Published in final edited form as:

J Alzheimers Dis. 2014 ; 40(1): 1–17. doi:10.3233/JAD-131544.

Late-Onset Alzheimer's Disease, Heating up and Foxed by Several Proteins: Pathomolecular Effects of the Aging Process

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Abstract

Late-onset Alzheimer's disease (LOAD) is the most common neurodegenerative disorder in older adults, affecting over 50% of those over age 85. Aging is the most important risk factor for the development of LOAD. Aging is associated with the decrease in the ability of cells to cope with cellular stress, especially protein aggregation. Here we will describe how the process of aging affects pathways that control the processing and degradation of abnormal proteins including amyloid- β (A β). Genetic association studies in LOAD have successfully identified a large number of genetic variants involved in the development of the disease. However, there is a gap in understanding the interconnections between these pathomolecular events that prevent us from discovering therapies. We will compile pertinent links to elucidate how the biology of aging affects the sequence of events in the development of LOAD. Specifically, in this review we analyze the molecular-pathologic-clinical correlations of the aging process, the HSF1 and FOXO family pathways, A β toxicity, A β degradation, and the different clinical stages of LOAD.

Introduction

Late-onset Alzheimer's disease (LOAD) affects up to one-third of individuals by the age of 80 [1–2]. There is a strong interest in identifying pathways involved in the pathogenesis of AD in order to find new therapeutic targets for clinical trials. Evidence suggests that the pathophysiological process of AD begins years, if not decades, prior to the diagnosis of clinical dementia [3]. In this study, we will examine the development of AD and its

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relationship with the longevity pathways on the A β clearance and antiproteotoxicity systems. Clinical progression of AD evolves (presumably) from presymptomatic neurodegeneration, through mild cognitive impairment (MCI), ultimately to symptomatic AD [4–5]. MCI patients with primary memory deficits, aka “amnesic MCI”, have a significantly higher likelihood to progress to probable AD, with a conversion rate of 10–15% per year [2]. AD is associated with abnormal deposition aggregates consisting primarily of extraneuronal amyloid- β peptide (A β) (plaques) or intraneuronal hyperphosphorylated τ proteins (tangles) in the brain [6]. As the preclinical phase begins is presumed to begin decades before diagnosis of MCI, it would provide a critical opportunity for potential intervention with disease-modifying therapy [3]. This makes it a priority to elucidate links between the pathomolecular process that start during the preclinical phase and the onset of the clinical syndrome. A hypothetical intervention that delays the onset of AD by 5 years would result in a 57% reduction in the number of AD patients, and reduce the projected Medicare costs of AD from \$627 to \$344 billion dollars [7] (presuming no overall extension of lifespan due to reduction of AD).

Aging is the strongest risk factor in the development of LOAD [8]. It seems that the earliest events in the development of AD are controlled by longevity pathways [9]. These pathways control A β degradation and aggregation processes in the intra and extra-cellular space. These aging/longevity pathways can in theory be manipulated to prevent or delay the development of AD. Recent studies with transgenic mouse models suggest that amyloid-modifying therapies may have beneficial effects even once neuronal degeneration has begun [10]. Interestingly, recent clinical trials in the stages of mild to moderate dementia have failed to demonstrate clinical benefit, even in the setting of biomarker or autopsy evidence of decreased amyloid-burden [11]. These results may be an evidence of a failure of the amyloid hypothesis or a lack of understanding of the pathomolecular events in the development of AD; we believe the latter is a more reasonable explanation. In response to late-stage treatment failures, AD research is moving towards early stage clinical trials such as amnesic mild cognitive impairment (MCI) or even in patients with conditions currently deemed pre-clinical, such as subjective memory impairment (SMI) [12] or cognitive complaints (CC) [13]. The overarching therapeutic objective of “preclinical studies” would be to discover treatments for early pathologic processes (e.g. lower A β burden) in order to prevent subsequent neurodegeneration and eventual cognitive decline. For these reasons, we demonstrate new molecular targets for therapies that will focus on the preclinical stages of AD by slowing down its main risk factor, elements of the aging process itself.

Pathomolecular-Clinical Correlations

Early LOAD is associated with abnormal protein degradation and aggregation within specific areas of the brain, and this specificity helps with differential diagnosis. The A β deposition often begins in the temporal cortex particularly on the hippocampus [14], which causes cognitive impairment associated with MCI. Frontal and occipital brain regions are spared from early neurodegenerative pathology. Ensuing specific cognitive deficits and strengths in early stages suggest the pathologic process and help differentiate between AD and other types of dementia including Fronto-temporal (FTD) and Subcortical Ischemic Vascular Dementia (SIVD).

