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As vias de sinalização das secretases no processamento de APP - uma abordagem OMICA para a saúde neuronal.

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular, realizada sob a orientação científica da Doutora Odete da Cruz e Silva, Professora Associada com Agregação do Departamento de Ciências Médicas da Universidade de Aveiro e coorientação do Doutor Diogo Trigo, Investigador Auxiliar do Instituto de Biomedicina da Universidade de Aveiro.

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palavras-chave α-secretase, β-secretase, γ-secretases, Ácido Retoinco, Proteina Percursora Amiloid-β, Secretaseoma da APP, Processamentro da APP, Peptido Amiloid-β, Doença de Alzheimer, Neurodegeneração.

resumo

A proteína precursora da Doença de Alzheimer (APP), uma proteína central na Doença de Alzheimer (AD), é metabolizada de forma altamente complexa por uma série de secretases, que podem levar às clivagens α, β e γ. As capacidades tóxicas dos fragmentos resultantes são uma consequência direta da primeira clivagem: a β-secretase (BACE1) induz uma clivagem amiloide enquanto, contrariamente, a clivagem da α-secretase (ADAM10 e ADAM17) não tem o mesmo impacto patológico na AD. As α-secretases são, há algum tempo, alvo de estudo como um potencial alvo terapêutico para prevenir ou reverter os eventos bioquímicos iniciais da AD, uma vez que podem competir com BACE1 pela primeira clivagem de APP. A literatura demonstra que o recetor (RAR) do ácido retinoico (RA) altera a atividade das secretases, promovendo efeitos anti amiloides: regulação negativa do peptídeo β-amiloide (Aβ), libertado pelas secretases β e γ, e regulação positiva da α-secretase. Além disso, o RA reduz a neuroinflamação e promove o crescimento de neurites.

Para verificar experimentalmente o impacto do RA, a linha de células neuronal SH-SY5Y foi usada. As células foram diferenciadas e tratadas durante 48 horas com retinoides agonistas e antagonistas de diferentes isoformas do RAR. O tratamento sugere alterações nas proteínas diretamente associadas à AD, que podem ser benéficas ou patológicas, dependendo da isoforma do RAR estimulada.

Posteriormente, o secretaseoma da APP foi obtido e analisado. Dados experimentais obtidos por técnicas de dupla hibridação em leveduras foram incorporados potenciando novos interactores. Os alvos, a partir de uma abordagem holística e sistémica, foram identificados como proteínas de potencial interesse para a melhor perceção do impacto da estimulação dos RAR e da diferença decorrente de cada uma de suas isoformas individuais.

Dados da análise de espectrofotometria de massa da fração insolúvel de fibroblastos humanos com AD também foram introduzidos na pesquisa por alvos do ponto de vista holístico e de medicina de sistemas. Coincidentemente, alguns alvos encontram-se em comum quando comparados com a rede de secretases da APP apresentando-se como possíveis pontos de importante valor no contexto das vias de neurodegeneração da AD.

Em conclusão, as abordagens bioinformáticas fornecem resultados globais e dinâmicos sobre mudanças moleculares e interações proteicas sob diferentes condições, de particular interesse para patologias complexas como a AD. As alterações terapêuticas induzidas devem ser compatíveis com a homeostase e não apenas alterar a origem específica do problema. A combinação de bioinformática e ferramentas de *wet lab* fornecem uma potencial forma de abordagem para patologias complexas, com resultados promissores no estudo específico dos efeitos resultantes da estimulação dos RAR como uma potencial terapia na AD.

Keywords α-secretases, β-secretases, Ƴ-secretases, Retinoic Acid, Amyloid-beta precursor protein, APP secretaseome network, APP Processing, Amyloid-β peptide, Alzheimer´s Disease, Neurodegeneration.

abstract

The Alzheimer's Amyloid Precursor Protein (APP), a central protein in Alzheimer´s Disease (AD), is metabolized in a highly complex fashion by a series of sequential secretases, which can lead to α, β, and γ-cleavage, accordingly. The toxic abilities of the resulting fragments are a direct consequence of the first cleaving secretase: β-secretase (BACE1) induces an amyloid cleavage while, α-secretase (ADAM10 and ADAM17) cleavage does not have the same pathological impact in AD. The latter is currently being studied as a therapeutic target for preventing or reverting the initial biochemical events of AD, as it may compete with BACE1 for APP's first cleavage. Literature demonstrates that retinoic acid (RA) receptor (RAR) alters secretase activity, suggesting anti-amyloid effects: downregulation of Amyloid-β-peptide (Aβ) releasing secretases (β and γ-secretase) and upregulation of the beneficial α-secretase. In addition, RA reduces neuroinflammation and promotes neurite growth.

To experimentally verify the impact of RA, the SH-SY5Y neuronal cell line was used. Differentiated cells were treated for 48 hours with retinoids agonists and antagonists for different RAR isoforms. Treatments suggest alterations in proteins directly associated with AD, that can be either benefitial or pathlogical, depending the RAR isoform being stimulated.

The APP secretaseome was compiled and analyzed. The data from a YTH system was introduced and new potential interactors described. Targets from a holistic and systems aproach were identified as potencial interest proteins for further understanding the impact of stimulating each RAR isoform.

Data from mass spectophotometry analysis of the insoluble fraction of human fibroblasts from AD patients was also introduced in the search for targets in the holistic and systems medicine perspective. Coincidentelly, some targets were found in common when compared with the APP secretase network, being possible important proteins in the context of AD neurodegeneration pathways.

In conclusion, bioinformatic approaches provide global and dynamic results on molecular changes and interactions under different conditions, of particular interest for complex pathologies such as AD. Induced therapeutic changes must restore homeostasis and not only alter the specific source of the problem. The combination of bioinformatics and *wet lab* tools provides a putative gateway, with promising results in the specific study of effects resulting from the stimulattion of the retinoid acid receptor as a potential therapy for AD.

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Chapter I – Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease and the main cause of dementia in the elderly population [1], that affects about 6% of individuals over the age of 65 [2]. Thought subject to extensive research, the main mechanisms causing AD are still, to some extent, uncertain, with the majority of cases occur spontaneously. Still, family aggregation of AD (FAD) can arise, caused by autosomal dominant mutations in the genes coding for the amyloid precursor protein (APP) and the Presenilins (PSEN1 and PSEN2) [3] among other relevant AD related genes .

AD is characterized, biochemically, by the abnormal intracellular deposition of hyperphosphorylated Tau, forming the neurofibrillary tangles, and the extracellular accumulation of the 38 to 43 amino-acid long amyloid beta (Aβ) peptide. The mechanisms involving Aβ processing and prevention are a current topic of exhaustive research. The main players involved in Aβ production are β and γ-secretases. APP cleavage by α-secretase prevents Aβ accumulation [3]. Noticeably, APP ectodomain shedding is completed by two alternative proteases: α and β-secretases that, under specific conditions, may compete for APP cleavage, in a competition that decides Aβ generation [4]. The first fragment released is sAPP, α or β, depending on the cleaving protease. Subsequently, γ-secretase cleavage will result in the generation of p3 or Aβ, correspondingly and in accordance with the first cleaving secretase.

APP is ubiquitously expressed in all tissues, with especially high levels in the brain. It is a single-pass transmembrane protein with a large extracellular N-terminus and a short cytosolic C-terminus [5]. Following protein synthesis in the endoplasmic reticulum (ER) associated polyribosome, APP is N-glycosylated in the ER and transported to the Golgi apparatus for O- and N-glycosylation, phosphorylation and sulphonation at tyrosine sites [5].

APP belongs to the type-I transmembrane mammal protein family that includes APP-like protein 1 (APLP1) and 2 (APLP2), all processed as described above. In humans, the gene for the APP-unique Aβ domain is located on chromosome

21. It contains 18 exons spanning 290 kilobases [6], with three major isoforms resulting from alternative splicing: APP695, APP751 and APP770 [7]. The two largest isoforms contain a 56 amino acid Kunitz Protease Inhibitor (KPI) domain in their extracellular regions, and are expressed in most tissues [8], while APP695, lacks the KPI domain, and is principally expressed in neurons [9]. The KPI domain is described to play an essential role in APP dimerization, impacting the regulation of the sub-cellular location, secretory pathway, and subsequent processing of KPI-containing APP isoforms [10].

Furthermore, the APP770 isoform contains the OX-2 antigen domain. Studies demonstrate increased protein and mRNA level of APP751 and APP770 in AD brains as well as an association with increased Aβ deposition [11]. Prolonged activation of extra-synaptic NMDA receptor in neurons can shift APP expression from APP695 to KPI-containing APP isoforms, complemented by an increased production of Aβ [12]. APP695 has been shown to associate with NMDA receptors in a way that involves regulation of the intracellular trafficking mechanism by APP [13]. Such findings propose a dysregulation in splicing of APP RNA [11] and allocate APP isoforms, facilitating access to the γ-secretase complex [12], an important factor in AD pathogenesis.

Although the inherent biological role of APP is of paramount importance in Alzheimer's research, its physiological function remains somewhat elusive. The most substantiated and certified role for APP is in synaptic formation and repair, with its expression being upregulated during neuronal differentiation and following neural injury [14]. Functions in cell signalling, long-term potentiation, cell adhesion, and transport, are currently only suggested and supported limited research [15], [16]. In particular, resemblances in post-translational processing have invited comparisons to the signalling role of the surface receptor protein Notch, a combined substrate of several APP secretases [17].

Extensive literature analysis corroborates the above-mentioned general theory and adds to APP's multimodal function. The APP protein family can function as a cell surface receptor-like protein and ligand, mediating several physiological or pathological effects either from the cell surface or via the released proteolytic

fragments [18]. However, APP is not restricted to the Central Nervous System (CNS) with studies revealing a presence in spermatozoa [19] and marking it as an important sentinel protein for male reproduction [20], suggesting a wide range of yet unravelled functions, for the APP protein family.

The importance of phosphorylation in AD is widely accepted [21]; even though some research has correlated α-secretase phosphorylation with increased alpha cleavage and amyloid protection [22] suggesting its activation by phosphorylation, the most significant impact of this event appears to occur as a consequence of APP phosphorylation [23]. APP cytoplasmatic domain phosphorylation has been suggested to influence its trafficking, with several studies demonstrating that phosphorylation of APP on threonine 668 (Thr668) by MAPK8 can promote β-cleavage by facilitating secretase-APP interaction [24], [25], as seen in figure 1. In addition, several other kinases are described to phosphorylate APP at Thr668. However, this theory remains unconfirmed, as other studies demonstrate that Thr668 phosphorylation decreases extracellular Aβ and γ-secretase activity [26].

1.1 α-secretases

Over 30 ADAMs (a disintegrin and metalloprotease) have been described, nearly 20 of which associated with the nervous system [27]. Numerous molecular functions have been credited to ADAM transmembrane proteins, namely cell adhesion and integrin-binding properties, but these metalloproteases are also important to intracellular signalling pathways, affecting several mediator molecules. ADAM 10 is associated with neurogenesis and axonal growth during embryonic development [28]. Distinctive to ADAMs, but not typical of other metalloproteases, is the ability to exhibit cleavage-dependent activation of other proteins, including the transforming growth factor α (TGFα), NOTCH1 (Neurogenic locus notch homolog protein 1), IL6-receptor (IL6R).

Although the trans-Golgi network is a strong candidate as one of the sites of α-cleavage [27], a membrane-bound endoprotease at the cell surface has been

observed to have an α-secretase-like activity [14], [29]. The precise subcellular localization of α-secretase is uncertain. Nevertheless, it is challenging to articulate the localization of α-secretase exclusively to the late secretory pathway with the concept that α-secretase stimulation inhibits β-secretase processing [30], an idea extensively debated in the literature.

APP cleavage by α-secretase occurs within the Aβ peptide, between Lys-16 and Leu-17. The precise cleavage site is believed to be primarily determined by an α-helical conformation and membrane distance from the hydrolysed bond. The function exerted by α-secretase on APP results in a membrane-anchored carboxyterminal fragment, termed C83, and the extracellular release of the large soluble fragment sAPPα [31]–[33].

The α-secretase-derived soluble APP N-terminal fragment, sAPPα is associated with neurotrophic and neuroprotective functions, further supporting the therapeutic value of increased APP α-secretase cleavage [34], as discussed further below. sAPPα is constitutively secreted from cells: studies using protein kinase C (PKC) stimulation by phorbol esters show an increase in sAPPα release, demonstrating that APP α-secretase cleavage can be either constitutive or regulated by phosphorylation, thus suggesting the existence of different α-secretase proteases [35].

The anti-amyloidogenic α-secretase ADAM10 directly competes for APP at the cell surface [36]. The ADAM protein family is characterized by the presence of some conserved amino-acid domains, including an N-terminal signal sequence, required for directing the proteins to the secretory pathway, followed by a pro-domain, responsible for proper protein folding, a metalloprotein domain, a disintegrin domain, a cysteine-rich region, a transmembrane domain, a cytoplasmic domain, and, with the exception of ADAM10 and 17, an EGF-like domain [37], [38].

The diversity of the ADAM protein family is increased by alternative splicing of several ADAM genes. ADAMs are separated into two groups: the catalytically inactive ones, including proteases lacking a functional Zn-binding active site, acting via other mechanisms such as protein folding or protein interaction, and the

catalytically active group, which contains proteases with a Zn-binding active site: ADAM10 and ADAM17 (also described as TACE) [37], [39].

1.1.1 Cleavages by α-secretases

α-secretase cleavage is independent of protein sequence. The cleavage is determined by an α-helical conformation, at 12 to 13 residues distance from the membrane hydrolysed bond [39]. Like ADAM10, ADAM17 targets a broad range of substrates implicated in several important physiological pathways and mechanisms. ADAM17 was first reported to be responsible for the proteolytic activation of the membrane precursor of TNFα [1]. Notch receptors, ligands, cadherins, IL-6 receptor, and EGF receptor ligands are also cleaved by α-secretases, as well as several other type I transmembrane proteins, in a process contributing to the release of their extracellular domains.

A number of ADAM proteases reportedly exhibit α-secretase activity [40], as selective interference of individual ADAM10, ADAM17 and ADAM19 genes, both in cell and animal models, had no noticeable impact on non-amyloid APP processing [41], [42]. In 2010, ADAM10 was shown to have the most relevant α-secretase activity in neurons [43]. In recent work, we have described that even though several ADAM proteases are described to exert activity towards APP, only ADAM10 and ADAM17 have a direct APP interaction, with most of the other ADAM proteins playing a supporting role (da Cruz e Silva et al, submitted).

1.1.2 Retinoic Acid impact in α-secretase

Retinoic Acid (RA) metabolism and signalling are vital for neuronal health and several studies have described its impairment in AD patients, who present decreased serum and Cerebral-Spinal Fluid (CSF) levels of RA precursors, such as vitamin A and carotenoids [44], [45]. Furthermore, Aβ has been demonstrated to reduce cellular RA synthesis [46].

Contemplating the promising results of promoting α-secretase expression for AD therapy, functional studies depicted two potential RA responsive elements in the ADAM10 promotor region, 203 and 302 bp upstream of the gene translation start site [47]. Promotor reporter assays in neuroblastoma cells treated with all–trans RA

(atRA) showed a significant increase in ADAM10 transcriptional activity, mRNA, and protein levels and, consequently, sAPPα secretion [48]–[50]. RA receptor (RAR) activation results in increased α-secretase activation, as is the case for ADAM10 [51]. Moreover, RA presence, and consequent stimulation of the α isoform of this receptor (RARα) activates several kinase proteins, including PKC [50], [52], inducing anti-amyloid effects, via direct RARα guiding to responsive promotors [53] and through direct activation of α-secretase via phosphorylation [54], demonstrated in figure 1.

Although α-secretases are complex therapeutic targets, due to their large substrate number and the range of signalling pathways in which they are involved, they are, nonetheless, prime candidates contributing towards unravelling Aβ deposition. However, most studies regarding AD, Aβ formation, or APP proteolytic processing, focus on β and γ-secretases and their influence on APP cleaving, lacking a holistic approach encompassing α-secretases and their supporting interactors [55].

1.2 β-secretases

BACE1 (beta-site APP cleaving enzyme 1) is the fundamental protein with β-secretase activity [56]. This secretase plays a central role in Aβ production, being the first and rate-limiting processing stage of APP contributing towards the amyloidogenic pathway [57]. These conclusions are drawn from repeated and well validated experiments knocking out BACE1, which completely halts Aβ formation [58]–[60].

BACE1, a membrane-bound aspartyl protease, has structural similarities to the pepsin family [61], containing two active site motifs at amino acids 93 to 96 and 289 to 292 in the luminal domain [62]. Each of these motifs contain a highly conserved signature sequence of aspartic proteases, D $^{T}/_{S}$ G $^{T}/_{S}$ in which the aspartic acid residue is essential for catalytic activity. In addition, BACE1 has four putative N-linked glycosylation sites and six luminal cysteines, which allow for the formation of up to three intramolecular disulphide bonds.

β-secretase activity is highest in the late-secretory pathway compartments: the Golgi apparatus, trans-Golgi network (TGN), secretory vesicles, and endosomes [63]. Its expression levels are highest in the brain but is nonetheless ubiquitously expressed and present in most tissues, with the pancreas being a close second. The relevance of pancreatic BACE1 is not yet understood, but it is marked by low activity and high levels of an mRNA splice variant, lacking the exon 3, resulting in a different isoform [64].

The therapeutical value of β-secretase has been widely investigated, with progress being made in regards to achieving an inhibitor [65], [66], that although promising, has not yet reached the potential to specifically inhibit the cleavage of APP alone, not interfering with other BACE1 substrates. In fact, there are approximately 68 putative substrates cleaved by β-secretase, most of them are type I transmembrane proteins, like APP [67]. As such, specifically inhibiting only one of them is a particularly challenging task.

