Extracellular RNA in Alzheimer's disease

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Abstract: Extracellular vesicles (EVs) are released from all cells and represent a heterogeneous mixture of species, differing in size, function, cargo and cellular origin. EVs contain a diverse cargo, including a multitude of, predominantly non-coding, RNA species (exRNA). Alterations in exRNA have been associated with various diseases, including Alzheimer's disease (AD). AD is a progressive neurodegenerative disease of unknown aetiology which is often associated with diagnosis in the later stages of disease and poor therapeutic options. Given the huge number of people affected by AD, both directly and indirectly, the disease has a significant social and economic burden. Thus, there is a critical need for novel mechanistic and therapeutic avenues of discovery. Hence exRNA “fingerprints” may assist with earlier, more reliable diagnoses and greater insight into disease mechanisms. However, given the large variation in study populations (stage of AD, genetic background) and analysis methods (e.g., biofluids, RNA extraction protocols), there is limited concordance in the published literature. Most studies focus on miRNA alterations, and several independent studies have reported downregulation of miR-125b, miR-29b, miR-342, miR-107, miR-142 and miR-15b in plasma/serum/blood and also miR-29c, miR-127 and miR-139 in CSF. Going forward, as exRNA consortia are assembled, protocols are standardised and novel technologies are leveraged, this field holds great promise for AD research.

Keywords: exRNA; extracellular vesicles (EVs); Alzheimer's disease (AD); biomarkers

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Extracellular RNA (exRNA)

RNA was once thought to have an exclusively intracellular localisation, yet recent discoveries have upended this dogma (1). Various populations of RNA have been discovered in the extracellular milieu, collectively termed exRNA. These RNA subtypes include both protein-coding RNA (mRNA), alongside a multitude of non-coding RNAs, namely microRNAs (miRNA), long noncoding RNAs (lncRNAs), circular RNAs (circRNAs), small nucleolar RNA (snoRNAs), small nuclear RNAs (snRNAs), transfer RNA (tRNAs), ribosomal RNAs (rRNAs) and piwi-interacting RNAs (piRNAs) (2). However, the presence of ribonucleases renders the extracellular environment unfavourable for RNA (3). As such, protection against degradation is afforded in two main ways. Firstly, RNA can

be bound in ribonucleoprotein complexes, such as that of Argonaute2 and miRNAs (4). Less frequently RNA can be found as part of lipoprotein complexes (5). Secondly, RNAs can be transported by extracellular vesicles (EVs) (6-8).

EVs

Comprehensive overviews of EV biogenesis and release have been recently published (9-11). However, in brief, EV is an umbrella term encompassing a heterogeneous assortment of vesicular species released from cells (*Figure 1*). These can be broadly categorised into exosomes and microvesicles (MVs) (10), although some investigators prefer ectosome as an alternative nomenclature for the latter (9,12). The term exosome was originally used to

Figure 1 Extracellular vesicles are constitutively released from cells. Endocytosed material progresses from the early to late endosomes, leading to the formation of multi-vesicular bodies (MVBs). RNA can be loaded into these vesicles. Fusion of the MVB with the plasma membrane leads to the release of exosomes into the extracellular milieu. Alternatively, microvesicles, into which RNA can also be loaded, can be released by blebbing directly from the plasma membrane.

describe small vesicles released from reticulocytes (13), with an estimated size of 40–160 nm (9). The possible levels of exosome heterogeneity are well summarised by Kalluri and LeBleu: size, content, function and source (9). Exosomes are derived from intraluminal vesicles contained within endosomal intermediates, multi-vesicular bodies (MVBs), and released from the cell upon MVB fusion with the plasma membrane (14,15). Biogenesis of exosomes can be driven by the ESCRT machinery, acting in a stepwise manner (16–18), although ESCRT-independent exosome release is possible (19). One mechanism for the latter requires ceramide, the generation of which is dependent on neutral type II sphingomyelinase mediating the hydrolysis of sphingomyelin to ceramide (20). Tetraspanins, especially CD63, have also been implicated in ESCRT-independent exosome biogenesis and cargo selection (21). Exosome secretion has been demonstrated in many different cells types and functionally implicated in both physiological and pathological processes (10,11). Although the calculation of exosome release rate is fraught with confounding difficulties, a recent study has estimated a release rate in three breast cell lines. Two breast cancer cell lines (MCF7 and MDA-MB-231) had a release rate of approximately 60–65 exosomes/cell/hour, which was much lower than a comparable non-cancer cell line (MCF10A). In addition to showing the differences in exosome release between

cell lines, this study also illuminated the substantial heterogeneity in exosomes species. Exosomes were captured from MCF10A cells by anti-CD63 probes and only 31% were also immunopositive for CD9, while almost none were immunopositive for CD81. By contrast, in MDA-MB-231 breast cancer cells, almost all exosomes captured by anti-CD63 probes were also immunopositive for CD9 and CD81 (22).

MVs are generally much larger, predominantly ranging in size from 50–1,000 nm (10) and have also been implicated in intercellular communication, most often in cancer cells (23). In contrast to exosomes, MVs are formed by plasma membrane budding and fission, followed by vesicle release into the extracellular milieu (24). MVs are by no means a homogenous population, and incorporate distinct extracellular species such as apoptotic bodies, migrasomes and arrestin-domain-containing protein 1 (ARRDC1)-mediated microvesicles (ARMMs), which have been shown to bud directly from the plasma membrane (10,25,26). Plasma membrane rearrangement is required for MV biogenesis, specifically alterations in protein and lipid composition alongside changes in Ca^{2+} levels. Enzymatic processes dependent on the latter include scramblases, calpain and aminophospholipid translocases. Altogether, these changes cause physical deformation of the plasma membrane and alterations in actin cytoskeleton structure

which favour MV formation and release (10,23,27).

