

***IN VIVO* CHARACTERIZATION OF EXPRESSION OF ADAM10, AN ENZYME ACTING AS ALPHA-SECRETASE CLEAVAGE OF APP.**

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Introduction

The complete understanding of the pathological impact *in vivo* of alterations in expression/level and/or activity of key molecular elements still represent a challenge in the field of Alzheimer Disease (AD).

Amyloid precursor protein (APP) processing represents a crucial step in the molecular cascade of events leading to Alzheimer's disease (AD) pathogenesis. APP can be metabolised in two alternative ways. APP is delivered to the surface membrane, where it is subject to proteolytic cleavage by α -secretase, ADAM10 (Lammich et al., 1999; Postina et al., 2004), which occurs within the sequence of A β , thus precluding the formation of the amyloidogenic fragment. APP molecules that fail to be cleaved by α -secretase can be internalised into endocytic compartments and subsequently cleaved by β -secretase (BACE) (Vassar et al., 1999) and γ -secretase (De Strooper et al., 1998) to generate A β . These two proteolytic pathways are differentially compartmentalised within the cells, being alpha-secretase localised either in the Trans-Golgi network or in the plasma membrane (Lammich et al., 1999), whereas BACE activity is mainly confined to the endoplasmic reticulum and the endosomal/lysosomal system (Vassar et al., 1999).

Strategies to treat AD are aimed at preventing the formation of A β peptides. Therefore, β - and γ -secretases that generate A β peptides by sequential cleavage of APP are obvious targets for the development of specific inhibitors (Vassar and Citron, 2000). Alternatively, increasing α -secretase activity in the brain provides an attractive strategy, since proteolysis of APP within the A β sequence precludes the formation of A β peptides.

Though molecular biology and *in vitro* studies had a tremendous impact in our knowledge and they represented a crucial advancement in our understanding of the

disease, clear-cut data obtained in accessible cells or biological fluids were still lacking until few years ago.

Platelets appear immediately as a reliable peripheral cellular system where to analyse APP metabolism, a key event in AD pathogenesis, since they show numerous alterations typical for neurodegeneration (Zubenko et al., 1999) (Zoia et al., 2004) (Ripovi et al., 2000) (de Silva et al., 1998) (Bosetti et al., 2002) and appear to be the primary source of A β in human blood (Chen et al., 1995). In the last few years we investigated if a correlation between levels of platelet's APP forms and Alzheimer disease could be detected. We reported that patients with sporadic AD show an alteration of APP forms expression in platelets when compared with age-matched controls and with patients with non-AD dementia (Di Luca et al., 1996).

Furthermore, platelets contain the same APP processing enzymes found in neurons (Abraham et al., 1999; Colciaghi et al., 2002), BACE and ADAM10. We reported that in AD patients the alteration of APP forms ratio in platelets is accompanied by a significant modification of the two main enzymatic protagonists involved in APP metabolism (Colciaghi et al., 2004). We demonstrated an unsetting between α - and β -secretase activity in AD patients in vivo, with β -secretase activity being predominant as assessed by measurements of APP fragments- i.e. sAPP α released from activated platelets and the membrane-attached APP C-terminal fragments CTF83 and CTF99 produced by α - and β -secretase activity, respectively (Colciaghi et al., 2002) (Zimmermann et al., 2005). It is possible that an alteration of the concerted interplay among amyloid cascade actors occurs in AD with a concomitant decrease of α - and increase of β -secretase activity.

Moreover, we found a marked alteration of APP, BACE and ADAM10 in platelets already in the very early stages of the disease where dementia can be barely inferred by neuropsychological assessments (Colciaghi et al., 2004). Therefore, platelets provide a reliable tool to identify the pathological process already before the onset of clinical dementia and reflect the central pathogenic development.

Aim

ADAM10 will be characterized in peripheral cells of patients affected by Alzheimer Disease.

We already described a modification in the protein level of ADAM10 in platelets of mild AD patients and in the very early stages of the disease (Colciaghi et al., 2004)

Is ADAM10 protein reduction a consequence of a decrease of ADAM10 transcription or an increase of ADAM10 protein degradation?

In order to answer this question, here we propose to assess the expression of ADAM10 mRNA level in a selected population of AD patients and control subjects recruited at the Department of Medical Sciences-Neurology (University of Brescia, School of Medicine).

