Serum microRNAs in patients with genetic amyotrophic lateral sclerosis and pre-manifest mutation carriers

Axel Freischmidt,1 Kathrin Müller,1 Lisa Zondler,1 Patrick Weydt,1 Alexander E. Volk,2 Anže Lošdorfer Božič,3 Michael Walter,4 Michael Bonin,4 Benjamin Mayer,5 Christine A. F. von Arnim,1 Markus Otto,1 Christoph Dieterich,3 Karlheinz Holzmann,6 Peter M. Andersen,1,7,8 Albert C. Ludolph,1,8 Karin M. Danzer1 and Jochen H. Weishaupt1

1 Department of Neurology, Ulm University, Ulm, Germany
2 Institute of Human Genetics, Ulm University, Ulm, Germany
3 Max Planck Institute for Biology of Ageing, Cologne, Germany
4 Department of Medical Genetics, University of Tübingen, Tübingen, Germany
5 Institute for Epidemiology and Medical Biometry, Ulm University, Ulm, Germany
6 Genomics-Core Facility, University Hospital Ulm, Centre for Biomedical Research, Ulm, Germany
7 The Institute of Pharmacology and Clinical Neuroscience, Umeå University, Umeå, Sweden
8 Virtual Helmholtz Institute RNA dysmetabolism in Amyotrophic Lateral Sclerosis and Fronto-temporal Dementia, Germany

Correspondence to: Prof. Dr. Jochen H. Weishaupt,
Ulm University,
Department of Neurology,
Albert-Einstein-Allee 11,
89081 Ulm, Germany
E-mail: jochen.weishaupt@uni-ulm.de

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Knowledge about the nature of pathomolecular alterations preceding onset of symptoms in amyotrophic lateral sclerosis is largely lacking. It could not only pave the way for the discovery of valuable therapeutic targets but might also govern future concepts of pre-manifest disease modifying treatments. MicroRNAs are central regulators of transcriptome plasticity and participate in pathogenic cascades and/or mirror cellular adaptation to insults. We obtained comprehensive expression profiles of microRNAs in the serum of patients with familial amyotrophic lateral sclerosis, asymptomatic mutation carriers and healthy control subjects. We observed a strikingly homogenous microRNA profile in patients with familial amyotrophic lateral sclerosis that was largely independent from the underlying disease gene. Moreover, we identified 24 significantly downregulated microRNAs in pre-manifest amyotrophic lateral sclerosis mutation carriers up to two decades or more before the estimated time window of disease onset; 91.7% of the downregulated microRNAs in mutation carriers overlapped with the patients with familial amyotrophic lateral sclerosis. Bioinformatic analysis revealed a consensus sequence motif present in the vast majority of downregulated microRNAs identified in this study. Our data thus suggest specific common denominators regarding molecular pathogenesis of different amyotrophic lateral sclerosis genes. We describe the earliest pathomolecular alterations in amyotrophic lateral sclerosis mutation carriers known to date, which provide a basis for the discovery of novel therapeutic targets and strongly argue for studies evaluating presymptomatic disease-modifying treatment in amyotrophic lateral sclerosis.
**Preclinical miRNA signatures in ALS**

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**Keywords:** amyotrophic lateral sclerosis; microRNA; mutation carriers; SOD1; C9ORF72

**Abbreviations:** ALS = amyotrophic lateral sclerosis; qRT-PCR = quantitative real-time PCR

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**Introduction**

The symptomatic phase of the fatal neurodegenerative disease amyotrophic lateral sclerosis (ALS) has been well characterized with regard to clinical progression (Roche et al., 2012; Ravits et al., 2013) as well as imaging (Sach et al., 2004; Turner et al., 2007) or electrophysiological studies (de Carvalho and Swash, 2010). However, little is known about the long preclinical period preceding manifestation of symptoms (Turner et al., 2013). Especially the nature of first molecular alterations and reasons for the delay to the appearance of first clinical symptoms are unclear. Such insights could have profound implications for the understanding of ALS disease mechanisms and for the development of therapeutic targets involved in the earliest pathomolecular cascades. Moreover, the discovery of novel ALS genes will lead to the identification of an increasing number of asymptomatic ALS gene carriers, boosting the discussion about presymptomatic treatment with e.g. riluzole, the only approved disease modifying ALS drug. Riluzole is thought to delay degeneration of motor neurons primarily by a reduction of glutamate release and excitotoxicity (Cheah et al., 2010), but has only a modest effect on survival when treatment is started after onset of disease (Miller et al., 2012). Earlier commencement of neuroprotective treatment might correlate with higher protective efficacy. To determine a reasonable time point for treatment initiation of mutation carriers, however, it will be important to know whether the prodromal phase is relatively short or precedes the disease for years or even decades at the molecular level. The latter, for example, has been shown for Alzheimer’s disease (Kok et al., 2009).

