10 : 10	24 ± 3	290 ± 96	95.1 ± 2.7	36.2 ± 5.8	285 ± 52	0.1317 ± 0.0342
MCI	6	65.7 ± 9.8	3 : 3	27.6 ± 2.1	268 ± 71	96.2 ± 2.0	34.9 ± 2.1	246 ± 42	0.1458 ± 0.0284

Table.1. Sample population characteristics and platelet secretase activity results.

° Values are expressed as Mean ± Standard Deviation of Mean

* % Purity was calculated by expressing the number of platelets as a percentage of total number of platelets + red blood cells + white blood cells.

Variable	n	α-secretase activity	β-secretase activity	α/β Secretase Activity Ratio
Age (AD)	20	ρ = 0.23	ρ = -0.19	ρ = 0.24
		P = N.S	P = N.S	P = N.S
MMSE	24	ρ = 0.12	ρ = 0.00	ρ = 0.10
		P = N.S	P = N.S	P = N.S
CSF Aβ	18	ρ = -0.11	ρ = -0.20	ρ = 0.07
		P = N.S	P = N.S	P = N.S
Total Cholesterol	16	ρ = -0.29	ρ = 0.07	ρ = -0.26
		P = N.S	P = N.S	P = N.S
HDL Cholesterol	16	ρ = -0.23	ρ = -0.22	ρ = -0.11
		P = N.S	P = N.S	P = N.S
LDL Cholesterol	16	ρ = -0.29	ρ = -0.02	ρ = -0.11
		P = N.S	P = N.S	P = N.S
Triglycerides	16	ρ = -0.34	ρ = 0.51	ρ = -0.67
		P = N.S.	P = < 0.05	P = < 0.01

Table 2. Relationship between platelet secretase activities and variables including Age, MMSE Score, CSF Aβ, Total Cholesterol, HDL Cholesterol, LDL Cholesterol and Triglyceride levels. Correlation was tested for using the Pearson correlation test. The level of significance was set at $P \leq 0.05$. N.S = Not Significant