Given the overlapping size of exosomes and MVs, in addition to the presence of some common biogenesis factors, sorting machineries and tetraspanins (10), the nomenclature small EV (sEV) has been encouraged for small vesicles of uncertain origin (28).

Mechanisms of RNA loading

The mechanisms underpinning specific RNA loading into EVs is not well understood (29). It seems clear that the formation of vesicles results in substantial non-specific incorporation of RNA (30). Incorporation of specific RNAs into EVs likely utilises a range of mechanisms (31). Firstly, miRNAs may be transcriptionally modified to guide their fate. Adenylation of miRNA promoted cellular localisation, whereas 3' end uridylation promoted trafficking of RNA to exosomes (32).

In addition to direct transcript modification, RNA binding proteins (RBPs) have a key role in RNA trafficking to exosomes (31,33). There is a huge number of RNA elements that are recognised by RBPs, as comprehensively reported by phase III of the ENCODE project (34). In another study, using a miRNA bait, isolated RBPs were subjected to proteomic analysis revealing several putative exosomal RNA-bound RBPs. Prominent RBPs identified included major vault protein, HSP90AB, XPO5 and hnRNP family members (33). Another study using exosomes isolated from cultured HEK293 cells found several miRNA which were present in exosomes, but not detectable in cells. Furthermore, the exosomal loading of one of these (miR-223) was dependent on YBX1 (35). Subsequent work identified a more wide-ranging role for YBX1 in defining exosomal RNA composition (36). However, in MDA-MB-231 cells, exosome loading of miR-122 was found to be YBX1-independent, but dependent on the Lupus La protein (37). In another study, exosome loading of miR-193a was dependent on MVP (38). The binding between RBPs and target miRNAs may be driven by consensus miRNA sequence motifs. The RBP hnRNPA2B1 binds target miRNA through GC-rich tetranucleotide motifs, which drive exosomal loading. Furthermore, this function is dependent on the SUMOylation of hnRNPA2B1 (39). An alternative hexanucleotide miRNA motif was subsequently identified as a binding target of SYNCRIP, an interaction in which SYNCRIP SUMOylation was important (40). Other studies have implicated different RBPs in exosomal miRNA loading, such as GW182 (41), Ago2 (42) and, potentially,

connexin 43 (43,44). Taken together, these findings show that RBPs can drive the loading of specific miRNAs into exosomes. However, different proteins bind to different complements of miRNAs. Despite substantial recent advances, there is still a great deal of uncertainty about the specific mechanisms driving the trafficking of RNA to EVs (29).

Although miRNA motifs have been the focus of the majority of cargo selection research, there is some evidence to support specific trafficking of mRNA to exosomes. A 25 nucleotide “zip code” has been identified in the 3'UTR, which is present in many mRNAs that are trafficked to EVs. The key features of this “zip code” are a CUGCC core presented on a stem loop structure and a binding site for miR-1289. Increased cellular expression of miR-1289 potentiates target mRNA loading in exosomes, suggesting that miRNA can modulate mRNA trafficking to EVs (45).

Alzheimer's disease (AD)

AD is a progressive neurodegenerative disease of predominantly unknown aetiology, although approximately 1–2% of cases are autosomal dominant. It is the most common form of dementia, predominantly affecting those aged over 65 (termed late-onset AD; LOAD) (46). Cases are typically characterised with deficits in learning and memory alongside a range of other symptoms, which may include difficulty with complex attentional tasks, altered behaviour and impairments in language and executive function (47). However, there is a degree of symptomatic variability between individuals (46). Historically, diagnosis has proved very challenging (48), although this has improved markedly with positron emission tomography (PET) and various fluid biomarkers (46,49,50).

The canonical neuropathological hallmarks of AD are extracellular plaques of the peptide amyloid β ($A\beta$) and intracellular tau tangles (46,51). $A\beta$ has been recognised as the main constituent of the AD hallmark neuritic plaques since the mid-1980s (52,53). $A\beta$ species are formed from amyloidogenic processing of the amyloid precursor protein (APP) (Figure 2). Initially, membrane-bound APP is cleaved by the β -secretase (BACE1), to generate extracellular sAPP β and β -CTF (aka C99), the latter containing the $A\beta$ region (54). The β -CTF is subsequently cleaved by the multiprotein γ -secretase complex presenilin (PSEN), presenilin enhancer (Pen), anterior pharynx defective (Aph) and nicastrin. Given the multiplicity of PSEN and Aph isoforms, various different γ -secretase complexes are

Figure 2 Amyloid precursor protein is processed by amyloidogenic or non-amyloidogenic pathways. The APP holoprotein is sequentially cleaved in either the amyloidogenic or non-amyloidogenic pathways. The former is mediated by the β-secretase and results in the generation of extracellular sAPPβ and CTFβ. The latter is then cleaved by the γ-secretase complex to yield AICD and Aβ species. In the non-amyloidogenic pathway, the α-secretase cleaves APP, resulting in the formation of the extracellular sAPPα and CTFα. Subsequent γ-secretase cleavage of CTFα releases AICD and p3.

