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EXOSOMAL BIOMARKERS IN DOWN SYNDROME AND ALZHEIMER'S DISEASE

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Abstract

Every person with Down syndrome (DS) has the characteristic features of Alzheimer's disease (AD) neuropathology in their brain by the age of forty, and most go on to develop AD dementia. Since people with DS show highly variable levels of baseline function, it is often difficult to identify early signs of dementia in this population. The discovery of blood biomarkers predictive of dementia onset and/or progression in DS is critical for developing effective clinical diagnostics. Our recent studies show that neuron-derived exosomes, which are small extracellular vesicles secreted by most cells in the body, contain elevated levels of amyloid-beta peptides and phosphorylated-Tau that could indicate a preclinical AD phase in people with DS starting in childhood. We also found that the relative levels of these biomarkers were altered following dementia onset. Exosome release and signaling are dependent on cellular redox homeostasis as well as on inflammatory processes, and exosomes may be involved in the immune response, suggesting a dual role as both triggers of inflammation in the brain and propagators of inflammatory signals between brain regions. Based on recently reported connections between inflammatory processes and exosome release, the elevated neuroinflammatory state observed in people with DS may affect exosomal AD biomarkers. Herein, we discuss findings from studies of people with DS, people with DS and AD (DS-AD), and mouse models of DS showing new connections between neuroinflammatory pathways, oxidative stress, exosomes, and exosome-

mediated signaling, which may inform future AD diagnostics, preventions, and treatments in the DS population as well as in the general population.

Keywords

Down syndrome; Alzheimer's Disease; Neurodegeneration; Exosomes; Neuroinflammation; Oxidative Stress

INTRODUCTION

Down syndrome (DS) is the most prevalent intellectual disability with an incidence of approximately one in 700 live births in the U.S., leading to 6,000 new babies born with DS each year in the U.S. (<https://www.ndss.org/Down-Syndrome/What-Is-Down-Syndrome/>). DS is usually due to three copies of human chromosome 21 (Hsa21) (Zigman 2013), also referred to as trisomy 21 (T21). Individuals with DS exhibit a high incidence of cognitive impairment and high comorbidity with Alzheimer's disease (AD) (Epstein 1990; Hartley et al. 2015; Wisniewski et al. 1985; Glasson et al. 2014; Krinsky-McHale and Silverman 2013). People with DS have three copies of the amyloid precursor protein (*APP*) gene (Lee et al. 2017), which resides on Hsa21, resulting in elevated levels of amyloid beta ($A\beta$) peptides that form amyloid plaques (Glasson et al. 2014) at least two decades prior to the onset of the clinical symptoms of AD (Hartley et al. 2015; Head et al. 2001; Head et al. 2016; Lemere et al. 1996). In addition, hyper-phosphorylated species of Tau (P-Tau) progressively accumulate intra-neuronally throughout the brain, resulting in the formation of neurofibrillary tangles (NFTs) (Stoltzner et al. 2000; Mondragon-Rodriguez et al. 2014). Individuals with DS concurrently display enhanced microglial activation (Head et al. 2016; Wilcock and Griffin 2013; Wilcock et al. 2015), elevated oxidative stress, and mitochondrial dysfunction (Pagano and Castello 2012; Perluigi and Butterfield 2012). Collectively, these pathological hallmarks forge a pathway to AD in people with DS (DS-AD) leading to widespread and progressive neurodegeneration of basal forebrain cholinergic neurons (BFCNs), locus coeruleus (LC) noradrenergic (NE) neurons, certain hippocampal neuronal populations (Lockrow et al. 2012; Millan Sanchez et al. 2012; Salehi et al. 2009), and neurons in certain cortical areas (Sadowski et al. 1999; Teipel et al. 2004), similar to the neurodegeneration observed in idiopathic AD (Wisniewski et al. 1985).

By the age of forty, amyloid plaques and NFTs are present in the brain of persons with DS in sufficient numbers to allow a *post mortem* pathological diagnosis of AD (Head et al. 2001; Stoltzner et al. 2000; Wisniewski et al. 1985; Prasher et al. 2010), even though the pathological trajectory toward DS-AD is established much earlier. Beta amyloid pathology has been detected in *post mortem* brain samples from people with DS as young as 15 years of age (Lemere et al. 1996). Significant changes in Tau protein have been observed in the DS fetal brain (Milenkovic et al. 2017), with NFTs observed early in life (Butterfield et al. 2014; Perluigi et al. 2015; Hartley et al. 2015). Despite the fact that AD neuropathology occurs decades prior to the development of dementia in DS-AD, this process has remained relatively unexplored in younger individuals with DS. Critical barriers for studying the underlying mechanisms of DS-AD include the relative absence of well-characterized *post*

mortem brain tissue available for study, and of reliable, *ante mortem* measurements of pathological cascades that can be used as AD biomarkers in the clinic. The term “biomarker” refers to objective indications of a medical state that can be measured in a reproducible manner. Cerebrospinal fluid (CSF) biomarkers of AD include the A β peptides, A β ₁₋₄₀ and A β ₁₋₄₂, different species of P-Tau, pro-inflammatory cytokines, including IL-6, TNF- α , and IL-1 β , and pro-NGF, which have been detected decades prior to dementia onset in both the general population (Bayer-Carter et al. 2011; Counts et al. 2017; Jack et al. 2012;) and in people with DS (Perluigi et al. 2014; Prasher et al. 2010). However, the use of CSF biomarkers is not always practical due to its invasiveness and potential risks for post-lumbar puncture (LP) headaches. In addition, the LP procedure is more difficult to conduct in patients with DS, especially after the onset of dementia.