AD Molecular Biology and Markers

Amyloid Plaques—The distinguishing histopathological features of AD consist of cerebral plaques that consist primarily of A β [15], dystrophic neurites in neocortical terminal fields [16], and prominent neurofibrillary tangles in medial temporal-lobe structures [17]. Loss of neurons and white matter, congophilic (amyloid) angiopathy, inflammation, and oxidative damage are also present. A β peptides are natural products of metabolism consisting of 36 to 43 amino acids. Monomers of A β are much more prevalent than the aggregation-prone and damaging A β_{42} species [18]. A β originates from proteolysis of A β PP by the sequential enzymatic actions of β -site A β PP-cleaving enzyme 1 (BACE1), the primary β -secretase, and the γ -secretase protein complex, which has presenilin 1 at its catalytic core [19]. Carboxy-terminal cleavage of A β by γ -secretase results in the generation of multiple peptides, the two most common being 40- and 42-amino acid A β (A β_{40} and A β_{42} , respectively). A β_{40} comprises 90–95% of secreted A β and is the predominant species recovered from cerebrospinal fluid [20]. In contrast, less than 10% of secreted A β is A β_{42} . Despite the relative paucity of its production, A β_{42} is the predominant species found in plaques and is deposited initially [21], perhaps due to its ability to form insoluble amyloid aggregates more rapidly than A β_{40} [22–23]. An imbalance in intracellular A β clearance causes A β to accumulate, and this decreased degradation may be an initiating factor in LOAD [24]. This pathomolecular process starts in the preclinical stage and our hypothesis considers it likely to require the onset of the aging process.

Intracellular A β —Accumulation of A β , as seen in AD, could be due to increased generation, decreased clearance, or a combination of both events. Recently, decreased clearance has been recognized as the cause of A β accumulation in LOAD [24]. Accumulation of intracellular A β (iA β) is an early event in AD, synaptic loss and iA β detection occurred before the appearance of A β plaques [25]. The most current hypothesis states that extracellular A β (eA β) causes toxicity and cell death [26]. This fails to explain why cultures of human primary neurons are resistant to the administration of eA β [27]. In this study, they used 100 nM fibrillar A β 1–42, which triggered upregulation of apoptotic protein Bax but not neuronal death. Since current findings suggest a more important role of oligomeric A β 1–42 compared to fibrillar A β 1–42, it could be the reason. In a recent study we observed 40% loss of cell viability in rat primary neurons upon 100 nM oligomeric A β 1–42 treatment for 72 h [28]. Nevertheless directly injecting intracellular A β is much more effective. Compared to 100 nM eA β , direct microinjecting 1 pM iA β induces 50–70% cell death in human neurons [29]. Most importantly, in AD transgenic mouse models, deposition of iA β precedes eA β and NFT formation and synaptic dysfunction [30]. Thus eA β deposition alone is inadequate to account for the LOAD pathology. A complementary explanation is that iA β causes toxicity and neuronal dysfunction. A β accumulates intracellularly in AD patients, it localizes in multivesicular bodies, an organelle originating from the endosomal pathway [31]. Changes in the endosomal-lysosomal pathway are amongst the earliest changes in AD [32]. A β can be degraded from the intracellular compartment by activation of clearance pathways [33]. Therefore, new therapeutics targeting iA β could be an effective treatment for LOAD [34].

Neurofibrillary Tangles—Neurofibrillary tangles (NFT) are mainly aggregates of abnormally hyperphosphorylated τ protein [35]. The soluble τ protein is abundant in axons; it promotes assembly and stability of microtubules and vesicle transport [36]. Hyperphosphorylated τ is insoluble, lacks affinity for microtubules, and self-associates into paired helical filament structures [37]. Like A β oligomers, intermediate aggregates of abnormal τ molecules are cytotoxic [38] and impair cognition [39]. NFTs also contain significant percentage of hyperphosphorylated neurofilaments (NF) [40–41] and collapsin response mediator protein 2 (CRMP2) [42]. Neurofilaments promote an increase in axonal diameter in myelinated fibers, thereby increasing nerve conduction velocity. NF is phosphorylated physiologically in axonal compartments; however, they are hyperphosphorylated in AD and form aggregates in cell bodies. In AD, hyperphosphorylation of medium NF (13 sites) and heavy NF (10 sites) occurs at conserved KXXXP motifs located on the C-terminal tail domain, and is ~4–8 fold higher than in age-matched controls [41]. CRMP2 protein is also hyperphosphorylated in AD and co-localizes with NFT in human AD brains. Hyperphosphorylated CRMP2 is highly resistant to dephosphorylation and is used as a marker of NFT [42].