possible. The γ-secretase complex can cleave at various sites in APP, giving rise to several different Aβ species, predominantly Aβ₁₋₃₈, Aβ₁₋₄₀ and Aβ₁₋₄₂, the latter being the most aggregation prone (46,55,56). The γ-secretase cleavage also released the amyloid precursor protein intracellular domain (AICD), which can traffic to the nucleus and act as a transcriptional regulator (57-59). However, APP is predominantly cleaved at the cell surface by a disintegrin and metalloprotease domain-containing protein 10 (ADAM10) (60). This non-amyloidogenic cleavage is within the Aβ region and as such prevents its generation. The α-cleavage generates extracellular sAPPα and α-CTF (aka C83), followed by γ-secretase cleavage of α-CTF to generate AICD and p3 (56). However, it seems that AICD is only functional when generated in the β-cleavage pathway (57). Various strands of evidence led to the development of the amyloid cascade hypothesis, which states that Aβ deposition in the brain is the initiating step in AD pathogenesis (61). Data seem to support a role for Aβ in AD (46), but there are also clear inconsistencies in the amyloid hypothesis, especially around the correlation of neuropathological changes with amyloid burden (62-64) and the failure of several amyloid-centric therapies (46). There is a wide-ranging evidentiary basis

for the role of hyperphosphorylated, aggregated tau in AD pathogenesis (46). Unlike Aβ, tau pathology shows good correlation with the progress of cognitive dysfunction (62). The physiological role of tau is unclear, but it does function as a microtubule-binding protein (65). The role of tau in AD is complex and has been reviewed elsewhere (66,67).

MicroRNA (miRNA)

MiRNA are short (18-25 nt) non-coding RNAs which function in post-transcriptional gene regulation. This is achieved via miRNA binding to target sequences in the 3'UTR of mRNA, usually resulting in down-regulation of mRNA and protein (68). Various studies have investigated alterations in extracellular miRNA levels in AD (69). There are important differences between cited studies, including the miRNA source (serum, plasma or CSF), purification of EVs (i.e. different methodologies or no purification), method of RNA isolation and downstream analysis techniques. Inter-study disparities in these parameters can cause significant variation and biases in the resulting data (70,71). In addition, data can be further confounded by sample contamination, such by blood (72) or non-human RNA, predominantly bacterial rRNA (71). Cells have been also shown to release

more than one exRNA profiles, termed low-density and high-density exRNA. These two sub-populations are enriched in different RNA subtypes (73).

Further studies have predominantly focused on miRNA, identifying changes in many miRNAs. Various recent systematic reviews have summarised the literature in this area. Herrera-Espejo *et al.* reported 54 studies comparing circulating miRNA between control and late-onset AD. These encompassed various biofluids, including CSF, serum, plasma, blood, monocytes, and lymphocytes. Overall, analysis showed 271 miRNAs with significant expression changes, but none reached the authors' threshold of appearing in four or more independent studies. This was in contrast to brain tissue (i.e. non-circulating miRNA), which revealed 11 miRNA which were significantly altered in four or more independent studies. In addition, the miRNA in different circulating biofluids is often contradictory (74). As published by Nagaraj *et al.*, reducing the threshold to reporting in two or more studies reveals 36 altered miRNA in blood fractions, of which 15 are upregulated, 5 are downregulated and the remainder are discordantly reported. The top six miRNA in terms of the number of independent reports are miR-125b, miR-29-b, miR-342-3p, miR-107, miR-15b and miR-142-3p, which are all downregulated, although a recent study has shown miR-15b upregulation (75). However, in CSF, the majority of multiply reported miRNAs are discordant. Those which are concordant are miR-29c, miR-127-3p, miR-139-5p and miR-9-5p, which appear in 2–3 independent reports (76) (Table 1 and Figure 3).

Further reviews have reiterated the heterogeneity in results and the consequent difficulty in establishing meaningful AD miRNA biomarkers (8,105–108). Reflecting on this challenge, Kosikova *et al.* report miRNA biomarker panels, comprising anything between two and thirteen different miRNAs. Some of these panels have reached 95% accuracy in detecting AD samples. However, the authors and others note the technical difficulties in validating miRNA hits from next generation sequencing, which may lead to the high number of contradictory findings in the literature (106,109). The accuracy of miRNA biomarker panels may be improved by concomitant analysis of the CSF A β : tau ratio (110).

miR-125b

Multiple studies have linked extracellular miR-125b with AD (76), but potential mechanisms by which miR-125b may be involved in AD are generally not addressed. However,

several cell-based studies have investigated putative roles for miR-125b in disease progression. In cultured cells and rat primary neurons, miR-125b induced neuron apoptosis, reduced neurite outgrowth, potentiated inflammation, including induction of prostaglandin-endoperoxide synthase 2 (PTGS2) and cyclin-dependent kinase 5 (cdk5) (111,112). The latter is a known tau kinase (113) and the ability of miR-125b to drive tau phosphorylation (Ser404) via cdk5 was demonstrated (114). In addition, miR-125b is also able to antagonise the tau phosphatases PPP1CA and DUSP6, alongside the anti-apoptotic factor Bcl-W (115). The activity of miR-125b can be antagonised by lncRNA MALAT1 (111). Recent data also suggest that miR-125b is able to block the protective effects on dendritic spines of melatonin 2 (MT2) receptor agonism. In an APP/PS1 mouse model, a miR-125b sponge reduced dendritic deformations and learning/memory impairments (116). Additional reports have suggested that miR-125b may be induced by reactive oxygen species (ROS) formation (117) or A β (118). A recent study has also linked miR-125b to cortical thickness, glucose metabolism and cognitive performance (119).