There is an urgent need for the development of reliable blood-based AD biomarkers for the DS population. Biomarkers that reflect AD pathology at early ages would be of considerable clinical value because neuroprotective therapies may eventually be useful in younger individuals with DS. With the goal of delineating early pathological processes in the CNS by non-invasive means, recent studies have focused on the development of methods for isolating and characterizing exosomes, which are extracellular vesicles released by a cell when an intermediate endocytic membrane compartment called the multivesicular body (MVB) fuses with the plasma membrane and releases its intraluminal vesicles (referred to hereafter as exosomes) into the extracellular space. Most cell types in the body release exosomes (Barile and Vassalli 2017; Fiandaca et al. 2015; Kapogiannis et al. 2015; Vingtdeux et al. 2012), which can be found in blood, CSF, urine, and medium from cells grown in culture (Barile and Vassalli 2017). Exosomes contain proteins, messenger RNAs (mRNAs), and microRNAs (miRNAs) that reflect their cellular origin, and they play a prominent role in cellular signaling, expulsion of toxic proteins, and transfer of cellular pathogens to other cells (Barile and Vassalli 2017). Relevant to studies of AD biomarkers, CNS-derived exosomes are present in biological fluids (blood, CSF, and urine) and circulate in the interstitial space, both in the brain and in the periphery (Barile and Vassalli 2017).

In addition to their role as carriers of cell-based biomarkers, accumulating evidence suggests that secreted exosomes may serve as vehicles for the transport of a wide range of proteins and immune markers, thereby potentially initiating or exacerbating pathogenic processes by fusing with recipient cells, including neurons (Barile and Vassalli 2017). Because of this, the cargo carried by CNS-derived exosomes may serve as markers of underlying CNS changes that occur in advance of changes in circulating proteins. Because CNS-derived exosomes have unique surface markers that reflect their cell of origin, targeted examinations of neuron-, astrocyte-, or endothelial cell-derived exosomes can be employed (Fiandaca et al. 2015; Kapogiannis et al. 2015; Goetzl et al. 2016; Goetzl et al. 2017). After targeted selection using antibodies directed against the cell-type-specific surface marker(s) for an exosome population of interest, examination of specific exosomal cargos may be used to probe this unique biological niche that may provide a “liquid biopsy snapshot” of current cellular processes. This approach may be particularly useful in the clinic, where patient blood samples are readily acquired for standard clinical testing needs, but also for research purposes by consortiums of biobanks worldwide with extensive blood sample collections from well-characterized subjects. In this review article, we will detail current

conceptualizations of exosome biomarkers from people with DS, review recent data on the role of CNS-derived exosomes in AD pathological cascades, discuss interplay between exosome cargo and cellular pathways in the brain, and examine the potential for exosomes to serve as and/or inform therapeutic targets.

Elevated Levels of CD81 in Neuron-Derived Exosomes from People with DS

Several studies have shown that classical markers of exosomes, including the tetraspanin membrane proteins Cluster of Differentiation (CD) 63 and CD81, are enriched in preparations of total exosomes and can be used in the normalization of subsequent analyses of other biomarkers (Booth et al. 2006; Raposo and Stoorvogel 2013). As discussed, expulsion of toxic proteins may be one function of exosomes. In the context of DS-AD, where levels of toxic A β peptides and aberrant P-Tau proteins are significantly elevated in neurons, one may postulate that upregulation of exosome release could serve as a useful mechanism for decreasing the levels of these deleterious proteins. In our recent study, which was a collaboration between several DS clinics both in the U.S. and abroad and with the Goetzl laboratory at the National Institute of Health (NIH), we hypothesized that neuron-derived exosomes might be more abundant in people with DS relative to an age-matched control population without DS (referred to herein as a 'control' population), which could serve to downregulate the increased levels of A β peptides and aberrant P-Tau proteins in neurons from people with DS. This assumption was based on previous work by others, showing increased production of exosomes from cells exposed to amyloid *in vitro* (Chiarini et al., 2017). To test our hypothesis, we isolated neuron-derived exosomes as previously described (Hamlett et al. 2016), and quantified CD81 by enzyme-linked immunosorbent assay (ELISA) (Cusabio, Waltham, MA) against a standard curve of recombinant CD81 (Origene Technologies, Rockville, MD). Briefly, plasma samples were incubated with protease and phosphatase inhibitor cocktails, centrifuged, and the supernatants were mixed with ExoQuick polymer solution (System Bioscience, Mountain view, CA) and incubated at 4°C for 1 h to precipitate exosomes (Hamlett et al., 2016). The two cohorts included a population of participants with DS (8-62 years old) and a population of age-matched control participants (8-77 years old). Both populations were age- and gender-matched (Hamlett et al. 2016).

We quantified the size of the neuron-derived exosomes using nanoparticle tracking analysis (Nanosight, Malvern, Salisbury, UK), which revealed an average vesicle size distribution slightly over 100 nm (Figure 1A) for multiple exosome preparations, which is within the 40-110 nm size range reported in previous studies (Barile and Vassalli 2017). To isolate neuron-derived exosomes, samples were incubated with mouse biotinylated anti-human CD171 monoclonal antibody (clone 5G3, neural cell adhesion molecule L1 [L1CAM], eBioscience, San Diego, CA), allowing isolation of only neuron-derived exosomes from the plasma sample (Hamlett et al., 2016; Kapiogannis et al., 2014). We quantified the CD81 levels in neuron-derived exosomes from both DS and control populations (Figure 1B) and found that the neuron-derived exosomes from participants with DS had 39% higher average levels ($1,433 \pm 87$ pg/ml) of the CD81 exosomal marker relative to neuron-derived exosomes from the control participants ($1,027 \pm 87$ pg/ml). A Student's t-test revealed that the difference was significant ($p < 0.01$). We further divided the DS and control groups into two