Synaptic Failure—The cognitive symptoms of AD may be primarily due to synaptic failure [18]. Memory deficits in clinical LOAD are associated with degeneration of the hippocampus, which extends tissue changes associated with MCI, in which there is an early decreased hippocampal synaptic plasticity [43]. Hippocampal synapses begin to decline in patients with MCI, in whom remaining synaptic profiles show compensatory increases in size [44]. In mild AD, there is a reduction of the presynaptic vesicle protein synaptophysin [45]. With advancing disease, synapses are disproportionately lost relative to neurons, and this loss is the best correlate with dementia [46]. A β oligomers mediate synaptic dysfunction by binding and self-association to structures (membrane receptors, mitochondria, etc) leading to synaptic dysfunction and memory impairment [47]. While A β accumulation is the earliest event in the development, it is not sufficient to produce the clinical AD syndrome. The cognitive decline of AD occurs only in the setting of synaptic dysfunction and/or additional neurodegeneration, including paired helical filament τ formation and neuronal loss [48]. Evidence also suggests that additional factors, such as cognitive reserve, white matter alterations, dopaminergic depletion, cerebrovascular disease, and Lewy bodies, alter the relationship between the neuropathological and clinical manifestations of AD. We also recognize that a substantial subset of individuals can have all of the classic neuropathologic features of AD at autopsy but never show signs of dementia during life [49]. It remains unknown whether these individuals would have manifested clinical symptoms should they have lived longer or their individual adaptation mechanisms to loss of synaptic plasticity would have indefinitely prevented clinical LOAD.

Neurodegeneration—In Alzheimer's disease the pathomolecular sequence of events show that neuronal loss is a relatively late event, typically following synaptic dysfunction, synaptic loss, neurite retraction, and the appearance of other abnormalities such as axonal transport defects [50]. Evidence suggests that although cell death itself occurs late in the degenerative process, the pathways involved in cell death signaling play critical roles in neurodegeneration, both in sub-apoptotic events such as synapse loss and in the ultimate

neuronal loss itself [51–53]. Neuroinflammation, typified by the accumulation of activated microglia and reactive astrocytes, plays an initial role in neurodegeneration, and A β activates microglia and astrocytes localized around A β plaques. The severity of glial activation correlates with the extent of brain atrophy [54] and cognitive decline [55]. Glial biochemical markers are elevated in the brains of patients with AD [56]. Initially, phagocytic microglia engulf and degrade A β . However, chronically activated microglia release chemokines and a cascade of damaging cytokines such as interleukin-1, interleukin-6, and tumor necrosis factor α (TNF- α) [57]. In common with vascular cells, microglia express receptors for advanced glycation end products, which bind A β , thereby amplifying the generation of cytokines, glutamate, and nitric oxide [58]. A β and glial activation also stimulate the classic complement pathway [59]. Tangles and plaques contain complement cleavage products, C1q and C5b-9, indicating that opsonization and autolytic attack are under way [57]. Stimulated astroglia also release acute-phase reactants, alpha1-antichymotrypsin, alpha2-macroglobulin, and C-reactive protein, which can both aggravate and ameliorate AD.

Cytokines stimulate a variety of intracellular signalling pathways by receptor binding. These cytokines have been implicated in AD, including the activation of protein kinase C, c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (p38/MAPK), PI3 kinase, and extracellular signalling-related kinase (ERK) as well as activation of caspase-1 and -3 [60]. Elevated A β induces apoptosis-related events in synapses and dendrites in AD through caspase activation [61]. In addition, A β -induced caspase-3 activation causes abnormal processing of the microtubule-associated protein τ in models of AD, and these fragments directly reduce neuronal viability [62].

An important factor in degeneration is the loss of calcium regulation in Alzheimer's disease, elevated concentrations of cytosolic calcium stimulate A β aggregation and amyloidogenesis [63]. A β_{42} also increases calcium stores in the endoplasmic reticulum and the release of calcium into the cytoplasm [64]. Also increased glutamate level leads to increase cytosolic calcium, which in turn stimulates calcium-release channels in the endoplasmic reticulum. It has been shown that a chronic state of excitatory amino acid (glutamatergic) receptor activation aggravates neuronal damage in LOAD [65]. However, the evidence of excessive excitatory amino acid mechanisms in AD is modest. A β forms voltage-independent cat ion channels in lipid membranes [66], resulting in calcium uptake and degeneration of neurons [67].

