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Extracellular microRNAs in human circulation are associated with miRISC complexes that are accessible to anti-AGO2 antibody and can bind target mimic oligonucleotides

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MicroRNAs (miRNAs) function cell-intrinsically to regulate gene expression by base-pairing to complementary mRNA targets while in association with Argonaute, the effector protein of the miRNA-mediated silencing complex (miRISC). A relatively dilute population of miRNAs can be found extracellularly in body fluids such as human blood plasma and cerebrospinal fluid (CSF). The remarkable stability of circulating miRNAs in such harsh extracellular environments can be attributed to their association with protective macromolecular complexes, including extracellular vesicles (EVs), proteins such as Argonaut 2 (AGO2), or high-density lipoproteins. The precise origins and the potential biological significance of various forms of miRNA-containing extracellular complexes are poorly understood. It is also not known whether extracellular miRNAs in their native state may retain the capacity for miRISC-mediated target RNA binding. To explore the potential functionality of circulating extracellular miRNAs, we comprehensively investigated the association between circulating miRNAs and the miRISC Argonaute AGO2. Using AGO2 immunoprecipitation (IP) followed by small-RNA sequencing, we find that miRNAs in circulation are primarily associated with antibody-accessible miRISC/AGO2 complexes. Moreover, we show that circulating miRNAs can base-pair with a target mimic in a seed-based manner, and that the target-bound AGO2 can be recovered from blood plasma in an ~1:1 ratio with the respective miRNA. Our findings suggest that miRNAs in circulation are largely contained in functional miRISC/AGO2 complexes under normal physiological conditions. However, we find that, in human CSF, the assortment of certain extracellular miRNAs into free miRISC/AGO2 complexes can be affected by pathological conditions such as amyotrophic lateral sclerosis.

Argonaute | extracellular microRNA | human plasma | cerebrospinal fluid | AGO2

MicroRNAs (miRNAs) are abundant inside cells, where they regulate gene expression posttranscriptionally, but are also found in circulation as remarkably stable extracellular constituents of body fluids such as human blood plasma and cerebrospinal fluid. We observed that these circulating miRNAs are predominantly associated with free Argonaut 2 (AGO2) complexes that are accessible to immunoprecipitation by AGO2 antibodies and are able to base-pair with complementary target RNAs, suggesting that, under normal physiological conditions, circulating miRNAs are largely contained in functional miRNA-mediated silencing complexes (miRISCs). However, we observe that, under certain pathological conditions, more miRNAs become associated with complexes other than free AGO2/miRISC, suggesting that the assortment of circulating miRNAs into distinct complexes offers a potential new dimension to miRNA-based diagnostics.

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portion of miRNAs in blood plasma and/or cell culture media could be separated from EVs by ultracentrifugation (7) and fractionated together with AGO2 in subsequent ultrafiltration (5, 20). It is possible that the yield of miRNAs recovered from EVs could depend on whether the particular protocol used to enrich for EVs can effectively distinguish between miRNAs encapsulated within EVs or, alternatively, miRNAs associated with the exterior surface of the EVs (Fig. 1).

The underlying mechanisms by which cells export miRNAs are poorly understood, and it is not known which extracellular carrier complexes, such as EVs or free AGO2, may reflect active versus passive export of miRNAs from cells. In some disease contexts (14, 21, 22), cells can release miRNAs passively due to injury, chronic inflammation, apoptosis, or necrosis. There is also evidence that cells can actively export miRNAs by selectively packaging them into particular carriers (23–27), perhaps to maintain cell homeostasis or send signals to recipient cells. For example, certain cancer cell lines appear to selectively export let-7 family miRNAs into the extracellular milieu via EVs, conceivably to maintain their oncogenic state (28). Similarly, miR-126 released into apoptotic bodies by cells during atherosclerosis is reported to communicate paracrine alarm signals to vascular cells, which in turn produce protective chemokines (24).

The apparent stability of miRNAs in circulation supports the hypothesis that extracellular miRNAs could reflect a mode of RNA-mediated intercellular communication, such that miRNAs produced in one cell could function to regulate gene expression in another cell. In this regard, there is evidence that miRNAs in circulation could be functional in recipient cells, either in cell culture or in the intact mouse (15, 29–32). However, the concentration of extracellular miRNAs in blood plasma is in the order of ~5 pM (33), far less than the concentrations considered to be minimal for intracellular function, which are estimated to at least ~26 pM (34), or even in the nanomolar range when considering intratarget pool competition in the cytoplasm (4). Therefore, the sparse concentration of miRNAs in circulation would need to be dramatically concentrated upon uptake in order to function in a recipient cell. However, it is possible that the miRNAs detected in circulation may correspond to a diluted sample of more concentrated (and thus potentially functional) populations of extracellular miRNAs in the interstitial milieu of tissues.