Paradoxically, BACE1 knockout mice are viable, fertile, and do not show any morphological or developmental alterations [58]–[60]. Nevertheless, corroborating the above mentioned problem, these animals show subtle behavioural phenotypes, with a mild memory impairment and spontaneous activity changes [68], [69]. Therefore, these findings shed new light in the viability of BACE1 inhibition, reinforcing the previously explained challenge.

The role of BACE1 in the myelination process can explain the phenotypes presented by animal models [70]. This enzyme is expressed in high levels during post-natal stages, acting on the NRG1 (Neuregulin-1) signalling pathway and the proteolytic process, believed to facilitate signalling pathways towards myelinization [71]. All BACE1 knockout animal models consistently present elevated hypomyelination [70], [72], indicating that BACE1 interferes in central nervous system myelination. Despite these *in vivo* results, this claim remains controversial. BACE1 has also been described to be relevant to the function and regulation of voltage-dependent sodium channels [73], although with minor hypothetic impact in behavioural changes.

1.2.1 Retinoic Acid impact in β-secretase

Although not to the extension described above for ADAM10, BACE1 activity also correlates with RAR stimulation. Treatment with atRA was shown to modify both BACE1 expression and activity, with no impact in its homologue BACE2 [50]. Interestingly, BACE1 mRNA levels were significantly increased, but no impact was observed on overall protein quantity or activity [50]. A different study, however, reports BACE1 expression levels to be reduced upon atRA treatment [74]. The same authors describe this interference in BACE1 expression to be mediated by NFkB (figure 1), with its DNA binding site upstream of the BACE1 promotor region, in addition to the direct effect of RAR. In fact, disruption of NFkB increases BACE1 transcription and reverses the effects of the atRA treatment [74].

1.3 γ-secretases

γ-secretase is a multiprotein complex consisting of presenilin (PSEN1 and 2), nicastrin, Aph-1, and Pen-2, with PSEN proteases, each containing the two catalytic aspartates that mediate peptide bond scission [75], thus enabling APP cleavage within the transmembrane domain (TMD) [76]. This cleavage occurs in two critical aspartyl residues within TMDs 6 and 7 of PSEN1 and/or 2 [77]. Though the specific function of each component of this secretase complex has been under intense research and scrutiny over the last decade, consensus has not been attained regarding their importance for AD and their ability to interfere with secretase activity [78]–[81].

Like ADAM and BACE secretases, PSEN1 and 2 are involved in the processing of type-1 transmembrane proteins, including APP [82]. Their genes, with closely related and conserved structures, were discovered as loci mutated in a large proportion of human pedigrees with inherited early AD onset. These two genes (PSEN1 and 2) encode the catalytic proteins of γ-secretase complexes that cleave transmembrane proteins within lipid bilayers, including beyond APP, the Notch receptor, E-cadherin, Nectin1 and others [83], [84].

An aspect of significant importance regarding γ-secretases is that intramembrane processing of APP by this complex is not restricted to a single cleaving site. The γ-secretase complex is widely accepted to cleave at several sites within the TMD of their targets, with 3 cleavages separated by approximately three amino acids each, in the specific case of APP [85]–[88]. Under physiological conditions, this last secretase cleavage is unprecise, occurring between amino acids at positions 37 to 43 of the Aβ peptide. This variation is highly significant for AD pathology, directly and proportionally associated with Aβ aggregation and deposition capacities, resulting in increased toxicity [89]. These multiple cleavages are postulated to be a stepwise cleavage mechanism performed by the secretase, and extensive, recent work has begun to show that this phenomenon may be a general characteristic of all γ-like-secretases [90]–[92]. The difference between the three cleavages is of immense importance to AD and may hold the key to combined therapeutic strategies [65].

1.3.1 Retinoic Acid impact in γ-secretase

The impact of RA in AD secretases is not restricted to α and β-secretases and affects the γ-secretase complex. Its activity is largely reduced upon RA treatment [93], validated by the extreme increase of γ-secretase substrate, C99 [93]. This might be considered an important objection against the use of RA-induced changes, since the cellular accumulation of C99 is postulated to have a cytotoxic effect, as discussed below. But the same research describes this unique inhibition of γ-cleavage to significantly reduce Aβ secretion. In addition to transcriptional alterations, RA regulates several signalling pathways, including kinases, resulting in alterations upon γ-cleavage. The γ-secretase complex is regulated by the ERK-pathway [94], and the effects of RAR stimulation upon γ-secretase were confirmed, revealing that RA-mediated γ-secretase inhibition requires ERK activation [93]. A more targeted analysis of the impact of atRA treatment in PSEN1 and 2 revealed that, mRNA levels of PSEN1 are increased, but PSEN2 remains unaltered, when compared with two isoforms of BACE secretase, [50]. Furthermore, similar effects between BACE1 and PSEN1 extent to mRNA levels as they increase in both proteins and, paradoxically, the protein levels remain largely unchanged with a significant loss of activity, as depicted in figure 1

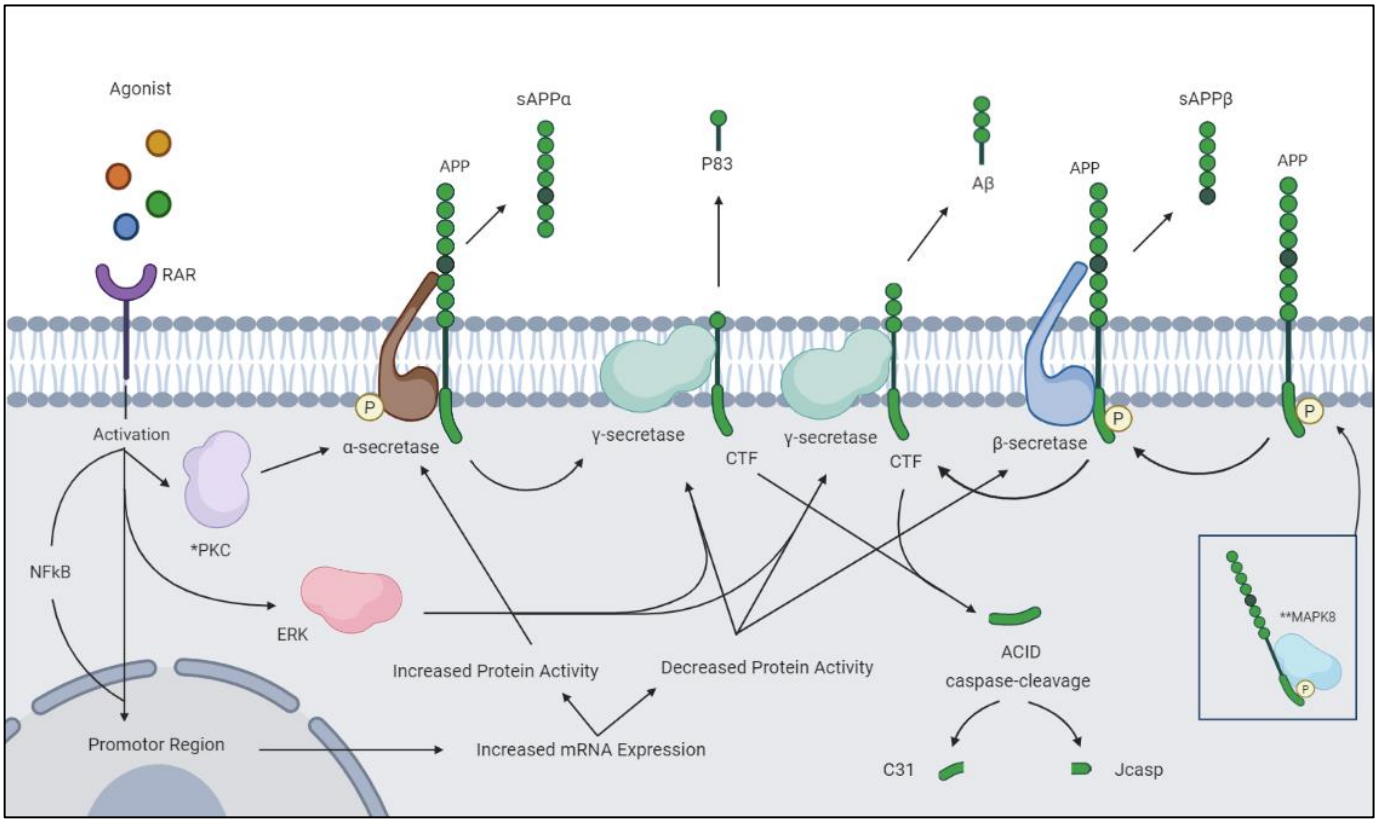


Figure 1 – Integrated retinoid signalling impact on APP processing and consequent fragment production. *Treatment with atRA modifies the gene promotor region for each of the secretases. This stimulus can be either direct* [93] *or indirect, being mediated by NFkB* [74]. *The activation of several kinases by RA receptors can have a direct impact on α-secretase activation, contributing to augmented non-amyloidogenic APP processing. ERK pathway activation by RAR stimulus leads to loss of γ-secretase activity, even though its mRNA levels are increased; the same observations are described for the β-secretase BACE1. APP´s 3 key structural elements are represented appropriately: circles representing the extracellular domain; linear representing the transmembrane domain; rectangular representing the intracellular domain. *PKC is exemplified since it is the most relevant kinase activated by RAR, but other kinases are explained to take part in RAR-indued activation of α-secretase. **MAPK8 is here denoted to phosphorylate APP at Thr668 since it is the kinase most frequently depicted to produce this effect, but several other kinases have been shown to phosphorylate at this site.* (Adapted from: Vitória, J.M. *et al,* submitted.)

1.4 Function of APP derived proteolytic fragments

Over time, the proteolytic fragments of APP have been the focus of intense research towards unravelling AD. Their roles spread over a wide range of pathways, both physiological and pathological. The cleavage of full-length APP results in a considerable number of peptides, which can be secreted or intermediary (the latter not impeditive of cellular function or impacting cellular physiology).

1.4.1 sAPP

Following full length APP cleavage by α-secretase, the large ectodomain sAPPα, containing the N-terminus, is released to the extracellular medium, leaving the TMD and the shorter, cytoplasmatic C-terminal (CTFα/C83) integrated within the cellular membrane. Many studies attribute a trophic role to sAPPα, and lower levels of this secreted fragment were found in the CSF of AD patients [95]. The lower levels of sAPPα have been correlated to impaired spatial memory performance, both in AD patients and animal models [96], [97]. *In vivo* and *in vitro* evidence suggests that sAPPα positively contributes to memory normalization and, especially, neuronal survival in non-pathologic models [98]–[101]. This proteolytic fragment is also notable for its effects in attenuating the normal rate of neuronal cell death induced by glutamate and Aβ [102]. Domains within the sAPPα sequence promote neurite outgrowth, some of which heparin-binding sites. Functionally, sAPPα has been reported to be an enhancement factor for neurite outgrowth processes [103].

Consistent with these findings, intraventricular brain delivery of sAPPα after traumatic neuronal injury significantly reduces cellular death and axonal injury, and improves the outcome of motor skills in *in vivo* models, with the same results following ischemic damage [104]–[111]. Finally, the phenotypical effects of APP-deficient rat models were visibly contracted with sAPPα expression in APP-null and sAPPα-knock experiments [112], from which one can infer that the phenotypical characteristics of APP-deficient rodents are due to the absence of sAPPα.

Although mostly linked to positive outcomes, overexpressed sAPPα also corelates with tumorigenesis as some cancer cell lines secrete significant amounts of this proteolytic fragment [102], [113]. The mechanism by which sAPPα induces

tumorigenesis is postulated to be via various MAP kinases or PKC signalling pathways [114], reiterating that although distinct pathological processes are involved, altered phosphorylation pathways are of great importance in AD-related events and can be influenced by some released APP fragments.

The differences between sAPPα and sAPPβ amino acids stretch appears to be the key to their different effects on cell physiology. The amino acids present in sAPPα, but not in sAPPβ, have been associated to neurotrophic properties [115], [116]. Nevertheless, sAPPβ has been somewhat included in the neuroprotective effect of sAPPα, although with an enormous decrease in potency and neuroprotective impact registered in terms of excitotoxicity, when compared to sAPPα [115]. This smaller neuroprotective influence has been proposed to be correlate with the lack of a short, 5-16 amino acid sequence, present in sAPPα but part of Aβ in the case of sAPPβ.

Despite the beneficial effects of both sAPP fragments, an important shared characteristic is the ability to activate microglia via the MAP Kinase pathway [115], with increases NFkB, IL-1β, reactive oxygen species, and other inflammatory cytokines reported to increase activity upon sAPP treatment in cell lines [117].

1.4.2 CTF

The complementary fragment (CTF) resulting from the shedding of sAPP remains in the cell and is designated CTFα and CTFβ, or C83 and C99, respectively. CTFα/C83 lacks the protective 16 amino acid stretch embedded in the last part of sAPPα. Alternative β-cleavage includes these amino acids in first part of CTFβ /C99, resulting in diminished sAPPβ protective effects.

Lacking the protective amino acid sequence, the CTFα/C83 fragment is predicted to be toxic, should its cellular pool increase. This toxic effect results from the toxic properties of the intact cytoplasmatic C-terminal, contrary to the secreted sAPP fragments; CTFβ/C99 might be more beneficial to cellular physiology than C83, as it better suited for γ-cleavage [117]. Little is known about the properties of C83; however, its effects are less beneficial than its amyloid homologous, since C99 is readily cleaved by γ-secretase [118].

Along with Aβ, CTF fragments in *in vitro* studies were described to have early neurotoxic effects [119]. These findings were corroborated by *in vivo* experiments, reporting general cortical atrophy, loss of hippocampal granule cells, and impaired working memory [105], [120], [121]. Additional studies demonstrated behavioural changes [122], [123], suggesting the induction of early AD pathways, mediated by CTF fragments. Additionally, CTF fragments have been implicated in ion channel activity alterations, disrupting calcium and potassium channels when overexpressed, compromising homeostasis and signal conduction [124], [125]. The question whether the neurotoxicity exerted by CTF fragments is direct or a consequence of secondary cleavages remains unanswered.

1.4.3 Aβ and P3

Aβ generation depends on APP proteolytic cleavage by two proteases: β-secretase and ϒ-secretase, both considered prime therapeutic targets [63]. However, the major physiological route of APP processing is via the α-secretase pathway, cleaving the C-terminal side of residue 16 of the Aβ sequence, generating an 83-residue C-terminal fragment (C83) [126]. The subsequent cleavage by γ-secretase releases a short peptide (p3) which includes the C-terminal region of the Aβ peptide, concluding the non-pathologic cleavage of the CTFα/C83 fragment (Fig. 1) [56].

BACE1 facilitates APP transport and complex formation with γ-secretase, resulting in the stepwise cleavages of APP. Altered BACE1 levels and activity impact Aβ40 and Aβ42 degradation, involving a common Aβ34 intermediate [127]. As such, Aβ34 can be considered a good predictor of Aβ turnover in patients with mild cognitive impairment (MCI) in individuals with increased CSF Aβ levels.

Aβ was originally described as an irregular and toxic protein type, restricted to the brain of aged or demented humans, but eventual detection of soluble Aβ species in the conditioned medium of cultured cells refuted this idea and implied a physiological function for Aβ. Even though excessive Aβ production results in synaptic dysfunction and synapse loss, low levels of Aβ had been shown to increase hippocampal long-term potentiation and improve memory, representing a novel, positive and modulatory role on neurotransmission and memory [128].

At the picomolar level, Aβ can also salvage neuronal cell death induced by inhibiting Aβ generation with inhibitors of β or γ-secretases [129], possibly affecting neuronal excitability by regulating the expression of potassium ion channels [130]. Aβ aggregation in peripherical muscle cells has recently been proposed to trap excess free copper and reduce copper-mediated cytotoxic effects [131].

There is strong evidence demonstrating that Aβ overproduction results in a neurodegenerative cascade leading to synaptic dysfunction, the formation of extracellular senile plaques and ultimately neuronal loss in affected areas of the brain [132]. There are two key toxic species of Aβ: Aβ40 and Aβ42, with the last being more hydrophobic and, as such, more prone to aggregate formation. However, Aβ42, individually, only accounts for around 10% of total Aβ peptide [133]. Previous work on FAD validated that mutations in PSEN proteins consistently increased the ratio of Aβ42/40 [134]–[136], suggesting that ratio elevation is an aggravated risk for AD pathogenesis, probably by providing the seeding for Aβ assembly into oligomers, fibrils and, ultimately, amyloidogenic plaques [135].

Even though the majority of Aβ is secreted extracellularly, it can also be generated in subcellular compartments, such as the ER, Golgi and endosome/lysosome complex, and extracellular Aβ can be internalized by the cell for degradation pathways. The existence of intracellular Aβ implies that it may accumulate within neurons and contribute to disease pathogenesis, especially neuronal death. A recent study suggests that internalized Aβ can aggregate within the cell and disturb the vesicular membrane, thus increasing its pathological effect and neurotoxicity [137], [138].