Pathomolecular Sequence of Events of the Aging Process on Clinical AD

A β is generated by the sequential enzymatic cleavage of the amyloid- β protein precursor (A β PP) in the endoplasmic reticulum (ER)/intermediate compartment [68], the medial Golgi saccules [69], and the trans-Golgi network (TGN) [70–71]. Furthermore, A β_{40} is generated exclusively within the TGN and packaged into post-TGN secretory vesicles [70–71], while A β_{42} could be made and retained within the ER in an insoluble state, before entering the TGN and being packaged into secretory vesicles [72]. A β is recognized as an abnormal protein and reversely translocated back to the cytosol [34]. A β is ubiquitinated and sent to

the proteasomes [73] or to the chaperone-mediated autophagy (CMA) system for degradation [74].

The ubiquitin-proteasome and autophagy-lysosome pathways are the two main routes of protein clearance in eukaryotic cells. Since the proteasome activity decreases with aging [75], inefficient degradation and clearance of A β would result in iA β accumulation. This chronic proteasome inhibition causes an excessive activation of the lysosomal system demonstrated by an increase in autophagy [76]. Alternately, iA β is internalized into Autophagic Vacuoles (AVs) for degradation [77].

Age-related decline in overall proteolytic activity has been observed in almost all organisms studied, and specific age-related defects in the different proteolytic systems have been reported [78]. The molecular basis of this age-related decline offers an explanation for the late-onset observed for many neurodegenerative diseases [79]. The earliest event in the aging process is the collapse of proteostasis, produced by a sharp decline of the heat shock response (HSR) in cell cultures and animal studies [80–83]. Therefore, it is important to correlate these initial molecular events with the development of AD (Fig. 1). For example, in humans, the HSR attenuation begins after the third or fourth decade of life [84]. Thereafter, abnormal protein accumulation occurs [83] in the form A β accumulation in plaques after the age of 30 years [85]. Interestingly, this occurs after the onset of the attenuation of the HSR (Fig. 2A). Then, after a couple of decades of A β accumulation, the clinical signs of dementia are more commonly observed when individuals are over 50 years of age [86]. These various situations are interrelated and tend to correlate with age (Table 1), although strict age causation is likely to be an overly simplistic model.

The general picture that emerges from these observations is a model that favors the following pathomolecular sequence of events: firstly, HSR attenuation, which leads to proteasome and CMA dysfunction with increased iA β , resulting in an inefficient compensatory activation of AV, and this AV accumulates in the cytoplasm and ends up releasing ubiquitin tagged eA β that forms toxic oligomers (Fig 2A). At this point, multifunctional factors, such as FOXO3, are activated, which form non-toxic neuritic plaques and thus prevent neurodegeneration from A β oligomers. Later, the age-related attenuation of FOXO3 is initiated and inflammatory/oxidative stress directs FOXO3 toward the nucleus, an apoptotic pathway. FOXO3 stops forming plaques with the subsequent increase in extracellular toxic oligomers which causes neurodegeneration and the onset of clinical AD (Fig. 2B).

It is important that HSR is regulated at the transcriptional level by HSF1 [87]. At the molecular level a decreased HSF1-DNA binding is responsible for the age-related attenuation of the HSR and heat shock proteins (HSP) expression, especially HSP70 [88–89]. The chaperone HSP70 is very important for the intracellular degradation of A β [90]. Also, the regulation of aging and the maintenance of proteostasis in invertebrates require the activation of the transcription factor FOXO [91]. In a recent study, the overexpression of HSP70 preserved proteostasis during aging but additional FOXO-dependent pathway responses were required [92]. In the same study, the overexpression of FOXO delayed protein accumulation and increased lifespan in *Drosophila* [92]. Therefore, we believe that

in humans an age-related decline in activation of a member or members of the mammalian FOXO family is required to allow proteotoxicity and neuronal degeneration (Fig. 1). This is supported by the fact that, in invertebrates FoxO regulates [93] the expression of several autophagy genes which progressively decline during aging in muscles [92, 94]. This is also supported by the extrapolation of data from *C. elegans* (flatworm) on the age-related effects of the HSF1 and FOXO pathways [9]. This *C. elegans* transgenic model for AD revealed that FOXO and HSF1 are required for A β degradation and proteotoxicity prevention. They used HSF1 and FOXO (DAF16) interfering RNA (RNAi) in worms to demonstrate the function of these pathways. HSF1 regulated disaggregation, whereas the FOXO mediated the formation of less-toxic high-molecular weight aggregates. This process may be analogous to the human disease, when organisms would be young and both pathways are functional, but there are no signs of disease. Later when humans develop age-related attenuation of HSF1 analogous to the inhibition of worm HSF1 by RNAi, the formation of less-toxic high-MW aggregates would be inhibited and formation of toxic oligomers and clinical symptoms would appear. It should be noted that *C. elegans* APL-1, the native A β PP homologue, does not, itself produce A β , and this aggregate-inducing activity has not yet been specifically replicated in mammals.