To analyse mRNA level, Real Time quantitative PCR (qPCR) will be used to assess alterations in transcript levels. These experiments will allow us to test whether the reduction of ADAM10 levels observed in AD patients will be accompanied by a reduction in mRNA levels.

Materials and Methods

Characteristics of the Subjects. Patients were enrolled at the Neurology Unit of the Department of Medical Sciences of the University of Brescia, School of Medicine, with all subjects being recruited and diagnosed with probable AD by Prof. Padovani and his group according to National Institute of Neurological Disorders and Stroke-Alzheimer Disease and Related Disorders Association (NINCS-ADRDA) criteria. Control subjects (C) were drawn from a series of either healthy subjects or non-demented patients hospitalised in the Neurology Unit. Exclusion criteria for all groups were the following: head trauma, metabolic dysfunctions, haematological diseases, alcohol abuse, drug abuse, delirium, mood disorders, and treatment with medications affecting platelet functions, i.e., anticoagulants, antiplatelet drugs, serotonergic agonists-antagonists, and corticosteroids. All subjects included had a standardised clinical workup based on neurological examinations, laboratory blood and urine analysis, neuropsychological assessment including a Mini Mental State Examination (MMSE) and a clinical dementia rating (CDR), and neuroimaging study (Head Computed Tomography and/or Magnetic Resonance Imaging). Before enrolment, subjects or their legal caregivers filled out an informed consent, after the nature and possible consequences of the study were explained.

Platelets' preparation. Personnel carrying out platelet preparation and subsequent analysis were blind for diagnosis and treatment of subjects. 27 ml of blood were

collected into 1 vol. 3.8% sodium citrate (in the presence of 136 mM glucose), mixed gently and centrifuged at 200 g for 10 min. The time interval between blood drawing and the first centrifugation was never longer than 20-25 min. Platelet rich plasma was separated from blood cells using a plastic pipette, carefully avoiding the drawing in of the buffy coat. Platelets were collected by centrifugation at 500 g for 20 min. Platelet pellets were washed twice with Tris-HCl 10 mM pH 7.4 and resuspended in an ice-cold lysis buffer (L-buffer) containing Tris-HCl 10 mM pH 7.4, EGTA 1 mM, Phenyl-Methyl-Sulfonyl Fluoride (PMSF) 0.1 mM, and a complete set of protease inhibitors (Complete ®; Roche, Mannheim, Germany). Homogenates were then subjected to 3 rounds of freeze thawing followed by 15 seconds of sonication at 0°C.

Isolation of total RNA from human platelets. Total RNA was isolated from Control subjects and platelets' pellets of AD patients by the Trizol, following the manufacturer's instructions (Invitrogen). Isolated RNA was dissolved in 12 µl diethylpyrocarbonate (DEPC)-treated water. The RNA samples had sharp ribosomal RNA bands with no sign of degradation (Agilent Technologies, 2100 Bioanalyser).

Reverse transcription. Total RNA, 400 ng in 4 µl water, was DNase I treated (0.5 U DNaseI, Amplification Grade, Invitrogen), reverse transcribed into first strand cDNA with 100 U/µl of Superscript III (Invitrogen) and 50 ng random hexamer primers, during 50 min at 50 °C. To the resulting cDNA sample, 15 µl of 10 mM Tris–1 mM EDTA was added, bringing the final volume to a total of 35 µl. From all samples a 1:20 dilution was made and used for qPCR analysis. All cDNA samples were stored at -20 °C until analysis.

qPCR primer design. qPCR primer sequences were designed using PrimerExpress V 2.0 software (PE Applied Biosystems, Warrington, UK). The length of the amplicons was kept as close as possible to 100–200 bp and the melting temperature of the primers was set at 59–60 °C. Details of the primers and the GenBank Accession Nos. are given in Table 1. Specificity of the primers was confirmed by BLAST searching. The length of the resulting amplicons was verified by agarose gel electrophoresis.