age categories: 1) below 35 years of age (DS population: mean age = 19 ± 2.2 years, $n = 14$; Control population: mean age = 15 ± 2.5 years, $n = 9$), and 2) above 35 years of age (DS population: mean age = 51 ± 0.8 , $n = 33$; Control population: mean age = 55 ± 1.7 , $n = 28$) (Figure 1C). A two-way ANOVA (Karyotype $\times$ Age) revealed that having DS was the only primary driver of CD81 levels, with no significant effects found for age. Tukey's posthoc adjusted comparisons revealed significantly increased CD81 levels for individuals with DS, in both the younger ($p < 0.05$) and older ($p < 0.05$) groups with DS compared to age-matched controls. These findings suggested that neuron-derived exosomes were more abundant in blood samples from people with DS, regardless of their age, but could also suggest that soluble CD81 levels are elevated per exosome, since elevated soluble CD81 has been observed for example in patients with chronic liver inflammation (Welker et al., 2012). This result is interesting, as similar observations have been made in a mouse model of DS (Perez-Gonzalez and Levy 2016). A recent manuscript by Guix et al. (2017) shows that, unlike other tetraspanin protein family members that interact with γ -secretase, Tetraspanin-6 (TSPAN6), which has been shown to be upregulated in prefrontal cortex of AD patients, is instead enriched in MVBs and ILVs where it recruits the exosome-forming adaptor protein syntenin and modulates A β production independent of γ -secretase (Guix et al. 2017). Specifically, upregulation of TSPAN6 expression leads to increased secretion of exosomes that contain A β , suggesting that an increase in exosome secretion may participate directly in AD pathology by increasing A β production and blocking its degradation via the autolysosomal pathway (Guix et al. 2017). Future studies will further investigate the cellular consequences of increased exosome secretion and/or increased production of CD81, both for inter-region spread of pathology in AD and DS-AD and as a potential target for drug intervention.

Exosomes Contain Early Biomarkers of DS-AD Pathology

Studies of exosomal cargos in aging adults with AD have focused primarily on neuron-derived exosomes. Neuron-derived exosomes, identified by L1CAM, are involved in APP processing, which plays a key role in AD neuropathology (Haass et al. 1995; Perez-Gonzalez et al. 2012; Rajendran et al. 2006; Sharples et al. 2008), and increasing evidence suggests that neuron-derived exosomes may play a crucial role in A β clearance (Yuyama et al. 2015). Exosomes receive APP from early endosomes after cleavage into A β peptides, which may then be secreted from the cells in exosomes (Yuyama et al. 2015). Neuron-derived exosomes contain toxic A β peptides and Tau, which they may transmit to neighboring cells, to other brain regions, and to the circulatory system, suggesting that neuron-derived exosomes extracted from either plasma or CSF can specifically assess relevant neuropathological processes within CNS neurons (Jaunmuktane et al. 2015; Vingtdeux et al. 2012; Fiandaca et al. 2015; Kapogiannis et al. 2015). Other findings suggest that AD biomarkers carried by neuron-derived exosomes relay critical information about clinical severity and staging, such that they may be used to predict the onset of dementia as early as 10 years in advance of symptoms in patients with sporadic AD or frontotemporal dementia (FTD) (Fiandaca et al. 2015; Kapogiannis et al. 2015).

Despite accumulating evidence that exosome biomarkers offer an early predictive indication of the development of AD, these biomarkers had not been analyzed in individuals with DS

previously. In our collaborative study with several other research groups, we hypothesized that neuron-derived exosomes from blood from people with DS would have elevated levels of A β peptides and phosphorylated-Tau (P-Tau) relative to those from controls, which could represent a preclinical phase of AD in DS (Hamlett et al. 2016). As described in Hamlett et al. (2016), we compared levels of AD biomarkers in neuron-derived exosomes obtained from plasma from a population of participants with DS (8-62 years old) compared to a population of control participants (8-77 years old). The two groups were age- and gender-matched. Three neuron-derived exosome biomarkers, including A β ₁₋₄₂, P-T181-Tau, and P-S396-Tau, were significantly elevated in the DS population relative to the control population (Student's t-test, $p < 0.0001$ for each biomarker; $n = 37$ for control participants, and $n = 47$ for participants with DS). As described, we divided the control and DS groups into two age categories: 1) below 35 years of age, and 2) above 35 years of age, and found highly significant effects of DS, both in the young and older DS groups (both $p < 0.0001$) compared to age-matched controls. These findings demonstrated that this set of neuron-derived AD biomarkers in the exosomal cargo were all significantly elevated in people with DS compared to age-matched controls, regardless of age. All three neuron-derived AD biomarkers had at least a 2-fold elevation in levels for people with DS. Extrapolation of the linear regressions (biomarkers by age) suggests that the neuron-derived exosome biomarker levels may be elevated already at birth in DS, but this analysis needs to be examined in expanded studies. In addition to potential age effects, we also examined gender differences in exosomal biomarkers, but did not detect any significant differences between male and female participants for the A β ₁₋₄₂ or the P-S396-Tau levels (Hamlett et al. 2016). However, there was a significant gender effect for the P-T181-Tau levels, only in the control group ($p < 0.05$) and in the DS group with participants older than 35 years of age ($p < 0.05$) (Hamlett et al. 2016). Interestingly, older women in the control group had 34% higher P-T181-Tau levels than men, whereas the older men in the group with DS had 34% higher P-T181-Tau levels than women in the group with DS, suggesting a potentially unique interaction between gender and age with this particular AD biomarker among people with DS. Surprisingly, older men (> 35 years of age) with DS had on average 275% higher P-T181-Tau levels than age-matched men in the control group. Future studies will have to be conducted to determine whether other factors, such as hormone levels, may influence the changes in exosomal P-T181-Tau levels based on age and gender. It would therefore be interesting to assess exosomal markers in males versus females as a function of sex hormone levels in future studies.

We further divided the adult DS group into two categories: 1) those with no cognitive impairment (NCI), and 2) those with early symptomatic dementia or with fully symptomatic dementia indicative of DS-AD (Figure 2). A β ₁₋₄₂ levels were significantly decreased (Student's t-test, $p < 0.05$) in adults with DS and dementia symptoms, relative to the DS group with no cognitive impairment (Figure 2A). Both DS groups had significantly elevated A β ₁₋₄₂ levels compared to the age-matched non-DS control group ($p < 0.0001$). P-T181-Tau levels were not significantly different between the two DS groups (Hamlett et al. 2016). By contrast, P-S396-Tau levels were significantly elevated (Student's t-test, $p < 0.01$) in participants with early or fully symptomatic dementia compared to those with DS and no cognitive impairment (Figure 2B). Again, the P-S396-Tau levels were significantly elevated

in both DS groups compared to age-matched non-DS controls. Taken together, these data demonstrated that neuron-derived exosome levels of A β ₁₋₄₂ and P-Tau follow different trajectories in adults with DS with symptoms of dementia, suggesting that these differences may be useful for the diagnosis and prognosis of AD dementia.