Interestingly, activation of FOXO family members can also lead to cell death via upregulation of Bcl-2 interacting mediator of cell death (Bim) and FAS ligand (FasL) under neurotoxic conditions. Although gaps exist in our understanding of the mechanisms by which FOXO family proteins tilt the balance in favor of cell death, SIRT1-mediated regulation of FOXO family proteins offers an intriguing possibility. SIRT1 deacetylates invertebrate FOXO, which induces transcription of stress-resistance genes [95] and prevents induction of apoptosis genes [96]. Thus, it appears that SIRT1 plays a crucial role in tipping the balance of FOXO functions away from cell death towards stress resistance. Importantly, SIRT1 levels are significantly reduced in AD patients as compared to controls and correlate well with toxic A β and NFT accumulation [97]. These findings further support our model that age-related attenuation and/or altered FOXO signaling (due to reduced deacetylation) are critical in promoting proteotoxicity in LOAD pathogenesis.

It is therefore of interest to understand the functions of the HSF1 and FOXO pathways and their role in regulating HSP expression during ubiquitin–proteasome, CMA degradation, and defense against proteotoxicity. The key regulatory role of HSF1 in the ubiquitin proteolytic pathway was established using HSF1 null cells and the capability of exogenous HSF1 to restore the HSF DNA binding activity and inducible HSP70 expression upon proteasome inhibition [98]. HSF1 activation pathway leads to enhanced expression of HSP70 and other molecular chaperones. These molecular chaperones work in tandem with the ubiquitin–proteasome pathway for protein quality control to prevent the accumulation of abnormal proteins, including A β [99–100]. Interestingly, intracellular A β also prompts the rapid induction of stress-inducible HSP70 protein in neurons [101], most likely due to activation of HSF1 [102].

Chaperones such as HSP70 limit the accumulation of damaged proteins by suppression of misfolding and enhancing chaperone mediated folding [103–104], promoting the enzymatic removal of covalent modifications [105], and stimulating clearance by ubiquitin-mediated,

proteosomal [106], and autophagic processes [9, 107]. The ubiquitin-proteasome system is the primary mechanism for disposal of stress-denatured proteins [108–109] as well as being responsible for the majority of normal protein turnover [110]. The fact that the HSF1, FOXO, and proteasome pathways are implicated in several neurodegenerative diseases [111–114], including A β degradation [106], raises a potential therapeutic role for HSF1 and FOXO in AD and other neurodegenerative diseases.

Therefore, the enhancement of the HSF1 and appropriate FOXO pathways simultaneously as a new therapeutic strategy to improve the proteasome-mediated and CMA degradation of A β has been suggested recently [115]. Interestingly, the Rpn4, ubiquitin, an E3 ligase, and a ubiquitin-conjugating enzyme were identified in a genome-wide screen for HSF1-regulated genes in yeast [116], raising the possibility that HSF1 could affect expression of components of the ubiquitin-proteasome system in stressed cells, in addition to the well-characterized upregulation of HSP gene expression [98, 117]. This raises the question if reversing the attenuation of the HSR would reverse the age-related ubiquitin-proteasome decline.

HSF1 has variable effects on expression of proteasome subunits in mammalian cells, depending on the experimental conditions and cell type being investigated. Oxidative stress had no effect on proteasome expression in liver epithelial cells [108] but did increase expression of proteasome subunits in neuroblastoma cells [87]. Repeated, mild heat shock resulted in significant upregulation of proteasomal catalytic activities and 11S subunit levels in human fibroblasts [118], but acute, mild heat shock led to decreased proteasome messenger RNA (mRNA) levels, inhibition of proteasome complex assembly, decrease in chymotrypsin-like activity, and reorganization of the intracellular distribution of proteasomes [119]. Overexpressing a constitutively active form of HSF1 in mouse embryonic fibroblasts (MEF) did not increase the level of proteasomal proteins expression or proteasomal activity [120]. When the ubiquitin-proteasome network is down-regulated, certain heat shock proteins such as HSP70, among other molecular chaperones, are induced [117]. Proteasome inhibition leads to the activation of all members of the heat-shock-factor family [121].