Primer name	Gene	Gen Bank	Forward	Reverse
ADAM10 ₉₋₁₀	ADAM10	NM_001110.2	CTTTTGCTCACGAAGT TGGACA	TGTCCCCAGATGTTGCTCT TG
ADAM10 ₁₁₋₁₂	ADAM10	NM_001110.2	AGATGAATGCTGCTTC GATGC	AAGGACCTTGACTTGGACT GCA

ADAM10 _{5'UTR}	ADAM10	NM_001110.2	GCGGAGGTCTGAGTTT CGAA	AGGAGAAACGGCGAAGCA C
β-Actin1	β-Actin	NM_001101.2	CCTTCTACAATGAGCT GCGTGT	ACAGCCTGGATAGCAACG TACA
β-Actin2	β-Actin	NM_001101.2	GCTCCTCCTGAGCGCA AG	CATCTGCTGGAAGGTGGA CA
HPRT	HPRT	NM_000194.1	ATGGACAGGACTGAA CGTCTTG	TGATGTAATCCAGCAGGTC AGC
GAPDH	GAPDH	NM_017008	TGCACCACCAACTGCT TAGC	GGCATGGACTGTGGTCAT GA
EF1α	EF1α	NM_001402	AAGCTGGAAGATGGC CCTAAA	AAGCGACCCAAAGGTGGA T
Cyclop	Cyclop	NM_021130	GCTCGCAGTATCCTA GAATCTTTGT	CTGCAATCCAGCTAGGCA TG
RPLPO	RPLPO	NM_053275	GTCGGAGGAGTCGGA CGA	AGCCTTTATTTCTTGTTT TGCA
CD2	CD2	NM_001767	CCAGCCTGAGTGCAA AATTCA	AAAACGAGCAGTGCCACA AAG
CD20	CD20	NM_152866	TATTTCCGGATCACTC CTGGC	GAAATGGCAGCAAAGAGG CTC
ADAM10 _{intron}	ADAM10	NM_001110.2	GACTGAGGTTTGCCTT TCGGT	TTAGCCCCTGCATCCTTTC A
β-Actin _{intron}	β-Actin	NM_001101.2	TGCTTTTTCCAGATG AGCTC	AATACACACTCCAAGGCC GCT

Table 1 Gene nomenclature, GenBank accession number and primer sequences.

Real-time quantitative PCR. In short, transcript levels were derived from the accumulation of DNA concentration-dependent SYBR green fluorescence in an ABI Prism 5700 Sequence Detection System (Applied Biosystems). The PCR conditions were as follows: 1·SYBR Green PCR buffer, 3 mM MgCl₂, 200 mM dATP, dGTP, dCTP, and 400 mM dUTP, 0.5 U AmpliTaq Gold, 2 pmol primers, and 2 μl of the 1:20 dilution of the cDNA in a total volume of 10 μl. At the end of the PCR run, the temperature of the sample was slowly raised from 60 to 95 °C while continuously collecting fluorescence data. This allows for the construction of a dissociation curve of the amplified DNA. These curves showed a single amplified product and the absence of primer–dimer formation. Non-template controls were included for each primer pair to check for any significant levels of contaminants. Primers designed against ADAM10 intronic sequences were used for each sample to check genomic DNA contamination. These samples always resulted in at least in a difference of 8

cycles of the cycle threshold (Ct) values compared to template containing samples. All samples were analysed together on a single 96-well plate.

For all primer pairs, the linearity of the qPCR assay (commonly referred to as amplification efficiency (E)) was determined on a cDNA dilution series. The Ct values obtained from the dilutions are expressed as the logarithm of the dilution factor: $Ct = \log(\text{dilution factor})$. This allows for a transformation of the observed changes in Ct to the linear domain. The slope of the best-fit line allowed us to determine the efficiency E according to the following equation: $E = 10^{-(1/\text{slope})}$. The reaction efficiencies had values between 1.8 and 2.0 for all assays. The qPCR Ct values were converted to absolute amounts of cDNA present in the sample E^{-Ct} and presented as $C * E^{-Ct}$ with $C = 10^{10}$.

Normalization. All cDNA synthesis reactions were performed on 400 ng total RNA and it may be expected that the cDNA input in the qPCR is not different among the groups and that in fact normalization for the amount of cDNA would not be required. This assumption was tested with a selection of reference genes. The remaining candidate reference genes were subjected to the geNorm-assisted analysis to select the most optimal set of reference genes (Vandesompele et al., 2002) (Tricarico et al., 2002)

Data analysis and statistical evaluation. Data are expressed as mean \pm standard deviation. Statistical evaluations were performed according to a one-way analysis of variance followed by Bonferroni as a post hoc comparison test.

