REVIEW

Unraveling the complexity of neurodegeneration in brains of subjects with Down syndrome: Insights from proteomics

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Down syndrome (DS) is one of the most common genetic causes of intellectual disability characterized by multiple pathological phenotypes, among which neurodegeneration is a key feature. The neuropathology of DS is complex and likely results from impaired mitochondrial function, increased oxidative stress, and altered proteostasis. After the age of 40 years, many (most) DS individuals develop a type of dementia that closely resembles that of Alzheimer's disease with deposition of senile plaques and neurofibrillary tangles. A number of studies demonstrated that increased oxidative damage, accumulation of damaged/misfolded protein aggregates, and dysfunction of intracellular degradative systems are critical events in the neurodegenerative processes. This review summarizes the current knowledge that demonstrates a "chronic" condition of oxidative stress in DS pointing to the putative molecular pathways that could contribute to accelerate cognition and memory decline. Proteomics and redox proteomics studies are powerful tools to unravel the complexity of DS phenotypes, by allowing to identifying protein expression changes and oxidative PTMs that are proved to be detrimental for protein function. It is reasonable to suggest that changes in the cellular redox status in DS neurons, early from the fetal period, could provide a fertile environment upon which increased aging favors neurodegeneration. Thus, after a critical age, DS neuropathology can be considered a human model of early Alzheimer's disease and could contribute to understanding the overlapping mechanisms that lead from normal aging to development of dementia.

Keywords:
Brain / Down syndrome / Neurodegeneration / Oxidative stress / Proteasome

1 Introduction

Down syndrome (DS) is the most common chromosomal abnormality with an estimated 70–80% prenatal lethality and an incidence of 1:700 births. This syndrome results from the presence of an extra complete or segment of chromosome 21 and is termed trisomy 21 (Ts21) [1]. The third chromosome is predominantly of maternal origin [2]. This genetic defect is responsible for the specific congenital phenotypes such as craniofacial abnormalities, small brain size, accelerated aging, and cognitive decline. The chromosomal region between the carbonyl reductase (CBR) and transcriptional regulator ETS (E26 transformation specific) related gene loci is described as the DS critical region (DSCR) and is likely the major determinant of

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Abbreviations: Aβ, amyloid beta; **AD**, Alzheimer's disease; **AF**, amniotic fluid; **APP**, amyloid precursor protein; **Cα**, collagen alpha; **CAT**, catalase; **CBR**, carbonyl reductase; **CSTB**, cystatin B; **DS**, Down syndrome; **DSCR**, Down syndrome critical region; **ES**, embryonic stem; **ETS**, E26 transformation specific; **GPX**, glutathione peroxidase; **4-HNE**, 4-hydroxy-*trans*-2-nonenal; **13-HPODE**, 3-hydroperoxy-9Z,11E-octadecadienoic acid; **MMP2**, matrix metalloproteinase 2; **OS**, oxidative stress; **SOD**, superoxide dismutase; **SP**, senile plaques; **UCH-L1**, ubiquitin-carboxy-hydroxyl lyase 1

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multiple neurological features of DS [3]. However, genes outside the DSCR can also be involved and contribute to explain the wide variability of DS phenotypes [4]. Two major hypothesis have been proposed: (i) the "gene dosage hypothesis" is based on the fact that the increased dosage of Chr21 genes is the direct cause of the phenotypical alterations of DS [5–7]; (ii) "the amplified developmental instability hypothesis" suggests that the variability of phenotypic features may become much more complex when considering the effects of overexpression of trisomic genes on dysomic genes, which, in turn, may gain aberrant expression and contribute to some clinical manifestations [7]. The integrated view of these two hypothesis results in a dysregulated scenario in which a subset of dosage-sensitive genes are consistently amplified and lead to different phenotypic features.

The major neurological deficits that afflict DS individuals are intellectual disability and neuropathological changes leading to an early onset of Alzheimer's disease (AD) [8, 9]. Numerous cellular and systemic abnormalities in the DS nervous system have been reported, but other unknown factors could contribute to the range of neurological changes in DS [10].

Considerable evidence demonstrates the link between the DS phenotype and an increased risk of development of AD [11]. The incidence of dementia among DS patients is 8% in the age range 35–49 years, 55% in the age range 50–59 years, and 75% above the age of 60 years, but AD neuropathology is present in all DS individuals by the age of 40 [12]. Senile plaques (SPs) and neurofibrillary tangles, the pathological hallmarks of AD, and also cholinergic and serotonergic reduction [13, 14], have been detected in DS brain. However, although depositions of amyloid beta (Aβ) plaques have been observed in young DS individuals, even in the fetus [15, 16], clinical manifestation of dementia clearly is manifested many years later.