However, another study reported miR-125b to be protective in primary cortical neurons via repression of the pro-apoptotic factors BAK and p53 and that 17 β -estradiol is neuroprotective through induction of miR-125b expression (120). In line with this, miR-125b reduced A β toxicity in N2a cells and primary mouse neurons by reducing apoptosis and ROS formation, including the suppression of BACE1 expression (121).

miR-29b/miR-29c

In N2a cells overexpressing APP₆₉₅, delivery of pre-miR-29b was shown to suppress BACE1 expression by approximately 80%, leading to a reduction in A β _{1–42} of approximately 50% (122). Levels of miR-29b are suggested to be reduced in AD and this has been correlated with increased Sp1 mRNA in PBMCs (85). Sp1 has been implicated in a variety of neuroinflammatory processes in neurodegeneration (123). Furthermore, knockdown of miR-29b in mouse brain resulted in ataxia (124).

In an AD mouse model (APP_{Swe}/PS1 Δ E9), miR-29c was increased, although not miR-29a or miR-29b. This was correlated with a decrease in expression of the axonal guidance protein NAV3, which has a miR-29c binding motif in its 3'UTR. The authors thus suggested that NAV3 downregulation may be associated with neurodegeneration (125). In another

Table 1 Key miRNA identified in multiple studies

miR	Biofluid	Change	Change (CSF)	References	Role in AD
miR-125b	Plasma/serum/ exosome- depleted serum ^a	Downregulated, but upregulated in one study (77) ^a	<2 independent reports	(77-84) ^a	Contradictory. Reported to induce apoptosis and reduce neurite outgrowth, but also repress pro-apoptotic factor and reduce A β toxicity
miR-29b	Plasma/serum/ blood/PBMC	Downregulated, but upregulated in one study (77)	<2 independent reports	(77,83,85-87)	Suppression of BACE1 expression, but also downregulation of axonal guidance protein NAV3
miR-29c	N/A	<2 independent reports	Downregulated	(88-91)	Suppression of BACE1 expression, but also downregulation of axonal guidance protein NAV3. Promotes learning and memory in SAMP8 mice
miR-342	Plasma/serum	Downregulated	<2 independent reports	(83,92-94)	Antagonism of miR-342 reduced amyloid burden. Can downregulate ankyrin G, leading to axonal dysfunction
miR-107	Plasma/blood	Downregulated	<2 independent reports	(95-98)	Reduced deleterious effects of A β on cultured cells. May regulate tau phosphorylation through cdk5 and NF- κ B function
miR-15b	Plasma/serum	Downregulated, but upregulated in one study (77)	<2 independent reports	(75,77,92,94,99,100)	Suppressed BACE1 expression and A β generation, as well as reducing NF- κ B activity
miR-142	Plasma	Downregulated	<2 independent reports	(99-101)	Can mitigate the effects of A β on PSD-95 expression. Also shown to reduce neuropathic pain
miR-127	N/A	<2 independent reports	Downregulated	(79,102)	Exacerbated OGD-induced cell injury. Inhibits axonal regeneration after spinal cord transection
miR-139	N/A	<2 independent reports	Downregulated	(79,102)	Over-expression reduced performance in Morris water maze, but also shown to reduce cellular response to pro-inflammatory stimuli
miR-9	Plasma/serum/ blood	Discordant	Downregulated	(79,86,93,95,103,104)	Reduced A β -induced dendritic spine loss. Antagonism caused memory impairment and LTP suppression

Key miRNA are listed, i.e., those reported as having altered circulating expression in ≥ 2 independent studies (76). ^a, in one study (77), miR-125b is only reported as upregulated in exosome-depleted serum.

Figure 3 miRNA are altered in biofluids in AD. Several miRNA are altered, concordantly, in several independent studies. This may be either in plasma/serum/blood (^a) or CSF (^b). For some miRNA (^c), the disparity in arrow size reflects reported downregulation in 4–7 independent studies and upregulation in one study. CSF, cerebrospinal fluid.

study, miR-29c was downregulated in AD brain samples. Furthermore, miR-29c suppressed BACE1 expression in SH-SY5Y cells and although A β was not measured, there was a decrease in sAPP β (126). In blood samples, miR-29c was found to be negatively correlated with BACE1 expression and further, that miR-29c could promote learning and memory in SAMP8 mice (88). In agreement with this, miR-29c over-expression reduced escape latency in the Morris water maze in A β ₁₋₄₀-injected rats (78). This rescue of learning and memory in A β ₁₋₄₀-injected rats was recapitulated by treatment with miR-29-containing exosomes isolated from cultured cells (127). Yang *et al.* also demonstrated reduced miR-29c in AD CSF samples and showed miR-

29c was negatively correlated with DNA methyltransferase 3 (DNMT3), but positively correlated with brain-derived neurotrophic factor (BDNF) (89).

miR-342

The development of AD has been associated with miR-342-3p in post-mortem tissue and 3xTg mouse models. Antagonism of miR-342-3p in transgenic AD mice resulted in improved cognitive performance and reduced hippocampal amyloid burden. Treatment of HT22 cells with supraphathological levels of A β increased levels of miR-342-3p via a mechanism dependent on c-jun N-terminal kinase (JNK) (128). In mice expressing the AD-linked Swedish APP mutant (APP_{Swe}), miR-342-3p was found to be significantly decreased. This resulted in increased expression of chitinase-3-like-1 (CHI3L1), leading to endothelial cell inflammation and vascular smooth muscle cell activation (129). In various PS1 transgenic AD mouse models, miR-342-5p was shown to downregulate ankyrin G expression, occasioning axonal dysfunction in cultured neurons isolated from the transgenic mice. Furthermore, exogenous ankyrin G addition improved the cognitive performance of 12 month old, but not 18 month old, APP/PS1 mice (130,131).