Results and Discussion

RNA was isolated from platelets' pellets obtained from 17 Control subjects, 20 AD patients (MMSE = 20.95 ± 3.63) and 15 patients with Mild Cognitive Impairment (MCI). Subjects included in these three groups were age and sex matched.

Total RNA was reverse transcribed and the resulting cDNA was used for real-time quantitative PCR (qPCR) analysis.

Real-time qPCR is a powerful and accurate method to detect alterations in the level of transcript in tissues or cell cultures. It is generally accepted that between cDNA samples systematic differences in assessed transcript level are found that are caused by differences in RNA input, efficiency of the reverse transcription, etc.

Normalization procedures for qPCR assays are commonly applied to correct the outcome of qPCR for such errors. The most frequently used procedure is to express the level of the gene of interest relative to the level of a set of so-called reference genes. The proper way of selecting the optimal set of reference genes for a particular tissue or experimental setting is an issue of constant deliberation (Foss et al., 1998; Vandesompele et al., 2002). However, even after normalization a considerable inter-individual variation in expression level remains.

In order to determine the normalization factor, transcript levels of several genes frequently selected for normalizing PCR, were analysed: β -Actin (with 2 sets of primers β -Actin1 β -Actin2), hypoxanthine phosphoribosyl transferase (HPRT), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Elongation Factor 1 α (EF1 α), Cyclo and RPLPO (Table1).

Fundamental to a reference gene is that its expression is abundant and essential for basic maintenance of cells and typically a housekeeping gene is selected. However, the fact that a gene is a housekeeping gene does not necessarily mean that it will behave as a reference gene in a particular paradigm, because its multifunctional nature suggests versatility in the mechanisms regulating its expression.

The expression ratio of two ideal internal reference genes is identical in all samples, regardless of the experimental condition. Variation in the expression ratios of two genes reflects the fact that one or both of the genes are not constantly expressed. Using the data from all 52 cDNA samples and from all 7 studied genes, for each gene the pair-wise variation in relation to all other genes was determined as the standard deviation of the logarithmically transformed expression ratios.

The internal gene stability measure M was defined as the average pair-wise variation with all other control genes. Stepwise exclusion of the gene with the highest M value identified the order of the genes from the least stable to the most stable gene (Fig 1A). This analysis is facilitated by the use of the virtual basic applet GeNorm developed by Vandesompele et al. (Vandesompele et al., 2002).