To date, mutations in over 20 different genes have been implicated in ALS pathogenesis, with a hexanucleotide expansion in C9orf72 and mutations in the genes coding for SOD1, TARDBP and FUS being the most frequent. Although familial ALS comprises ~10–15% of all cases, the majority of ALS is considered sporadic without a known family history of ALS. However, in ~10% of sporadic ALS cases mutations in known ALS genes are detected (DeJesus-Hernandez et al., 2011; Renton et al., 2011, 2014; Al-Chalabi et al., 2012).

In the recent and ongoing identification of novel ALS genes, RNA metabolism has emerged as one of the common functional denominators. Several ALS-related proteins are involved in the processing of messenger RNAs (Chen et al., 2004; Sreedharan et al., 2008; Kwiatkowski et al., 2009; Couthouis et al., 2011; Kim et al., 2013), but also in the biogenesis of microRNAs (miRNAs) (Buratti et al., 2010; Kawahara and Mieda-Sato, 2012; Morlando et al., 2012). MiRNAs are key regulators of transcriptome plasticity. They are centrally involved in the cellular adaptation to various stressors (Mendell and Olson, 2012) and can be part of the molecular response to various pathogenic insults (Viader et al., 2011; Liu et al., 2013; Zhang et al., 2013). Thereby, miRNA expression ‘fingerprints’ can reflect activation of specific pathogenic pathways.

Importantly, miRNAs are found in the plasma, serum and other body fluids bound to specific proteins or as exosomal cargos (Xu et al., 2012). Despite being rapidly degraded in post-mortem brain tissue (Sethi and Lukiw, 2009), miRNAs are exceptionally stable in serum and other body fluids (Mitchell et al., 2008; Grasedieck et al., 2012). Additionally, almost all genes implicated in familial forms of ALS known to date are expressed also outside the CNS [McCord and Fridovich, 1969; Aman et al., 1996 or Petryszak et al., 2014 (http://www.ebi.ac.uk/gxa)]. We therefore hypothesized that, despite their unclear causal contribution to neurodegeneration, circulating miRNA patterns could reflect earliest pathomolecular alterations in ALS. As a proof of principle, we could recently demonstrate the downregulation of a small subset of TDP-43-binding candidate miRNAs in the serum and peripheral cell lines obtained from patients with ALS (Frischmidt et al., 2013). Furthermore, there is a growing list of miRNAs detected to be dysregulated in the CNS and the periphery of mouse models and patients with various neurodegenerative diseases (Goodall et al., 2013). Specifically in ALS patients, dysregulated miRNAs have been detected in spinal cord (Campos-Melo et al., 2013) and leucocytes (Butovsky et al., 2012; De Felice et al., 2012). In the ALS SOD1-G93A mouse model, for example, upregulation of muscle-specific miR-206 was evident in plasma even before the onset of symptoms (Toivonen et al., 2014), and miR-206 was shown to be involved in regeneration of neuromuscular synapses that slows down disease progression (Williams et al., 2009). Moreover, intraventricular delivery of an inhibitor of miR-155 (anti-mir-155), a miRNA that was upregulated in spinal cords of SOD1-G93A mice and patients with sporadic ALS, could significantly extend survival and disease duration of ALS model mice (Koval et al., 2013). Interestingly, the protein product of another gene linked to motor neuron degeneration (HNRNPA2B1) was recently shown to carry miRNAs towards exosomal release (Kim et al., 2013; Villarroya-Beltri et al., 2013).

Consequently, we performed a comprehensive characterization of circulating miRNAs, comparing serum miRNA profiles of patients with familial ALS, asymptomatic mutation carriers and age-matched healthy control subjects. Our data reveal distinct miRNA profiles in the serum of patients with familial ALS as well as at an early phase in presymptomatic mutation carriers that are largely independent of the underlying disease gene. These findings represent the earliest known pathomolecular alteration in ALS, and suggest the evaluation of pre-manifest disease modifying treatment.