2 Oxidative stress (OS) in DS: Implications for development of AD

Several studies demonstrated the involvement of OS in accelerated senescence and in neuropathology, characteristic features of DS [17, 18]. Different markers of oxidative damage are elevated in brain tissue from DS [19, 20]. It is likely that increased OS in the fetal stage can modify processes such as neurogenesis, differentiation, migration as well as survival [7, 12, 13]. In later life stages, OS is mainly involved in the neuropathology and may contribute to the development of AD in DS patients [108–110]. Interestingly, the cause of increased OS conditions has to be searched by mapping Chr21, where a number of genes, directly or indirectly, lead to overproduction of ROS, enhanced oxidative damage, and possibly ROS-induced cell death. Among the most relevant contributory factors to OS, CuZn superoxide dismutase (SOD1), amyloid precursor protein (APP), the transcription factor ETS-2, S100B, CBR all map on Chr21. SOD1 is one of the major enzymes of the antioxidant family to protect against ROS, through the dismutation of $O_2^{\bullet -}$ to O_2 and H_2O_2, the latter in turn neutralized by catalase (CAT) and by glutathione peroxidase (GPX) to water [21]. Nevertheless, both CAT and GPX are generally expressed at lower levels compared with other tissues [22] and this may account for reduced antioxidant defense in DS. Thus, an imbalance between the first and second steps results in accumulation of H_2O_2, increased hydroxyl radical formation that damages membrane lipids, proteins, and nucleic acids. ROS play an important role in cell death induced by different stimuli by activating death receptor pathway, p53 and also the mitochondrial death pathway [12, 13, 17, 23–26]. Accordingly, SOD1 levels are approximately 50% higher than normal in a variety of DS cells and tissues, including erythrocytes, B and T lymphocytes, and fibroblasts. The systemic increase of SOD1 is also accompanied by an increase of SOD1/GPX or the SOD1/(GPX + CAT) activity ratio in erythrocytes from DS children, adolescents, and adults [27]. The fact that SOD1 overexpression may play a key role in the cellular homoeostasis has been demonstrated by Shin et al., which found that transgenic mice overexpressing wild-type human SOD1 (Tg-SOD1) displayed mitochondrial alterations (swelling and vacuolization), and learning and memory deficits [28].

In line with these studies, neurons of DS patients are exposed to high intracellular ROS, which in turn induce lipid peroxidation [23]. However, as also shown by a proteomics study from Gulesserian et al. [29], OS in fetal DS is not only a consequence of SOD1 overexpression, which alone cannot explain the generalized increase of OS markers, but appeared to be exacerbated by low levels of antioxidant enzymes, such as glutathione transferases and thioredoxin peroxidases.

One of the genes triplicated in DS and animal models is that codifying the APP. As expected, in DS individuals, the increased expression of this gene leads to increased production of Aβ peptide [30–32], the major component of amyloid plaques found in all DS individuals over 40 years of age. Both the levels of Aβ(1–42) and Aβ(1–40) were higher in DS plasma than controls [33] and the ratio of Aβ42/Aβ40 was lower in DS than in controls. Recently, the same group demonstrated that among adults with DS, decreasing levels of plasma Aβ42, a decline in the Aβ42/Aβ40 ratio, or increasing levels of Aβ40 may be putative markers of conversion to AD, possibly reflecting compartmentalization of *A*β peptides in the brain [34]. To better understand the toxic role APP, recent studies from Anandatheerthavarada et al. [35] showing that full-length APP may be neurotoxic, mostly at the mitochondrial level, should be discussed in this context. Further support to this "revisited APP theory" came from evidence showing that mice overexpressing wild-type human APP develop neuronal pathology similar to AD, but without robust Aβ deposition in the hippocampus [36]. Overexpression of APP may promote mitochondrial dysfunction independently of aberrant Aβ deposition.

S100B, an astroglial-derived Ca^{2+}-binding protein acting as a neurotrophic factor on neurons and glial cells, is also encoded on Chr21. S100B is involved in the regulation of energy metabolism in brain cells by stimulating the enzymatic activity of fructose-1,6-bisphosphate aldolase and phosphoglucomuatse [37]. It modulates the proliferation and differentiation of neurons and glia, and it interacts with many immunological functions of the brain. S100B exerts a protective effect as long as its intracellular concentration is at physiological levels. However, once secreted, its local concentration dictates its beneficial or detrimental effects. At nanomolar concentrations neuroprotective effects prevail, while at micromolar concentrations neurodegenerative or apoptosis-inducing effects are observed [37, 38]. In both DS and AD, astrocytic S100B is expressed at high levels, and the increased expression correlates with the accumulation of neuritic plaques across brain regions in AD [39, 40]. This strong correlation found between numbers of activated S100B-positive astrocytes and the numerical density of amyloid plaques supports the idea that S100B is an important element in the accumulation of plaques in DS and AD. It has also been shown that Aβ stimulates the synthesis of both S100B mRNA and S100B protein in astrocyte cultures [41]. It is likely that chronic overexpression of S100B promotes increased neuronal and neuritic βAPP expression with consequent increased amyloid deposition, as well as abnormal growth of neurites in Aβ plaques, as seen in middle-aged DS patients [39].

ETS-2 is a transcription factor with important functions in cancer biology, bone development, and immune response. ETS-2-dependent transcriptional activity is initiated by OS and it is involved in differentiation, maturation, and signaling cascade [24]. The major outcome of ETS-2 overexpression is the activation of neuronal apoptotic cell death and this specific pathway seems to be particularly relevant to explain the reduced incidence of solid tumors occurring in DS individuals [42]. Conversely, overexpression of ETS-2 has been hypothesized to be an important contributor to the increased susceptibility of DS cells to apoptotic stimuli that might, at least in part, be responsible for the thymic and splenic hypoplasia and conceivably other pathophysiological features shared between ETS-2 transgenic mice and individuals with DS [43].

By mapping Chr21, another candidate gene that may be involved in OS is that codifying the enzyme CBR. Indeed, this enzyme catalyzes the reduction of free carbonyl compounds to their corresponding alcohols. Protein carbonyls, including reactive aldehydes such as HNE, can also be detoxified by aldehyde dehydrogenase, which catalyzes their oxidation to carboxylic acids. Protein levels of both these enzymes were found to be increased in different brain regions of both DS and AD patients, indexing the cell response to increase carbonyl production [44]. Further, our group demonstrated that CBR is an oxidatively modified protein in brains of subjects with amnestic mild cognitive impairment [45].