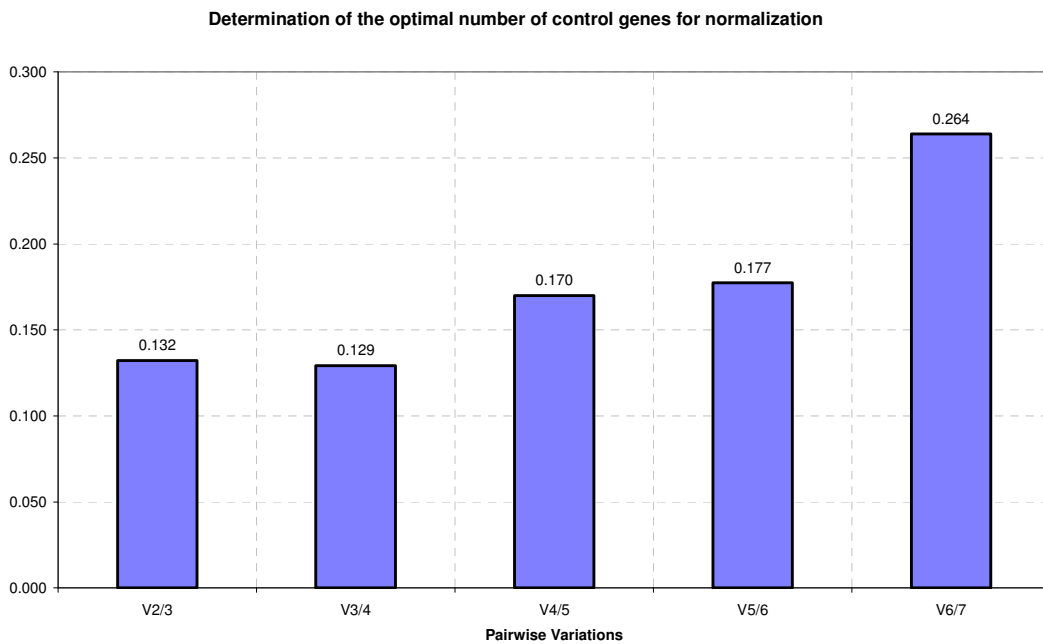
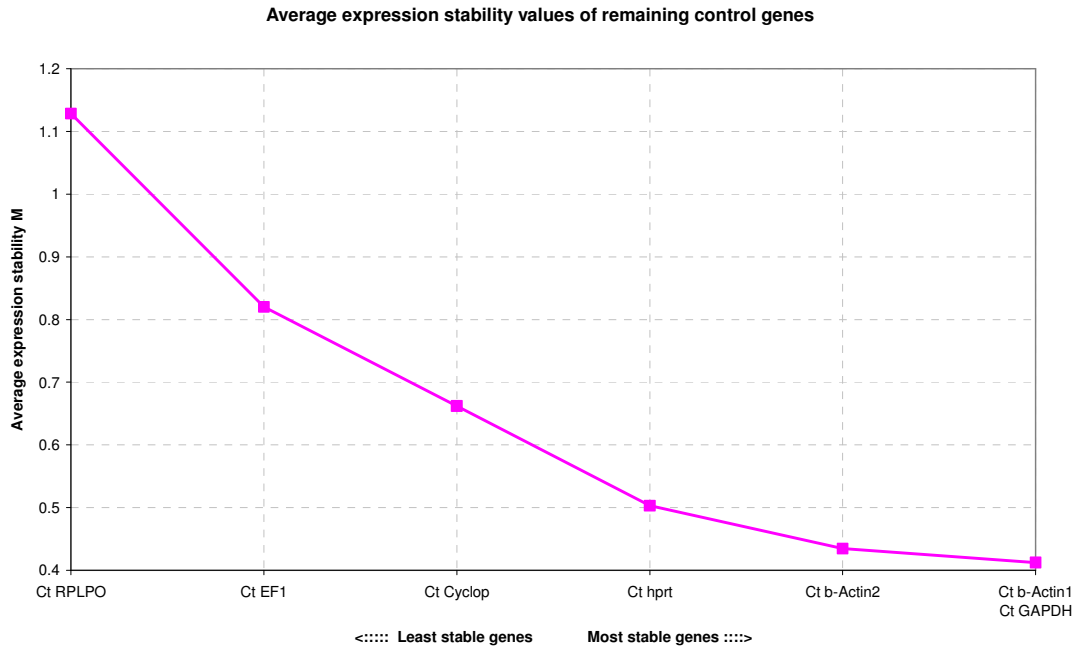


Fig.1 (A) The internal gene stability measure M of analyzed housekeeping genes. **(B)** Pairwise variation ($V_{n/n+1}$) analysis to determine the number of control genes required for accurate normalization. Note the increase between $V_{3/4}$ and $V_{4/5}$ caused by the addition of gene 5 cyclop.

Subsequently, normalization factors are calculated based on the two most stable genes (V_2) and compared to the normalization factors based on the three most stable genes (V_3) The pair-wise variation (V) was calculated between these normalization factors; an increase in the V value means that the added gene has a significant effect and should be excluded for the final normalization factor. After the suggested cut-off

value of 0.15 by Vandesompele et al.,(43) β -actin1, β -actin2, HPRT, and GAPDH resulted the most optimal set of reference genes (**Fig.1B**).

Therefore, a normalization factor based on the expression levels of β -actin1, β -actin2, HPRT, and GAPDH was calculated by using the geometric mean of the Ct.