Intraneuronal Aβ immunoreactivity has indeed been described in the hippocampal and entorhinal cortical regions of MCI patients, areas prone to early AD pathology [139]. In Down Syndrome patients, who have a third copy of the APP gene due to the extra chromosome 21, the build-up of intracellular Aβ precedes extracellular plaque formation [140]. Additionally, the level of intraneuronal Aβ declines with extracellular Aβ plaque accumulation [141]. These results have been consistently confirmed by transgenic mouse models, revealing intracellular Aβ accumulation as an early event in the neuropathological phenotype, with

intraneuronal Aβ levels decreasing as extracellular plaques accumulate [142]. Inhibition of dynamin-mediated Aβ internalization was also described to reduce Aβ-induced neurotoxicity, but the same was not verified for clathrin-mediated Aβ internalization [119].

p3 is a somewhat homologous peptide to Aβ, although with very different physiological effects. This peptide is a subsequent product of α-cleavage, cleaved inside the Aβ sequence, and by γ-secretase. p3 has been reported in plaques and pre-amyloid deposits [143], [144], and being a small peptide (3kDa), it is theorized to exert some extent of neurotoxicity [145]. p3 can also induce apoptosis in cell models, although not as dramatically as Aβ42 [146]. Similar to Aβ, the p3 fragment is described to promote inflammatory responses in various cell models, through the production of proinflammatory cytokines [147].

1.4.4 AICD

Both α and γ cleavage secrete fragments, though with considerable differences. Common to both cases is the intracellular domain of APP, denominated AICD (APP Intracellular Cytoplasmic/C-terminal Domain). AICD release has been shown to have transactivation activity and regulate transcription of multiple genes, including GSK3-β, BACE1, p53, EGFR, KAII, LRP1 and APP itself [148]–[153], and was described to induce apoptosis and sensitize neuronal cells towards toxic stimuli [154], [155]. Besides these effects, the main function of this fragment appears to be to facilitate the interactions with various cytosolic interactors, when in the full-length APP. APP-containing complexes regulate protein intracellular trafficking and signal transduction related events. The regulation of APP interactions with cytosolic factors appears to be, in turn, regulated by protein phosphorylation [156].

In its cleaved form and once secreted, AICD can be subjected to direct caspase cleavage (predominantly caspase 3), releasing a fragment containing the last 31 amino acids of the APP sequence, (C31). In addition, as seen in figure 1, another fragment, called Jcasp, is generated [157], [158]. These 2 fragments are neurotoxic and can initiate a detrimental cascade [159], but Jcasp is thought to play a smaller role in cellular toxicity [158].

1.5 Retinoic Acid Receptors

RA exerts a profound effect on homeostatic properties and signalling pathways, ranging from physiological functions, such as tissue development, to pathological alterations. As established above, understanding RAR stimulation is of paramount importance for unravelling RAR stimulation in AD, changing APP cleaving secretases in a way that may be beneficial and a strong candidate for therapeutic translational research.

Most studies on RA-induced changes in secretases are carried out using *at*RA. However, the influence of isoform-specific RAR simulation is well defined in neuronal impact. Nevertheless, the overall significance in the context of AD is, to some extent, elusive. Furthermore, RAR activation is a possible therapeutic candidate for testing in AD models as it reduces neuroinflammation and contributes to neuroregeneration, adding paths in which it can promote neuronal health and rehabilitation [160].

RA can act via two families of nuclear receptors. RAR, the RA receptor, has three isoforms (α, β and γ) and can be activated by both *at*RA and 9-*cis*-RA enantiomer [161]. RXR, the retinoic X receptor, also has three isoforms and is activated by 9-*cis*-RA [162]. Both families can form heterodimers as RAR/RXR.

1.5.1 RAR

Each receptor isoform produces several splice variants, as is the case with other type II nuclear receptors. Agonists, once bound to RAR, result in the detachment of the corepressor proteins, leading to receptor activation. Recruitment of coactivator proteins, in turn, promoting downstream gene transcription, is also a key factor in the RAR mechanism. The expression of RAR genes themselves, is shown to be under epigenetic regulation through promoter methylation events [163].

RAR-α is also in the nuclear receptor subfamily 1, group B, member 1 (NR1B1) [164]. RARα activation plays a central role in cell growth, differentiation, and organ formation during embryonic development. RARα signalling has been linked to various pathways, especially in early embryonic development. It plays a

central role in regulating neural differentiation, via the expression of Neurog2 (pro-neural induction factor Neurogenin 2) [165].

RARβ or NR1B2 (nuclear receptor subfamily 1, group B, member 2), is also a cytoplasmic nuclear receptor, directed to subnuclear compartments once activated [166]. This receptor mediates cellular signalling in cell growth, differentiation, and embryonic events. Unexpectedly, it is theorized to limit the growth of many cell types by regulating gene expression.

RARγ or NR1B3 (nuclear receptor subfamily 1, group B, member 3) is rather less well understood. It produces effects over physiological processes with functions in embryogenesis and cell differentiation, resembling the other isoforms in RAR family.

1.5.2 RXR

Retinoid Receptors (RXR) are also nuclear receptors (NR2B3) is a natural participant in the negative feedback system related to RA, mediating antiproliferative effects. Unlike the RAR family, RXR is only activated by 9-*cis*-RA, consequently increasing DNA binding and transcriptional function, with direct effect on their respective response elements [167]. This family of receptors is important to the signal transduction resulting from RAR stimulus, as it forms heterodimers RXR/RAR that act as transcription factors.

1.6 Project framework

The ability of RAR stimulation to alter APP cleaving secretases, inducing anti-amyloidogenic effects, is of great importance, but the impact on AD-related risk genes has not been addressed. These are considerations which may hold the key to understanding the value of RAR signalling as a therapy. Of note, RA improves neuronal health and reduces inflammation resulting from pathological events, such as neurotoxic protein aggregates or acute traumatic events. The hypothesis and objectives in this thesis are governed by these considerations.

The hypothesis postulated for the present work is that **RAR stimulation induces changes in key signalling proteins beyond the APP-cleaving secretases towards a protective effect in the context of AD**.

The specific objectives outlined for the current work are as follows:

- Verify the impact of RAR stimulus in pivotal proteins without a direct role in APP-cleavage.
- Distinguish between RAR isoform specific alterations.
- Identify pivotal proteins that affect APP-cleaving interactions.
- Take an OMICS approach to integrate potential signalling pathways related to APP secretases.
- Identify the potential benefit of modulating signalling pathways through RAR modulation.
- Identify potential modulation targets in a disease associated state.
- Integrate RAR induced alterations in the targets identified in the disease associated state.

To carry out the work plan and meet the above-mentioned objectives, four strategies were used:

- Measure the effect of RA on preselected AD relevant targets (ADAM 17 being one of them) using a neuronal cell line SH-SY5Y.
- Analyse the ADAM17 interactome, which was previously obtained by yeast-two-hybrid (YTH) methodology.
- Apply a bioinformatics approach to elaborate the AD secretaseome.
- Compare with the insoluble fraction from fibroblasts of control and AD patients under basal conditions and in the presence of RAR stimulation (given time limitations only a pilot was carried out to test the conditions to be applied).

Chapter II – RA induced alterations in selected AD relevant targets

2.1 Material and Methods

2.1.1 Cell Line Culture, Differentiation and Treatment

Cellular culture and manipulation were performed using a class II air flow cabinet, within a Biosafety Level II facility. The human neuroblastoma cell line SH-SY5Y was cultured in 60mm plates with MEM:F12 (1:1) with 10% foetal bovine serum (FBS). Cells were kept in an incubator, at a temperature of 37ºC, 5% CO and 95% humidity. Cells were passaged when approximately 70-80% of confluence. Stock passages were preserved at -80ºC in culture medium supplemented with DMSO (10%). Cell thawing proceeded in a quick manner, minimizing exposure to DMSO. Once defrosted, cells were slowly resuspended in culture medium, preventing osmotic shock, centrifuged, resuspended in fresh culture medium and placed in the incubator.

Cells were differentiated before experimentation (adapted from [168]). Differentiation medium of MEM:F12 (1:1) 1% FBS supplemented with 10 µM of RA was changed every 48 hours for a total of 3 changes. Following differentiation, cells were treated, with 10^{-7} M of **RA (***at***RA)**, 10^{-7} M **AM580**, 10^{-7} M **Ch55** and 10^{-7} M **BMS493** for 48 hours, in addition to **control** (untreated).

RA (*at*RA) ((2E,4E,6E,8E)-3,7-dimethyl-9-(2,6,6-trimethylcyclohexen-1-yl) nona-2,4,6,8-tetraenoic acid) was used with to promote pan-RAR stimulation. AM580 (4-(5,6,7,8-Tetrahydro-5,5,8,8-tetramethyl-2-naphthalenyl) carboxamidobenzoic acid) was used to activate RARα. Ch55 (3,5-Di-tert-butylchalcone) was used to activate RARα and RARβ [169]. The RAR pan-antagonist BMS493 ((E)-4-(2-(5,5-dimethyl-8-(phenylethynyl)-5,6-dihydronaphthalen-2-yl) vinyl) benzoic acid) was also included in the conditions tested.

For microscopy imaging, cells were cultured in the manner described above, and exposed to the different treatments upon reaching 50% confluence. For protein

level quantification by western blotting, as described below, cells were differentiated and treated when approximately at 90% confluence.

2.1.2 Microscopy Imaging of Neurite Length

Imaging took place in 12mm cell culture dishes. After treatment, cells were washed in PBS and imaged with an Olympus IX81 Phase Contrast Microscope, 10 individual images were collected from two cell dishes for each individual treatment. Measurements were carried out using the public domain image processing software ImageJ. Data was processed in the GraphPad Prism version 4.0 software in the form of fold-increase.

2.1.3 Protein Extraction and Gel Electrophoresis

Cell lysate was obtained using RIPA protocol. RIPA buffer supplemented with a protease inhibitor cocktail was used. Once RIPA buffer was added to the cell dish cells were lysed with the help of a scraper, for 3 minutes and, posteriorly, transferred to a microtube. Subsequently, to obtain homogenous samples, cell lysates were vortexed for 15 seconds every 2 minutes for 10 minutes, while kept on ice. Samples were stored at -30ºC.

Protein content was quantified with the BCA (bicinchoninic acid) assay. This method is based on protein reduction of Cu^{2+} to Cu^{+}. The reaction takes place in an alkaline environment (the biuret reaction), producing a purple colour by chelating with two molecules of BCA for one cuprous ion, which permits the measurement of absorbance at 562 nm.

Samples and standards were incubated with working reagent (200 µL mixture of reagent A with reagent B 50:1) and incubated for 30 minutes at 37ºC. Absorbance at 562 nm was measured. Samples were normalized and quantified.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate sample proteins by molecular weight. 12% acrylamide resolving gel was used, with a 5% acrylamide loading gel. SDS-PAGE was caried out in a Mini Gel Tank (Cat. No. A25977 Pub. No. MAN0010862, TermoFisher), following

the recommended protocol. The system was initially ran for 15 minutes at 80 V for sample gel entry, followed by 125 V for approximately 1.5 hours.

2.1.4 Western Blotting and Statistical Analysis

Proteins were then transferred to a solid nitrocellulose membrane through electrical induction. Transfer was caried out in the Mini Blot Module System (Cat. No. B1000, TermoFisher) according to the respective protocol. First transfers were performed at 30V for 2 hours, but subsequent transfers were caried out overnight at 20V, to transfer heavier proteins 75 kDa. After transfer, membranes were blocked with 5% milk TBS-T solution, overnight, at 4ºC.

Membranes were incubated with Ponceau S solution for 5 min, washed with deionized water and scanned in the GS-800 calibrated imaging densitometer. Staining was then removed by washing 1x TBS.

For protein detection, membranes were incubated with primary antibodies, described in table 1, overnight at 4ºC. Next, membranes were washed with 1x TBS-T 3 times for 5 min and incubated with the secondary antibody (Table 1), for 2 hours at room temperature. Membranes were then washed as before and scanned with a Li-Cor Odyssey Scanner.

Immunoblot quantification was performed using ImageJ, and results were analysed using GraphPad Prism version 4.0 software in the form of fold-increase.

Table 1 – Antibodies used for western blotting experiments.

Primary Antibodies
Anti-APP (Mouse) 22C11, TermoFisher Scientific (1:1000)
Anti-TAU (Rabbit) #PA5-27287, Invitrogen (1:500)
Anti-APOE E4 (Mouse) MA5-16146, Invitrogen (1:2000)
Anti-GRB2 (Mouse) 610111, Biosciences (1:5000)
Anti-GSK3 (Rabbit) AB9258, Merk Millipore, (1:500)
Anti-p62/SQSTM1 (Rabbit) P0067, Merk (1:1000)
Anti-HSP60 (Mouse) 4B9/89, Invitrogen (1:1000)
Anti-TACE/ADAM17 (B-6) (Mouse) sc-390859, Santa Cruz Biotechnology, (1:1000)
Secondary Antibodies
Anti-Mouse Secondary (Goat) 926-32280 IRDye 800CW, LI-COR (1:15000)
Anti-Rabbit Secondary (Goat) 926-68071 IRDye 680RD, LI-COR (1:15000)

The statistical analysis software used was GraphPad Prism version 4.0. Data was edited and expressed as the numerical fold-increase results of three independent experiments. Statistical significance analysis was determined by t-Test for independent groups, with the presumed requirements validated automatically. $p < 0.05$ was considered statistically significant.

2.2 Results

2.2.1 Neurite growth

One described way to measure the anti-inflammatory and, more importantly, the neuroregeneration effects of RAR stimulation is by assessing the neurite growth [170]. Phase-contrast microscopy is an important for biomedical research, as it reveals many cellular structures that are otherwise invisible or hard to identify by bright-field microscopy. Thus, this was the approach used to measure neurite outgrowth in Sh-Sy5Y cells upon exposure to the different treatments.

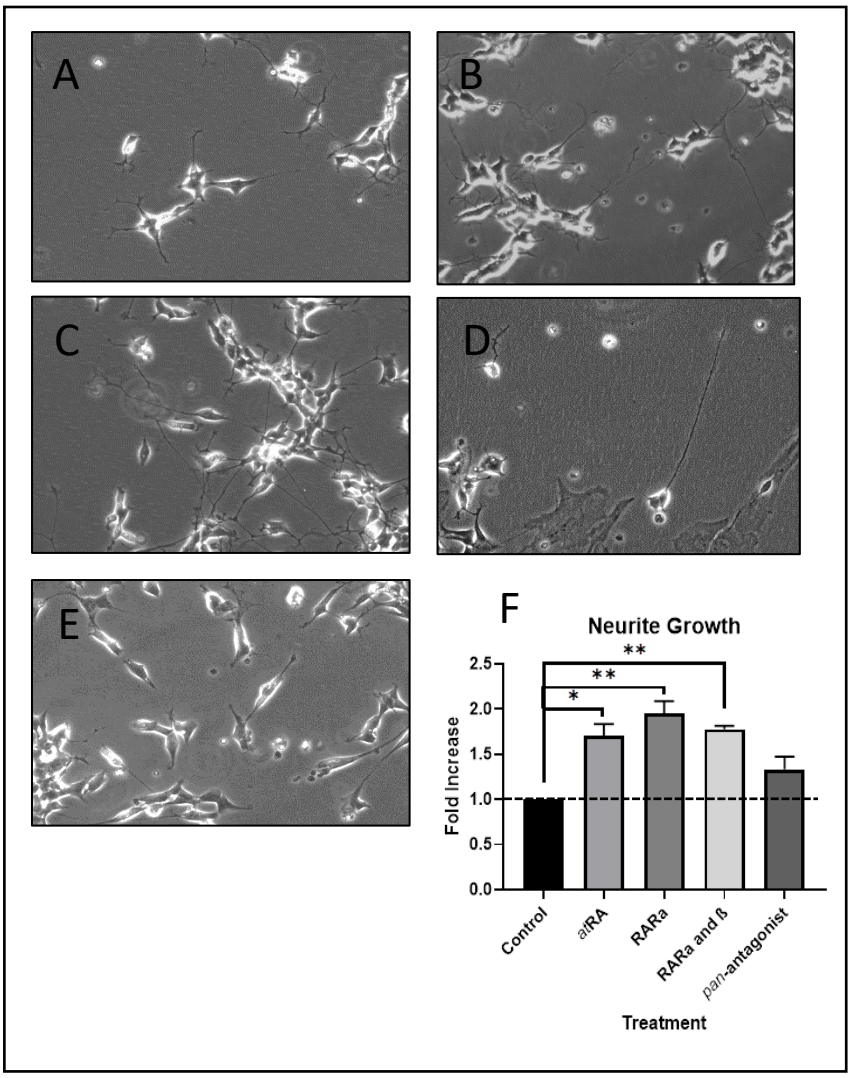


Figure 2 – Fold-increase of neurite growth as a result of RAR stimulation. *A - Control. B - atRA stimulation. C - RARα stimulation. D - RARα and β stimulation. E – treatment with RAR*

pan-antagonist. F – Data graph representation and statistical analysis of the results, presented in fold-increase of the neurite length, compared to the control.

Results of the neurite outgrowth following treatment with retinoids (Figure 2) show a significant increase following treatment with *at*RA, RAR-α agonist (AM580) and RAR-α/β agonist (CH55) (Figure 2F). Treatment with the *pan*-antagonist (BMS493) showed no result in terms of neurites growth (Figure 2F). In fact, specific stimulation of RAR-α resulted in the biggest increase in neurite length.

To validate that these observations indeed result from RAR activation, agonists were co-incubated with pan-antagonist (Figure 3). As expected, a statistical significant increase in neurite length was prevented in every agonist group, in the presence of the antagonist.