3 Oxidized proteins and degradative systems: The route toward neurodegeneration?

Accumulation of OS products and insoluble protein aggregates, such as Aβ and other oxidized proteins, results from increased production and/or reduced efficiency of protein turnover systems [46]. There are different pathways for general protein degradation, including lysosomal proteases, calcium-dependent proteases, mitochondrial proteases, and the proteasomal system [46]. However, the majority of intracellular proteins (more then 70%) are degraded via the intracellular proteasomal system [47], whereas exogenous proteins, which enter the cell by endocytosis, and a number of intracellular proteins are degraded largely within lysosomes. Degradation into the lysosomes seems to be nonspecific [46]. In contrast, proteasomal degradation is characterized by a high degree of specificity toward its substrates through the activity of highly specific enzymatic system, such as ubiquitin ligases.

Protein degradation is predominately catalyzed by the proteasome, which is responsible for the clearance of defective, denatured, or in general damaged proteins as well as for the regulated degradation of short-lived proteins [48]. The 26S proteasome is a large protease complex composed of a catalytic 20S subunit (20S core particle) and a 19S regulatory particle that caps one or both ends of the 20S proteasome [49, 50]. The assembly and activity of the 26S proteasome is tightly regulated by a large number of loosely associated proteins that function as regulators or cofactors [51, 52]. The 20S proteasome contains four heptameric rings in a barrel-like structure localized in both cytoplasm and nucleus. It is composed of two sets of 14 different subunits and has a molecular mass of 670–700 kDa [53, 54]. The subunits form a cylinder of four rings, each containing seven subunits. It has three proteolytic centers, in each, half located within a hollow cavity of the cylinder, showing either peptidyl-glutamyl peptide hydrolyzing, trypsin-like, or chymotrypsin-like activity [55].

In order to be recognized by the proteolytic complex, protein substrates usually undergo a covalent modification, known as ubiquitination. This chemical modification requires the covalent attachment of an 8.6-kDa protein, ubiquitin, to the epsilon-amino group of a lysine residue of the substrate protein [56]. A number of enzymes participate to ubiquitination, namely activating enzyme (E1), a carrier/conjugating enzyme (E2), and conjugating enzymes (E3) [57]. Ubiquitin can also be covalently attached to itself to form an ubiquitin chain on a substrate protein that is then targeted for degradation by the 26S proteasome. A direct interaction of some subunits of the proteasome with the multiubiquitin chain facilitates the proteolytic degradation of polyubiquitinated proteins [58]. However, removal of ubiquitin is also necessary to allow it to recycle and specific deubiquitinating enzymes are responsible for this step [59]. Recycling of ubiquitin is essential for the function of the

proteolytic machinery and is achieved by ubiquitin C-terminal hydrolases, ubiquitin isopeptidases, or deubiquitinating enzymes.

Interestingly, a defective protein ubiquitination could result in reduced intracellular protein degradation, also in the presence of adequate proteasome activity. Several changes in intracellular protein ubiquitination with age have been reported, showing that aged cells have less free ubiquitin and more ubiquitin-protein conjugates than young cells [60]. The fact that changes in protein ubiquitination do not always indicate changes in protein degradation rates may reflect the participation of ubiquitin in intracellular processes other than protein degradation [61]. It is likely that conjugation with ubiquitin competes with other types of PTMs among which phosphorylation, acetylation, as in the case of p53, are fundamental for its activation [62].

In order to prevent protein aggregation, oxidized proteins have to be efficiently degraded. Therefore, specific systems are required to both recognize and degrade damaged/misfolded proteins. The proteasomal system is the major proteolytic system responsible for the removal of oxidized proteins. Since one of the main functions of the proteasome is the removal of oxidatively damaged proteins, proteasomal activity is regulated by OS. After protein exposure to oxidants, increased susceptibility to proteolytic attack by various proteases is well documented [63]. Thus, oxidation processes correlate with intracellular proteolysis [64]. However, oxidized proteins accumulate within cells if oxidative damage is faster than the rate of proteolysis. It is generally accepted that intracellular protein turnover declines during aging, while oxidatively modified and damaged proteins accumulate [65–70]. This accumulation of oxidatively modified and ubiquitinated proteins and the general decline in protein turnover have raised the possibility that proteasome function is impaired with age. The aggregates thus formed are called plaques, aggresomes, age pigments, or Lewy bodies, depending on their composition and location. Changes in proteasomal function have also been observed in senescent cells, whereas proteasome inhibition in young cells induces premature senescence [71].

DS fetal brains have a selective upregulation of the proteasome zeta chain and isopeptidase T [72]. Very recently, our group demonstrated using redox proteomics that ubiquitin-carboxy-hydroxyl lyase 1 (UCH-L1) is a target of oxidative damage in DS brains, with a reduction of its enzymatic activity [73]. Indeed, UCH-L1 is responsible for recycling of ubiquitin through hydrolysis of peptide-ubiquitin bonds and processing of ubiquitin precursors, but it also has ubiquitin ligase activity [40]. We suggest that aberrant ubiquitin hydrolase and/or ligase activity for the identified oxidative modifications of UCH-L1 might lead to dysfunction of the neuronal ubiquitination/deubiquitination machinery, causing synaptic deterioration and neuronal degeneration in DS as well as demonstrated in AD brains [41–43]. In parallel, we found increased expression levels of the 20S subunit; however, the trypsin-like, chymotrypsin-like, and caspase-like activities were decreased in DS brain compared with age-matched controls. Increased expression may be a signal to activate protein degradation in affected neurons to prevent accumulation of toxic aggregates, but at the same time the enzyme is target of oxidative damage and its function is impaired and the total proteasome activity reduced.