Molecular chaperones of the 70-kDa heat shock protein family (HSP70) are involved in protein folding, protein translocation, and protein degradation [122–124]. Other proteins are necessary for HSP70 function, and the protein BAG-1 associates with the proteasome in an ATP dependent manner and promote binding of constitutive heat shock protein 70 (HSC70) and HSP70 to the proteolytic complex. BAG-1 can apparently act as a coupling factor between the HSP70 chaperone system and the protein degradation machinery [125]. Also, the membrane-stabilizing effect of HSP70 and its related molecules are involved in lysosomal function. HSP70 can be efficiently endocytosed and localized to the lysosomes, and binding of HSP70 to a lysosome-specific lipid stabilizes lysosomes [126]. HSP70 has a positive effect on the lysosomal integrity by binding specifically to BMP [127]. In cancer cells, [126], Kirkegaard et al demonstrated that in acidic environments, HSP70 binds with high affinity and specificity to BMP, facilitates an activity of ASM, and thereby stabilizes lysosomes. HSP70 also serves cytoprotective roles as a guardian of the lysosomal membrane integrity by assisting sphingomyelin degradation or maintaining proper protein folding and recycling as a chaperone. However, calpain mediated cleavage of HSP70, especially after its

carbonylation due to oxidative stress can induce lysosomal rupture. Furthermore, HSP70 dysfunction activates nuclear factor-kappaB (NF- κ B) signaling that can also promote neurodegeneration [128].

Lysosomes are involved in protein degradation through autophagy. Autophagy is the cellular process that mediates the degradation of $\text{iA}\beta$, and studies in aging models have revealed that a failure of the autophagic system results in marked accumulation of abnormal proteins leading to dysfunction and cell death [107, 129]. Different types of autophagy have been described, depending on the mechanism that mediates the delivery of cytosolic cargo to lysosomes for degradation. The three main types of autophagy in mammals are macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA) [130–131]. CMA and proteasome defective cells maintain normal rates of long-lived protein degradation by up-regulating macroautophagy, the major form of autophagy. Macroautophagy is an active pathway for turning over $\text{A}\beta$ PP and generating $\text{A}\beta$ peptide [132]. Constitutive upregulation of macroautophagy is unable, however, to compensate for all CMA and proteasome functions [133–134]. Thus, this limitation leads to long term protein accumulation such as $\text{A}\beta$ plaque formation. In human neurons, serum starvation, which strongly induces autophagy, elevates $\text{A}\beta$ levels threefold [135].

The $\text{A}\beta$ generated in AVs is presumably delivered principally to lysosomes and degraded by cathepsins, which have the necessary cleavage specificity [136]. Cathepsin B deletion in mice, for example, elevates $\text{A}\beta$ levels in brain, whereas increasing cathepsin B expression has the opposite effect [137]. The presence of undigested proteins in lysosomes could be responsible for their impaired ability to fuse and/or degrade the autophagosome contents. A decrease in the lysosomal levels of the CMA receptor (LAMP-2A) is the primary defect responsible for the diminished CMA activity during aging [138]. Normal CMA activity is initially maintained (during middle age) by increasing the amount of luminal chaperone. At advanced ages, the levels of the receptor are so low that compensation by the chaperone is no longer possible [139].

CMA has shown to be defective in several neurodegenerative diseases, and it involves the facilitated direct entry into lysosomes of selected cytosolic proteins containing a chaperone-mediated autophagy-targeting motif [133]. This also denotes the existence of crosstalk among different forms of autophagy [134]. In CMA, cytosolic proteins containing a KFERQ motif are selectively targeted to the lysosomal lumen for degradation. Notably $\text{A}\beta$ PP and synuclein, a protein implicated in Parkinson disease, contain this targeting sequence [140] and impaired CMA-mediated degradation contributes to accumulation of $\text{A}\beta$ in AD [141].

Interestingly, the age-related FOXO attenuation leads to an increase in oxidative stress which causes an age-related lysosomal dysfunction. FOXO controls the SOD and catalase genes [142], so its attenuation during aging causes increased oxidative injuries. This oxidative stress damages the lysosome and may lead to macroautophagy inhibition if the result of sustained oxidative stress is a net compromise in lysosome function [143].