It is not surprising that A β ₁₋₄₂ levels were elevated early in DS because the *APP* gene resides on Hsa21 (Glasson et al. 2014; Lee et al. 2017), and previous studies have shown elevated levels of amyloid peptides in plasma from children and young adults with DS (Obeid et al. 2016). Interestingly, findings from the Dominantly Inherited Alzheimer Network (DIAN) study (e.g., Fagan et al., 2014), an international registry of individuals at certain risk for developing autosomal dominant AD, suggest that early biomarkers predict the onset and trajectory of dementia in vulnerable non-DS populations. The DIAN studies showed early high levels of A β in the CSF, followed by reduced A β levels in CSF after onset of dementia, demonstrating that early blood or CSF biomarkers can be used to mark or predict the onset of AD (Fagan et al. 2014; Bateman et al. 2012). Because the *TAU* gene is not known to be affected by trisomy 21, it is more surprising that the P-Tau levels, including both of the phosphorylation sites examined, are elevated early in DS. Hyperphosphorylation of Tau protein, particularly at the carboxyl terminus, has been shown to be one of the earliest pathological events in both AD and in DS-AD (Mondragon-Rodriguez et al. 2014), and may occur prior to the accumulation of brain fibrillary P-Tau. In our study, we found that neuron-derived exosome levels of both P-T181-Tau and P-S396-Tau were significantly increased in younger people with DS compared to age-matched controls, and that this hyperphosphorylation profile persisted into late adulthood with DS-AD dementia (Hamlett et al. 2016). The highest levels of exosomal P-S396-Tau were found in participants diagnosed with dementia (Figure 2B), suggesting that P-Tau continues to accumulate in exosomes as AD pathology progresses.

There are several genes on Hsa21 that may contribute to Tau pathology. Hyperphosphorylation of Tau can be influenced by dysregulation of alternative splicing of Tau reported in DS as well as in AD (Iqbal et al. 2013). The dual-specificity tyrosine-phosphorylation-regulated kinase 1A (*DYRK1A*) gene and regulator of calcineurin 1 (*RCAN1*) gene, are both located on Hsa21, both proteins are highly expressed in the DS brain, and have both been implicated in the dysregulation of Tau phosphorylation associated with early onset AD in DS (Yin et al. 2017; Shi et al. 2008). Interestingly, Dyrk1A-mediated phosphorylation of RCAN1 induces a targeted phosphorylation cascade that leads to Tau phosphorylation (Jung et al. 2011). Dysregulation of these genes is likely involved in early Tau pathology observed in DS and may help to explain why abnormal levels of P-Tau are seen so robustly in neuron-derived exosomes. Our data support previous observations about P-Tau in AD-related progression in adults with DS, but also suggest that significant elevations occur, at least in neuron-derived exosomes, at as early as 8 years of age in children with DS. These data have recently been supported by a study from Milenkovic et al. (2017) demonstrating changes in Tau phosphorylation even in fetuses with DS, further suggesting that alterations in Tau may contribute to the development of early pathology in the DS brain as early as during fetal development (Milenkovic et al. 2017).

Toxic derivatives of Tau in exosomes can be secreted by either neurons or glia and spread the pathology between different brain regions in AD, thereby propagating AD pathology in a hierarchical manner (Wang et al. 2017). Specifically, Wang and collaborators (2017) showed that Tau is released by cultured cells over-expressing Tau via exosome release, and that exosomal Tau is hyperphosphorylated whereas cytosolic Tau in the cells of origin is not. Furthermore, depolarization of neurons led to the release of exosomes with P-Tau cargo. The investigators showed that the exosomes could mediate transneuronal transfer of Tau, depending on synaptic connectivity in an *in vitro* model (Wang et al. 2017). These data are fascinating and can potentially lead to a much better understanding of how AD pathology spreads between brain regions, although it is important to highlight that these studies remain in an early stage of inquiry. Astrocyte-derived exosomes from patients with AD have also been purified by Goetzl et al. (2016) using glial surface markers (Goetzl et al. 2016). They displayed higher levels of both A β and p-Tau, as well as the β -site amyloid precursor protein cleaving enzyme 1 (BACE-1) than neuron-derived exosomes from the same samples. It has long been known that treatment of cells in culture with A β leads to P-Tau overexpression within the cells and recently it has also been shown that A β treatment of astrocytes leads to increased production of exosomes with P-Tau cargo (Chiarini et al. 2017), thus providing an opportunity to study cell-cell interactions within the CNS with respect to AD pathology by examining both astrocyte- and neuron-derived exosomes and their cargo in blood.

Exosomes and Oxidative Stress Associated with DS

Proteins that are modified by oxidation have long been thought to be exclusively degraded by the proteasome system (Dunlop et al. 2002). However, recent evidence suggests that autophagy is involved in the removal of heavily oxidized proteins (Kiffin et al. 2004). If these removal systems become dysfunctional, oxidized protein will accumulate, leading to cellular pathology. Emerging evidence indicates that the secretion, composition, and functional capacity of exosomes are all altered as AD pathology accumulates in the brain. Progressive alterations in exosomal proteomic cargo may underlie different pathological mechanisms associated with AD progression that are related to oxidative stress and other altered pathways in DS. This has recently been reported in the cancer field (Prieto et al. 2017), but, to our knowledge, it remains unexplored in neurodegenerative disorders. In the general population, the accumulation of oxidized proteins due to deficiencies in proteosomal degradation increases with age (Carrard et al. 2002; Szweda et al. 2002) but this process occurs earlier in people with DS, and has been observed in fetal cells with T21 (Butterfield et al. 2014; Busciglio et al. 2007; Guedj et al. 2014; Perluigi et al. 2014; Perluigi et al. 2011; Rodriguez-Sureda et al. 2015), and is persistent from childhood to later ages (Garlet et al. 2013). Thus, oxidative stress is a major etiological factor on the pathway to DS-AD (Pagano and Castello 2012). The superoxide dismutase 1 (*SOD1*) gene is encoded on Hsa21, within the so called Down syndrome critical region (DSCR) (Antonarakis 1998) and has been shown to have up to 50% increased activity in all DS tissues examined, including the brain (Sinet 1992), as well as in DS mouse models (Lockrow et al. 2012). The levels of SOD1 protein and mRNA are particularly high in hippocampal pyramidal neurons in DS, which are especially susceptible to the degenerative processes that occur in DS-AD (Furuta et al. 1995). SOD1 catalyzes the conversion of superoxide anions into hydrogen peroxide, both being natural reactive oxygen species (ROS) that are continuously generated by aerobic