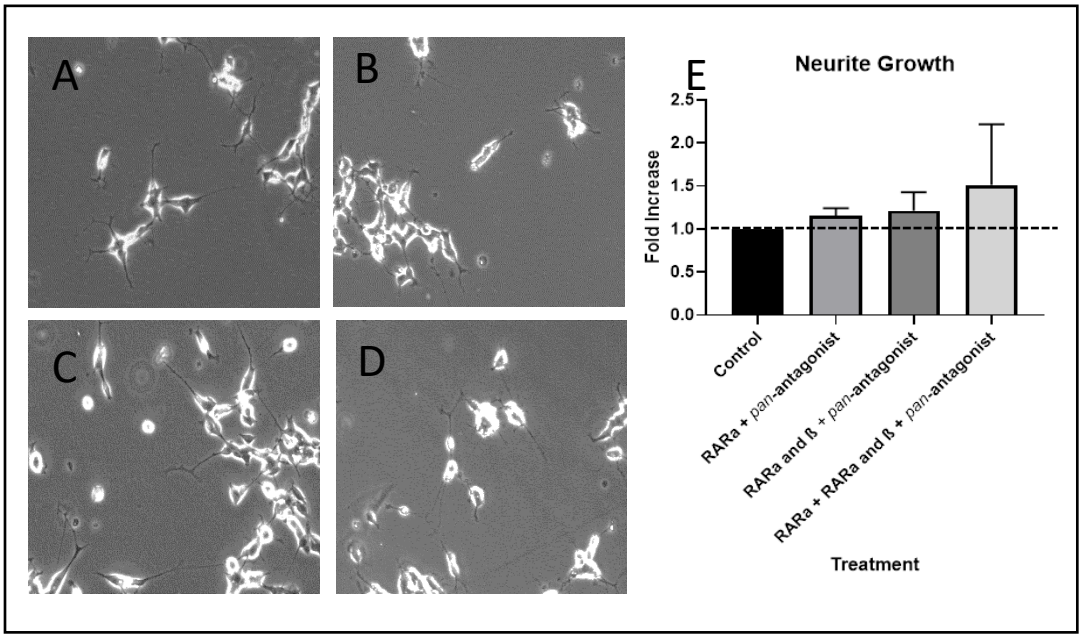


Figure 3 – Neurite growth fold-increase measurements from the combined stimulation. *A – Control. B - Co-treatment with RARα and RAR pan-antagonist. C - Co-treatment with RARα and β and RAR pan-antagonist. D – Co-treatment with RARα, RARα and β, and RAR pan-antagonist. E - Data graph representation and statistical analysis results.*

2.2.2 Western Blotting Analyses

Western blot analysis was carried for, proteins typically associated with AD, namely responsible for the formation of senile plaques and neurofibrillary tangles (APP and Tau). Proteins considered as risk-factors for AD, interfering with the aggregating abilities of Aβ or promoting β-cleavage, were also analysed (APOE E4, GRB2 and GSK3). Important for aggregation related diseases, ubiquitin protein p62 and chaperone HSP60 were studied. Finally, for the impact in α-cleavage, ADAM17 was also investigated.

2.2.2.1 APP

The physiological function of APP remains, to this day, somewhat elusive. Its most commonly accepted role is as a cell surface receptor related to synaptogenesis, neurite growth and neuronal adhesion [173]. As previously described, Aβ results from secretase processing of this protein, and extensive APP-focused research concerns its central role in AD.

This protein was investigated by western blot analysis (Figure 4). Protein levels showed only slight deviations upon treatment with any RAR agonist (Figure 4), however a significant, dramatic decrease was observed following treatment with RAR antagonist.

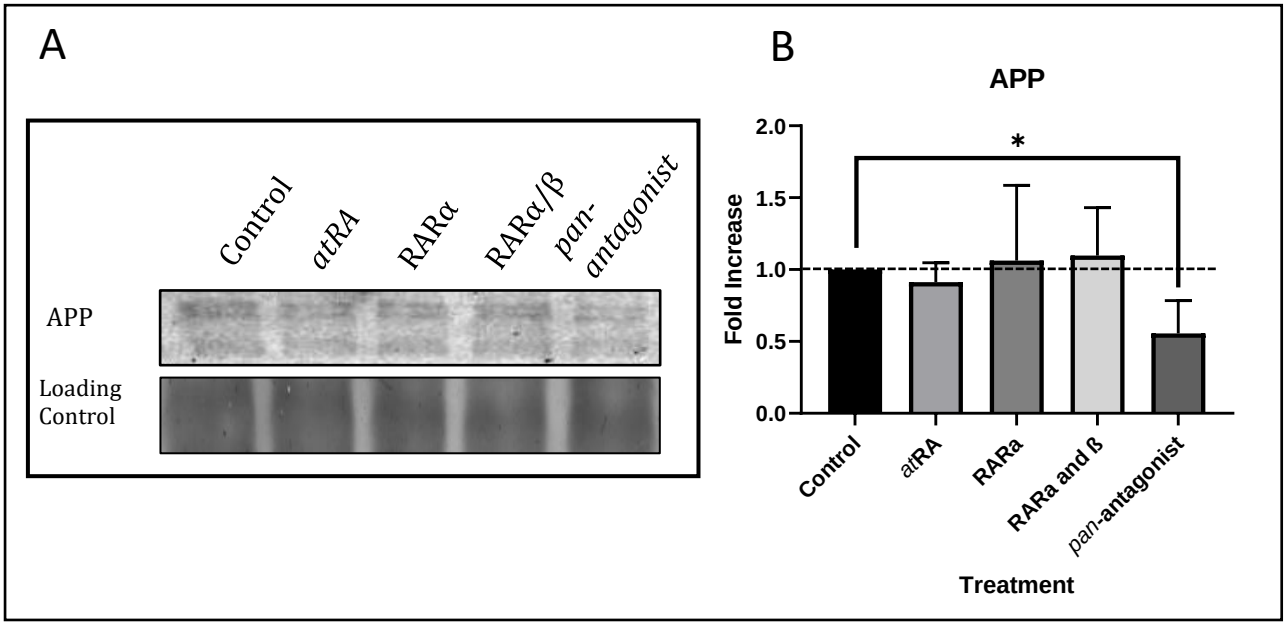


Figure 4 – Protein level alterations for APP*. A – Representative western blot protein band. B – Fold-increase data graph and statistical analysis results*.

2.2.2.2 TAU

Microtubule Associated Protein Tau is of great importance for AD, aging, and neurodegeneration. TAU assists assembly and stabilizes the microtubules, and is also involved in neuronal polarity [175]. It is associated with AD due to its presence in intracellular neurofibrillary tangles.

This protein was investigated by western blot analysis (Figure 5). Treatment with RAR-α/β agonist results in a significant increase in Tau protein levels (Figure 5), while other treatments did not produce any significant alterations.

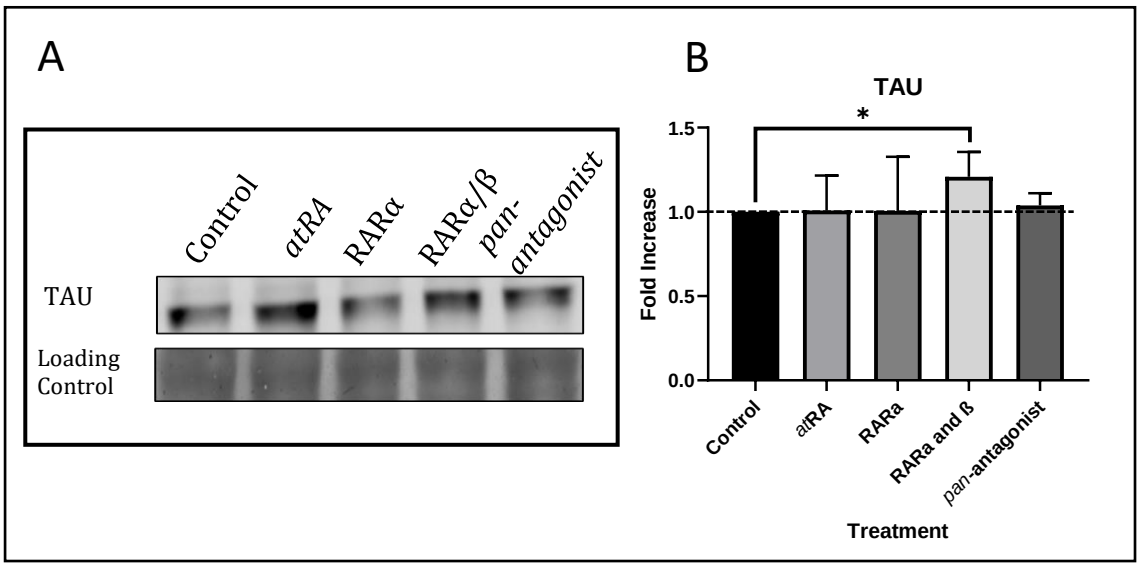


Figure 5 – Protein level alterations for TAU*. A – Representative western blot protein band. B – Fold-increase data graph and statistical analysis results*.

2.2.2.3 APOE E4

Apolipoprotein E isoform 4 (APOE E4) is a key protein in AD, widely described as a risk gene both by *in vitro* experiments and Genome Wide Association Studies (GWAS). This protein is associated with lipid particles transport, mainly functioning in lipid transport between organs via the plasma and interstitial fluids [171]. Increased APOE level is associated with cognitive decline and dementia-like symptoms, and it is thought to initiate and aggravate the toxic effects of Aβ aggregation [172].

This protein was investigated by western blot (Figure 6). APOE4 E4 protein levels were found to be elevated in SH-SY5Y cell lysates treated with RAR-α/β agonist (Figure 6B), but not statistically significant following treatment with atRA or RAR-α agonist. Although the latter did show more of an increase. The antagonist remained around control levels, although there was some variation.

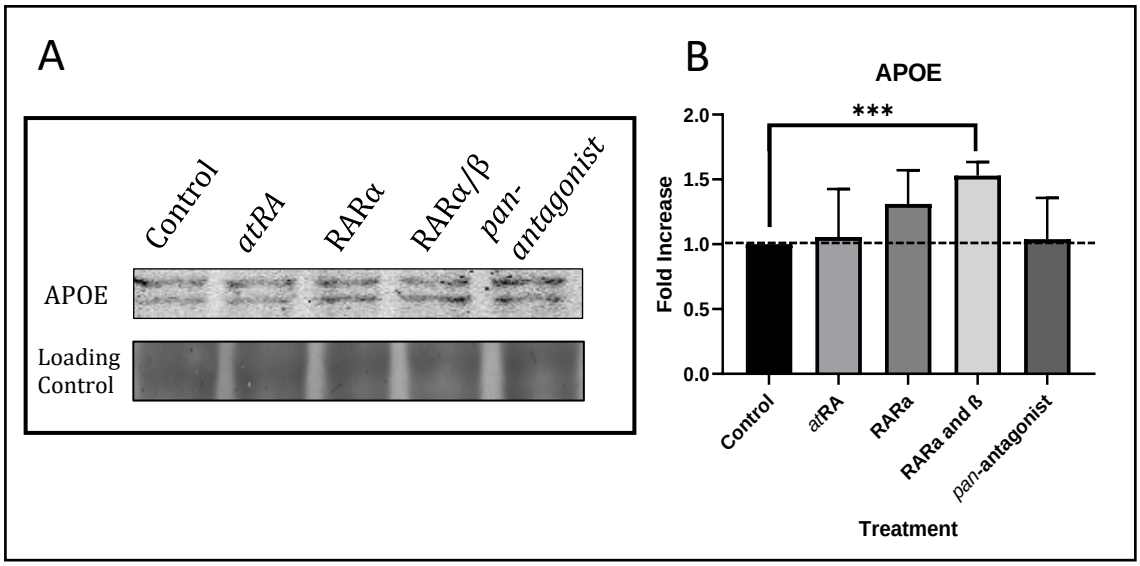


Figure 6 – Protein level alterations for APOE E4*. A – Representative western blot protein band. B – Fold-increase data graph and statistical analysis results*.

2.2.2.4 GRB2

Growth Factor Receptor Associated protein 2 (GRB2) initiates cellular phosphorylation events and is an important mediator of the Ras signalling pathway [174]. Like APOE, GWAS associate it with AD. Further understanding of potential roles for GRB2 is explored below (Figure 7).

This protein was investigated by western blot analysis (figure 7). GRB2 was shown to be significantly decreased following treatment with RAR-α agonist, this was not observed with atRA or RAR-α/β agonist. Although a tendency towards an increase was evident with *at*RA.

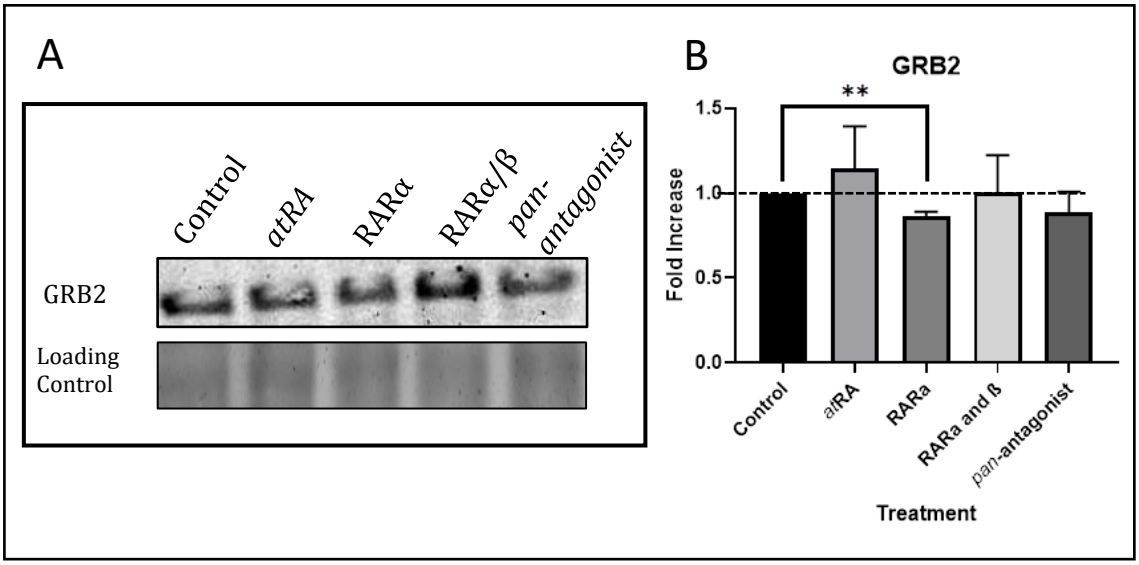


Figure 7 – Protein level alterations for GRB2*. A – Representative western blot protein band. B – Fold-increase data graph and statistical analysis results.*

2.2.2.5 GSK3

GSK3 (Glycogen synthase kinase 3) is a constitutively active kinase, first discovered acting as a negative factor in the hormonal control of glucose homeostasis. It has two isoforms, α (53 kDa) and β (46 kDa). Both isoforms have functions beyond hormonal control, influencing APP cleavage, either promoting the amyloidogenic or anti-amyloidogenic pathways.

This protein was investigated by western blot analysis (figure 8). GSK3α protein levels (53kDa) were found to be decreased in SH-SY5Y cell lysates treated with *at*RA (Figure 8B), but not significantly altered following treatment with RARα or RAR-α/β agonists.

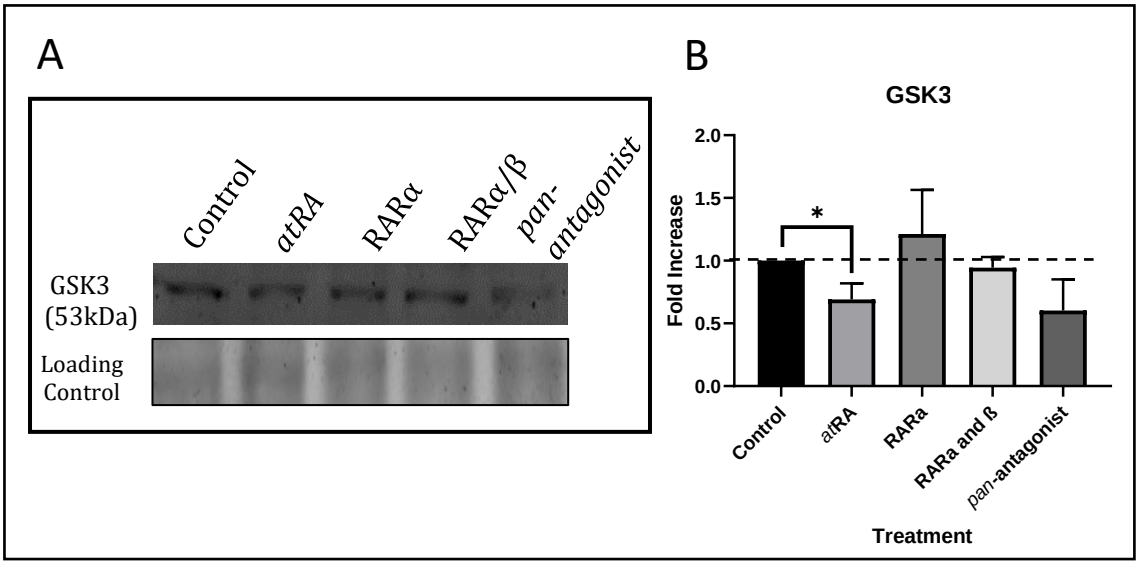


Figure 8 – Protein level alterations for GSK3*. A – Representative western blot protein band. B – Fold-increase data graph and statistical analysis results*.

2.2.2.6 p62

p62 is a ubiquitin bridge protein that acts as a receptor for selective cellular autophagy. It plays an important role in aggregate related pathologies, contributing to the constitutive degradation of misfolded and aggregated proteins [176].

This protein was investigated by western blot analysis (Figure 9). While no significant alterations were observed following treatment with RAR-α or RAR-α/β agonists, a significant decrease in protein levels of p62 was observed upon treatment of SH-SY5Y cells with *at*RA (Figure 9). Interestingly, a similar effect was observed following treatment with RAR *pan*-antagonist.