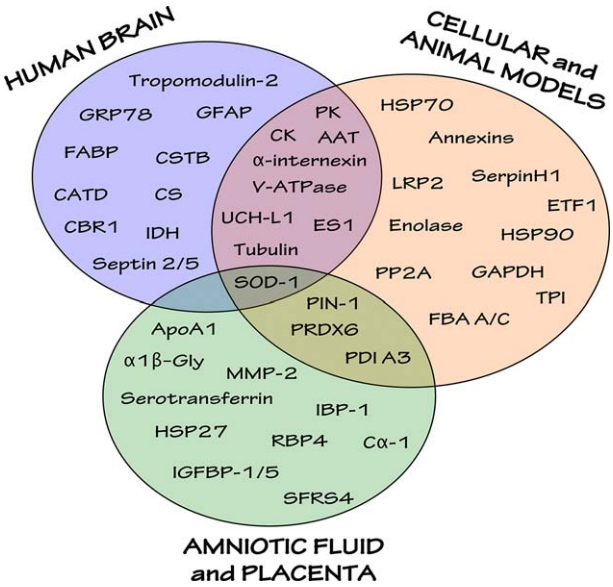

Figure 1. Venn diagram comparing the proteins identified with altered expression or increased oxidation by proteomics analysis of DS samples from different source (human brain, cellular and animal models, and AF and placenta). The proteins in the overlapping space are common to more than one study.

Though based on limited human samples, our findings together with results from other groups suggest that DS brain is characterized by increased OS combined with defective degradative systems, which in turn results in further accumulation of toxic protein aggregates. These mechanisms may play a crucial role in the development of Alzheimer-like dementia in DS population (Fig. 1).

4 Proteomics on human brain from DS individuals

Proteomics studies on DS samples started in 2000 with the work by Opperman et al. [74], which employed 2DE separation to analyze fetal brain samples. The first goal of this study was the production of a 2D map of the most abundant proteins from fetal human brain. The comparison of the expression profiles of DS and control subjects showed an 84% homology in protein expression between the two groups, with the most consistent changes in the expression pattern for β-tubulin, α- and β-actin. All the proteins found to be differentially expressed possess a structural role suggesting alteration of brain development during DS, consistent with some of the characteristic DS phenotypes.

By this first report, Lubec and co-workers initiated a comprehensive proteomics analysis of fetal DS brain [75] that

allowed identification and quantification of ten protein spots with different expression in DS versus control brain. Among these, hypothetical protein DKFZp564D177.1-human (fragment) and septin 6 showed decreased expression in DS. These proteins could be involved in the defective development of fetal DS brains. The other eight proteins (WD repeat protein 1, novel protein highly similar to septin 2 homolog, septin 5, septin 2, DJ37E16.5, hypothetical 30.2-kDa protein, neuronal protein NP25, and DC7 protein vacuolar sorting protein 29) did not reach significance between control and DS samples.

In 2002, the same group, using proteomics techniques, evaluated the protein expression levels of several enzymes involved in different metabolic pathways of intermediary metabolism in fetal DS and control brains [76]. The researchers demonstrated alterations of energy metabolism pathways as indexed by increased expression of mitochondrial aconitase and mitochondrial NADP-isocitrate dehydrogenase, and decreased expression of citrate synthase, of protein metabolism as indicated by decreased levels of aspartate aminotransferase, and of carbohydrate metabolism suggested by the increased expression of pyruvate kinase M1/M2. The authors suggested that brain intermediary metabolism is deranged during prenatal development of DS [76]. In 2004, the Lubec group applied 2DE and MALDI-MS to analyze the number of differently expressed spots between fetal DS and normal brain [77], and the authors identified three proteins encoded on Chr21: cystathionine-β-synthase, pyridoxal kinase, and ES1 (ES is embryonic stem) protein homolog, mitochondrial precursor. Among these, only ES1 showed a significant increase in DS.

In a following study in 2006 [78], Lubec and co-workers successfully identified nine proteins encoded on Chr21 with different expression levels between DS and control fetal brain: pyridoxal kinase; SOD1; CBR1; ES1; cystathionine-β-synthase; T-complex protein 1, theta subunit; cystatin B (CSTB); 6-phosphofructokinase, liver type; and glycinamide ribonucleotide synthetase. Some of the proteins identified were already found to be altered in the 2004 study [77], while three of these (CSTB, 6-phosphofructokinase, liver type; and glycinamide ribonucleotide synthetase) were newly identified in human fetal brain and might may play an important role in fetal brain development. Interestingly CSTB inhibits cathepsin B and blocks apoptosis; indeed, mice with a gene deletion of CSTB exhibit increased apoptosis of specific neurons [79]. Moreover, increased levels of cathepsin B with colocalization in SPs have been observed in brains of adult DS individuals and AD [80].

More recently, reports from Lubec laboratory [81] aimed to complement the previous studies on the fetal DS partial proteomes or altered expression levels of individual proteins that may play a role for the abnormal development of the DS brain. The major outcome of their work was to show altered protein pathways and cascades possibly involved in the pathological mechanisms of fetal DS brain development, before morphological changes are detectable. Their findings showed increasing levels of tropomodulin-2, and fatty acid binding protein in DS, and decreasing levels of tubulin α-1A chain, α-internexin, creatine kinase B type, RNA-binding protein K-Cl solute carrier 7 family 12, and retrotransposon gag domain containing protein 1. Proteins found to be deregulated were not previously observed; however, this novel pattern of alterations is consistent with previous studies that demonstrate that aberrant expression of proteins leads to the impairment of specific functions in DS such as synaptic plasticity, brain development, and energy metabolism, directly involved in DS pathology [74, 76–78].