respiration in mitochondria. For people with DS, hydrogen peroxide is produced in excess as a result of: 1) increased SOD1 activity, and 2) a protracted antioxidant defense response (Garlet et al. 2013). Elevations in ROS and oxidative stress manifest damage to many cellular biomolecules, including monoamines, proteins, lipids, and DNA (Chiu 2014), which sets the stage for multifaceted oxidation problems at the cellular level for people with DS. Therapeutic interventions to combat oxidative stress are needed for both the DS community and the control population. We have previously shown that the antioxidant Vitamin E was protective against memory loss and neurodegeneration in the Ts65Dn mouse model of DS (Lockrow et al. 2009). Vitamin E acts as a peroxy-radical scavenger, which disables the production of damaging free radicals in tissues by reacting with them to form a tocopherol radical, which is then reduced by a hydrogen donor and returned to a reduced state. Vitamin E has been tested in adults with DS with no effects on progression to dementia (Sano et al. 2016). However, Vitamin E reduced biomarkers of oxidative stress in children with DS (Mustafa Nachvak et al. 2014), suggesting that preventive treatment earlier in life may hold some promise. In a similar study including children and teenagers with DS, Vitamin E treatment improved the systemic antioxidant capacity during the intervention and also showed long-lasting protective effects in plasma (Garlet et al. 2013; Parisotto et al. 2014). Growing evidence suggests that oxidative stress may act as a converging point for stimulating autophagy, which is a catabolic cellular process that recycles cellular components and removes debris. Indeed, ROS may serve as one of the main intracellular signal transducers that sustains autophagy, but this important physiological process becomes inefficient during aging and in neurodegenerative diseases (Filomeni et al. 2015).

Autophagy is inhibited both in DS and in AD (Liu et al. 2009; Perluigi et al. 2015; Tramutola et al. 2016), and loss of normal autophagic function contributes to AD pathogenesis (*see also Nixon et al in the current Special Issue*). Other groups have shown that not only autophagy, but also endosomal pathways, are hampered in both DS and AD, resulting in enlargement of early endosomes that significantly affects protein trafficking in the cytosol (Cataldo et al. 2000; Cossec et al. 2012) and may also ultimately affect the secretion and cargo of exosomes, as autophagy has been implicated in endosome/exosome secretory pathways (Papandreou and Tavernarakis 2017). Under conditions of perturbed membrane trafficking and lysosomal deficiency, such as in DS, pre-lysosomal compartments may instead fuse with the plasma membrane directly and release their toxic cargo components, and/or exosome secretion may be upregulated when lysosomal pathways are dysregulated (Borland and Vilhardt 2017). Various inflammatory and signaling molecules and small RNAs are selectively packaged in exosomes, depending on the activity status of the cell. It is conceivable that exosomes can contribute to oxidative stress by carrying increased levels of certain microRNAs (miRNAs) shown to regulate key pathological pathways, including oxidative stress, autophagy, and inflammation. On the other hand, potential future therapies for DS-AD could be to actively deliver certain miRNAs to the brain, to activate autophagy and remove AD pathology. This approach has been studied for different types of cancer (Qu et al. 2017), but has not, to our knowledge, been applied to neurodegenerative conditions and may represent an exciting and novel use for exosomes. Thus, there is a strong connection between autophagy and oxidative stress, which in turn have significant direct and/or indirect effects on exosome cargo and on exosome secretion.

This is an expanding field that holds promise with regard to exosomal pathways and their role(s) in neurodegenerative pathways, as well as for the development of novel therapies for DS-AD, for AD, and for other neurodegenerative conditions.

Exosomes and Neuroinflammation Associated with DS

Since the 1980s, studies of *post mortem* brain specimens from AD patients have revealed significant elevations in neuroinflammatory markers (Potter et al. 2001; Bazan et al. 2002; Heneka et al. 2015; Ho et al. 2001; Shaw et al. 2013). Pro-inflammatory processes represent a normal innate immune response to pathogen invasion that is critical for initiating tissue repair and maintaining homeostasis. However, sustained inflammation has deleterious effects on neurological functioning and is associated with cognitive decline (Bettcher and Kramer 2014; Eikelenboom et al. 2012; Cunningham et al. 2009; Wyss-Coray 2006; Westin et al. 2012). People with DS have pervasive inflammatory issues apparent early in life that may underlie the high prevalence of DS-AD (Wilcock and Griffin 2013; Wilcock et al. 2015; Head et al. 2016). Inflammation is well-known to be a core pathological component of AD (Wilcock and Griffin 2013; Wilcock et al. 2015), but the role of central and peripheral immune functions in people with DS on the path to DS-AD needs further study. Rampant neuroinflammation, including activation of both microglia and astrocytes, is a hallmark in the DS brain, as well as in DS mouse models (Lockrow et al. 2012; Wilcock et al., 2013; Hartley et al., 2015). Circulating levels of inflammatory cytokines are also increased in blood isolated from people with DS (Rodrigues et al. 2014), and the inflammatory markers correlate with other AD biomarkers in adults with DS (Naude et al. 2015). We have shown that pro-inflammatory cytokines are also elevated in plasma from mouse models of DS (Figure 3), suggesting that the Ts65Dn mouse may serve as an acceptable model for studies of inflammation-related DS pathology. Recently, efforts have been undertaken to characterize the neuroinflammatory profile of DS in relation to normal aging adults with AD. Wilcock and colleagues (2015) discovered a distinct neuroinflammatory phenotype using brain tissue from adults with DS relative to brain tissue from aging adults with AD pathology (Wilcock et al. 2015). In addition, recent evidence may indicate that neuroinflammation observed in AD and in DS-AD may be associated with exosome secretion and transport.