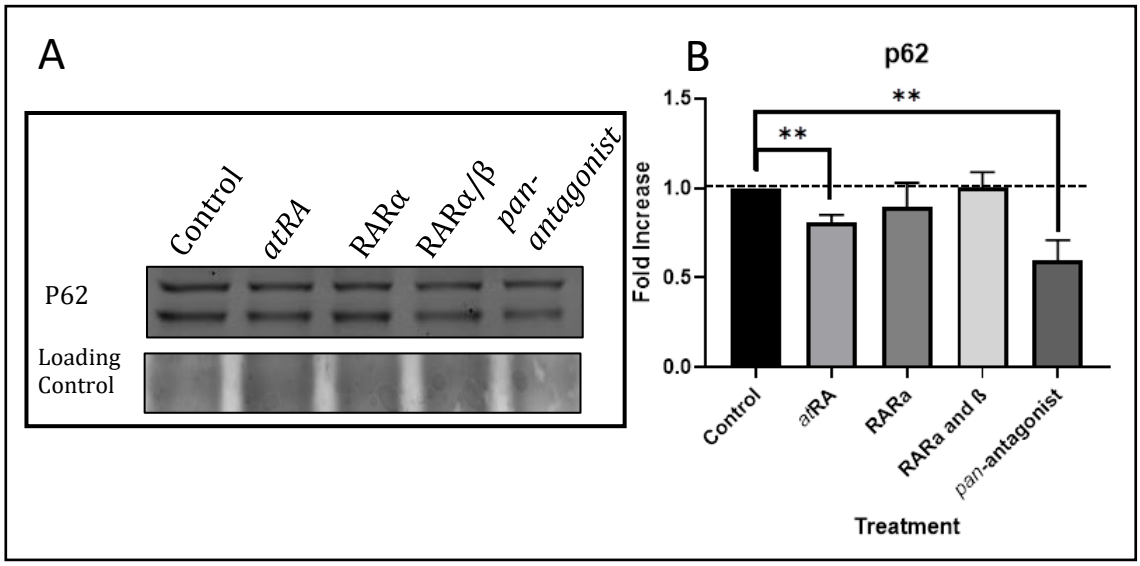


Figure 9 – Protein level alterations for p62*. A – Representative western blot protein band. B – Fold-increase data graph and statistical analysis results*.

Considering the observed effects of RAR modulation in ubiquitination and autophagy markers, a next logical step would be to investigate whether mitophagy was similarly altered.

2.2.2.7 HSP60

Heat Shock Protein 60 (HSP60) is a mitochondrial chaperone that identifies and stabilizes misfolded proteins with exposed hydrophobic residues, that can also be used as a mitochondrial marker [177].

This protein was investigated by western blot analysis (Figure 10). Treatment of SH-SY5Y cells with retinoids resulted in no statistically significant alterations to HSP60 levels (Figure 10).

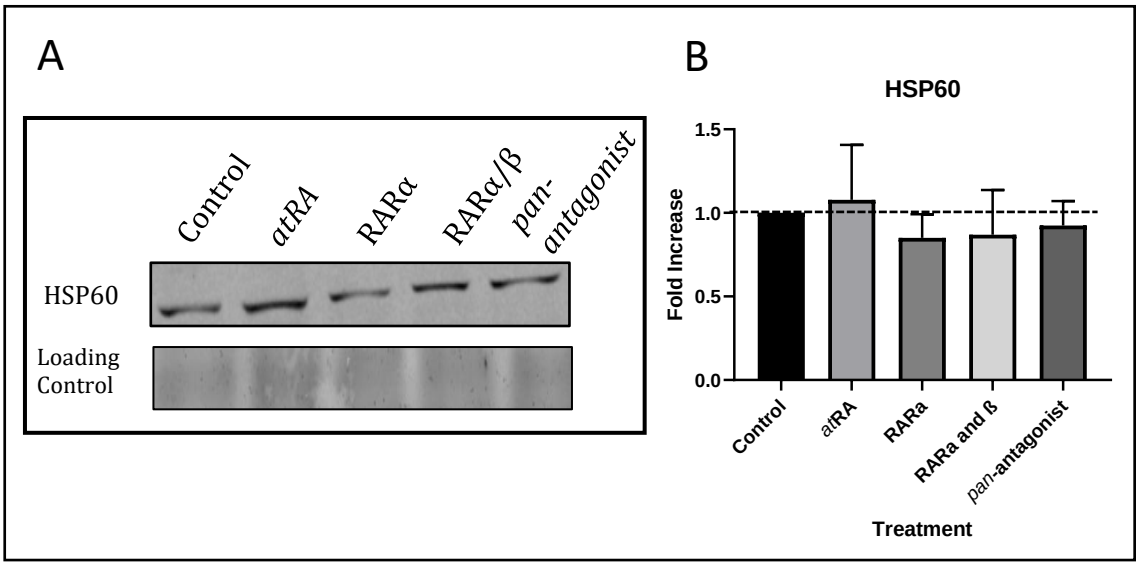


Figure 10 – Protein level alterations for HSP60*. A – Representative western blot protein band. B – Fold-increase data graph and statistical analysis results*.

2.2.2.8 ADAM17

ADAM17 is one of the main APP cleaving secretases. It remains a prime therapeutical target for favoring α-cleavage over the amyloidogenic pathway. In addition to cleaving APP, it is responsible for the cleavage of several type I transmembrane proteins, including TNFα and Notch. Like ADAM10, that has RAR responsive elements in the promoter region, ADAM17 was reported to increase in cell treated with *at*RA, as referred above.

This protein was investigated by western blot analysis (Figure 11). Treatment with RARα agonist results in a significant increase in ADAM17 protein levels, as seen in figure 11B, while treatment with *pan*-antagonist resulted in a significant decrease. Other treatments resulted in no significant alterations.

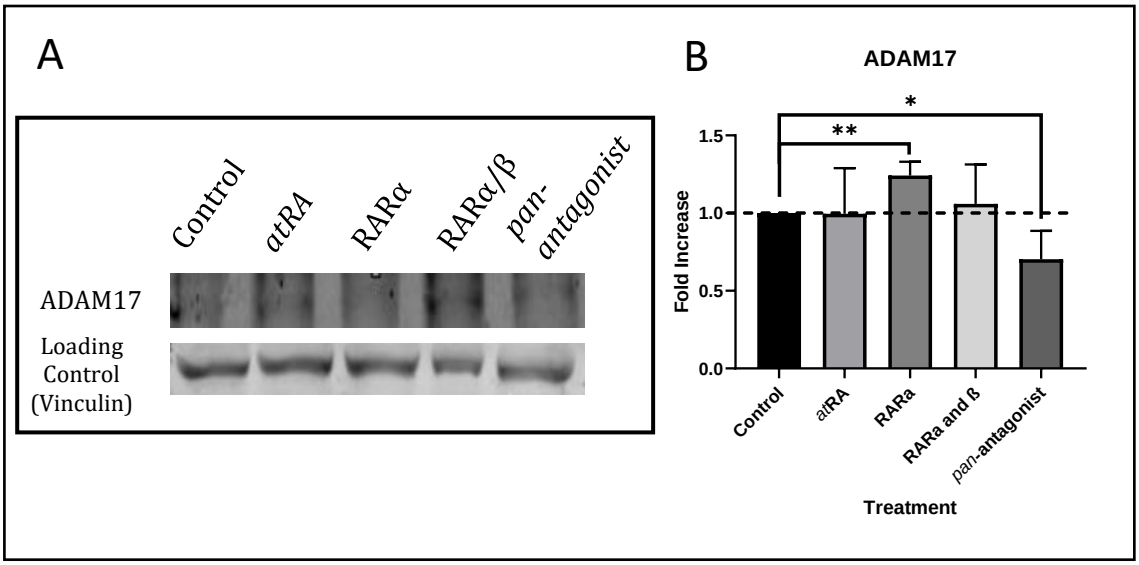


Figure 11 – Protein level alterations for ADAM17*. A – Representative western blot protein band. B – Fold-increase data graph and statistical analysis results*.

Given the results observed, where ADAM17 is clearly affected by RAR and an opposite effect is observed with the antagonist, this protein clearly merits further study. Additionally, in signal transduction mechanisms, in order to perform an array of functions, protein complexes are typically formed, an OMICS approach seems appropriate. Given that via an ongoing collaboration in the laboratory the interactome of ADMA17 was available resulting from a Yeast-Two-Hybrid experiment, this was subsequently analyzed.

Chapter III – Heading for a holistic and systems approach

Considering the array of α-, β-, and γ-secretase-related molecular functions and functional overlap between secretases and interactors, it is imperative to adopt a systems approach to better understand molecular functions and identify putative therapeutic targets focused on APP cleavage by secretases, for both amyloidogenic and non-amyloidogenic pathways. Thus, the ADAM17 interactome is here described and analysed using data from an unpublished yeast two hybrid (YTH) assay. The data was available due to an ongoing collaboration.

Subsequently an *in silico* approach, was used to reveal the APP secretaseome. Several interacting proteins, key for AD pathogenesis, are here identified as new potential therapeutical targets or likely biomarkers for interventions promoting the anti-amyloidogenic pathway. This work highlights pathway crosstalks that should be considered when designing therapeutic strategies for AD pathogenesis.

3.1 ADAM 17 Interactome

3.1.1 Methods

The YTH system is used to study protein-protein interactions. It consists in allocating, through transfection, a prey protein, i.e. ADAM17, attached to a repressor protein for the transcription of an operon, most commonly lacZ. Clones are then transfected with segments of a tissue DNA library, attached to an activation protein, inhibiting the repressor associated with the prey. Should the protein encoded in the segment from the DAN library and the prey protein interact, the operon repressor is released, and transcription activated. Positive clones resulting from segment-prey interaction are visualized in selective medium, considering the operon used. The fragments from positive clones are amplified and sequenced for protein identification. The data from the YTH for ADAM17 here presented were caried externally, resulting from a group collaboration. To complement this approach, the complete APP secretaseome was elaborated according to methodology referred in section 4.2.1.

3.1.2 Results

ADAM 17 interactome was considered on its own, as it is one of the main cleaving α-secretases. The YTH data was obtained using this ADAM17 as bait. The results revealed 14 ADAM17 interactors, with diverse cellular functions, were identified (Table 2).

Table 2. ADAM17 interactors identified in the Yeast Two-hybrid system. (Adapted from: da Cruz e Silva, O. *et al,* submitted.)

Protein	Gene	Uniprot
A-kinase anchor protein 9	AKAP9	Q99996
ATPase ASNA1	ASNA1	O43681
Uncharacterized protein C8orf46	C8orf46	Q8TAG6
Cadherin-2	CADH2	P19022
DnaJ homolog subfamily B member 6	DNAJB6	O75190
Neuron-specific calcium-binding protein hippocalcin	HPCA	P84074
Hippocalcin-like protein 1	HPCAL1	P37235
Leucine-rich repeat-containing protein 37A3	LRRC37A3	J3QTJ5
Neurogenic differentiation factor	NEUROD6	Q8IYR9
Serine/threonine-protein kinase PLK2	PLK2	Q9NYY3
cAMP-dependent protein kinase type I-alpha regulatory subunit	PRKAR1A	P10644
Small glutamine-rich tetratricopeptide repeat(TPR)-containing, alpha	SGTA	O43765
Zinc finger and BTB domain-containing protein 16	ZBTB16	Q05516
Cytochrome c oxidase subunit 1	MT-CO1	P00395

Of the 14 interactions identified by YTH, 3 are related to kinase function. Among them AKAP9 a novel ADAM 17 interactor here described for the first time to bind it (Table 2). A genome wide association study previously identified AKAP9 as a gene associated with AD [37]. With several isoforms, this protein is expressed in the cerebellum, hippocampus, and the cerebral cortex, and it localizes intracellularly to the centrosome and the Golgi apparatus. AKAP9 interacts with key signalling molecules such as GSK3α and is described, in literature, to coordinate cAMP responsive events, integrating and disseminating intracellular signals [38].

Using the procedures described below the ADAM17 interactome was complemented with interactions reported in public databases. The resulting interactome, consisting in the merged data from DB and the YTH, is represented in Figure 12A. For ADAM17 interactions, 20 further interacting proteins were retrieved from the IntAct database and added to the 14 experimentally obtained by

the YTH methodology. In addition, 10 interactors were added from the available literature [25], providing a total of 44 ADAM17 interacting protein.

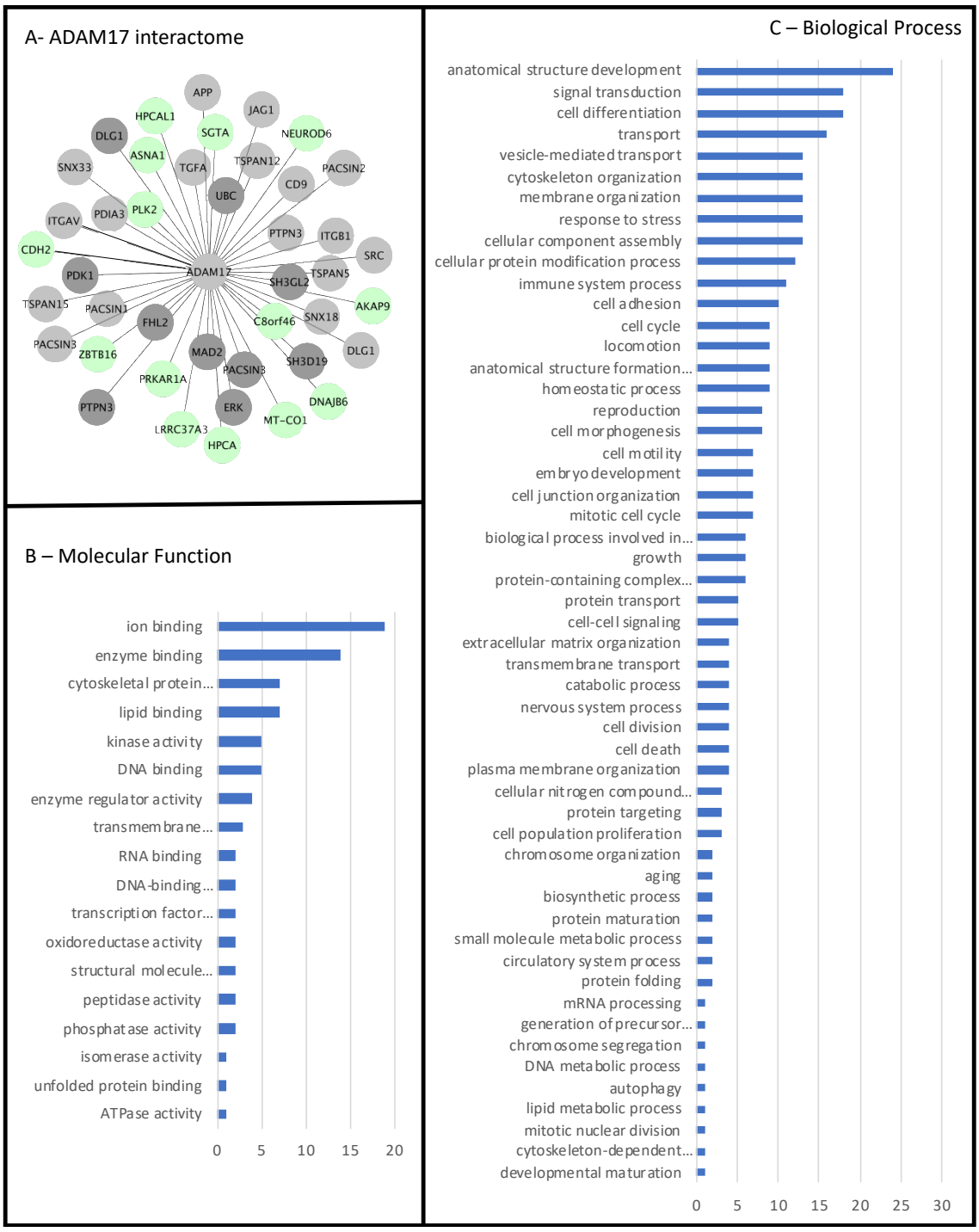


Figure 12. ADAM17 interactome and GO results. *A - ADAM17 interacting proteins, nodes filled in green signify the novel interactors identified in the YTH. Grey nodes were collected from the theoretical database search and darker nodes describe*

interactions extracted from the literature [37]. B- Gene ontology founded on molecular function for the ADAM17 interactome. C - Gene ontology for biological process of the ADAM17 interactome. (Taken from: da Cruz e Silva, O. *et al,* submitted – JJMV is co-author.)

Gene ontology (GO) analysis for the complete ADAM17 interactome (Figure 12B-C) identified the top three molecular functions as ion binding, enzyme binding, and cytoskeletal protein binding, and the top three biological processes as anatomical structure development, signal transduction, and cell differentiation. Overall, the interactome profile strongly associates ADAM17 to enzymatic activity and signal transduction, with kinase activity as the 5th Molecular function listed (Figure 12B). Biological processes from the complete interactome also result from GO (Figure 12C). Cellular transport (4th in GO for Biological process) and vesicle mediated transport (5th in GO for Biological process) are of particular importance. This is not particularly surprising, as ADAM17 is a central protein in the processing of APP. Other GO Molecular functions are represented (Figure 12B) and those associated with gene expression, namely DNA binding (listed 6th) and RNA binding (listed 9th), are of particular interest.

3.2 APP Secretaseome

For the elaboration of the APP secretaseome network, public databases and literature were consulted, searching for secretase-interactors.

3.2.1 Methods

For *in silico* studies, the main secretases involved in APP processing were ascertained in the KEGG Pathway DB for AD, map05010, accessed on the 4th of March 2020. ADAM10, BACE1, BACE2, PSEN1, PSEN2, and APP were selected [178]. The interactome for ADAM17 presented above was also included. IntAct DB was used for interactome search, accessed on the 4th of November 2020, using the UniProtID identifier for each protein. Other ADAMs, described in the literature as pertinent for APP processing, were also included: ADAM8, ADAM9, ADAM12, ADAM15, ADAM19 and the ADAM-regulator SH3D19 (EVE-1) [3], [37].