In this study, we comprehensively characterized the profiles of circulating miRNAs associated with AGO2 in human plasma and CSF by immunoprecipitating AGO2 under native conditions, followed by small RNA sequencing. Our studies show that a major fraction of miRNAs in circulation are primarily associated with AGO2 complexes that readily bind to anti-AGO2 antibodies under native conditions, and therefore are not enclosed within vesicles. We also find that a subfraction of the population of certain circulating miRNAs are inaccessible for AGO2 immunoprecipitation and therefore apparently are associated with other complexes, such as other Argonaute proteins or the interiors of extracellular vesicles.

We find that, for particular miRNAs in CSF samples, the AGO2 association profiles can differ significantly under pathological conditions such as elevated intracranial pressure (ICP) or amyotrophic lateral sclerosis (ALS) compared with healthy CSF samples, suggesting that AGO2 immunoprecipitation can potentially enhance the resolution of miRNA biomarker screening.

We assessed the target binding potential of miRISC in circulation by introducing an affinity matrix-linked target mimic into human plasma samples under native conditions and subsequently assessing depletion and recovery of the complementary miRNA via quantitative real-time PCR (qRT-PCR) and small-RNA sequencing. By using complementary target RNA against two distinct miRNAs, we show that the miRNAs associated with free miRISC/AGO2 in circulation can base-pair with their RNA targets in a seed-guided manner, and therefore could potentially function in the event that they were to be taken up by cells in sufficient quantities.

**Results**

miRNAs Can Be Depleted and Recovered from Human Blood Plasma by Native Immunoprecipitation with Anti-AGO2 Antibody. In order to identify plasma miRNAs associated with free, antibody-accessible AGO2, we immunoprecipitated AGO2 from 20 μL of human blood plasma samples using 10 μg of anti-AGO2 antibody with overnight incubation (Fig. 2A, **SI Appendix, Fig. S1, and Materials and Methods**). Anti-AGO2 Western blot analysis showed a band at ~97 kDa, which is the expected molecular weight of AGO2, in the AGO2 immunoprecipitation, while there was no such band evident for control IgG immunoprecipitation (Fig. 2B).

To confirm that the 97-kDa band reacting with anti-AGO2 in our Western blots was indeed immunoprecipitated AGO2 and not cross-reacting protein(s), we conducted mass spectrometry analysis of gel sections cut at specific ranges of molecular weights (Fig. 2C). Mass spectrometry analysis confirmed the presence of AGO2 protein in AGO2 immunoprecipitation at the correct molecular weight section (slice C, 75 to 100 kDa) and the absence of AGO2 in the control IgG immunoprecipitation. AGO2 protein was only detected in the gel slice corresponding to ~75 to 100 kDa and not at any other molecular weight (Fig. 2D and **Dataset S1**).

qRT-PCR analysis showed that miR-16 was depleted from plasma by AGO2 immunoprecipitation compared with control IgG immunoprecipitation and that the depleted miR-16 could be recovered from the immunoprecipitated beads (Fig. 2E). This indicates that miR-16 in human blood plasma is largely associated with antibody-accessible AGO2. To comprehensively identify the miRNAs associated with these complexes, we conducted small-RNA sequencing of RNA remaining in the supernatant after AGO2 immunoprecipitation and RNA recovered from the AGO2-immunoprecipitated beads. Quantitative analysis of RNA-seq data was conducted by normalizing the UMI counts of the circulating miRNA across the samples to a standard quantity of spike-in miRNA (cel-mir-39) that was added to each sample.
at the Trizol extraction step, thus allowing a direct measurement of depletion and recovery (Materials and Methods). We observed that essentially all of the miRNAs detected by RNA sequencing were at least partially depleted from plasma by AGO2 immunoprecipitation compared with control IgG immunoprecipitation (Fig. 3A), suggesting that many plasma miRNAs are associated with antibody-accessible AGO2. Many of the depleted miRNAs seem to be efficiently recovered from the anti-AGO2 beads, with some exceptions,
especially among the less abundant miRNAs (Fig. 3B). Across all miRNAs detectable in human blood plasma samples (n = 2) by our small RNA sequencing, we observed an average depletion of 78% (Fig. 3C). We identified 304 miRNAs that were depleted at least 50% by anti-AGO2 immunoprecipitation and 19 miRNAs that were less than 50% depleted by AGO2 immunoprecipitation (Fig. 3C). Of the 130 miRNAs that we detected by small RNA sequencing in 5 biological replicates, 114 miRNAs were depleted less than 50% in a statistically significant manner, and the remaining 5 miRNAs showed an average depletion of less than 50% but did not meet statistical significance (SI Appendix, Fig. S2, and Dataset S2).