While exosomes mediate vital processes required for normal cellular function, they are also involved specifically in the pathogenesis of neuroinflammatory disorders, both infectious and neurodegenerative in nature (see e.g. Gupta and Pulliam, 2014). Exosomes are capable of inducing pro-inflammatory effects, including antigen presentation, cellular migration, apoptosis induction, and inflammatory cytokine release (Wahlund et al. 2017). They can cross the blood-brain barrier to contribute to the spread of brain antigens from the periphery to the brain, or vice versa (Selmaj et al. 2017). Exosomes participate in intercellular communication in the brain. A recent study showed that neuro-derived exosomes carrying specific types of miRNAs can activate Toll-like receptors (TLRs) and thus trigger inflammation signaling in the brain either in neighboring cells or during long-distance cell-to-cell communication via exosome transport in the extracellular matrix (Paschon et al. 2016). Studies have also shown that activation of microglia, for example by the presence of amyloid, increases the release of exosomes, which may then directly participate in the

inflammatory process in the brain (Hooper et al. 2012). A recent study demonstrated that patients with lupus had increased exosomes in plasma compared to healthy controls, and when the lupus-originating exosomes were used to stimulate healthy peripheral blood mononuclear cells, this gave rise to a significant increase in IFN- α , TNF- α , IL-1 β , and IL-6 compared to the stimulation obtained from healthy exosomes (Lee et al. 2016). Our studies indicated that plasma from patients with DS and AD dementia contains elevated levels of exosomes (see Figure 1), but no studies have been undertaken as of yet to examine whether DS-derived exosomes also exhibit properties that increase pro-inflammatory cytokines in their target cells. Although still in an early stage of study, exosomes represent a potentially novel cellular mechanism of neuroinflammation and AD-related pathology that may provide insights into immune trafficking using a non-invasive methodology; moreover, exosomes may represent a potential target for future therapeutic delivery (Barile and Vassalli 2017). Based on these intriguing novel findings on inflammation and exosomal cargo in the pathology of neurodegenerative disorders, future studies will be focused on this interaction.

Exosomes Transmit Interferon-induced Response

Four of the six Interferon receptors (IFNRs) are encoded on Hsa21 (IFNAR1, IFNAR2, IFNGR2, and IL10RB) and are overexpressed in diverse cell types derived from people with DS (Sullivan et al. 2016). Interferon (IFN) ligands are produced by cells in response to a variety of insults, mostly viral or bacterial infections. Through engagement of specific receptors in neighboring cells, IFNs can elicit an antiviral response, shut down protein synthesis, induce apoptosis, and activate nearby immune cells, including microglia in the brain. Engagement of the IFNRs triggers activation of protein kinases of the JAK family (JAK1/2 and TYK2) and downstream transcription factors in the STAT and IRF families, leading to induced transcription of hundreds of Interferon-stimulated genes (ISGs). Recently, it was shown that T21 consistently activates the IFN response, as evidenced by elevated expression of dozens of ISGs in diverse cell types, including circulating monocytes and T cells (Sullivan et al. 2016). Furthermore, it has been demonstrated that T21 cells are hypersensitive to various IFN ligands, that JAK kinases differentially impair the survival and proliferation, and that the ISG signature and defects in cell proliferation can be reversed with JAK inhibitors (Sullivan et al. 2016). Several additional lines of evidence indicate that triplication of the IFNR gene cluster in people with DS can contribute to the detrimental effects of T21. First, hyperactive IFN signaling has profound negative impacts on human development, as demonstrated by Type I Interferonopathies, a class of genetic conditions caused by mutations that lead to elevated IFN production, and which share many phenotypes with DS (Crow 2011; Crow and Manel 2015; Rodero and Crow 2016). Second, reducing IFN signaling in an early mouse model of DS (Ts16) with either anti-IFN antibodies or by crossbreeding with a mouse strain lacking two IFNRs improved embryonic development and increased survival of cortical neurons *in vitro* (Maroun 1995; Hallam and Maroun 1998; Maroun et al. 2000). Third, long-term administration of IFN ligands for the treatment of diverse medical conditions, such as multiple sclerosis or chronic viral infections, is known to produce several undesirable side effects, many of which resemble DS-associated co-morbidities, including wide-ranging psychiatric side-effects (Kovacs et al. 2016), depression (Kovacs et al. 2016; Lucaciu and Dumitrascu 2015), pulmonary hypertension (Savale et al. 2016), autoimmune hypothyroidism (Nair Kesavachandran et al. 2013), and cardiotoxicity

(Sonnenblick and Rosin 1991). Finally, a recent report of a segmental duplication of a region containing only 18 genes of Hsa21, including the four IFNRs, indicates that triplication of this small genomic region was sufficient to produce the developmental hallmarks of DS in the absence of complete T21 (Weisfeld-Adams et al. 2016).

Taken together, these findings support the hypothesis that triplication of the four IFNRs could synergize with *APP* triplication in the genesis of early-onset AD in DS. In fact, several lines of evidence indicate that IFN signaling could accelerate the progression of AD. In animal models and human cell-based models, IFN signaling was shown to enhance APP expression and processing into the pathological amyloid peptide, A β ₁₋₄₂, leading to increased amyloid plaque formation (Hong et al. 2003; Minter et al. 2016). In mouse models of AD, IFN signaling mediates neuroinflammation, neuronal cell death, and cognitive decline downstream of amyloid plaque formation (Browne et al. 2013; Yamamoto et al. 2007; Minter et al. 2016; Taylor et al. 2014). In fact, knockout of either of the T21-related IFNAR1 or IFNGR2 receptors was sufficient to prevent disease progression in these models (Yamamoto et al. 2007; Minter et al. 2016). Furthermore, in a longitudinal study of inflammatory proteins in plasma, increased circulating levels of IFN- γ as well as key IFN-inducible cytokines, such as IL6 (Zimmermann et al. 2016; Jordan et al. 2007; Duddy et al. 1999), were found to correlate with faster AD progression (Leung et al. 2013). A strong correlation between higher IL6 levels and increased risk of cognitive decline was also observed in a longitudinal assessment of 1,040 individuals (Jenny et al. 2012). Altogether, these observations demonstrate the importance of IFN signaling for the development of DS-AD, and the mechanisms that may underlie IFN-mediated transmittance could be potential pharmacological targets.