miR-107

The miR-15/107 family includes miR-107, but also the AD linked miR-15b (132). Decreases in miR-107 have been shown in AD progression and are particularly associated with the APOE ϵ 4 allele (133). Analysis of AD patients and A β -treated neuroblastoma cells has suggested that miR-107 is downregulated in both cases (134,135). Transfection of neuroblastoma cells with miR-107 was able to reverse the deleterious effects of A β on cell viability, apoptosis, proliferation and expression of pro-inflammatory cytokines. This may have resulted from miR-107-mediated suppression of the FGFR2/PI3K/Akt pathway (134). These findings are supported by the A β -induced increase in SOX21-AS1, a lncRNA, which then acts as a miR-107 sponge in SH-SY5Y and SK-N-SH neuroblastoma cells. This downregulation of miR-107 leads to apoptosis and increased tau phosphorylation, which are partially recovered by SOX21-AS1 depletion (136). This paradigm of A β induction of lncRNAs and suppression of miR107 was recapitulated for the lncRNA NEAT1 in neuroblastoma cells (137). A β is able to exert a multitude of deleterious effects on the vasculature which collectively result in blood-

brain barrier (BBB) damage. In a cell model, these changes are reversed by miR-107, via suppression of endophilin-1 expression (138).

In human neuroblastoma and rat pheochromocytoma cells, miR-107 is suppressed by 6-hydroxydopamine treatment, a dopaminergic neurotoxin. Subsequent transfection with miR-107 mimics reduces caspase 3 activation, LDH release and ROS levels, while increasing superoxide dismutase expression, potentially through repression of programmed cell death 10 (PDCD10) (139). The coumarin-derivative osthole can increase miR-107 levels, resulting in suppression of BACE1 expression (140,141) and reduced apoptosis of SH-SY5Y cells and primary neurons in response to APP overexpression. In APP/PS1 transgenic mice, osthole was able, via miR-107, to improve learning and memory alongside decreased A β burden (140). However, miR-107 may also suppress the α -secretase, ADAM10 (142). miR-107 has been shown to regulate the cdk5/p35 complex, which is able to phosphorylate tau. This suggests that miR-107 dysfunction may lead to increased tau phosphorylation via increases cdk5/p35 activity (143).

In transgenic mice, miR-107 has also been reported to suppress NF- κ B and SYK, leading to improvement in spatial memory and reduced apoptosis (144). In the transgenic Tg19959 mice, cofilin expression is increased and miR-107 is reduced, the former being a major constituent of the Hirano bodies seen in AD brains. Restoration of miR-107 levels suppresses the translation of cofilin and reduces the abundance of neuronal cofilin rod-like inclusions (145). In wild type C57 mice, injection of A β ₁₋₄₂ aggregates led to spatial memory dysfunction, impaired long term potentiation (LTP) and loss of pyramidal neurons in the CA1 region of the hippocampus. These changes were underpinned by suppression of BDNF expression and TrkB signalling. Collectively, these pathological changes were reversed by the administration of miR-107 mimics (146).

miR-15b

Although miR-15b is in the same family as miR-107 (132), far fewer mechanistic studies have been published. However, miR-15b was found to be significantly reduced in sporadic AD brain compared to control. This was associated with an increase in BACE1 mRNA and protein. In SH-SY5Y cells, miR-15b was able to decrease BACE1 expression via binding the *BACE1* 3'UTR. However, the overall picture is complicated by the protective potential of

anti-miR-15b transfection against A β toxicity in SH-SY5Y cells (147). A subsequent study also showed binding of miR-15b to the *BACE1* 3'UTR and repression of *BACE1* expression in SH-SY5Y cells. In these cells, Li and Wang also showed miR-15b transfection to reduce sAPP β , A β ₁₋₄₀ and A β ₁₋₄₂ alongside a concomitant increase in sAPP α . In addition, a miR-15b mimic was shown to bind the *NF- κ B1* 3'UTR and reduce its expression from a reporter construct. This targeting of NF- κ B signalling was associated with suppression of APP_{Swe}-induced increases in TNF α , IL-1 β , IL-6 and PGE2 expression (148). In HEK293 cells, curcumin has been suggested to increase miR-15b levels, which consequently suppresses APP expression and A β levels (149).

miR-142

In two independent human sample sets and a mouse AD model (APP/PSEN1 Δ E9), miR-142 was downregulated. Furthermore, in SH-SY5Y cells, A β ₁₋₄₂ treatment caused reduced PSD-95 expression, but this effect was mitigated by miR-142 transfection. No miR-142 binding motif was discerned in the PSD-95, so it is possible miR-142 modulates expression of a PSD-95 binding partner rather than PSD-95 itself (150). In a rat model, miR-142 was shown to reduce neuropathic pain via targeting of soluble guanylate cyclase. The authors suggested this may have relevance for people with AD who suffer from neuropathic pain (151). Conversely, Zhang *et al.* showed A β could upregulate miR-142 in human neuronal cells resulting in neurotoxicity, which was suppressed by circHDAC9 expression (152). In addition to its implication in several biomarker and cellular studies, a polymorphism in miR-142 has been associated with a reduced risk of AD. A particular variant (rs2526377:A>G) affects the promoter activity of miR-142 and reduces its expression. The study suggests that this may lead to depression of genes such as *PICALM* and *TGFBR1*, which could be beneficial in AD (153). Interestingly, miR-142 was also the target of a novel nanobiosensor, which couples SYBR Green fluorescence with graphene oxide quenching to sensitively detect miRNA in body fluids (154).

miR-127

No mechanistic studies are available in the published literature linking miR-127 to AD. However, overexpression of miR-127 did exacerbate cell injury induced by oxygen-glucose deprivation (OGD) in PC12 cells (155).