Very recently, our laboratory focused its work on the study of protein carbonylation in cerebral cortex of young DS subjects (±24 years old) compared to age-matched controls [73]. We employed a redox proteomics approach obtaining data about specific protein oxidation and its effects on protein function. Our study aimed to shed light on molecular pathways perturbed by OS, which may play a key role in the neurodegenerative phenomena occurring in DS. Our analyses showed increased carbonylation (normalized to expression levels) of six proteins identified by MS/MS analysis as UCH-L1; cathepsin D; 78-kDa glucose-regulated protein 78; V0-type proton ATPase subunit B, brain isoform; glial fibrillary acidic protein; and succinyl CoA:3-ketoacid-coenzyme A transferase 1 mitochondrial. Interestingly, the majority of these proteins are members of the intracellular quality-control system including glucose-regulated protein 78; UCH-L1; cathespin D; V0-type proton ATPase subunit B, brain isoform; and glial fibrillary acidic protein. Our proteomics findings suggested that chronic exposure to OS might participate to impair the proteostasis network, which is fundamental in maintaining correct protein homeostasis. In support to this hypothesis, we measured proteasome activity and autophagic flux, demonstrating the reduced functionality of both these degradative pathways that may eventually contribute to Aβ deposition and tau hyperphosphorylation. Both of these phenomena are already present in DS brain at a young age [17, 82]. Thus, it is reasonable to suggest a close relationship between OS and protein misfolding in DS brain, implying that these events might be central for the increased onset of AD in DS subjects.

5 Proteomics on animal and cellular models of DS

The first proteomics study on a DS model, by Kodota et al., was performed in 2004 on TT2F mouse ES cell lines containing a single hChr21 (TT2F/hChr21) [83]. The chimeric mice generated from TT2F/hChr21 cells presented a wide variety of phenotypic traits of human DS, including impairment in learning, emotional behavior, hypoplastic thymus, and cardiac defects [84]. The authors applied 2DE separation to describe the distinctive proteomic signatures between TT2F and TT2F/hChr21. Eighteen significantly altered proteins in TT2F cells with an extra hChr21 were identified. Among these proteins, SOD1 and CCT8, encoded on Chr21, were already found aberrantly expressed on human

brain studies [78, 85]. Of the other remaining proteins, six of them were matrix and structural proteins (annexin 4; plastin-3 T-isoform; keratin complex 2; Vil2, microtubule-associated protein RP/EB 2; and calponin 3); three were heat shock/stress proteins (HSP84–1, HASP70, and HSP86–1); three were degradation proteins or translational regulators (UCH-L1, eukaryotic translation elongation factor, and ubiquitin thioesterase); two were nuclear transcriptional factors (heterogeneous nuclear ribonucleoprotein H1 and heterogeneous nuclear ribonucleoprotein); and two were enzymes for energy and macromolecular metabolism (vacuolar ATPase subunit a isoform 1 (ATP6v1a1) and vacuolar ATPase subunit b isoform 2 (ATP6v1b2)). To better understand the expression patterns of these altered proteins throughout neuronal differentiation, the corresponding spot intensities in the 2DE gel in TT2F and TT2F/hChr21 cells at day 0 (D0), day 3 (D3), day 6 (D6), and day 10 (D10) of differentiation were also analyzed. Both protein subunits Atp6v1a1 and Atp6v1b2 of the vacuolar ATPase proton pump, which mediate acidification of intracellular organelles for energy production and convention, as well as autophagy, were overexpressed. Three proteins, ubiquitin thioesterase, Eef1D, and UCH-L1 involved in protein catabolism or translation regulation were underexpressed. HSPs, HSP84–1, HSP70, and HSP86–1, demonstrated a stage-specific suppression on D0, D3, and D6, respectively. However, HSP84–1 protein expression did not change significantly in TT2F/hChr21 cells between D3 and D6. Splicing regulatory elements, heterogeneous nuclear ribonucleoprotein and heterogeneous nuclear ribonucleoprotein, displayed contradictory expression patterns of overexpression and underexpression, respectively. Actin-related (plastin-3 T-isoform and Vil2), intermediate filament (keratin complex 2), and phospholipid-related (annexin 4) cytoskeleton proteins were overexpressed across all stages of differentiation, whereas microtubule-related (Vil2, microtubule-associated protein RP/EB 2) and calmodulin-related (calponin 3) architectural proteins were underexpressed. Interestingly, results concerning HSPs, UCH-L1, and vacuolar ATPase are consistent with human data [73, 86], supporting the involvement of protein unfolding/misfolding, the proteostasis network, and energy metabolism in the altered developing DS brain.

In 2006, a study from the Lubec laboratory [87] analyzed the quantitative variations of proteins in WT mice and the 141G6 mouse model of DS. 141G6 mice were generated, by inserting yeast artificial chromosomes containing a fragment of the human DSCR-1 region into the murine genome. Two-dimensional proteomics analyses demonstrated that expression levels of a series of identified proteins were significantly altered in 141G6 mice. Among these, interesting results were obtained with regard to α-and β-tubulin, HSP60 and 90, peptidyl-prolyl cis-trans isomerase A, aspartate aminotransferase, ATP synthase, V_0-ATPase, creatine kinase, fructose-bisphosphate aldolase, γ enolase, phosphoglycerate kinase 1, pyruvate kinase, isozyme M2, dihydropyrimidinase-related protein 1 (also called CRMP2), dihydropyrimidinase-related protein 4, laminin receptor, UCH-L1, voltage-dependent anion channel 1–2, ES1 protein homologue, electron-transfer flavoprotein, a part of complex I of the respiratory chain, nuclear ribonucleoprotein A2/B1. The above-listed proteins correlate with results obtained in reports on human brain and DS animal models [73,83,86], suggesting aberrant expression of proteins belonging to antioxidant response, chaperone system, cytoskeleton, proteostasis network, and metabolic pathways, processes implicated in neurodegeneration and cognitive decline known to occur in DS. Moreover, the results obtained in this study are consistent with alterations observed in AD brain [88], supporting once again the concept that DS and AD share some common mechanisms of alterations linked both by genetic and biochemical similarities that translate into protein dysfunctions [82].