Since AKAP9 (A-kinase anchoring protein 9), appeared to be an important facilitator between ADAM17 and APP, as derived from the ADAM17 interactome, its interactome was also included (data collected on the 5th of November 2020). For simplicity, data from the database is represented in the gene name format, although interactions occur at the protein level.

The resulting interactomes were introduced in Cytoscape 3.8.2, an open source software platform for visualizing complex networks [179] and interactomes were merged, after removing chemical interactions. Nodes described by Edward et al, 2009 [37] were included (coloured dark grey, or light Gray if the node was previously present in the database interactome). Nodes from the YTH system were also added (light green) (Table 1). Nodes demonstrating genes in common with GWAS for AD (EFO_0000249, 25th of January 2021) are identified with a red circumference. The network was organized using the glayCluster app in Cytoscape 3.8.2, and the result was analysed in terms of gene ontology.

3.2.1.2 Methodology to predict the kinases that phosphorylate APP, ADAM17 and AKAP9

The most relevant secretases for APP cleavage seem to be ADAM17 and BACE1 [180], [181], and the function of ADAM17 appears to be regulated via AKAP9 [182]. Thus, an *in silico* search, targeted for Homo Sapiens species, was performed in the UniProt database for APP, ADAM17, BACE1, and AKAP9 amino acid sequences. Each sequence was submitted to the phosphorylation prediction NetPhos 3.1 server on the 25th of November 2020, for ADAM17 and AKAP9. APP´s amino acid sequence was submitted to the phosphorylation database Phospho-ELM [183]. Putative phosphorylated kinases were identified and integrated into the results.

3.2.2 Results

3.2.2.1 APP secretaseome network

To establish the full APP secretaseome, the interactomes for ADAM17, ADAM 10, ADAM8, ADAM9, ADAM12, ADAM15, ADAM19, SH3D19/EVE, BACE1, BACE2, PSEN1, PSEN2, and APP were merged, and results from the YTH system were also

incorporated (Table 1). SH3D19/EVE was added as it is described to be necessary for ADAM sheddases activity, acting as a regulator. The glayCluster app was used to merge the network and resulting clusters are presented in figure 13.

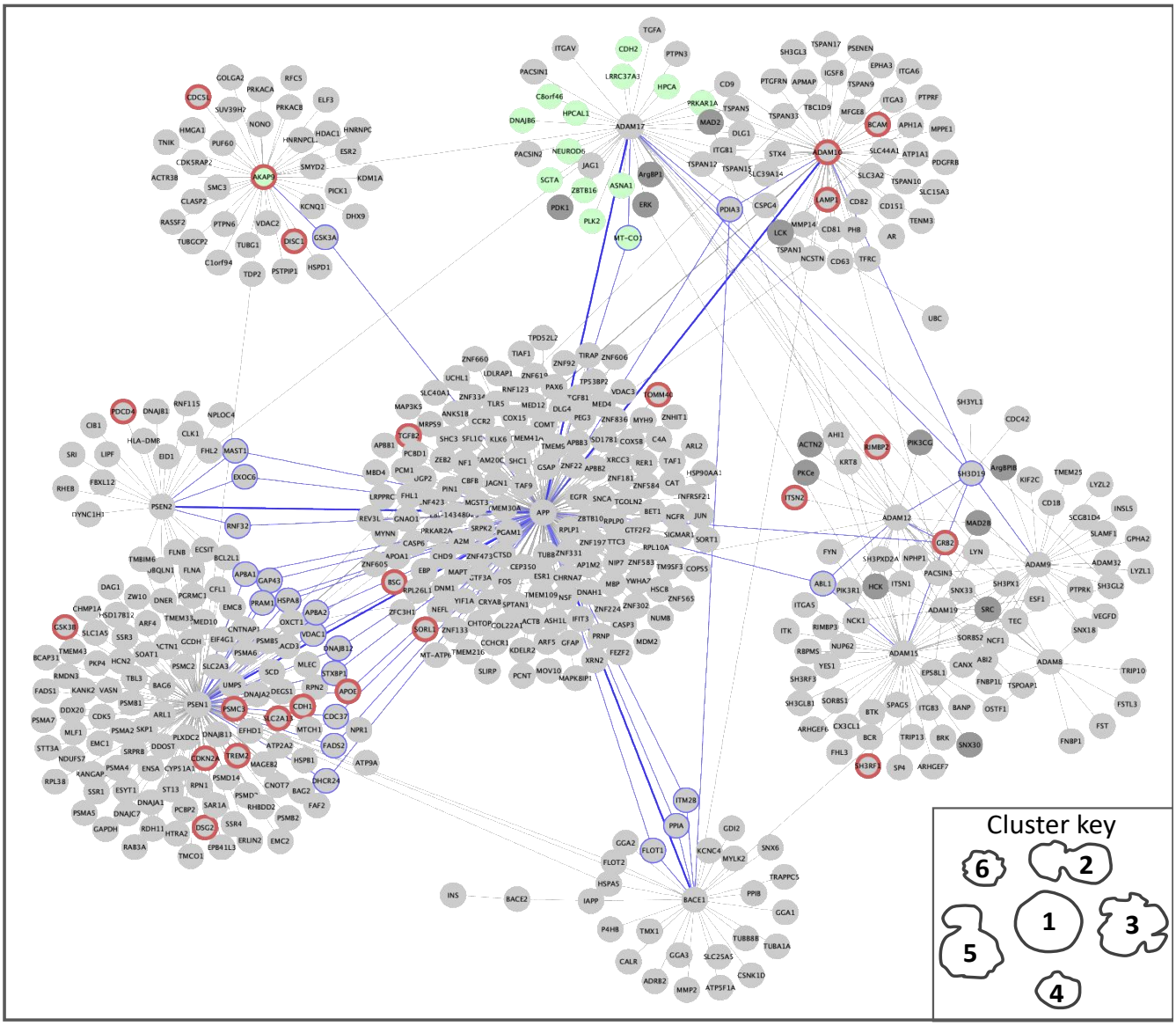


Figure 13. Result of the APP secretaseome network. *The interactomes of APP and its secretases were merged and AKAP9 as well as SH3D19 interactomes were included as described above. Theoretical human interactors form IntAct database, for APP and each selected secretase are represented in light grey. Supplementary interactions described in Edward et al, 2009* [37]*, were added and marked in dark grey. New interactors arising form YTH for ADAM17 were added and highlighted in light green. Nodes representing genes in common with GWAS for Alzheimer's Disease EFO_0000249 are emphasized in a red*

circumference. Clusters were arranged and numbered as shown in the cluster key. (Taken from: da Cruz e Silva, O. *et al,* submitted.)

Six clusters can be distinguished (Figure 13). A central cluster representing APP (1) and the typical APP cleaving α-secretases, ADAM 17 and ADAM10, cluster close together, constructing cluster 2. A total of twelve nodes are shared by both ADAM 17 and ADAM10, among them the SH3D19/EVE node, considered in this perspective as the most important (Figure 14). SH3D19/EVE seems to be fundamental for ADAM activation and function.

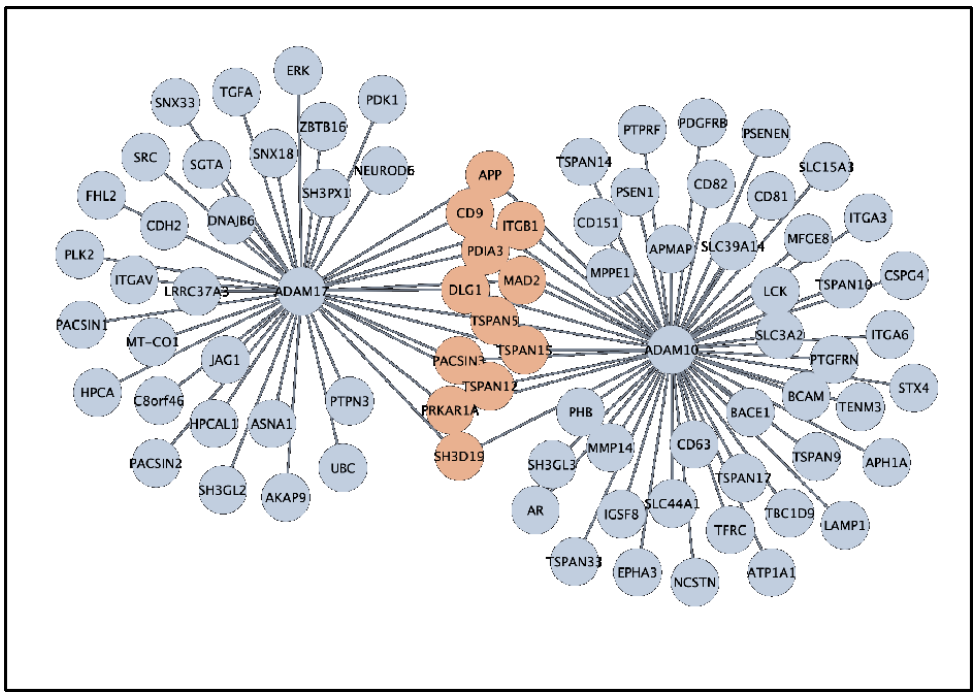


Figure 14. ADAM10 and 17 interactomes. *Shared proteins are signified as orange nodes.* (Adapted from: da Cruz e Silva, O. *et al,* submitted.)

The supporting ADAM interactomes (ADAM 8, ADAM 9, ADAM 12 ADAM 15, ADAM 19 and SH3D19) all compose cluster 3. Remarkably, SH3D19 clusters with the ADAMs, forming a functional bridge, being justifiable as the former is considered to play a critical role in regulating these metalloproteases. Cluster 4 represents the

β-secretase interactome and PSEN 1 and PSEN 2 combined to common cluster 5. A final cluster 6 shelters the AKAP9 interactome (Figure 12).

Genes from the APP secretaseome network found to be in common with a list of AD-risk genes, as described in the methods section were highlighted. Some risk genes were mapped to AKAP9, APP, and both the ADAMs clusters. Surprisingly, no risk genes were identified within the BACE1 direct interactome. Differently, a significant number of nodes, corresponding to AD risk genes mapped to the PSEN secretase, cluster 5, as, of the 23 risk genes in this network, 9 bind directly to presenilins (Figure 13).

3.2.2.2 Gene Ontology analyses of the APP secretaseome

Given the array of proteins covering the APP secretaseome, Gene Ontology (GO) analyses of this network was carried out (Figure 15). Taken together four Molecular functions/Biological processes appear to be central: namely signal transduction, enzymatic activity, cellular transport, and gene expression related events.

Signal transduction is the first output for biological process, supported by cellular protein modification (listed 6th). Moreover, the 8th Molecular function is Kinase activity (Figure 15A and 15B). The importance of protein phosphorylation in mediating intracellular processes is evident in literature and validated here with the impacts in signal transduction further explained below. Enzymatic activity is similarly well represented, especially with respect to Molecular function (Figure 15A). The considered most relevant Molecular functions identified in the GO analyses include, ion binding (listed 1st), enzyme binding (listed 2nd), enzyme regulator activity (listed 6th) and peptidase activity (listed 10th).

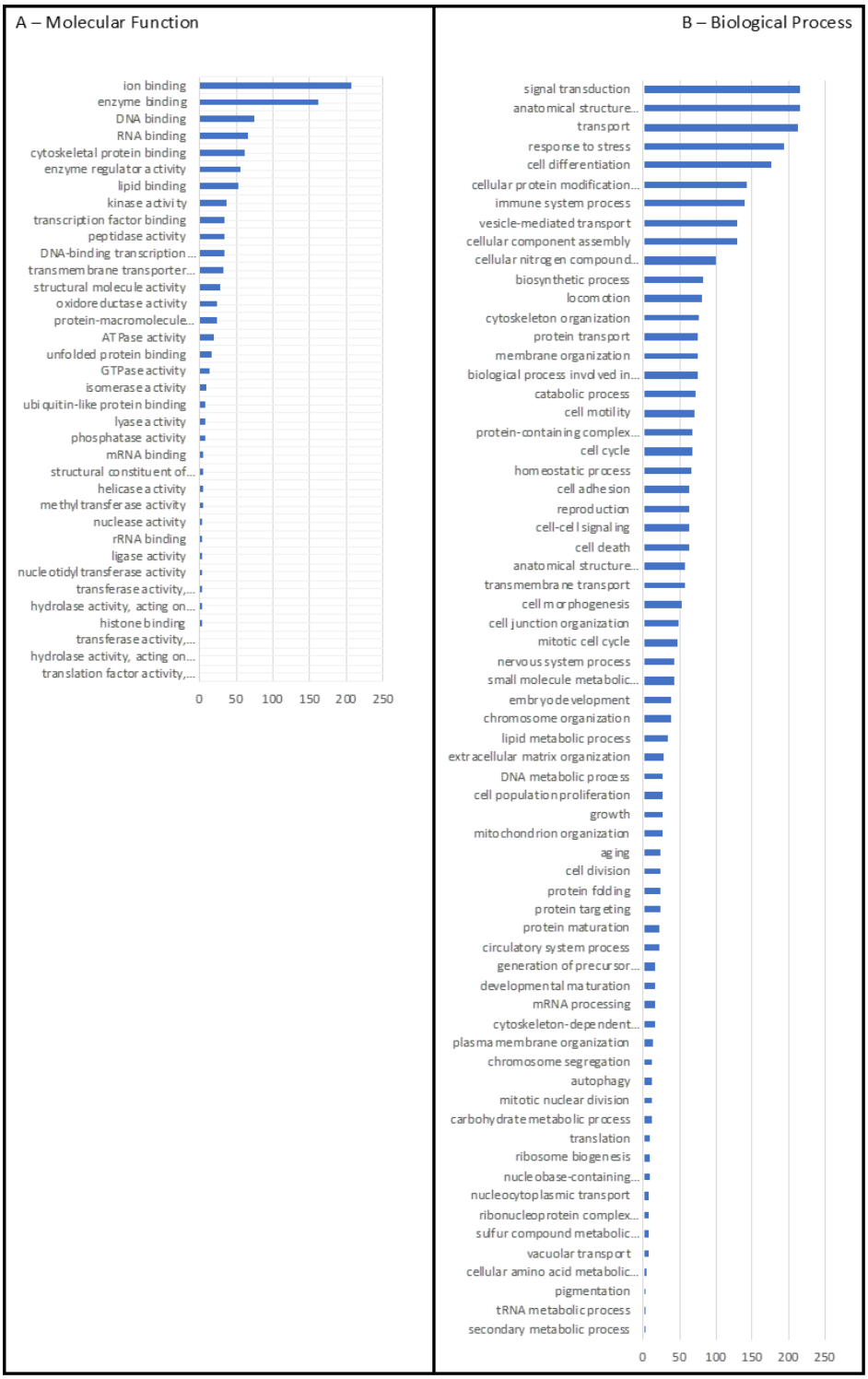


Figure 15 – Gene Ontology of the complete APP secretaseome*. A – represents the results for the function of Molecular function. B – represents the results for the function of biological process.* (Taken from: da Cruz e Silva, O. *et al*, submitted.)

Granted the central role of APP processing in its secretaseome network, it is expected to represent cellular transport in the GO analyses as one of the most common. In addition, biological process transport is listed third, followed by vesicle mediated transport (listed 8th), cellular component assembly (listed 9th) and protein transport (listed 14th), relevance of which involves intracellular transport, supported by some of the top Molecular function. Among them cytoskeletal protein binding (listed 5th) and transmembrane transporter activity (listed 12th).

Gene expression related actions are also well represented in the APP secretaseome, mainly with respect to the Molecular function-based GO, possibly with corelation with the feedback mechanisms exerted by proteolytic APP fragments. Coherently, DNA binding appears as the 3rd top Molecular function and is immediately followed by RNA binding. Other relevant Molecular functions described are transcription factor binding (listed 9th) and DNA-binding transcription factor activity (listed 11th). Many other Molecular functions and biological processes are represented in the APP secretaseome, however to a much lesser extent.

3.2.2.3 APP connected to secretases

APP can interact directly with secretases or via bridging proteins. The connection between APP and secretases is portrayed in figure 13 and identified with blue lines, thick for direct, and thin if an intermediate node/protein/gene is involved. APP interacts directly with ADAM17, ADAM10, BACE1, PSEN1, and PSEN2 (Figure 13, Table 3 below). APP interaction with supporting ADAMs 12 and 15 can occur via ABL1 and GRB2. SH3D19 does not bind to APP but, importantly, binds to several ADAMs, which could indirectly modulate APP processing. AKAP9 in cluster 6, warrants a specific mention, as not only can it bind APP directly, but it can also bind to ADAM17, bridging several of its interactors to ADAM17. This relationship is further explored below.

Table 3 - APP interacting bridge proteins. **Indicates genes present in GWAS EFO_0000249, thus AD risk factors*. (Taken from: da Cruz e Silva, O. *et all,* submitted.)