Recently, exosomes were found to be mediators of cell-to-cell transmission of IFN- α -induced activity *in vitro*. Inhibition of exosome biogenesis *in vitro* or inhibition of exosome release with an *in vivo* mouse model impaired the cell-to-cell transfer of IFN- α -induced antiviral activity against HBV *in vitro* (Li et al. 2013). Furthermore, this study revealed that treatment with IFN- α changes the cargo content of mRNAs and miRNA in exosomes, which were presumed to be primary communicative agents on antiviral activity via alterations in gene expression. Another study revealed that exosomes modulated IFN- γ -induced innate immune responses to viral infection *in vivo* (Kouwaki et al. 2016). IFN- γ modulation of immune response may involve dendritic cells (DCs), which control T-cell-mediated immune responses and modulate adaptive immune responses. In support of this, a recent study demonstrated that IFN signaling resulted in viral RNA transmission to DCs by exosomes, which again induced the innate immune response in these cells (Robbins and Morelli 2014). However, whether the etiological agent was indeed viral RNA remains to be answered. Overall, these results provide novel insights into the molecular mechanisms underlying exosome-mediated innate immune response via IFN signaling. We postulate that all sources of exosomes could potentially mediate IFN-induced signaling in those with DS, but that CNS-derived exosomes may have a higher interface for cell-to-cell transmission in the brain and thus a stronger impact on the path toward DS-AD.

Induction of pro-inflammatory cytokines via the pyroredoxan/exosome system

While many factors influence microglia status and cytokine production, oxidative stress is known to impact both AD pathology and microglial cytokine production. However, the mechanisms behind this interplay are not well understood in the brain. However, a novel pathway of protein secretion mediated by cysteine oxidation has recently been revealed to underlie the importance of redox-dependent signaling mechanisms on inflammation, with specific targeting of cytokine production. Pyroredoxans (Prdxs) are a family of antioxidant proteins that protect cells from oxidative damage via reduction of peroxides but also act as pro-inflammatory signals (Bianchi 2007). A recent study showed that cysteine oxidation of specific residues of Peroxiredoxins 1 and 2 causes them to localize in exosome cargo where they can then be released from human embryonic kidney cells. Once released in exosomes, Prdxs can induce production of inflammatory cytokines potentially throughout the circulatory system (Riddell et al. 2010). Relative to DS, oxidative stress-enhanced exosome secretion could be predicted to manifest elevations in Prdxs-mediated signaling via an exosome shuttle. Since Prdxs-mediated signaling is a key inflammatory driver in the brain (Shichita et al. 2012), studies of this system would be valuable for those with DS, and have not been undertaken.

GM-CSF as Potential Treatment for AD-DS; Role for Exosomes

At least 20 years in advance of any clinical symptoms — and unbeknownst to most who will eventually be diagnosed with AD — the pathogenic pathway that characterizes AD is initiated. Therefore, a focus on the early, pre-clinical phase of AD may be optimal for the development of more effective approaches to AD diagnosis, treatment, and intervention. However, this approach is complicated by the fact that 90% of AD is sporadic and late-onset, making it difficult, if not impossible, to identify asymptomatic people with early AD pathology, and the autosomal dominantly inherited mutations in the *APP*, *PSEN1*, and *PSEN2* genes that lead to early AD are extremely rare. However, people with DS — who typically carry three copies of the *APP* gene — all develop AD pathology in the brain by age 40, and most develop AD dementia by age 60 and thus provide the best opportunity to examine AD development across the lifespan starting at an early age. Another advantage of studying AD in people with DS lies in the fact that their increased risk for AD is due primarily to the presence of a third copy of the normal *APP* gene (Prasher et al. 1998; Rovelet-Lecrux et al. 2006; Sleegers et al. 2006), and thus the altered physiology of people with DS more closely mimics the etiology and natural history of sporadic late-onset AD.

In our work, we have also focused on epidemiological evidence that, in contrast to people with DS who have an increased risk of developing AD, people with rheumatoid arthritis (RA) have an ~8-fold reduced risk of developing AD. We tested several protein cytokines upregulated in blood from RA patients and found that treatment with one such cytokine — the innate immune system stimulant granulocyte-macrophage colony-stimulating factor (GM-CSF) — reduced AD pathology and completely reversed the cognitive impairment in transgenic AD mice (Boyd et al. 2010). To assess the efficacy of GM-CSF in humans, we retrospectively studied patients who had undergone hematopoietic cell transplantation for cancer and found that patients treated with Leukine® (recombinant human GM-CSF) plus recombinant granulocyte CSF (G-CSF) showed significantly improved cognitive functioning

compared to those receiving either G-CSF alone or no bone marrow enhancement at all (Jim et al. 2012). Based on these findings, we are currently engaged in two related clinical trials to assess the ability of GM-CSF (Leukine®/Sargramostim) to reverse AD pathology in the brains of people with mild-to-moderate AD and to improve cognition. Notably, preliminary findings from our ongoing safety trial with mild-to-moderate AD subjects treated with GM-CSF for 15 days over a 19-day period show no drug-related serious adverse events (AEs), including none of the amyloid-related imaging abnormalities (ARIAs) seen in other trials. Taken together, these findings suggest that the long-time FDA-approved drug Leukine® has the potential to greatly impact AD and related dementias. Our overall hypothesis is that GM-CSF treatment induces changes in the immune system that slow down and/or reverse the development of AD. Furthermore, recent work from our lab suggests that GM-CSF also improves cognition in a mouse model of DS (i.e., Dp(16)1Yey/+), which lacks the classic features of AD, such as amyloid deposition (Ahmed, Wang, Boyd et al., in preparation).

A primary effect of GM-CSF in animal models of AD is to activate the innate immune system, including macrophages and/or microglia in the brain, to better phagocytose amyloid deposits. Such activation can occur directly, but recent evidence indicates that exosomes may provide an important amplification mechanism. Specifically, macrophages activated by GM-CSF generate increased numbers of exosomes, which, although they contain no GM-CSF themselves, when added to naïve macrophages, induce differentiation, increased phagocytic activity, and production of pro-inflammatory molecules, including miR-223 (Ismail et al. 2013). Interestingly, low levels of miR-223 in plasma is a biomarker of AD (Jia and Liu 2016), which apparently, GM-CSF should ameliorate. Other studies indicate that macrophages activated by exosomes secrete increased amounts of GM-CSF, providing a potential amplification of the original GM-CSF signal (Atay et al. 2011).