These depletion and recovery data suggest that most circulating miRNAs in our human plasma samples are predominantly associated with AGO2 complexes that are free to interact with anti-AGO2 antibodies under the conditions of our immunoprecipitation experiments. The poorly immunoprecipitated miRNAs could be associated with AGO2 complexes that have their anti-AGO2 binding epitopes masked by other proteins, or they could be associated with other RNA-binding proteins, such as AGO1/3/4, or they could be enclosed within vesicles (Fig. 1). It should be noted that our immunoprecipitation experiments are conducted using essentially native plasma, and, in particular, the immunoprecipitation conditions do not include any detergent and hence should maintain any extracellular membranous vesicles in their intact state.

Most of the miRNAs in Normal Human CSF Can Be Depleted by AGO2 Immunoprecipitation. We conducted AGO2 immunoprecipitation using 250 μL of human CSF samples from a cohort of healthy individuals (n = 3) and assessed mRNA depletion using small RNA sequencing (Fig. 4A). For the 203 miRNAs represented in our sequencing data, the average depletion was 62% (Fig. 4D). Out of the 155 miRNAs that were at least 50% depleted from CSF by anti-AGO2 antibodies, 126 miRNAs showed statistical significance, and, out of the 48 miRNAs that exhibited less than 50% depletion, 22 miRNAs showed statistical significance (Dataset S3). This suggested that the majority of the miRNAs in CSF samples from healthy individuals are associated with antibody-accessible AGO2 complexes.

The results reported above, showing immunoprecipitation of miRNAs from plasma and CSF using anti-AGO2 antibodies, suggest that a major portion of circulating miRNAs in blood plasma and CSF are associated with AGO2 that is accessible to bind anti-AGO2 antibody. However, mere recovery of miRNA from anti-AGO2 IP does not in itself confirm that the miRNAs are loaded into Argonaute in the standard manner, or even directly associated with Argonaute. If the miRNAs are loaded into AGO2, then there are two predictions: (i) circulating miRNAs should exhibit the capacity to bind to a target via seed-mediated base pairing and (ii) miRNAs and their AGO2 partner proteins should be present in an ~1:1 ratio in plasma or CSF. In the following two sections, we test these predictions.

Fig. 3. Plasma miRNAs can be depleted by AGO2 immunoprecipitation. (A) Small-RNA sequencing of RNAs extracted from plasma supernatant following control IgG (x-axis; n = 2) and AGO2 (y-axis; n = 2) immunoprecipitation. A cel-miR-39 spike-in was included to normalize the data to the original sample volume, allowing a direct comparison of the yields of miRNAs from control and experimental samples. Essentially all of the miRNAs detected in the sequencing dataset are below the diagonal line, indicating a general depletion of miRNAs from plasma by AGO2 immunoprecipitation. (B) Small RNA sequencing analysis of depletion vs. recovery in plasma. Depletion was calculated by subtracting AGO2 immunoprecipitated supernatant miRNA counts, and recovery was calculated by subtracting control IgG-immunoprecipitated miRNA counts from the AGO2-immunoprecipitated miRNA counts. (C) Distribution of the percent depletions by anti-AGO2 immunoprecipitation for 323 miRNAs (n = 2 samples). Average depletion was ~78% (gray dashed line); 19 of the 323 miRNAs were depleted less than 50% (blue dashed line). The positions of miR-16 and let-7a are labeled.
Circulating miRNAs Can Base-Pair Sequence-Specifically to a Target Mimic Oligonucleotide. In order to assess whether AGO2-associated plasma miRNAs are accessible for base-pairing with a target, we incubated 5 pmol of biotinylated 2′-O-methyl RNA oligonucleotides (conjugated to streptavidin beads) that were fully complementary to either miR-16 or let-7a in plasma for 15 min at room temperature (Fig. 5 A and B, SI Appendix, Fig. S3, and Materials and Methods).

qRT-PCR data showed that a target oligonucleotide complementary to miR-16 depleted ~90% of miR-16 from plasma, whereas let-7a was not depleted by the miR-16 target oligo (Fig. 5 C). Conversely, using a target oligo complementary to let-7a, we observed depletion (~50% via qRT-PCR) of let-7a from plasma and essentially no depletion of miR-16 (Fig. 5 C).

These results indicate that the miR-16 and let-7a complementary targets specifically depleted their cognate miRNAs from human blood plasma. Consistent with sequence-specific capture, miR-16 (but not let-7a) was recovered from miR-16 target oligonucleotide-conjugated beads (Fig. 5 D and SI Appendix, Fig. S4; recovery protocol described in Materials and Methods).