In 2009, Wang et al. [89] performed a proteomic study on Tc1 mice embryonic stem cells. The Tc1 DS model was constructed by introducing a single supernumerary human chromosome 21 into a mouse embryonic stem cell. It reproduces a large number of DS phenotypes including heart defects, learning difficulties, and a reduced cerebellar neuron count [90]. Using iTRAQ and absolute quantification, 52 proteins were identified to be differently expressed at least by greater than two SDs from the mean when an extra human Chr21 was present. A group of 15 proteins from this total were downregulated, and 37 showed higher expression in DS cells compared to parental cells. Among the proteins identified, several have direct associations with DS and the extra copy of Chr21 such as DSCR1, DSCR3, DSCR5, TIAM1, TTC3, DYRK1A, and APP [91, 92]. Other proteins are associated with the premature onset of AD. Indeed, alterations in CTSB, LRP2, and LRPAP1 expression levels are consistent with previous studies on Aβ formation and clearance in AD [80, 93, 94]. Overall, this study demonstrates the high correlation between expression differences occurring in embryonic stem cells from mouse and human DS fetal tissue. Thus, as the authors suggest, changes that are determined in embryonic stem cells of DS persist in adult life and are most likely involved in DS phenotypes.

In the same year, Fernandez et al. [95] analyzed the protein composition of synapses from Ts65Dn, a mouse model of DS. This model contains 50% of the genes homologous for HSA21 in three copies, exhibits craniofacial skeletal malformation and reduced cerebellar volume and granular and Purkinje cell densities. TS65Dn mice also display learning and behavioral deficits [96]. The authors employed synaptosomes or PSDs from the Ts65Dn cerebrum and evaluated synaptic protein profiles via two quantitative methods: Odyssey-based fluorescence Western blotting or iTRAQ technique. Results on synaptosomal fraction showed only modest changes in protein expression: increased levels of synaptojanin and decreased levels of ERC1/CAST2/ELKS, the PSD proteins PSD-95 and CaMKIIa, as well as the a1 subunit of the GABA receptor. In PSD preparations from Ts65Dn mice, the few synaptic proteins found to exhibit slight changes included Munc13, fragile X mental retardation protein, the b4 subunit of the voltage-dependent calcium channel, and liprin.

Overall, the biochemical data presented are consistent with other reports showing little change in the expression of proteins from synaptosomes and PSDs isolated from the cerebri of adult Ts65Dn mice [97–99]. However, shifts in the phosphorylation of a variety of synaptic proteins including pre- and postsynaptic scaffold proteins and receptors such as synapsin, piccolo, liprin, dynamin, PSD-95, or NMDA (*N*-methyl-D-aspartate) receptors were observed. The results of this study suggest that the trisomic condition serves primarily to change the functional state of synaptic proteins, but may not result in a fundamental reorganization of synapses. Thus, cognitive impairment in people with DS cannot be reduced to compositional changes at excitatory synapses, but is dependent on higher order deficits in neurons and astrocytes.

A study by Ishihara et al. [100] on primary cultured astrocytes and neurons from Ts1CJe mouse model of DS demonstrated an increased level of ROS and mitochondrial dysfunction using a redox proteomics approach. Ts1Cje carries a segmental trisomy of mouse chromosome 16. It has been shown that the overexpression of genes in the trisomic region of Ts1Cje occurs in a dosage-dependent manner [101]. Ts1Cje shows some DS-related abnormalities such as craniofacial alterations [102], spatial learning deficits [103], and reduction of hippocampal long-term potentiation. The authors identified in this study the putative target proteins that were modified by two lipid peroxidation derived products, 3-hydroperoxy-9Z,11E-octadecadienoic acid (13-HPODE), and 4-hydroxy-trans-2-nonenal (4-HNE). Eight proteins in total were identified as putative 13-HPODE- and 4-HNE-modified proteins: ATP synthase mitochondrial F1 complex b-subunit, α-enolase, and triosephosphate isomerase 1, identified as proteins modified by 13-HPODE; neurofilament light polypeptide, α-internexin, neuron-specific enolase, peroxiredoxin 6, phosphoglycerate kinase 1, and TPI1, modified by 4-HNE. The proteins identified in this study are classified into three categories—proteins involved in ATP generation, the neuronal cytoskeleton, and antioxidant enzymes—suggesting the dysfunction of these pathways as a consequence of oxidative damage. The results presented in this study are consistent with previous studies on lipid peroxidation in DS and AD [18, 104] and with decreased ATP content, antioxidant depletion, and cytoskeletal rearrangement [17, 73, 88], suggesting that these modifications could disturb energy metabolism and neuronal structure and thereby contribute to the impairment of cognitive functions.

6 Proteomics analysis on human amniotic fluid (AF) and placenta

AF comprises two main fractions, supernatant and amniocytes that have been in the past years objects of proteomics studies related to DS pathology. Supernatant AF proteins are produced and secreted by either the fetus or the placenta as the pregnancy progresses, and their expression pattern may be influenced by a pathological condition such as DS. Thus, the analysis of AF proteome might provide new insights into the molecular mechanism involved in pathology progression.