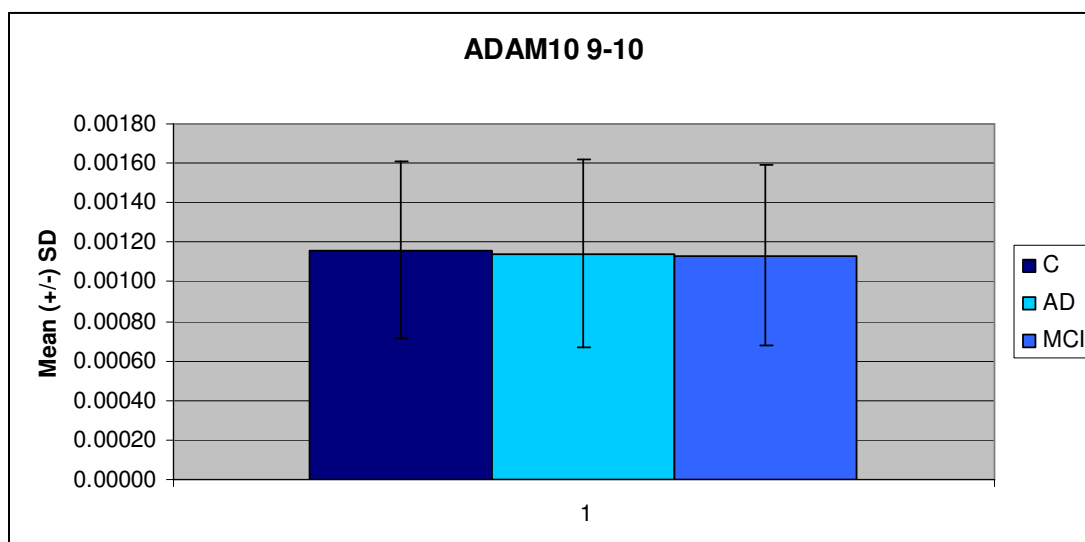
Genomic DNA contamination in each sample was evaluated by PCR amplification of intronic sequences, i.e. ADAM10 intron 7/8 and β -actin intron 2/3 (ADAM10_{intron} and β -Actin_{intron} primers).

qPCR amplification of CD2 transcript, a T-lymphocyte marker, and of CD20, B-lymphocyte antigen, was performed in order to detect Leukocyte presence in platelets' preparation (CD2 and CD20 primers).

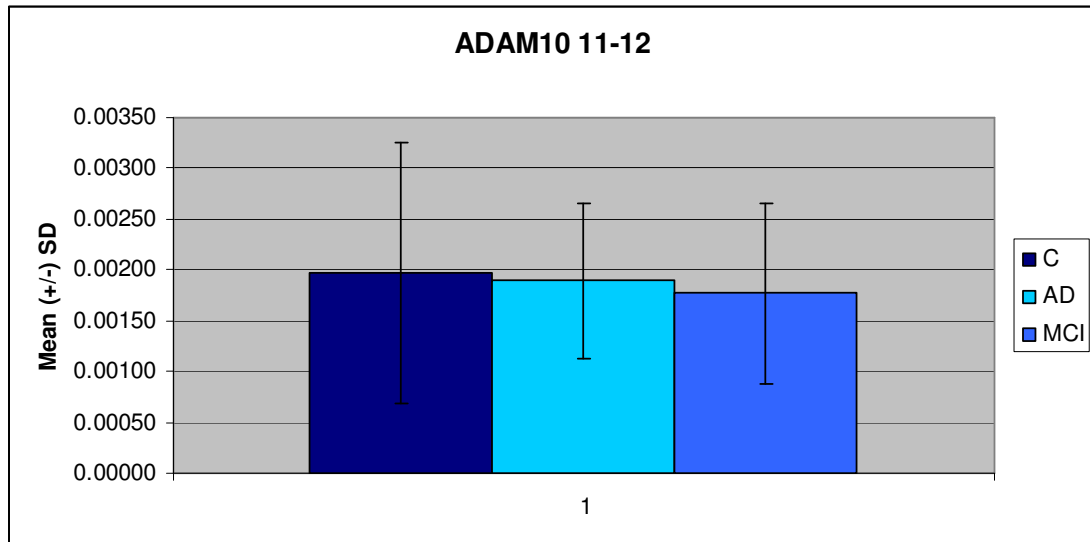
ADAM10 transcript level was assessed by qPCR with 3 different set of primers: primers ADAM10₉₋₁₀ and ADAM10₁₁₋₁₂ recognizing sequences in exons 9-10 and 11-12 and the set of primers ADAM10_{5'UTR} amplifying a sequence in the 5' untranslated region.