If the miR-16 target oligonucleotides were capturing miR-16 miRISC through Argonoule-facilitated (seed sequence-mediated) base pairing, then we expect that the miR-16 target should also deplete other members of the miR-16 seed family from plasma. We conducted qRT-PCR assays on RNA from plasma incubated with miR-16 target oligonucleotide or scrambled oligo to measure the specific depletion of a selected set of miRNAs, including the miR-16 family members miR-16, miR-15a, and miR-195 and other non-miR-16 family miRNAs, miR-451, miR-92, miR-223, miR-1249, miR-22, miR-126, miR-142-3p, miR-4454, and miR-191. We observed that the target oligonucleotides complementary to miR-16 depleted the miR-16 family, but did not appreciably deplete the non-miR-16 family miRNAs tested, with the exception of miR-191 (SI Appendix, Fig. S5; see additional discussion below regarding this partial depletion of miR-191 by miR-16 target oligo).

In order to comprehensively evaluate the sequence specificity of binding of circulating miRNAs with a defined target sequence, we conducted small RNA sequencing from the plasma supernatant (to assess depletion) and the bead-bound miRNAs (to assess recovery) after incubation of the beads containing the miR-16 target oligo. Small RNA sequencing analysis showed statistically significant depletion of miR-16 and the miR-16 seed family members miR-15a, miR-15b, and miR-195. Interestingly, we also observed depletion of members of another seed family of miRNAs, miR-103 and miR-107 (Fig. 6 A and B). Depletion of the miR-103/107 family can be accounted for by the fact that the miR-103/107 seed (GCAGCAU) is similar enough to the miR-16 seed (AGCAGCA) such that miR-16 target oligonucleotide contains a nucleotide 2–7 hexamer seed match to miR-103/107.

Fig. 4. The profiles of miRNAs depleted by AGO2 immunoprecipitation from healthy CSF samples and CSF from patients with ALS or ICP. (A) Small-RNA sequencing of supernatant following control IgG (x-axis; n = 3) and AGO2 (y-axis; n = 3) immunoprecipitation in CSF from healthy individuals. Almost all miRNAs are below the diagonal line, indicating a general depletion of almost all miRNAs in CSF from healthy individuals. (B) Small-RNA sequencing of supernatant following control IgG (x-axis; n = 3) and AGO2 (y-axis; n = 3) immunoprecipitation in CSF from ALS patients. Fewer miRNAs (than in A) are below the diagonal line for the ALS samples, indicating an increase in miRNAs refractory to immunoprecipitation in the CSF from ALS patients compared with healthy individuals. (C) Small-RNA sequencing of supernatant following control IgG (x-axis; n = 2) and AGO2 (y-axis; n = 2) immunoprecipitation in CSF from patients with ICP. Again, fewer miRNAs (than in A) are below the diagonal line for the ICP samples, indicating an increase in miRNAs refractory to immunoprecipitation in the CSF from ICP patients compared with healthy individuals. For A–C, the red dots represent the miRNAs that are refractory to depletion (P ≤ 0.05) in ALS CSF (B) but are efficiently depleted from control CSF (P ≤ 0.05 or P ≤ 0.1; A); the turquoise dots represent the miRNAs that are efficiently depleted (P ≤ 0.05) in healthy control CSF (A) but refractory to depletion (P ≥ 0.1) by AGO2 immunoprecipitation in CSF from ICP patients (C). miR-203b-5p is refractory to depletion in both ALS and ICP CSF samples compared with CSF samples from healthy individuals, and thus represented in an underlined (turquoise) red dot in A. (D–F) Distribution of miRNA depletion by AGO2 immunoprecipitation in healthy control CSF (D), ALS CSF (E), and ICP CSF (F). The average depletions (dashed gray lines) are ~62%, ~45%, and 48%, respectively. The UMI count for each sample is normalized to the spike-in cel-miR-39.
Similar to the qRT-PCR data reported above, our small RNA sequencing data also indicated a partial depletion (albeit not statistically significant by Deseq2 analysis) of miR-191 with the target oligo complementary to miR-16 (SI Appendix, Fig. S6). We note that miR-191 has partial complementarity in its seed region and 3′ supplementary region to the miR-16 target/capture oligo, which perhaps could account for the partial depletion of miR-191 by this miR-16 target oligonucleotide. Only the recovery (from beads) of miR-16 was statistically significant (Deseq2 analysis) compared to the incubation of a scrambled oligo (SI Appendix, Fig. S6 A and B) that did not have a specific target. We were unable to recover all of the miR-16 that was depleted, perhaps due to degradation during the lengthy recovery protocol that includes a high-temperature incubation (SI Appendix, Fig. S6).