Proteomics studies of AF samples from mothers carrying DS fetuses were initially performed by Tsangaris et al. [105] with the aim to understand the role of proteins' alterations in the biology of AF, the mechanisms involved in selective proteolysis, and to recognize putative markers for prenatal diagnosis. The 2DE methodology led to identification of seven proteins differentially expressed in pregnancies with DS fetuses compared with controls. Five of them were found to be upregulated in DS cases: alpha-1-microglobulin, collagen alpha I chain (Cα-1;), Cα III chain (Cα-3;), Cα V chain d (Cα-5;), and basement membrane-specific heparin sulfate proteoglycan core protein (PGBM). IGFBP-1 was found downregulated, and SFRS-4 was detected only in AF supernatant from cases with DS. Four proteins, Cα-1, Cα-3, Cα-5, and PGBM, appeared as fragments. Among the proteins identified, data from fragments of PGMB and IGFBP-1 are of interest in DS pathology. PGMB known as perlecan, has been reported to be involved in the chondrogenesis process, in the development of bones and to contribute to the integrity of cartilage [106, 107]. IGFBP-1 is involved in modulating the effects of insulin-like growth factors I and II, which have an important role in growth, development, metabolism, and apoptosis, with insulin being the primary determinant of IGFBP-1 expression. Moreover, this finding correlates with the increased incidence of insulin resistance and diabetes observed in DS subjects [108–110].

In 2010, Park et al. [111] analyzed AF supernatant proteins from mother with DS pregnancies using LC-ESI-MS/MS identifying 44 proteins differentially expressed. These proteins were divided into four groups: unique in DS cases, unique in CTR cases, downregulated in DS cases, and upregulated in DS cases. Consistent with previous studies, an increased expression of AFP and Cα-1 and a decreased expression of IBP were observed in the AF from the pregnancies with DS fetuses, supporting the role of these proteins as potential candidates for diagnosing a DS pregnancy through AF analysis. However, in this study, the authors reported 30 new AF proteins that were differentially expressed between the two groups and some of these are fundamental to the physiology of a pregnancy, such as decorin, hemopexin, and proteoglycan-2 proform. Decorin, uniquely expressed in DS cases, associates with extracellular matrix components, and by interacting with protein D surfactant protein may be involved in inflammatory response in pregnant women. In contrast, hemopexin, which binds to heme and plays an important role in heme transport and catabolism, is unique in normal cases and proteoglycan-2 proform was found increased in normal cases and associated with several proteins, such as pregnancy-associated plasma protein-A. Overall, this study reconfirmed that the global protein profile is altered in the AF from DS cases compared to normal and suggests that some of the proteins that were differentially expressed in the AF from DS cases such as AFP conceivably may be potential diagnostic tools.

In the same year, Diamandis laboratory utilized a bottom-up 2D fractionation strategy, involving strong cation-exchange followed by reverse-phase LC fractionation and MS/MS to analyze the AF proteome from DS pregnancies [112]. In this study, the authors recognized 60 proteins differentially expressed between DS and normal pregnancies, suggesting that some of these, such as the downregulation of APP or the overexpression of Tenascin-C, could represent potential AF prenatal biomarkers. The proteins identified in this study were more rigorously analyzed later by a multiplex SRM [113] assay restricting the field to 13 candidate markers of DS, among which 5 different proteins, not previously known biomarkers of DS, were as follows: bile salt-activated lipase, mucin-13, carboxypeptidase A1, and dipeptidyl peptidase 4 that showed a decrease in DS-affected AF, and matrix metalloproteinase 2 (MMP2) that showed an increase, in comparison to controls. Among the proteins identified, MMP2 was assayed by ELISA confirming its alteration in AF from DS subjects. Hence, the authors proposed that increased MMP2 together with increased APP during fetal development might be involved in the eventual pathogenesis of early-onset AD [112].

Our laboratory contributed to the study of AF proteome of mothers carrying a DS fetus employing a redox proteomics approach [114]. The ten proteins identified with increased carbonylation in AF of a DS fetus carrying mother compared to that of normal pregnancies [114] included ceruloplasmin, serotransferrin, complement component C9, a-1B-glycoprotein, kininogen-1, zinc-a-2-glycoprotein, Cα-2 and Cα-5, insulin-like growth factor-binding protein 1 (IGFBP-1), ApoA1, and retinol-binding protein 4. Interestingly, the proteins identified are involved in iron homeostasis, lipid metabolism, and inflammatory response, and the deregulation of these pathways might contribute to or exacerbate degenerative phenomena manifested in DS. Our study demonstrated that OS is an early event in the pathogenesis of DS [17, 30] and might contribute to the severity of DS phenotypes. The increased oxidation of specific proteins might lead to the impairment of multiple cellular pathways involved in DS clinical outcomes. Indeed, for example IGFBP-1 oxidative modification in the early phase of the disease might contribute to abnormal development of the brain. In addition, the increased oxidation of ApoA1, likely resulting in altered activity, correlates with recent reports highlighting that ApoA dysfunction may be linked to increased susceptibility to cognitive impairment characteristic of DS [115]. Accordingly, several markers of OS were analyzed in this study and were found to be significantly increased in the AF from DS pregnancies. Thus, the scenario of increased OS and oxidative damage proposed in this study highly correlates with some characteristic features of DS including early aging and neurological and cognitive impairment [82].

A recent study by Diamandis laboratory [116] analyzed amniocytes, the other major component of AF in addition to supernatant. Amniocytes derive from all three germ layers of the fetus, and some of these show stem cell like properties. The authors employed the SILAC technique to perform the quantization of amniocyte proteins showing a total of 60 proteins with different expression levels between DS and normal amniocytes. Twenty-nine proteins showed decreased levels in DS amniocytes, while thirty-one were increased in expression. Nine proteins were analyzed with a multiplex SRM assay in an independent set of trisomy 21 amniocyte samples, and two of them (SOD1 and nestin) showed a consistent differential expression. Although SOD1 is known to be altered in expression in DS [85], nestin, an intermediate filament protein, is new to the DS pantheon of altered proteins and its aberrant expression could be involved in the molecular pathogenesis of DS during fetal development.