These are the results obtained using the three primers sets:

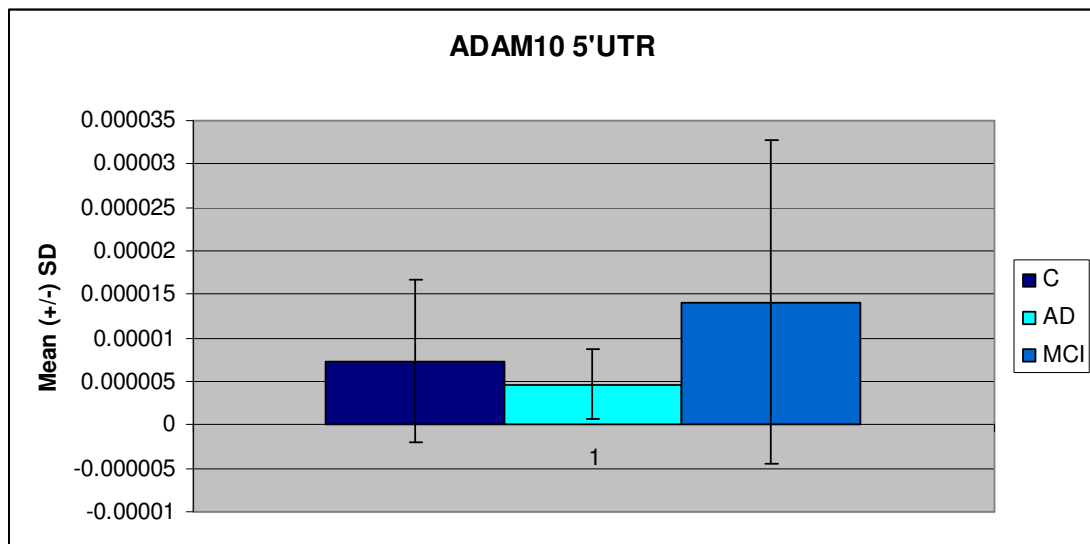
ADAM10 9-10: C=0.00116 \pm 0.0004, AD= 0.00114 \pm 0.0005, MCI=0.00113 \pm 0.0005;
p>0.05 AD vs C, MCI vs C



ADAM10 11-12: C=0.00197±0.0013, AD= 0.00189±0.0008, MCI=0.00177±0.0009;
p>0.05 AD vs C, MCI vs C



ADAM10 5'UTR: C= 7.73±9.48 10⁻⁶, AD= 4.692±4.14 10⁻⁶, MCI=15.4±18.9 10⁻⁶;
p>0.05 AD vs C, MCI vs C, *p*=0.047 AD vs MCI



No significant decrease of ADAM10 expression was observed in AD or MCI patients platelets compared to control subjects platelets.

Only when we used the set of primers ADAM10_{5'UTR}, amplifying a sequence in the 5' untranslated region, a significant difference of ADAM10 expression was detected

between AD and MCI patients' platelets. Despite of the above these results are not reliable since they present a high standard deviation and covariance values.

The data obtained in this study demonstrate that the decrease of ADAM10 protein levels in AD patients' platelets are not caused by a reduction of ADAM10 mRNA levels. A different mechanism is probably involved and it would concern translation or proteolysis phenomena.

In fact, ADAM10 activity is regulated at different levels (gene expression, maturation and trafficking) and by multiple signalling pathways.

From a gene expression point of view, ADAM10 promoter activity could be induced by vitamin A acid, since Sp1, USF, and retinoic acid-responsive elements to modulate its activity were identified in the core promoter (nucleotides -508 to -300) (Prinzen et al., 2005).

Several recent studies have examined the expression of ADAM10 in AD patients. ADAM10 protein levels were found to be reduced significantly in platelets of sporadic AD patients and sAPP α levels in platelets and cerebrospinal fluid of AD patients were also found to be reduced (Colciaghi et al., 2002).

Complementary to these findings is the observation that α -secretase activity was reduced in temporal cortex homogenates from AD patients (Tyler et al., 2002).

In contrast, ADAM10 mRNA levels were found to be *increased* two-fold in hippocampal and cerebellar sections of AD patients (Gatta et al., 2002). These results were from the brains of severe AD patients, and it is possible that in the later stages of the disease ADAM10 expression is increased as a defence mechanism or as a secondary effect of inflammation and reactive gliosis.

On the other hand, a study examining the effect of chronic hypoxia on APP processing found that α -secretase activity was reduced by 60% and the expression of ADAM10 by 50% in chronically hypoxic cells (Webster et al., 2002). In contrast, no change in the expression of the mRNA for this protein could be detected. Thus, they conclude that under chronic hypoxia the protein level of ADAM10 is regulated by post-translational mechanisms, most probably proteolysis, rather than at the level of transcription (Marshall et al., 2006).

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