Similarly, after spinal cord transection, miR-127 increased neuronal loss and cell apoptosis, while inhibiting axonal regeneration (156).

miR-139

There is only a single report exploring the possible mechanistic effects of miR-139 in AD. This study, by Tang *et al.*, shows increased APP and miR-139 expression in the accelerated aging SAMP8 mouse model. Over-expression of miR-139 in SAMP8 mice significantly reduces performance in the Morris water maze. However, miR-139 also suppresses cannabinoid CB2 expression, which is linked to microglial activation and miR-139 also reduced the response of cultured neurons to pro-inflammatory stimuli (157).

miR-9

A novel technology using two-photon fluorescent carbon dots showed miR-9 to be upregulated in early onset AD, but reduced in late onset AD (158). Chronic cerebral hypoperfusion has been shown to be symptom of AD and this caused increased miR-9 in a rat model. Treatment with miR-9 antagonists reduced memory impairment, dendritic spine loss and inhibition of long-term potentiation (159). In murine neural stem cells and human neuroblastoma cells, zoanthamine and osthole were suggested to be protective against APP-induced apoptosis, in a miR-9-dependent process (160-162). Treatment of primary hippocampal neurons with A β ₁₋₄₂ oligomers resulted in miR-9 downregulation and activation of the CAMKK2-AMPK pathway, leading to dendritic spine loss and tau phosphorylation (Ser262). Over-expression of miR-9 led to reduced CAMKK2 expression, dendritic spine loss and tau phosphorylation (163). In HeLa cells, luciferase reporter constructs were used to demonstrate the ability of miR-9 to repress *TGFBI*, *TRIM2*, *SIRT1* genes and in the case of the latter, co-transfection with miR-181c had an additive effect. The authors speculated that downregulation of miR-9 in AD may be a protective mechanism, to, for example, increase *SIRT1* expression (164).

Piwi-interacting RNA (piRNA)

First described in 2006, piRNA are 26-30 nt in length, with 2'-O-methylation at their 3' end and can promote gene silencing via interactions with Piwi proteins. Piwi proteins and piRNA can directly modify chromatin

structure and nuclear histone proteins. This is done through interaction with DNA methyltransferase (DNMT), which can methylate promoter regions, suppressing transcription (165).

In disease, alterations in piRNA have been overwhelmingly linked to cancer (165), although there are also some reported cellular alterations in AD (166) as well SNPs in piRNA-associated pathway genes (167). In order to improve accuracy of the extracellular miRNA signature in AD, piRNA have been used. An CSF AD signature was developed, comprising three miRNA (miR-27a-3p, miR-30a-5p and miR-34c-3p) and three piRNA (piR_019324, piR_019949, piR_020364). The accuracy can be further improved by concomitant CSF analysis of A β ₁₋₄₂, tau and phospho-tau (168), in line with the findings of others (110).

Long non-coding RNA (lncRNA)

LncRNAs comprise a class of non-coding RNAs that are in excess of 200 nt and account for a major part of the non-coding transcriptome (169). Although lncRNAs are dysregulated in AD brains (169), there has been limited evidence of this in circulating biofluids (170). An early study reported changes in lncRNA (RP11-462G22.1 and PCA3) between AD and healthy control samples although it is of note that neither lncRNA was discriminatory of AD from Parkinson's disease (PD) (90). However, these findings have not been subsequently validated and the majority of subsequent work in this field has focused on miRNA. Yet there have been some very recent reports focusing on lncRNAs, focusing on BACE1. Comparing control and AD plasma samples, BACE1-AS was significantly reduced in AD plasma. There was no reported change in other lncRNAs, specifically lnc 51A, lnc 17A and BC200 (171). However, another study showed a significant reduction of plasma BACE1-AS only in pre-AD samples relative to control, but a significantly increase in full AD samples. However, when exosomes were isolated, there was no significant change in BACE1-AS levels in AD (172). Targeting of BACE1-AS by siRNA increased *in vitro* proliferation of hippocampal neurons, as improving learning and memory in accelerated aging SAMP8 mice (173). Application of exogenous A β ₁₋₄₂ to either SH-SY5Y cells or C57/BL6J mice caused an increase in BACE1-AS, BACE1 mRNA and BACE1 protein, alongside increased A β ₁₋₄₀, all of which was mitigated by BACE1-AS siRNA (174).