Similarly, small RNA sequencing showed that let-7a and its family members, let-7b, let-7c, and let-7d, were depleted from plasma when a target fully complementary to let-7a was used (Fig. 5 C and D). Let-7a was depleted from plasma with a target oligo against let-7a, while miR-16 was not depleted. qRT-PCR assays indicated that let-7a was ∼50% depleted from plasma by the let-7a complementary target mimic oligonucleotide (Fig. 5C). However, small-RNA sequencing data showed that let-7a was ∼80% depleted by the same let-7a target mimic (SI Appendix, Fig. S7A). This apparent discrepancy between qPCR and small RNA sequencing could reflect a relaxed specificity of the qRT-PCR assay for let-7a, such that other (relatively less depleted) let-7 family members may have been captured.
detected in addition to let-7a. In this respect, we note that, in our small RNA sequencing data, the cumulative average depletion of let-7 family members (let-7a, let-7b, let-7c, let-7d, let-7e, let-7f, let-7g, and let-7i) was $\sim$34% (SI Appendix, Fig. S7).

These results using affinity matrix-linked target mimics against miR-16 and let-7a show that circulating miR-16 and let-7 retain the capacity to engage in sequence-specific target binding in a manner consistent with their association with active miRISC.

Stoichiometry of miRNA and Argonaute in Circulating miRISC/AGO2 Complexes. We quantified the amount of AGO2 immunoprecipitated from human plasma by anti-AGO2 antibodies using Western blotting and a purified human AGO2 protein as a quantification standard. The results indicate a concentration of antibody-accessible AGO2 in plasma of $\sim$35 pM (SI Appendix, Fig. S8). Similarly, we measured the amount of antibody-accessible AGO2 in CSF to be $\sim$100-fold less than that in plasma (SI Appendix, Fig. S9 A and B). This corresponds roughly to the relative levels of certain major miRNAs in CSF compared with plasma. For example, miR-16 was estimated to be $\sim$640 fM in plasma and $\sim$1.6 fM in CSF (SI Appendix, Fig. S9 C and D); miR-451 was estimated to be $\sim$2.6 pM in plasma and $\sim$15 fM in CSF (SI Appendix, Fig. S9 E and F).

We then quantified the amount of AGO2 recovered by oligonucleotides complementary to miR-16 or miR-451 (SI Appendix, Fig. S10). We determined that the amount of AGO2 recovered from 200 μL of plasma by each miRNA target oligonucleotide was similar to the amount of the respective miRNA in the same volume of plasma. miR-16 levels in plasma were determined to be about 640 fM (SI Appendix, Fig. S9C), which corresponds to $\sim$0.13 fmoles in 200 μL; the amount of miR-16-bound AGO2 recovered from 200 μL of plasma was similarly $\sim$0.1 fmoles (SI Appendix, Fig. S10 A and B). Likewise, miR-451 levels in plasma were determined to be an average of $\sim$2.6 pM (SI Appendix, Fig. S9E), which corresponds to $\sim$0.5 fmoles per 200 μL; the amount of miR-451-bound AGO2 recovered from 200 μL of plasma was similarly $\sim$0.56 fmoles (SI Appendix, Fig. S10 C and D). The essentially 1:1 ratio between the respective circulating miRNA and the AGO2 recovered by miRNA-specific target mimic supports the conclusion that circulating miR-16 and miR-451 are associated with bona fide miRISC.

Stability of Circulating miRISC Complexes against Sample Freeze/Thaw Cycles and during Incubations for Immunoprecipitation and Target Oligo Capture. Generally, our fresh plasma and CSF samples were aliquoted and frozen at $\sim$80 °C and thawed at the time...
of the experiments. Therefore, it was a formal possibility that some of the properties of circulating miRISC that we observed, such as their accessibility to immunoprecipitation by anti-AGO2 antibodies and base-pairing with target oligonucleotides, could be a consequence of their release from the protection of hypothetical higher-order complexes by a single cycle of freezing and thawing.

Accordingly, fresh (never frozen) plasma samples were collected from volunteers, split into aliquots, and, for each sample, one never-frozen aliquot was kept in ice (4 °C) for (immediate) further analysis while another aliquot was subjected to one cycle of freezing (−80 °C). Both the freeze-thawed aliquots and the fresh (never-frozen) aliquots were processed in parallel for AGO2 immunoprecipitation or target mimic oligo pull-down and subsequent small RNA extraction.

We observed very similar patterns of miRNA depletion by AGO2 immunoprecipitation from frozen vs. fresh aliquots of plasma, where essentially none of the miRNAs showed statistical significance (Deseq2) in depletion between fresh vs. frozen samples (SI Appendix, Fig. S11).

Likewise, we used a 2′-O-methyl oligonucleotide target mimic to pull down miR-16 from frozen and fresh aliquots of otherwise identical plasma samples and observed that the miR-16 family and the miR-107/103 family miRNAs were depleted (Deseq2) similarly from plasma, for both the fresh and the frozen samples (SI Appendix, Fig. S12).