Finally, GM-CSF has been shown to have other effects likely to be beneficial to the AD and/or DS brain (Boyd and Potter 2012). For example, GM-CSF induces the proliferation and differentiation of neural stem cells, which tend to migrate to areas of damage in the brain (Kruger et al. 2007; Kim et al. 2004), to increase cerebral angiogenesis, and to confer neuroprotection in animal models of stroke and Parkinson's disease (Kong et al. 2009; Schäbitz et al. 2008; Schneider et al. 2007; Todo et al. 2008). Thus, a single treatment with exogenous GM-CSF in AD or AD-DS patients may first act directly, and then indirectly via exosome-mediated amplification, to initiate diverse physiological responses with multiple benefits for cognition and brain recovery.

CONCLUSIONS

The average lifespan of individuals with DS has increased dramatically during the last few decades due to improved healthcare (Glasson et al. 2014; Zigman 2013), and many individuals with DS are now living well into their sixties or later. There are currently no successful medical treatments to prevent DS-associated AD (Hartley et al. 2015; Head et al. 2016). Therefore, the development of therapeutic or preventive agents for comorbid symptoms of AD is highly important for the vulnerable DS population. In order to prevent, or significantly slow down, neuron loss and AD progression in individuals with DS, it seems one will likely have to concurrently target several biological dysfunctions, including:

accumulation of A β peptides, excessive P-Tau levels, oxidative stress, neurotrophic losses, and neuroinflammation. As described in detail above, exosomes may be involved in these processes and can therefore potentially be used for prognosis, as a therapeutic, and/or as biomarkers of health and disease in DS-AD (Fiandaca et al. 2015; Lee et al. 2016; Selmaj et al. 2017).

Exosome cargo composition is highly affected by cellular homeostasis and pathology (Hooper et al. 2012; Prieto et al. 2017; Selmaj et al. 2017; Wahlund et al. 2017). As discussed in this review, toxic A β peptides and toxic species of P-Tau, neuroinflammation, and oxidative stress can all alter exosome content, secretion rate, and the cell-to-cell messages they carry. Several reports indicate that exosomes produced under pathological conditions can either confer protection or increased pathology to their target cells (Borland and Vilhardt 2017; Chiarini et al. 2017; Goetzl et al. 2016; Goetzl et al. 2017; Kapogiannis et al. 2015; Kouwaki et al. 2016; Lee et al. 2016; Li et al. 2013; Papandreou and Tavernarakis 2017). Further study of these processes will provide new insights into how AD pathology spreads throughout the brain and the role of exosomes in this process.

A CNS-derived exosome biomarker approach offers several benefits for interrogation of early events in AD pathogenesis in people with DS. Exosomal screening from blood samples allows insights into the profile of proteins secreted from neurons, as well as glial cells in the brain (Goetzl et al. 2016), and therefore may offer a “liquid biopsy” of ongoing pathological processes for different CNS cell types. This is particularly useful when working in the DS community where confounds of intellectual disability and functionality make a diagnosis of dementia more difficult. Outcome measures can be reliably obtained either from serum or plasma samples from a consortium of biobanks, since data are normalized against the same neuronal or glial exosomal marker. Finally, a CNS-derived exosome approach may prove useful in evaluating potential drug targets for early intervention of AD, while enabling reliable measures of therapeutic efficacy.

Recent studies have demonstrated the usefulness of this novel biomarker technology for prognosis as well as biological mechanisms for AD in the general population (Paschon et al. 2016; Rajendran et al. 2006; Riancho et al. 2017; Sharples et al. 2008; Vingtdoux et al. 2012; Xiao et al. 2017; Yuyama and Igarashi 2017), and can also be used for studying the propagation of pathology within the brain (Polanco et al. 2016; Winston et al. 2016). We are hopeful that this novel methodology can shed light on early events in the pathology in DS-AD and hopefully lead to novel intervention or prevention therapies.

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HIGHLIGHTS

- Exosome technology can detect AD biomarkers decades prior to the onset of symptoms in both DS and in age-matched control populations.
- Exosome signaling and release are affected by both oxidative homeostasis and the inflammatory status of the cell.
- Exosomes play a role in inflammatory processes and may serve as effective vehicles for the delivery of novel therapies targeting oxidative stress, inflammation, and amyloid pathology in the brain.

A. Exosome Size Histogram **B. Exosome Levels by Population** **C. Exosome Levels by Age Group**

Figure 1. CD81 levels were increased in those with DS (*unpublished data*)

(A) The neuron-derived exosome isolation technology captures L1CAM-positive exosomes that have an average size diameter of 117 nm, measured with Nanosight technology. Grey shading represents $\pm$ SEM from several exosome isolations demonstrating the reproducibility of the method. (B) CD81 quantification revealed that the DS cohort had over 39% more circulating neuron-derived exosomes than age-matched controls. (C) Two-way ANOVA of younger (< 35 years) and older (> 35 years) participants in the study revealed that only having DS had a significant effect on neuronal exosome secretion levels, and no age effects were observed. Error bars represent an average $\pm$ SEM. Cohort obtained from the Hamlett et al. manuscript (2016).

A. Neuronal exosome A β_{1-42} levels

B. Neuronal exosome P-S396-Tau levels

Figure 2. Effects of clinical diagnosis on AD biomarkers in neuron-derived exosomes

A β levels in neuron-derived exosomes were significantly reduced in people with DS with dementia compared to people with DS with no cognitive impairment (NCI), while both groups of people with DS had significantly higher levels of A β compared to the age-matched control group. On the contrary, P-S396-Tau levels were significantly elevated in people with DS with a dementia diagnosis compared to both the people with DS with NCI and the control group. *Modified from Hamlett et al., 2016.*

Figure 3. Cytokine levels in serum samples from male Ts65Dn mice that were 8 months of age. Note that not all cytokines were elevated in the Ts65Dn group compared to age-matched normosomic controls.