In parallel to AF, the analysis of protein alteration in placenta from DS pregnancies could represent a valid approach to highlight the mechanism of disease. In the study from Sun et al. [117], 17 protein spots were found to be differentially expressed by MALDI-TOF/TOF-MS. Seven proteins were found to be upregulated in pregnancies with DS fetuses, including annexin A2, ERp29, SOD1, proteasome subunit alpha type 2, HSP27, peptidyl-prolyl *cis-trans* isomerase A, and fibrinogen β chain. Three proteins were found to be downregulated in the placentas of pregnancies with DS fetuses, including PRDX6, enoyl-CoA hydratase mitochondrial, and protein disulphide isomerase 3. Interestingly, these data are consistent with the impairment of fatty acid metabolism in DS and with previous studies demonstrating the involvement of altered antioxidant response in DS [114]. Indeed, the aberrant levels of SOD1, HSP 27, ERp29, PRDX6, and protein disulphide isomerase 3 all support the role of oxidative damage in placenta alterations from mothers carrying DS fetuses and suggest further study of the possibility for these proteins to serve as biomarkers for prediction of DS.

A recent report from Cabras et al. [118] investigated the saliva of DS subjects by a top-down proteomic approach, revealing several differences regarding the saliva proteome composition with respect to control subjects. Among the results obtained, the most interesting differences observed between DS subjects and controls concerned the levels of S100A7, S100A8, and S100A12 proteins. Indeed, they found that the salivary concentration of S100A7, S100A8, and S100A12 was significantly higher in DS subjects with respect to controls, with S100A8 and S100A12 that significantly increased in DS subjects with age. Interestingly, both S100A7 and S100A12 levels correlate with the AD feature and increased OS observed in aged DS subjects [119–121].

A large part of proteomics studies on biofluids and DS pathology were conducted in the last decade on maternal plasma of DS pregnancies with the aim to increase the number of biomarker tools and improve sensitivity and specificity of prenatal screening for DS [122–129]. Some of these studies identified aberrant protein expression patterns that closely correlate with AF, suggesting common alteration pathways. However, the results of proteomics studies on maternal plasma, even if important for diagnostic purposes, are, with the possible exception of inflammation, difficult to link to the

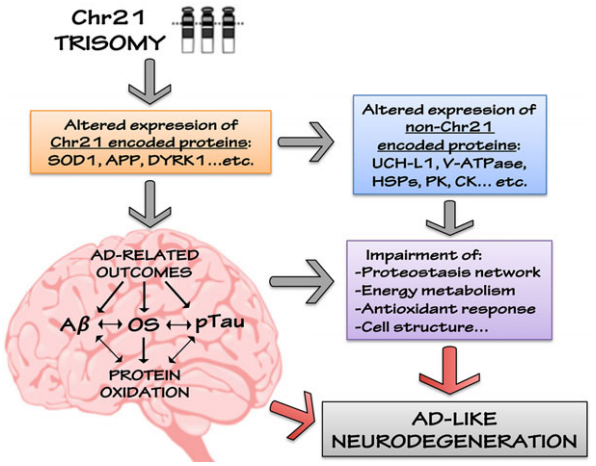

Figure 2. Putative scenario representing the contribution of altered protein expression of Chr21 and non-Chr21-related genes to the development of AD-like neurodegeneration in DS individuals.

molecular mechanisms involved in DS pathology, the object of this current review study.

7 Conclusion and future perspectives

In the last decade, DS neuropathology has become an attractive field of research for several reasons: (i) it can be regarded as a human model of accelerated aging; (ii) it allows correlation of well-characterized genetic defects to pathological phenotypes; (iii) it is a major cause of intellectual disability, thus permitting correlation among neurogenesis defects, brain development abnormalities, and cognitive decline; and (iv) neuropathological hallmarks are similar to AD, that is deposition of SPs and neurofibrillary tangles.

It is tempting to speculate that trisomy affects gene/protein expression outside Chr21 that results in multiple pathological phenotypes. Deregulation of several intracellular pathways occurs early in DS as demonstrated by studies performed on fetal brain and AF from DS pregnancy and plays an important role in brain development. In this scenario, OS, caused not only from overexpression of some Chr21 genes but also as a consequence of low levels of reducing agents and antioxidant enzymes, contributes to exacerbation of early neural pathological changes in DS brain. These include overproduction of Aβ, which accumulates into plaques across the lifespan in DS as well as in AD. Aβ-induced neuronal loss has been demonstrated to be a major contributor to cognitive dysfunctions observed both in DS and AD and further induce OS. It is likely that a vicious cycle occurs where increased Aβ production contributes to further ROS release and at the same time OS exacerbates plaques deposition. Within this frame, reduced activity of quality-control systems in affected neurons may be consonant with the reduced removal of toxic protein aggregates, not only Aβ aggregates but also those eventually formed by oxidized proteins. Accordingly, proteomics studies identified protein members of antioxidant systems, proteostasis network, energy metabolism, and maintenance of cell structure to be specific targets of oxidative modifications, likely leading to reduced activity (see Fig. 2). These findings contribute to explain which molecular mechanisms are defective in DS, as emerged either from animal or human studies, and we suggest that the failure of the above-mentioned functionalities is a hallmark of both aging and neurodegeneration. DS may be considered a protein deposition disorder, where toxic proteins aggregates are not efficiently removed. Impairment of energy metabolism and cell structure integrity together with reduced antioxidant defense makes neurons more susceptible to accumulate oxidative damage that culminates in cell death. Indeed, DS represents one of the most suitable models to study aging as the major risk factor for development of neurodegenerative diseases, particularly AD. On-going studies in our laboratory aim to unravel the complexity of neuropathology of DS and to test novel therapeutic strategies to counteract "pathological" aging.

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8 References

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