APP interacting protein	Secretase interactome	Binding detail	Molecular Function
ADAM17	ADAM17	Direct binding	Responsible for the proteolytic release of cell-surface proteins, among them p75 TNF-receptor, interleukin 1 receptor type II, p55 TNF-receptor, L-selectin, growth hormone receptor, JAM3 and APP [184]–[186].
MT-CO1	ADAM17	Bridge protein	Constituent of the cytochrome c oxidase, the last enzyme in the mitochondrial electron transport chain, driving oxidative phosphorylation [187].
GSK3A	ADAM17	Mediated via AKAP9*	A constitutively active protein kinase acting as a negative regulator in the hormonal control of glucose homeostasis [188], Wnt signalling, regulation of transcription factors and microtubules, by phosphorylating and inactivating glycogen synthase [189]. Requires primed phosphorylation of most of its substrates and facilitates APP processing and the generation of amyloid plaques found in AD [190].
ADAM10*	ADAM10	Direct binding	Proteolytic release of several cell-surface proteins like membrane-bound precursor of TNF-alpha, JAM3 [184], ephrin-A2, CD44, CDH2 and for constitutive and regulated alpha-secretase cleavage of APP [191].
PDIA3	ADAM17	Bridge protein	Catalyses the reorganization of -S-S- bonds in proteins with special importance for protein folding [192].
	ADAM10		
	BACE1		
ABL1	ADAM12	Bridge protein	Is a non-receptor tyrosine-protein kinase acting in many key processes such as cytoskeleton remodelling in response to extracellular stimuli, receptor endocytosis, autophagy, DNA damage response, apoptosis and other important pathways [193].
	ADAM15		
GRB2*	ADAM9	Mediated via SH3D19	Adapter protein providing an essential link between cell surface growth factor receptors and Ras signalling pathway [194]. It does not bind to phosphorylated epidermal growth factor receptor (EGFR), nonetheless inhibits EGF-induced transactivation of a RAS-responsive element, acting as a dominant negative protein over GRB2 and, suppressing proliferative signals, may trigger apoptosis [195].
	ADAM10	Mediated via SH3D19	
	ADAM12	Bridge protein	
	ADAM12	Mediated via SH3D19	
	ADAM15	Bridge protein	
	ADAM17	Mediated via SH3D20	
BACE1	BACE1	Direct binding	Responsible for the proteolytic APP cleavage at the N-terminus, between residues 671 and 672, leading to the generation and extracellular release of beta-cleaved soluble APP, and a corresponding cell-associated C-terminal fragment which is later released by gamma-secretase [196].
FLOT1	BACE1	Bridge Protein	A scaffolding protein in caveolar membranes. May participate in forming of caveolae or caveolae-like vesicles [197].
ITM2B	BACE1	Bridge protein	Plays a controlling role in the processing of APP and inhibits the amyloid-beta peptide aggregation and fibrils deposition [198]. It also involved in the induction of neurite outgrowth and functions as a protease inhibitor by blocking access of secretases to APP cleavage sites. Mature BRI2 functions as a modulator of APP processing and induces a strong reduction in the secretion of secretase-processed amyloid-beta protein 40 and amyloid-beta protein 42 [199].

APP interacting protein	Secretase interactome	Binding detail	Molecular Function
PPIA	BACE1	Bridge protein	Facilitates the cis-trans isomerization of proline imidic peptide bonds in oligopeptides [200]. In response to oxidative stress, it initiates proapoptotic and antiapoptotic signalling in ECs via activation of NF-kappa-B and AKT1 and the up-regulation of antiapoptotic protein BCL2.
PSEN1	PSEN1	Direct binding	Catalytic subunit of the gamma-secretase complex, an endoprotease complex that facilitates the intramembrane cleavage of integral membrane proteins such as Notch receptors and APP, but it requires the presence of the other subunits of the gamma-secretase complex for protease activity [201], [202].
APBA1	PSEN1	Bridge protein	Putative function in synaptic vesicle exocytosis and modulates APP processing [203].
APBA2	PSEN1	Bridge protein	Putative function in synaptic vesicle exocytosis and modulates the processing of APP [204].
APOE*	PSEN1	Bridge protein	Apolipoprotein that functions in lipoprotein-mediated lipid transport between organs via the plasma and interstitial fluids. It associates with all lipoproteins but shows preferential binding to HDL and binds to a wide range of cellular receptors that can also regulate neuron survival and sprouting [171]. APOE plays an important role in transcription regulation through a receptor-dependent and cholesterol-independent mechanism, that activates MAP3K12 and a non-canonical MAPK signal transduction pathway that results in enhanced AP-1-mediated transcription of APP [205].
CDC37	PSEN1	Bridge protein	Is a co-chaperone that binds to several kinases and promotes their interaction with the HSP90 complex with subsequent stabilization and elevation of their activity [206].
DHCR24	PSEN1	Bridge protein	Catalyses the reduction of the delta-24 double bond of sterol intermediates throughout cholesterol biosynthesis. It can also protect cells from oxidative stress by reducing caspase 3 activity during apoptosis induced by oxidative stress [207], [208].
DNAJB12	PSEN1	Bridge protein	Is a co-chaperone with HSPA8/Hsc70 essential to promote protein folding and trafficking, to avoid aggregation of proteins, and promote carriage of unfolded proteins to endoplasmic reticulum-associated degradation pathways [209]. It also has polypeptide-binding activities and can, together with DNAJB14, act as a chaperone that promotes maturation of potassium channels KCND2 and KCNH2 by stabilizing nascent channel subunits and assembling them into tetramers [210].
FADS2	PSEN1	Bridge protein	Involved in the biosynthesis of highly unsaturated fatty acids, catalysing the first and rate limiting step in this pathway. It desaturates tetracosapentaenoate to tetracosahexaenoate, which is then converted to docosahexaenoate, an important lipid for nervous system function [211].
GAP43	PSEN1	Bridge protein	Is associated with nerve growth and is a major component of growth cones that form the tips of elongating axons. Plays an important role in axonal and dendritic filopodia induction [212].
HSPA8	PSEN1	Bridge protein	Molecular chaperone concerned in an extensive assortment of cellular processes, including protection of the proteome beginning in stress, folding and transport of recently synthesized polypeptides and activation of proteolysis of misfolded proteins. Plays an essential role in the protein quality control arrangement, safeguarding the correct folding of proteins, the re-folding of misfolded proteins and controlling the targeting of proteins for subsequent degradation [213].
PRAM1	PSEN1	Bridge protein	Is thought to be involved in myeloid differentiation and in integrin signalling in neutrophils [214].

APP interacting protein	Secretase interactome	Binding detail	Molecular Function
STXBP1	PSEN1	Bridge protein	Contributes to the regulation of synaptic vesicle docking and fusion by interaction with GTP-binding proteins. It is also vital for neurotransmission and fixes syntaxin, a component of the synaptic vesicle fusion apparatus [215].
VDAC1	PSEN1	Bridge protein	Produces a channel through the mitochondrial outer membrane and the plasma membrane that allows diffusion of small hydrophilic molecules [216]. Involved in cell volume regulation, apoptosis and binds to various signalling molecules including phospholipid phosphatidylcholine and sterol cholesterol [217].
PSEN2	PSEN2	Direct binding	Is the second catalytic subunit of the gamma-secretase complex, an endoprotease compound that catalyses the intramembrane cleavage of integral membrane proteins such as Notch receptors and APP. Necessitates the other members of the gamma-secretase complex to have activity. Probable role in intracellular signalling and gene expression or in linking chromatin to the nuclear membrane [202], [218].
EXOC6	PSEN 2	Bridge protein	Element of the exocyst complex intricated in the docking of exocytic vesicles with fusion sites on the plasma membrane [219].
MAST1	PSEN 2	Bridge protein	Microtubule-associated protein crucial for precise brain development and appears to connect the dystrophin/utrophin network with microtubule filaments via the syntrophins [220].
RNF32	PSEN2	Bridge protein	Involved in protein-DNA and protein-protein interactions. It was found to be expressed during spermatogenesis, almost certainly in spermatocytes and spermatids. Several alternatively spliced transcript variants exist however, their functions are not clear [221].

APP is here shown to bind directly to ADAM17, but also indirectly via MT-CO1 (Table 3 and Figure 13), GSK3α, and AKAP9. Further analysis of both figure 13 and table 3 reveals that APP links to ADAM 10, and indirectly via PDIA3. Through binding to PDIA3, APP can correlate with ADAM17, ADAM10 and BACE1. As such, PDIA3 reveals as a potential key player in α-cleavage, potentially influencing substrate and enzyme interaction. APP does not bind directly to other ADAM secretases, doing so via bridging proteins. ABL1 can connect to ADAM12 and ADAM15 and, perhaps more important, considering GRB2, decreased upon RARα stimulation, can bridge to ADAM9, ADAM10, ADAM12, ADAM15 and ADAM17. As such, GRB2 is centrally situated regarding APP α–secretase cleavages. Furthermore, GRB2 binds to SH3D19, which in turn links to ADAM9, ADAM10, ADAM12, ADAM15, and ADAM17, creating a bridged-link, mediated by two nodes between the main participants in α-cleavage. AKAP9, GRB2, and SH3D19 all appear to have an important role in the APP secretaseome network, with respect to ADAM-mediated APP cleavage.

In light of the data here presented it follows that the western blot analysis, presented in the previous chapter should also include AKAP9 in future studies.

Concerning β-secretase cleavage, APP binds directly to BACE1, as anticipated. It can additionally associate via FLOT1, ITM2B and PPIA (Figure 13, Table 3). Relative to γ-secretase, the possibilities are wider. APP links to PSEN2, both directly or via the bridging proteins EXOC6, MAST1 and RNF32. Interestingly, PSEN1 linkage can also be direct or through 11 possible bridging nodes/proteins (APBA1, APBA2, APOE, CDC37, DHCR24, DNAJB12, FADS2, HSPA8, PRAM1, STXBP1 and VDAC1), some of which are associated with altered γ-secretase cleavage and AD (Table 3). In fact, APOE connects with the PSEN interactome, a hub for GWAS described risk genes for AD, as seen in figure 13 and table 3.

3.2.2.4 Kinases predicted to phosphorylate APP, ADAM17 and AKAP9

Results from the prediction for kinases phosphorylating APP, ADAM17 and AKAP9, using the methodology described in section 3.2.2.4, showed that all three proteins can potentially be extensively phosphorylated. The kinases GSK3β, MAPK8 and CDK1 were shown to potentially exhibit activity on the APP amino-acid sequence query. ADAM17 query demonstrated sequence compatible with the activity of a wide range of kinases, with the most frequent being PKC. The kinases GSK3, PKA and SRC were described to phosphorylate ADAM17. AKAP9 was also verified to be extensively phosphorylated, where PKA and PKC are potentially the most common kinases. GSK3 was also predicted to phosphorylate AKAP9. The integrate consequences of these results, considering the interactions visible in the network (Figure 13) are discussed below.

3.3 Implications derived from the APP Secretaseome

Protein phosphorylation modulates APP cleavage [8] and is widely associated with AD. Submitting APP´s sequence to Phospho.ELM [183] resulted in two predicted phosphorylation sites for GSK3β and MAPK8, consistent with

literature [222]. The resulting phosphorylated APP is facilitated for β-cleavage by BACE1 [24], and can alternatively also promote α-APP cleavage [223] (Figure 16).

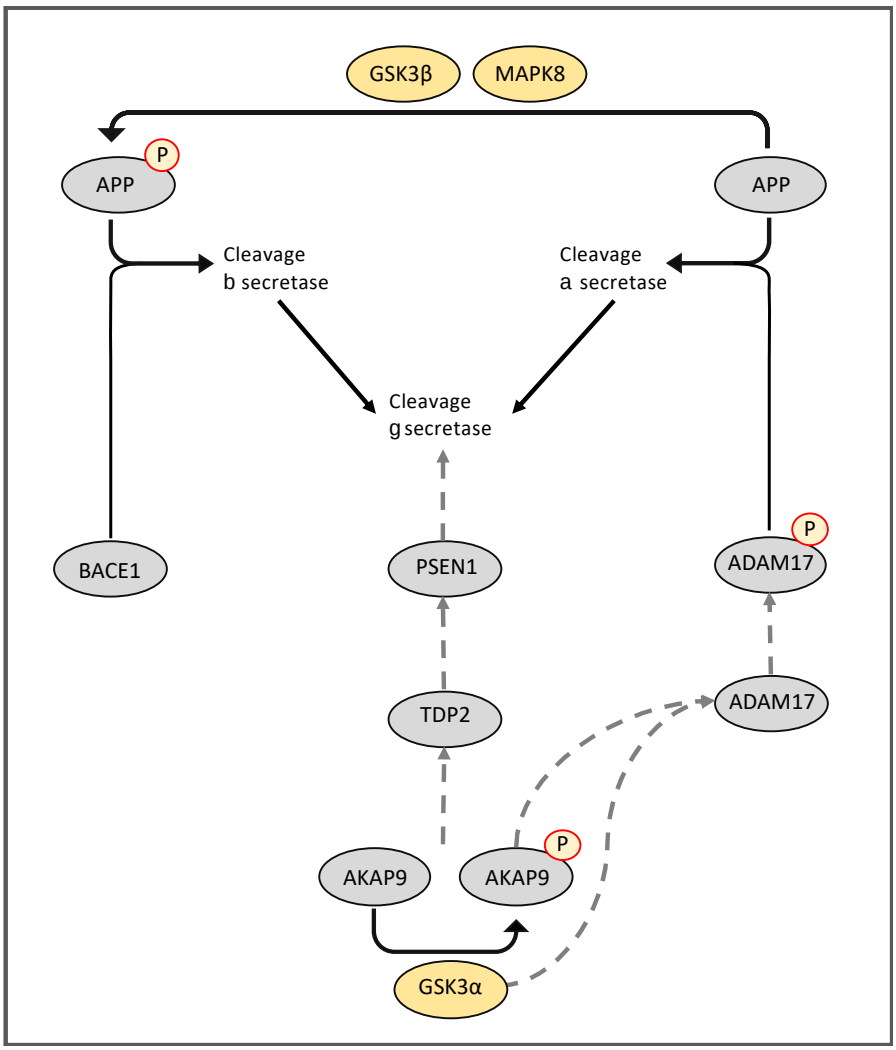


Figure 16 – Phosphorylation events mediating regulation of APP cleavage. *Predicted phosphorylations and their possible effects, obtained from databases. The probable phosphorylation of ADAM17 may be mediated by active AKAP9 or just by GSK3α. The two possibilities are shown with dashed lines. Solid lines depict published interaction and broken lines putative interactions from the work presented in this thesis* (Taken from: da Cruz e Silva, O. *et al*, submitted.)

ADAM17 and AKAP9, are described to be phosphorylated at serine, threonine or tyrosine residues [224], which promotes their activation. However, the most interesting prediction for this work is that GSK3α can phosphorylate ADAM17 at

Serine 180, 355, and 519, and AKAP9 at Serine 2953. ADAM17, once activated by phosphorylation [225] can act on APP as an α-secretase (Figure 16).

Intriguingly, the same phenomenon was described for the AKAP protein family [226]. As shown in the secretaseome network (Figure 13), there is no direct link between GSK3α and ADAM17, leading to the hypothesis that, due to AKAP9's molecular function, it may act as an adaptor for kinases other than PKA [227]. As such, it is possible that phosphorylation of ADAM17 could be mediated by activated AKAP9 and the model presented in figure 16 could represent a novel mechanism for ADAM17 activity modulation via AKAP9. AKAP9-ADAM17 interaction is identified as a novel interaction in the YTH screen (Figure 12 and Table 2). Still, AKAP9 could associate with PSEN1 via TDP2, an important component of the γ-secretase, an hypothesis further strengthened by the fact that GSK3β can also phosphorylate PSEN1.

Chapter IV – Implementing model systems for further studies in AD relevant human cell lines

It is already established that AD modifies several pathways within the cell. The analysis of those altered paths are a prime source for modulation targets, aiming to promote health. A final consideration can made as to what extent the search for alterations in insoluble protein fractions, can help in further understanding AD related alterations. The procedure may be useful to identify protein changes, suggesting modifications in cellular pathways associated with the disease state. Identifying these alterations is of paramount importance as they might act upon signalling pathways which, in turn, can be modulated towards a protective effect, alike seen with RAR stimulation altering pivotal proteins.

An adequate approach would be to use mass-spectrophotometry data, from insoluble protein fractions of human cells having undergone the different treatments. This is the next long-term goal for this project, however given the time constraints what is presented below is a pilot experiment to start to establish the optimal conditions to be applied.

4.1 Methods

Fibroblasts form individuals with 41, 69, and 80-years-old and from AD patients with 53 and 72-years-of-age were collected and cultured. Once confluent, the fibroblasts were lysed, and the insoluble fraction analysed by mass-spectrophotometry. From the resulting dataset “All Proteins”, a subset “Master Proteins”, with those most commonly present within the insoluble fraction, was further analysed. The data is presented in the form of inter-group ratio, with the cut-off set at 0.5/1.5 fold increase and organized in 5 different relations to determine relative alterations. Structural proteins were marked as yellow, for the lesser impact on signalling pathways and, as such, were not considered in the target search.

Special attention was given to proteins with inconsistent behaviour, both increasing and decreasing with no apparent correlation with age and AD. To better express inter-group alterations, Venn diagrams were elaborated, using the online

Venn diagram tool: Bioinformatics & Evolutionary Genomics - Calculate and draw custom Venn diagrams (http://bioinformatics.psb.ugent.be/webtools/Venn/ - accessed in June 2021).

4.2 Results

Data from an unpublished mass-spectrophotometry analysis of the insoluble fraction of fibroblasts from human donors (both healthy and AD patients) was analysed and is here presented. Results are shown as Veen diagrams which considered relative protein increase and decrease (Figure 17 and Figure 18).