To evaluate whether the stability of circulating miRNAs detectable in plasma can be appreciably affected by freeze-thawing or by the incubation conditions associated with our immunoprecipitation and target mimic pull-down experiments, we compared the abundance of miRNAs via small RNA sequencing between otherwise identical aliquots of fresh plasma samples (never frozen; maintained on ice) that were either processed immediately for RNA extraction (“Fresh” in SI Appendix, Fig. S13) and frozen (−80 °C) and thawed (SI Appendix, Fig. S13 A and B) or never-frozen plasma samples left at room temperature (−22 °C) for 30 min (which corresponds to the target mimic pull-down conditions; SI Appendix, Fig. S13 C and D) or never-frozen plasma samples left overnight at 4 °C (which corresponds to the immunoprecipitation conditions; SI Appendix, Fig. S13 E and F). In none of these comparisons did we detect appreciable changes (Deseq2 analysis) in miRNA profiles; the only noted exception was that miR-145-3p levels were decreased during the 30-min room temperature incubation.

These findings indicate that abundances of miRNAs in our plasma samples, and the accessibility of miRNA:carrier complexes to immunoprecipitation by anti-AGO2 or target mimic pull-down, is not an artifact of using frozen samples, nor are there appreciable changes in miRNA abundance over the course of our experimental incubations (SI Appendix, Figs. S11–S13).

**miRNA Depletion by AGO2 Immunoprecipitation of CSF from Normal Controls Compared to Patients with CNS Pathologies.** We conducted AGO2 immunoprecipitation using CSF samples from amyotrophic lateral sclerosis (ALS) patients and observed a pattern of miRNA depletion that differed from that observed in healthy CSF samples. Fewer miRNAs were efficiently depleted from ALS CSF compared with CSF from healthy individuals (Fig. 4 A–D). Overall, most miRNAs detected in normal CSF were depleted to varying levels by AGO2 immunoprecipitation (average depletion of ~62%; n = 3), while certain miRNAs in ALS CSF appear to be resistant to depletion by AGO2 immunoprecipitation (average depletion of ~45%; n = 3). For example, miR-203b-5p, miR-150-5p, and miR-676 were ~54% (P = 0.02), ~64% (P = 0.01), and ~59% (P = 0.0003) depleted, respectively, in healthy control CSF by AGO2 immunoprecipitation, while miR-203b-5p was not depleted at all (0%; P = 5.0E-324) and miR-150-5p and miR-676 were only ~31% (P = 0.03) and ~36% (P = 0.003) depleted, respectively, in ALS CSF samples. Similarly, miR-3168 and miR-625-3p were 69% (P = 0.1) and 61% (P = 0.01) depleted, respectively, following AGO2 immunoprecipitation in healthy control CSF samples, but appear to be refractory to depletion (P = 0.058) in ALS CSF (Fig. 4B and Datasets S4 and S5). These observations suggest that certain miRNAs may be associated with either of the two broad classes of immunologically distinguishable complexes in CSF, depending on the pathophysiological conditions. Under normal physiological conditions, CSF miRNAs appear to be primarily associated with miRISC/AGO2 complexes that are accessible by anti-AGO2 antibodies, while, under certain pathological conditions, CSF miRNA may be associated with complexes that are unable to interact with AGO2 antibodies.

We also conducted AGO2 immunoprecipitation in 400 μL of CSF from patients with elevated intracranial pressure (ICP). AGO2 immunoprecipitation from ICP CSF (Fig. 4C) resulted in a pattern of miRNA depletion that was different from normal CSF. Similar to what was observed in ALS CSF, certain miRNAs were resistant to AGO2 immunoprecipitation (average depletion of ~48%; Fig. 4F). For example, miR-203b-5p, miR-205a-5p, miR-152-5p, miR-1911-5p, and miR-138-2-3p were each more than 50% depleted (P ≤ 0.05) in healthy control CSF, while they were completely refractory to depletion (P ≤ 0.05) in ICP CSF samples. Similarly, miR-9-5p and miR-125a-5p were also more than 50% depleted (P ≤ 0.05) by AGO2 immunoprecipitation in healthy control CSF but were less than 50% depleted (P ≤ 0.05) in the ICP CSF samples (Datasets S6 and S7).