Circular RNA (circRNA)

Exonic circular RNAs (circRNAs) represent a substantial class of regulatory non-coding endogenous RNAs. These are mostly expressed from protein-coding genes, comprising single or multiple exons. They are generated by backsplicing, which happens in a reversed orientation as compared with canonical splicing (175). CircRNAs have been a recent focus of exRNA investigation. Using microarrays on peripheral blood mononuclear cells (PBMCs) from control and AD donors, Li *et al.* identified 4,060 differentially expressed circRNAs (1,990 upregulated and 2,070 downregulated). Overall, gene ontology analysis of these data identified MAPK, mTOR, AMPK, and WNT as key signalling pathways. Eight circRNAs were selected for qPCR validation and this confirmed the upregulation of circRNA_000843, circRNA_101618 and circRNA_405619 (176).

CircHDAC9 was shown to be downregulated in serum of patients with MCI or AD, as compared to control. A mechanistic model was suggested, whereby this downregulation is induced by A β species and decreased circHDAC9 leads to increased miR-138 and consequently SIRT1 and ADAM10 protein, which reduces non-amyloidogenic processing of APP, leading to increased A β (177). The ability of A β to suppress circHDAC9 has been corroborated by a recent study which linked this to increases in miR-142-5p. The effects of A β on circHDAC9 and miR-142-5p were shown to be reversed by berberine (152), a plant alkaloid (178) which has been suggested as a possible therapeutic in AD (179).

mRNA

The role of extracellular mRNA is unclear, although some functional effects have been suggested (30). Few studies have assessed altered extracellular mRNA profiles between control and AD samples, but Gui *et al.* identified seven altered mRNAs (APP, α -synuclein, tau, NF-L, DJ-1/PARK7, fractalkine, and neurosin). Of the mRNAs identified, only tau was altered in AD (a significant decrease), but not Parkinson's disease (90). APP mRNA was also identified as being enriched in AD by a recent study, but this did not reach statistical significance (180). This study by Yan *et al.* identified presymptomatic increases in phosphoglycerate dehydrogenase (PHGDH) extracellular mRNA with progression to AD. PHGDH serum exRNA and brain protein expression increases in AD were validated

by using five additional published cohorts. Mechanistically, the authors tentatively suggest that, as PHGDH catalyzes serine biosynthesis in the brain, which is required for glycine production, there may be a link to excitotoxicity (180). Furthermore, in another report, mRNA for IL-6, IL-12, and TNF α were all elevated in exosomes secreted by macrophages following treatment with A β . Interestingly, the response was significantly enhanced in macrophages isolated from aged patients (>65 years) (181).

Modification of the actual mRNA may result in altered trafficking to EVs in AD, as this can be influenced by 3' end modification (32). In AD, there are examples of alternative polyadenylation (APA), such as increased neocortical expression of a long *COX-2* isoform in AD. In this case, the alternative polyadenylation generates a more unstable transcript which is rapidly degraded (182). Subsequent transcriptomic analyses have revealed widespread APA alterations in AD brains (183) and that 3' poly(A) regions can be lengthened or shortened in AD, depending on the transcript (184). Such changes in APA can be responsive to cellular stress, as arsenite treatment caused a global reduction in poly(A) tail length (185). This treatment can also cause aggregation of RBPs, such as TDP-43 (186).

RNA-binding proteins in AD

It is unclear exactly why exRNA profiles are altered in AD, but one potential route is through RBP dysfunction, such that RNA trafficking to EVs is dysregulated. One example of this is the AD association of SNPs in *TARBP2* (95), which encodes a protein component of the RISC-loading complex alongside Dicer and Argonaute 2 (187). In addition to SNPs which may affect function of the translated protein, RBPs are often incorporated into cellular inclusions in neurodegeneration. Examples of this in AD include TDP-43 (188) and RBM45 (189) and in both cases inclusion formation can be driven by a variety of cell stresses (189,190). Recent work has suggested that, along with TDP-43, the RBPs FUS and matrin 3 are present in cytoplasmic inclusions in AD patients and pR5 (tau P301L) mice (191). The putative RBP, connexin 43, and also several hnRNP family members have also been strongly linked with AD (44,192,193).

In iPSC-derived human neurons, 24 h treatment with 1 μ M oligomeric A β_{1-42} resulted in several proteomic changes germane to exRNA. In AF22 cells (wild type APP), hnRNPF was reduced by 50% on A β_{1-42} challenge, alongside significant reductions in expression of Musashi

homolog 2 and TDP-43, which are all RBPs. None of these changes were significant in the ADP2 neurons, which harbour a V717I mutation in APP (194). Furthermore, YBOX1 phosphorylation is increased by A β_{1-42} (194), which may have significant effects on its ability to traffic RNA to EVs (195). In AD entorhinal cortices, there is evidence of global aberrant splicing, alongside selective reduction in the levels of hnRNP A/B proteins (196). In addition, Sackmann and Hallbeck also identified A β_{1-42} -induced alterations in expression of proteins linked to vesicular trafficking and fusion (194).

Taken together, these data suggest a multitude of potential mechanisms for the dysregulation of RNA trafficking to EVs in AD. These include (I) altered APA; (II) SNPs in genes in RNA trafficking/processing machinery; (III) sequestration of RBPs in cellular inclusions; (IV) stress-induced downregulation of RBP expression; (V) altered posttranslational modification (e.g., phosphorylation) of RBPs and (VI) dysfunctional vesicular trafficking and fusion. These possible processes are concomitant with large scale alterations in the cellular expression profiles of RNA subtypes, but especially mRNA and miRNA (68).

exRNA stoichiometry and cell entry

It has previously been suggested that most EVs do not carry biologically significant numbers of miRNA, specifically that approximately 100 EVs would be required to isolate one miRNA (197). This was corroborated using a novel methodology in cell culture supernatant, focusing on miR-21 and miR-221 (198). These findings conflict with data from neural stem cell cultures, where at least 10 copies of miR-1246 were found per EV (199). However, as highlighted by O'Brien *et al.*, such attempts at stoichiometric analysis face considerable technical confounders (29,200). However, the potentially low numbers of miRNA per vesicle may be offset by the number of vesicles released by an individual cell. Although the rate of EV release is likely highly variable between cells and dependent on conditions, HUVECs were shown to release 37 vesicles per cell under basal conditions (201). Additionally, in a disease like AD, the disease course is so long that exRNA could exert various functions for decades.