Translating invertebrate FOXO activity to an appropriate human analogue should be done with some care. Just as *Drosophila* APPL and *C. elegans* APL-1 are reflected in not a single

mammalian protein but a three-protein family, consisting of A β PP and A β PP-like proteins 1 (APLP1) and 2 (APLP2), the invertebrate FOXO has a four-member family homologue in mammals, consisting of FOXO1, FOXO3, FOXO4, and FOXO6, with both overlapping and distinct functions, and function of an individual FOXO family member in terms of neuroprotection can reverse depending upon circumstance [144]. The effects of individual family members may be counter our model. For example, FOXO1 expression is positively correlated to AD and associated cognitive decline in humans [145]. Likewise, exclusion of FOXO3 from the nucleus due to calorie restriction attenuated memory loss and other deficiencies in the AD transgenic mouse model A β PP_{SWE}-Tg2576 [146]. On the other hand, FOXO3 protects quiescent cells from oxidative stress [142], and it is our contention that it is not FOXO3's nuclear activity but its cytosolic or extracellular activities that would be neuroprotective. Likewise, while nuclear FOXO3 is apoptotic, and overall restriction of FOXO3 levels attenuates the BACE1 processing pathway [146], it may be an oversimplification to equate A β with AD, particularly since A β neurotoxicity is as much a factor of its tertiary and quaternary structures as its mere presence. Likewise, A β has been found to potentially have multiple non-pathogenic functions, including acting as a transcription factor [147]. The specific biochemical stages of "pre-AD" have not been adequately elucidated, and relative contributions of A β , oligomeric A β , and amyloid plaque to the disorder are not yet characterized.

Decreased autophagic activity is associated with neurodegenerative disorders, such as Parkinson's [148] and AD [149]. In addition to the well-documented lysosomal dysfunction in AD [150], a decrease in autophagy has recently been found [132]. The hallmark feature of AD pathology is the presence of enormously swollen "dystrophic" neuritis in which autophagic vacuoles (AV) progressively accumulate and become the predominant organelle [132]. In normal brain cells, autophagosomes are usually found in low numbers. However, in a large number of brain cells from AD patients, autophagosomes are very prominent. In AD brains, diminished autophagosome-lysosome fusion results in accumulation of A β -filled autophagosomes, which constitute a major intracellular storage of the toxic protein [151]. This probably indicates activation of autophagy but with impaired maturation and/or impaired autophagosome-lysosome fusion [152], which eventually leads to apoptosis. Excessive generation of A β in AD brain cells is also driven by increased levels of the γ -secretase complex within autophagosomes [153]. eA β can also be cleared from the brain through interaction with lipoprotein receptors [154]. ApoE, a lipid transporter, also has a role in the clearance of A β from the brain, and ApoE ϵ 4 is shown to be less effective, compared to ApoE ϵ 2 and ApoE ϵ 3 variants [155].

Conclusions

Although the current model of AD views A β peptide accumulation as etiologic and suggests that abnormal accumulation of A β ₄₂ is an early event in the pathophysiologic cascade, we would like to add that age-related attenuation in the HSF1 and FOXO pathways play an even earlier role than A β accumulation and proteotoxicity respectively in the predisposition to LOAD (Fig. 3). Recently, it has been shown that the clearance of A β ₄₂ is in fact the primary inciting event in LOAD. Both autopsy and biomarker studies similarly suggest that A β ₄₂ accumulation increases with advanced aging, the greatest risk factor for developing LOAD.

Therefore the aging process is required for the development of LOAD. The initial event in the aging process should be correlated to the initiation of LOAD, first initiated by the attenuation of HSF1 leading to decreased degradation of A β , facilitated by decreased chaperone production, CMA degradation, and proteasome activity. This will cause increased iA β levels which will activate autophagy systems. At this stage we can see increased AV in pathologic studies.

This increase in autophagy cannot be sustained and the iA β is released into the extracellular space (ECS). In the ECS A β forms toxic oligomers, but the activation of certain FOXO family members would convert these oligomers in non-toxic plaques. This process explains the temporary lag between plaque formation and clinical symptoms. As long as certain of the FOXO family pathways prevent the formation of oligomers, there would be no AD clinical symptomatology. Later, attenuation (e.g., age-related) of appropriate FOXO family members would appear, and clinical symptoms develop.

Longevity pathways are crucial to the development of AD. A β clearance and antiproteotoxic pathways have been unequivocally associated with LOAD. However, the complex features of LOAD, especially its convoluted molecular biology and (epi)genetics, have prevented the resolution of the age-related specific associations within AD. In order to clarify the role of the aging process on the A β clearance and antiproteotoxic pathways, we postulate a new model in which the aging process would precede A β accumulation, and attenuation of HSF1 is an “upstream” event in the cascade that results in excess iA β and synaptic dysfunction, which may lead directly to cognitive impairment and/or trigger “downstream” neurodegeneration and cell loss. Specific host factors, such as the activity of FOXO family pathways, would mediate the response to A β toxicity and the pace of progression towards the clinical manifestations of AD.

As a result of these observations, a genetic and molecular model is proposed here, in which assessment of the activity of the HSF1 and FOXO3 (as a suggested family member) would give information of sequential preclinical biomarkers of AD pathology. This biomarker model parallels the pathomolecular sequence of events in AD, and is particularly relevant to tracking the preclinical stages of LOAD of patients at high risk for developing this disease.