**Discussion**

We observed that almost all of the miRNAs in normal human plasma could be immunoprecipitated by AGO2 antibodies, suggesting that the majority of plasma-borne miRNAs are associated with miRISC/AGO2 complexes that are free to interact with anti-AGO2 antibodies (Fig. 1). Across 5 independent samples of plasma small RNA sequencing, only 11 miRNAs were less than 50% depleted by AGO2 immunoprecipitation; apparently, a substantial fraction of these 11 miRNAs (miR-155, miR141-3p, miR-34a, miR-10b, miR-223-3p, miR-10h, miR-125a-5p, miR-125b-5p, miR-29b-3p, miR-30e-3p, miR-30a-5p, and miR-126-3p) are associated with complexes in which AGO2 is absent or inaccessible to anti-AGO2 antibodies (Fig. 1). It is possible that the antibody-inaccessible fraction of these 11 miRNAs reflects enclosure within EVs, as these same miRNAs have been previously reported to be exosome-associated (15, 35–39). It should be noted that, in our experiments, some portion of the miRNAs that were not immunoprecipitated with AGO2 could be associated with AGO1, AGO3, or AGO4; we did not assess the contribution of these other Argonuases to circulating miRISC, as our antibody was specific to AGO2.

In support of the conclusion that miRNAs in circulation are predominantly miRISC-associated, we find that the two miRNAs that we tested (let-7 and miR-16) can base-pair with a complementary 2′-O-methyl oligonucleotide target mimic in a sequence-specific manner. Moreover, the binding of circulating miRNAs to a target mimic appears to be mediated by seed nucleotide (nucleotides g2 to g8) base pairing. In particular, we observed that miR-16 family members with identical seed sequences but divergent nonseed (nucleotides g9 to g22) sequences were depleted from plasma using a target oligo complementary to miR-16. Further, miR-107/miR-103 family members, which fortuitously match a portion of the miR-16 target through their seed sequence, are also efficiently depleted from plasma along with miR-16. The ability of circulating miRNAs to engage in seed-mediated base
pairing to a target mimic oligonucleotide is consistent with bona fide miRISC-mediated target recognition (40–44). Also consistent with the association of circulating miRNAs with active miRISC, we find that the amount of AGO2 recovered from plasma by a target mimic oligo is similar to the amount of the cognate miRNA in the plasma sample, suggesting a 1:1 ratio between a circulating miRNA and its AGO2 partner.

In contrast to the miR-16 family members detected, which were efficiently depleted from plasma by a miR-16 complementary 2′-O-methyl target mimic oligonucleotide, let-7 family members were less efficiently depleted by a cognate let-7a target mimic. One interpretation of this observation is that a portion of some circulating miRNAs (exemplified by the let-7 family) may be sequestered within vesicles or in higher-order protein complexes that mask the miRNA in such a way that inhibits the base pairing with a target mimic. An alternative explanation for inefficient depletion of a miRNA from plasma using target mimics could be the presence of an endogenous cRNA including unreleased passenger strand already annealed to the miRNA. However, we think that this latter explanation is unlikely for two reasons. First, we did not observe appreciable representation of passenger strand read counts relative to guide strand reads in our datasets (SI Appendix, Tables S3–S5). In particular, the let-7 family member with the highest fraction of passenger strand detected in plasma was let-7d (4.83% of the two strands), while it was 61% depleted by 2′-OM pull-down (SI Appendix, Table S5). Second, our RNA sequencing data of plasma supernatant from control experiments (SI Appendix, Table S6) and of plasma mRNA immunoprecipitated by anti-AGO2 antibody (SI Appendix, Table S7) did not indicate an appreciable representation of mappable RNA species other than miRNA.

We observed that the miRNA profile in plasma was not quantitatively affected by freeze-thawing the sample, nor by the incubation times and conditions of our immunoprecipitation and target mimic experimental conditions. Other researchers have also observed that the levels of miRNAs associated with exosomes is not affected in frozen plasma compared with fresh plasma, suggesting that EVs are stable during freeze-thawing (7). The association of circulating miRNAs with miRISC/AGO2 complexes could in part account for the remarkable stability of miRNA in body fluids (5, 6), as miRNA bound to AGO2 is held in a conformation where the phosphodiester backbone appears to be protected from nucleases by interactions with AGO2 (45). Moreover, AGO2 loaded with a miRNA appears to adopt a conformation that is resistant to proteases, such as thermolysin (46).

Our findings that plasma-borne miRNAs are associated with miRISC and can sequence-specifically bind to complementary target oligonucleotides implies that these extracellular miRNAs could, in principle, contribute to cellular posttranscriptional gene regulation, provided that they were reinternalized into cells in sufficient quantities. However, it is important to consider that the intracellular concentrations of a miRNA required for sufficient quantities. However, it is important to consider that the intracellular concentrations of a miRNA required for gene regulation is estimated to be appreciably greater than ~20 pM, the Kd for estimated to be appreciably greater than ~20 pM, the Kd for miRISC::target binding (34, 47), and perhaps even in the nanomolar range (4). Our data confirm previous estimates by others (48–50) that the total concentration of circulating miRNAs in blood plasma is approximately ~5 pM. In our plasma samples, the most abundant miRNAs, miR-451 and miR-16, are present in concentrations of ~2.5 pM and 0.5 pM, respectively, indicating that even these relatively abundant circulating miRNAs would need to be concentrated 10 to 1,000 fold upon import into the cytoplasm of recipient cells in order to be functional. Further in vivo studies are necessary to determine whether certain circumstances could make it possible for cells to import miRNAs at functional levels from the extracellular milieu.