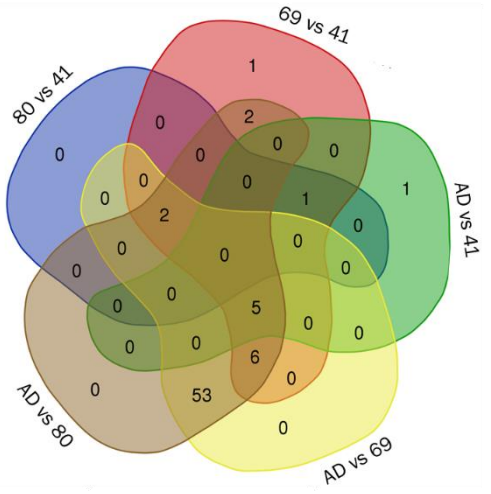
The alterations in the ratios are observable when comparing healthy aging with disease associated states (Figures 17A and 18A). Some proteins present a behaviour correlated not only with age but also pathology (Figures 17B and 18B).

4.2.1 Increases

As expected, AD seems to produce a clear alteration in proteins present in the insoluble fraction, as the number of proteins increased in AD patients is greater those from healthy donors. In fact, 53 proteins are commonly increased for AD, compared to normal aging from 69 and 80 years of age (Figure 17A-B), and the number of structural proteins present in the aggregates is also considerably higher, with ACTA2 being elevated in cells from both groups of AD patients when comparing with cells from 41-years-old donors.

Five proteins were found to be increased in cells from both groups of AD patients and 69-years-old donors, when compared to cells from 41-years-old donors (Figure 17A-B); of these, ATP5F1A, a mitochondrial protein involved in the ATP synthesis, is of special interest, as energy disruption is a feature of the normal aging process.

A



B

Grouping	Total	Elements
69 vs 41 AND 80 vs 41 AND AD vs 69 AND AD vs 80	2	TUBB2A, VTN,
69 vs 41 AND AD vs 41 AND AD vs 69 AND AD vs 80	5	ACTBL2, CKB, IDH2, DPYSL2, ATP5F1A,
69 vs 41 AND 80 vs 41 AND AD vs 41	1	ACTA2,
69 vs 41 AND AD vs 69 AND AD vs 80	6	ACTB, ATAD3A, EEF1A1, RIC8A, PKM, ACTG1,
69 vs 41 AND AD vs 80	2	LMNA, HBA1,
AD vs 69 AND AD vs 80	53	RBBP4, KRT6B, HSPD1, ILF2, CCT3, GNL3, ALB, TUBB, KRT1, HNRNPR, CCT6A, HNRNPH1, KRT14, DARS1, KRT10, TUBB4B, KHDRBS1, RBMXL1, H1-5, KRT9, MATR3, UMPS, RBMX, SHMT2, NOP58, MACROH2A2, DDX5, TUBB3, CARM1, CBS, HNRNPK, TUFM, KRT5, TUBA1B, H1-2, CRMP1, RPL4, PAICS, FSCN1, ATIC, ATP5F1B, TUBA1A, SMARCD1, PSMD12, NARS1, ALDH2, TUBB2B, DDX3X, NONO, KRT2, HNRNPL, HNRNPU, MACROH2A1,
69 vs 41	1	PLCB3,
AD vs 41	1	CALD1,

Figure 17 – Proteins increased in fibroblast insoluble fraction. *A – Venn Diagram of the proteins described to be increased. B – Expression of the obtain Venn Diagram in table form. Highlighted are proteins considered relevant, considering their function and behaviour. Green: Decreased in late-stage AD. Red: Increased in AD. Yellow: structural proteins, considered less relevant for lesser interference in signalling pathways.*

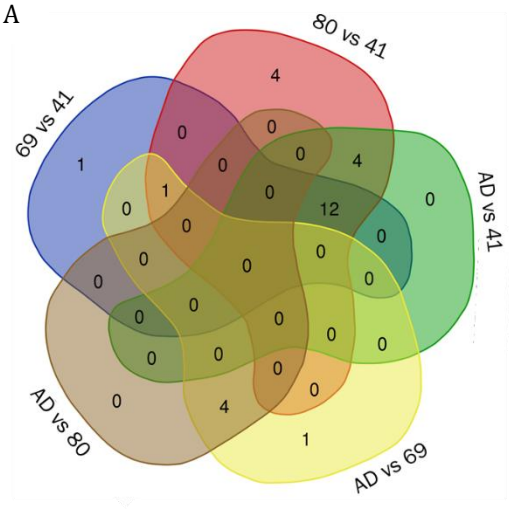
4.2.2 Decreases

The presence of some proteins in the insoluble fraction of cells from AD patients was decreased. Interestingly, this was observed in proteins reported to be increased in AD in the previous section, such as CALD1 (Figure 17 and Figure 18).

PDIA3, a pivotal protein that connects to several α-secretases, BACE1 and APP, present in APP secretaseome (Figure 13, Table 3), is here found to be decreased with healthy ageing and however, further decreased in AD (Figure 18).

The number of structural proteins decreased is considerably smaller when compared to those increased, corroborating the idea that structural proteins are less relevant for the aim of this research.

A



B

Names	Total	Elements
69 vs 41 AND 80 vs 41 AND AD vs 41	12	ILF2, FSCN1, TUBB3, KRT14, KRT10, KHDRBS1, TUBB4B, KRT5, PRSS1, KRT2, KRT9, KRT1,
69 vs 41 AND 80 vs 41 AND AD vs 69	1	PDIA3,
80 vs 41 AND AD vs 41	4	DDX5, ATAD3A, CCT3, NONO,
AD vs 69 AND AD vs 80	4	PLCB3, CALD1, ACTA2, VIM,
69 vs 41	1	ALB,
80 vs 41	4	RPL4, ACTB, EEF1A1, ACTG1,
AD vs 69	1	HBA1,

Figure 18 – Proteins decreased in fibroblast aggregates*. A – Venn diagram of the proteins described to be decreased. B – Expression of the obtain Venn Diagram in table form. Highlighted are proteins considered relevant, considering their function and behaviour. Green: Decreased in late-stage AD. Red: Increased in AD. Yellow: structural proteins, considered less relevant for lesser interference in signalling pathways.*

Chapter V – Concluding remarks

5.1 Discussion

The stimulation of RAR has been studied, due to its impact on APP processing, and while some aspects are currently well characterized, others remain unclear. From the work here presented, the stimulation of differentiated SH-SY5Y cells with *at*RA decreased p62, (Figure 9); bearing in mind the context of AD, this effect can be considered unfavourable, since p62 promotes proteostasis via ubiquitin-mediated autophagy. Furthermore, this finding re-enforces the relevance of RAR activation in other proteins, beyond those secretases with direct impact upon APP. These secondary alterations in proteins with important roles for neuronal health or key interactors in APP cleavage, resulting from single or combined isoform modulation, may explain the apparent contradiction with beneficial effects initially reported.

The activation of RAR-α alone showed more promising results. Treatment with RAR-α agonist, AM580, decreased GRB2 (figure 7). GRB2 is a risk gene for AD in GWAS (figure 12), and its main function should be considered, as it mediates the activation of the Ras pathway, promoting several phosphorylation events. Increased protein phosphorylation in AD is well characterized, as phosphorylated Tau forms neurofibrillary tangles and specific phosphorylation pathways can induce APP β-cleavage. Phosphorylation can also be argued to activate cleaving ADAMS, especially ADAM17 (Figure 1). Noteworthy, the phosphorylation paths that activate ADAM secretases are, to some extent, related to those that inactivate γ-cleavage. As such, the vast and complex importance of phosphorylation in AD-related cellular ways must be considered.

RAR-α specific activation also resulted in a tendency to decrease the mitochondrial HSP60 (Figure 10). This result is interesting, should it be confirmed by further experimentation, as the chaperone HSP60 is a mitochondrial marker that rescues misfolded proteins with exposed hydrophobic residues. However, the results here presented lack statistical significance, and further work is required before mitochondrial health can be considered altered.

Interestingly, treatment with agonist for both RAR-α and RAR-β was not as auspicious, as it significantly increased both APOE and Tau (Figures 5 and 6). APOE is, like GRB2, a risk gene for AD (Figure 12), and is associated with cognitive decline. However, a dramatic decrease in APOE would also be detrimental for neuronal health, as it is responsible for facilitating the lipid import and intake, crucial to normal neuronal metabolism. The observed increase is of importance because APOE is thought to initiate and aggravate the toxic abilities of secreted Aβ by directly influencing the aggregation capacity. Additional research is needed to further understand the special significance of RARα and the difference with RARα/β stimulation as well as to verify the effects in AD models.

The increase observed in Tau (Figure 5), is not particularly relevant on its own, as Tau is an important stabilizer of neuronal cytoskeleton, crucial for cell transport and integrity. However, together the fact that several phosphorylation pathways are activated by the RAR activation, and that Tau phosphorylated is released from cytoskeleton, aggregating, and inducing neurotoxicity, the significance of this increase grows substantially.

The variations between these results reflect the importance of the activated isoform. Differences between treatments with RAR-α and RAR-α/β agonists can be explained by the interference of the β isoform on RAR-α effects. However, the effects of RAR-β cannot be deduced from these results. As such, to infer if the unfavourable results are consequence of RAR-β stimulus or only observable upon the combined stimulation of RARα and β requires further investigation.

Furthermore, differences observed between *at*RA treatment and the combined stimulation of RAR-α/β cannot be justified by the additional activation of RAR-γ as it can produce different alterations once stimulated alone. Though the mentioned isoform is, to some extent, less described in literature, the effects of isolated stimulation should not be of underrated in the presented context.

The phenomenon by which APP phosphorylation induces β-secretase mediated cleavage, especially associated to Thr668, is well described [24], [25], given that phosphorylation at other sites can have similar effects [222]. Current research suggests that phosphorylation occurs via MAPK8 and GSK3B [228] with

the latter also interacting with PSEN1, central for the γ-secretase complex. This mediation through phosphorylation events should be further researched [229], [230] as α-secretase mediated cleavage is also mediated by phosphorylation [21], seen bellow. A systems approach was taken to the APP secretaseome elaboration, to better address APP cleavage events.

The newly described ADAM17 interactors, identified by the YTH system, are novel putative functions and potential modes of regulating protein cleavage events, particularly the interaction with AKAP9. AKAPs can modulate cAMP mediated signalling events, in a spatial and temporal perspective, as these proteins can anchor PKA, targeting it to other cAMP effector proteins [231] like adenylyl cyclases [232]. Both PKA and GSK3 are involved in phosphorylation events, and as previously described, hyperphosphorylated Tau deposits as neurofibrillary tangles in AD brains and APP is cleaved by secretases to produce Aβ which can also form deposits as senile plaques [133]. AKAP9 appears to be a critical bridging protein between ADAM17 and APP (Figure 13), which clearly asks for further investigation, unravelling phosphorylating events.

AKAP9 can be an adaptor protein for kinases other than PKA [227], [233], including GSK3β [234], thus creating a compartmentalized environment within the cell to bring various signalling transducing proteins to their targets [235] and fine-tune phosphorylation spatial and temporal regulation. As such, a possible regulation of ADAM17 activation being by GSK3α must be considered, particularly due to a predicted phosphorylation site for GSK3α in ADAM17 [224], with no direct contact identified between these two proteins (Figure 13). The chance of an incomplete network, due to inaccuracies in the database, be also considered. Otherwise, two scenarios can be speculated, as seen in figure 16. Figure 16 shows that interaction can be mediated by AKAP9, a path present in the secretaseome (Figure 13) and ADAM17, well established to be activated by phosphorylation. Other kinases could be involved in this regulation, such as PKC and p38 MAP kinase [22], [225]. Further analysis of the ADAM17 interactome network shows it to also interact directly with PDK1.

AKAP9 can be activated by phosphorylation [226], corroborated by sequence- and structure-based prediction of eukaryotic protein phosphorylation sites for various kinases, among them GSK3α [224]. As such, the formation of a complex forming between AKAP9 and GSK3α, allowing the easy activation of the adaptor, AKAP9, and ADAM17 is possible and likely.

Like GRB2 and APOE, AKAP9 is a risk factor for AD. As such, it is possible to assume that, as an alteration in AKAP9 or in its interactome not only affects PKA function, but are seen in Tau phosphorylation levels [233]. Alterations to AKAP9 interactions could also alter ADAM17 activation; this theory, if confirmed, would lead to another path for deregulation of APP α-cleavage, like GRB2. This adapter protein, providing a link between cell surface and internal signalling pathways, and identified as altered in AD, is the link between APP and ADAMs 12 and 15. If altered, it can deregulate and interfere with these interactions, compromising APP α-cleavage. Considering GRB2 and SH3D19 (EVE-1) interactions (table 3), an important bridge between APP and several ADAM secretases can be identified; GRB2 binding proteins are known to be important for signal transduction via kinases [236].

Figure 13, representing the expanded ADAM10 and ADAM17 interaction network, allowed the identification of several key proteins, including GRB2. Considering that APP can bind to ADAM10 via GRB2, which in turn can recruit SH3D19/EVE to ADAM10 and/or to ADAM17, suggests that SH3D19/EVE is a critical pivotal protein targeting to either of the two cleaving ADAM proteins.

This is of paramount importance, as individual ADAMs appear to be associated with distinct molecular and physiological functions. Therefore, as SH3D19/EVE and GRB2 appear to be central hub proteins, their interaction with ADAM proteins reinforces the importance of further studying these mechanisms. The SH3-binding site in ADAMs' cytoplasmic domain has previously been reported to allow the interaction and activation of SH3 domain binding proteins, like SRC and GRB2 [237]–[239]. This observation provides an explanation as to how extracellular events may activate intracellular signalling pathways: it is plausible that ADAM, binding to some of the above-mentioned signalling molecules, would promote

conformational changes and thus potentiate integrin binding. This proposed idea places ADAMs as critical bi-directional signalling molecules, linking the extracellular events with intracellular actions.

Mass-spectrophotometry results not only contribute to the holistic search for targets, but also allow the understanding of the behaviour of several key proteins, present in the insoluble fraction both in aging as in AD.

As seen in figures 17 and 18, the number of structural proteins in the insoluble fraction are increased with aging and, especially, with AD, in an extent vastly greater than those decreased. This is not surprising, as cytoskeleton proteins are described to lose some function over time, not contributing directly to the initial process. However, these structural proteins are not irrelevant and, as such, these structural proteins were included in the dataset, but distinguished, to better visualize the extent of the difference between the increases and increases.

Still comparing both increase and decrease groups, a selected group of proteins was found to be increased in some instances and decreased within other. CALD1 was increased in cells from AD patients, when compared with cells from 41 years old donors, and, interestingly, decreased in cells from AD patients when compared with cells from 69 and 80-years old donors (Figures 17B and 18B). KHDRBS1 has a somewhat mirror behaviour, being increased in cells from AD patients when compared with cells from 69 and 80-years old donors but decreased when compared with cells from 41 years old donor. Furthermore, this protein is also decreased in cells from 69 and 80-years-old donors when compared with cells from 41 years old donor (Figure 18B). KHDRBS1 can be inferred to decrease with the natural aging process, possibly as activity of pathways that require it, like transcriptional activity and respective regulation, are decreased. This possibility also corelates with the fact that it is increased in AD groups (Figure 17), as several regulatory events may be inefficient, resulting in the increased protein levels.

5.2 Concussion

As described in this work, a relevant approach to AD is promoting APP α-secretase cleavage, which may be crucial in developing novel therapeutic targets to

delay AD onset of AD. In addition, understanding of alterations to key proteins that further contribute to observed cellular alterations, seen in pathology, is of paramount importance. As such, the holistic approach is a significant candidate for modelling the proposed research, as it investigates global and dynamic molecular changes and interactions under different normal and pathological conditions, thus representing a promising approach for the study of AD, and other complex pathologies [33], [240].

Applying OMICS platforms range from the identification of genes (genomics), messenger RNA (transcriptomics), and epigenomic factors (epigenomics), to proteins (proteomics), metabolites (metabolomics), lipids (lipidomic), and it has attracted growing interest due to its association with different diseases [240]. Also, the study of protein interactions (interatomic) has showed promising results in understanding key pathways and functions of important proteins, playing key roles in several pathways, both physiological or associated with disease [20], [241].

For pathologies involving a large number of affected pathways and regulation systems, analysis and integration of data from different OMICS technologies is crucial for the full knowledge of the disease, supporting the development of personalized diagnostic and therapeutic tools. Several OMICS studies aim to determine novel pathways and networks, suggesting new pathologic mechanisms associated with the disease states and cross-linked with other diseases.

OMICS approaches, on their own, cannot distinguish whether the alteration of networks in molecules and markers are a cause or an effect of the disease. In this sense, the role of the candidate molecules to therapeutics, in various aspects of the disease cascade, needs to be replicated with a larger number of patients and with alternative techniques.

The notion of APP secretases as therapeutic targets for AD is demanding. As the three classes of secretases cleave a significant pool of substrates, not restricted to APP, affecting several signalling and metabolic pathways, purposefully altering their activity is a complex procedure with possible adverse outcomes. As such, selective inhibition of one or a group of secretases generates problems in maintaining the physiological pathways inherent to the normal cell function.

Besides, the build-up of several substrates can have unforeseen cytotoxic effects and thus be counter-productive for preventing neurodegeneration. Secretase alterations induced by RAR signalling are, to some extent, described in the specific context, lacking a holistic approach to determine whether observed changes occur only in the studied protein complex, or they have deeper effects, altering the AD interactome. This knowledge is of the upmost importance, as it allows determining whether RA-induced alterations are compatible with homeostasis and, consequently, a step closer to deciphering a potential retinoic therapy for AD.

In closing, the holistic and systems approach presented in this work focuses on several molecular players and not only on the underlying individual disease processes. This has the advantage of identifying signalling cascades and crosstalk in different pathways, appropriate to a specific molecular target, involving many fronts of the disease. In fact, the networks here presented comprise pertinent and state-of-the-art tools for the search and validation of novel therapeutic approaches, not only for AD but also other disorders involving the molecular players here identified.

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