Any preclinical biomarkers should be complemented by clinical biomarkers of brain A β amyloidosis, such as reductions in CSF A β ₄₂ and increased amyloid PET tracer retention. Elevated CSF τ is thought to be a biomarker of τ -mediated neuronal injury and is not specific for AD pathology [156]. At a more advanced disease, a decreased [¹⁸F]-fludeoxyglucose (FDG) uptake on PET in a characteristic temporal-parietal pattern is a biomarker of AD-related synaptic dysfunction [14]. When neurodegeneration is initiated, brain atrophy on structural MRI in a characteristic pattern involving medial temporal, paralimbic and temporal-partial isocortex serves as a biomarker of AD-related neurodegeneration.

This new preclinical biomarker model has the following features: 1) HSF1-DNA binding attenuation would risk abnormality due to effects of the aging process; 2) A β oligomer and amyloid plaque biomarkers would become abnormal, and a substantial A β load accumulates

prior to the appearance of clinical symptoms. So far as this model of brain amyloid deposition is necessary but not sufficient to produce the clinical symptoms of MCI and dementia, it requires an additional step to produce clinical symptoms, specifically 3) Attenuation of FOXO family pathways causing A β oligomer formation and neurodegeneration; 4) Finally, biomarkers of dysfunction, neuronal injury, and degeneration would be confirmatory later in the disease. The severity and change over time in these biomarkers correlate with clinical symptoms. Structural MRI would be the last biomarker to become abnormal.

Definitive studies to determine if asymptomatic individuals with HSF1 and FOXO family pathway attenuation associated with A β accumulation are indeed destined to develop AD dementia, and whether new therapies targeting these pathways will prevent cognitive decline are a needed strategy to prevent this epidemic disease.

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Fig. 1. Intermolecular Effects of HSF and FOXO3 in Non-Pathogenic Aβ Metabolism

Schematic representation of interlocking roles for heat shock factors and FOXO3 in Aβ metabolism. Healthy cell. HSF, HSP, and FOXO3 are all at normal expression and activity. A minority of AβPP is processed via the β-secretase pathway, producing Aβ, at least some of which is directed intracellularly for functions such as transcription factor activity. HSF binds to HSP and is activated. Active HSF (*) is directed to the nucleus where it upregulates (among others) HSP expression. HSP activation (HSP90) directs excess intracellular Aβ toward proteasomes, where it is degraded to amino acids (AA). FOXO3, among its multiple functions, catalyzes formation of amyloid plaque from Aβ. In addition, extracellular non-plaque Aβ is cleared by enzymes such as insulin-degrading enzyme (IDE).

Fig. 2. Intermolecular Effects of HSF and FOXO3 in Pathogenesis of AD

A) Initial breakdown of HSF processes. HSF activity is reduced due to age or environmental stressors. Part of the result is the reduction of the feedback loop that provides HSP90 for intracellular Aβ degradation. Excess intracellular Aβ is able to aggregate into toxic oligomers, producing reactive oxidizing species (ROS). **B)** "Recruitment" of FOXO3 into the cytotoxic cascade. ROS stimulates FOXO3, directing it to the nucleus, wherein it participates in apoptosis. Redirection of FOXO3 reduces production of plaque, permitting greater formation of toxic extracellular Aβ oligomers and interfering with enzymatic clearance of eAβ, permitting plaque buildup even though overall plaque catalysis has been reduced. Combination of neuronal cell death and extracellular events lead to clinical AD symptoms.

Table 1

Interlocking Age-Related Effects of HSF1, FOXO3, Aβ, and AD Risk

Age Group	Years	HSF1-DNA binding	FOXO3 ^a		Aβ		AD Risk
			cyto	nucl	plaque	oligo	
Adult	20–40						
Mid Life	30–50	↓			↑		
Late Mid Life	40–60	↓	↓	↑	↑	↑	
Early Old	50–70	↓↓	↓	↑	↑↑	↑↑	↑
Middle Old	60–90	↓↓↓	↓↓	↑↑	↑↑	↑↑	↑↑
Oldest Old	90+	↓↓↓	↓↓	↑↑(↑) ^a	↑↑(↑)	↑↑(↑)	↑↑(↑)

^a Location of FOXO3 would be either cytoplasmic (cyto—neuroprotective) or nuclear (nucl—neurodegenerative)
^b Aβ forms of note would be either plaque or higher-toxicity oligomers. Monomeric Aβ is not presented in this table.
^c Prevalence of AD may level off among the oldest old [157].