By analogy with synthetic miRNAs and siRNAs, whose in vivo delivery to cells are facilitated by the interactions of their ligand conjugates and the cell surface receptors (51, 52), uptake of natural extracellular miRISC could be mediated by cell surface proteins with the capacity to bind AGO2. Neuropilin-1 is one such cell surface protein suggested to bind AGO2 (53). It is also likely that the fate of some extracellular miRNAs could be determined by association (either internally or externally) with EVs (Fig. 1). Although we observed that the majority of plasma miRNAs are associated with free miRISC, based on their ability to readily interact with anti-AGO2 antibody and target mimics, we did not assess what fraction of circulating miRISC in our samples could be bound to the extracellular surfaces of EVs. Support for the association of miRISC with EVs comes from various reports, including the identification of AGO2 and GW182 in exosomal samples recovered from cell culture medium (25). However, previous studies that have reported EV-associated miRNAs have generally not included confirmation of whether the reported miRNAs/miRISC/AGO2 are enclosed within the EV interior or, alternatively, bound to the extracellular surfaces of EVs.

Circulating miRNAs have been investigated as potential diagnostic markers for numerous diseases, including cancer (11, 12), liver toxicity (14), and neurodegenerative disorders (10, 13). What has not been explored is the question of possible disease-related differences in the assortment of circulating miRNAs into distinct carrier complexes, such as free miRISC/AGO2 and EVs. It is not yet understood whether the assortment of extracellular miRNAs into particular complexes may reflect the cell type and/or pathophysiological status of their cells of origin. Numerous reports suggest that cells may produce elevated levels of EVs under pathological conditions, such as in the tumor microenvironment (54, 55). EV-associated miRNAs have been reported to be released through a ceramide-dependent secretion pathway (31), and accumulation of ceramide has been reported in spinal cords of ALS patients (56) and in brain cortices of Alzheimer’s disease patients (9).

We observed that extracellular miRNAs in CSF samples from healthy individuals were predominantly depleted by AGO2 immunoprecipitation, while CSF from ALS patients contained a subset of miRNAs that were inefficiently depleted by AGO2 immunoprecipitation. Similarly, CSF from patients with elevated intracranial pressure contained a higher portion, compared to normal CSF, of miRNAs that were not efficiently depleted by AGO2 immunoprecipitation. These observations suggest that pathological conditions may alter not only the overall profile of miRNA levels in CSF (57–59), but also the profile of miRNA carrier complexes. Further investigations will be required to characterize the origin and composition of anti-AGO2–refractory miRNA complexes in CSF and to determine their potential significance as a novel class of diagnostic biomarkers in the context of neurological diseases.

More broadly, we suggest that employing methods to resolve circulating miRNAs profiles into classes of carrier complexes such as those reported here—free miRISC complexes that are compliant with AGO2 immunoprecipitation and/or that can base-pair with a target mimic, and complexes that are refractory to these biochemical assays—could substantially increase the specificity of biomarker discovery for diverse pathological conditions. To this end, it will be important to further characterize in detail the properties and molecular compositions of extracellular miRNA complexes, both in normal and pathological contexts, and to uncover the cellular processes and export pathways that produce them.

**Materials and Methods**

Detailed materials and methods used in all experiments are provided in SI Appendix, SI Methods. AGO2 immunoprecipitation was conducted according
to Arroyo et al. (6), and 2'-O-methyl RNA oligonucleotide target mimic pull-down assay was conducted according to Jannott et al. (60). Plasma and CSF RNA was extracted according to Tanriverdi et al. (61), and qRT-PCR was conducted according to Geekiyange et al. (9). Small-RNA libraries were prepared using QIAseq miRNA Library Kit (Qiagen), and RNA sequencing was conducted using Illumina platforms. Small-RNA sequencing data analysis was conducted using the Qiangen pipeline, Deseq2 (62), Student t tests, and deblur platforms (63).

Human Plasma and CSF Sample Collection. Deidentified human plasma and CSF samples were collected in accordance with procedures and consent forms approved by the University of Massachusetts Medical School Institutional Review Board (IRB 130109_12 and IRB 140407_12). Deidentified healthy control and ALS CSF samples were obtained from the NEALS consortium (https://www.neals.org).

Data Availability. All study data are included in the article and SI Appendix.

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