The study of Stevanato *et al.* represents one approach to assessing the functionality of exRNA (199), but many others have been deployed. Although there is some discrepancy in the field, the assembled data generally favours the notion

that EV RNA cargo is functional (29,202).

Conclusions

RNA can be isolated from the extracellular milieu. It is predominantly non-coding (chiefly miRNA) and encapsulated in EVs. In AD, many attempts have been made to identify exRNA biomarker signatures that may be used as a diagnostic tool. These approaches vary from single miRNA biomarkers, to miRNA panels, lncRNAs, circRNAs and combinations of exRNA signatures with A β and phospho-tau data. Mechanistically, several of the exRNA species identified have been explored *in vitro* to develop understanding of the putative effects of exRNA on recipient cells. Understanding of the role of exRNA in AD is advancing apace, but this field, more than most, also brings with it a wide range of technical considerations with the potential to confound results.

Future perspectives

Research on exRNA has focused on two overlapping, but also distinct, areas: specifically biomarkers and *in vitro* mechanistic studies. It is likely the former that will bring more rapid benefits. This is due to the availability of samples from individuals living with AD and the observational nature of these studies, that is to say an understanding of exRNA function is not intrinsically required to develop an AD exRNA “fingerprint”. The principle, as outlined in dozens of research publications, is quite straightforward: biofluid isolation from large populations, followed by RNA sequencing and “fingerprint” development. However, the huge disparity in results shows clearly that simplicity of principle does not equate to simplicity of practice. Every stage of the process offers the potential to diverge from the protocols of others. Different studies display differences in MMSE score of study participants, biofluid, isolation of EVs (many studies don’t specifically isolate EVs) and RNA extraction technique, *inter alia* (76,203). Given these technical issues, elegantly detailed by O’Brien *et al.* (29), it should come as no surprise that exRNA studies in AD rarely provide concordant results (74). This also highlights the importance of arriving at a consensus in the EV field and, as such, standard protocols (i.e., MISEV guidelines) are vital (28). It seems clear that exRNA biomarker studies will only bring benefit with standardised protocols across multiple centres. In practice, this requires a consortium, given the lack of

incentive for individual investigators to repeat and validate the work of others. Thus the exRNA Atlas is a hugely positive development, a NIH-funded consortium of experts comprehensively assessing exRNA (204).

Independently of the technical difficulties of EV, AD research presents a slew of challenges. Foremost among these is the challenge of diagnosing AD, in the frequent absence of neuroimaging or biomarker assays. Further complicating this is the frequent coexistence of AD with other pathologies, including Lewy bodies and/or TDP-43 inclusions (188,205). These differences in pathology are accompanied by vast genetic differences (206). Taken together, these findings are highly suggestive of substantial pathological, biochemical and genetic heterogeneity in study populations with an AD diagnosis. This heterogeneity may confound efforts to reliably discover alternations in exRNA profiles associated with AD. As has been previously reported (76,203), as indicated by MMSE scores, some exRNA studies have a predominantly mild cognitive impairment (MCI, pre-AD) cohort, while other studies focus on late-AD cohorts.

The second focus of exRNA in AD is *in vitro* mechanistic studies. Certainly, published studies have shown that miRNA are capable of modulating, for example, BACE1, NF- κ B and learning/memory. The difficulty is finding an appropriate model system for assessing the relevance of these *in vitro* findings for AD in human patients. There is a multiplicity of unanswered questions, including how the exRNA profile changes through the AD disease course, the extent to which the exRNA can enter recipient cells *in vivo* over time (decades in AD) and whether transferred exRNA actually does have an effect on the translational programme. Transfection of cultured cells with high concentrations of a particular miR over a 24–48-hour time period, while not without its uses, is quite far removed from the physiology of an individual with AD. However, the rapid advance of analytical technologies may result in answers to these key questions. Substantial progress has been made on analysis of single EVs (207–212) and single cell transcriptomics (213), alongside the emergence of single cell proteome analytic techniques (214). Taken together, a nexus of these novel technologies may offer the ability to analyse the exRNA content of single EVs from individuals with AD throughout the disease course. This could be combined with single cell transcriptomic and proteomic analysis of recipient cells, *in vivo*, to understand the extent to which exRNA can exert functional effects.

Overall, the studies of exRNA in AD to date have

demonstrated both the potential and limitations of this field. The approach to date, predominantly consisting of individual investigators (or small groups) assessing single cohorts, has served its purpose in demonstrating the potential of exRNA biomarkers. Going forward, a new approach is imperative, developing large consortia (204), standardising protocols (28) and leveraging emerging technologies (210,213,214). This may then help realise the immense promise that this field holds.

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Footnote

Conflicts of Interest: The author has completed the ICMJE uniform disclosure form (available at <http://dx.doi.org/10.21037/exrna-21-2>). The author has no conflicts of interest to declare.

Ethical Statement: The author is accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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