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**Materials and methods**

**Patient cohorts and ethics statements**

Appropriate approval and procedures were used concerning human subjects. With informed written consent and approved by the national
medical ethical review boards in accordance with the Declaration of Helsinki (WMA, 1964), blood samples were drawn from control individuals, patients with ALS and pre-manifest ALS mutation carriers. Anonymous genotyping of ALS mutation carriers was performed after informed written consent and approved by the local medical ethical review boards. Patients were not informed about genetic results that were obtained for scientific purposes only, according to the written informed consent. All patients with ALS fulfilled the El-Escorial criteria for definite ALS.

Patients with familial ALS as well as asymptomatic mutation carriers were identified by sequencing of the SOD1 (van Es et al., 2010), PFN1 (Ingre et al., 2013) or FUS (Waibel et al., 2013) genes or by repeat-primed PCR (DeJesus-Hernandez et al., 2011) for detection of C9orf72 mutations. C9orf72 hexanucleotide repeat expansions of all patients with familial ALS and mutation carriers according to repeat-primed PCR (DeJesus-Hernandez et al., 2011) were confirmed by Southern blotting (Hubers et al., 2014).

Patients were considered sporadic cases based on a negative family history. Additionally, the two most frequent known causes of familial ALS, namely mutations in the SOD1 gene and a hexanucleotide repeat expansion in C9orf72, were excluded by Sanger sequencing (van Es et al., 2010) or repeat-primed PCR (DeJesus-Hernandez et al., 2011), respectively.

Sample preparation for miRNA microarrays

All serum samples were collected by the same centre and processed according to standard procedures immediately after blood drawing and stored at –80°C until further use.

For the miRNA microarrays total RNA was prepared in each case from 8–10 ml of serum using the QIAzol® lysis reagent (Qiagen) according to the manufacturer’s recommendations for serum with minor modifications. Notably, RNA isolation was performed in aliquots of 250 μl in 2 ml reaction tubes (32–40 tubes/patient). Larger volumes and consequently larger reaction tubes (15 or 50 ml) sometimes lead to incomplete phase separation during the first RNA extraction step and loss of RNA. In brief, after RNA extraction the aqueous phase was collected and total RNA precipitated by adding 1.4 volumes of isopropanol and incubating overnight at –20°C. Precipitated RNA was spun down and resuspended in RNase-free water. At this stage total RNA was still contaminated with serum protein. Therefore, the RNA was extracted a second time and purified using the miRNeasy Mini Kit (Qiagen). Total RNA yield was 405.7 ± 126.0 ng [mean ± standard deviation (SD)] and similar to the RNA amount derived from plasma samples (Burgos et al., 2013).

Microarray analysis

All array measurements of this study were run in one batch. After quality control using the Agilent 2100 Bioanalyzer (Agilent Technologies) and RNA 6000 Nano Kit (measurement of RNA concentration and demonstration of expected size distribution; Jacob et al., 2013), 200 ng of total RNA were labelled using the FlashTag™ Biotin HSR RNA Labeling Kit (Genisphere, LLC) according to the manufacturer’s instructions. After hybridization to Affymetrix™ miRNA 3.0 arrays, arrays were washed and stained according to the manufacturer’s protocol on a GeneChip Fluidics Station 450 (Affymetrix). The arrays were analysed by the Affymetrix GeneChip Scanner 3000 and the Affymetrix® Expression Console™ software.

Raw feature data were normalized using the RMA + DBAG (human only) algorithm and log2 intensity expression. Summary values for each probe set were calculated using Expression™ Console software (Build 1.3.1.187).

Microarray raw data can be accessed at Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/). GSE number is GSE52917. The miRNA-transcriptome analyses were performed using BRB-ArrayTools (http://linus.nci.nih.gov/BRB-ArrayTools.html) as published previously (Sunami et al., 2012; Kuehner et al., 2013).

Class comparison using microarray data

miRNAs showing minimal variation across the whole set of arrays were excluded from the analysis. miRNAs whose abundance differed by at least 1.5-fold from the median in at least 20% of the arrays were retained (n = 278 of 5639).

We identified miRNAs that were differentially expressed among the two classes using a two sample t-test. Resulting P-values were corrected for multiple testing using the multivariate permutation test to provide false discovery rate (FDR) detection (Korn et al., 2004). The multivariate permutation tests are based on permutations of the labels of which arrays are in which classes. A large number of random permutations are considered. For each random permutation, the parameteric tests are recomputed to determine a P-value for each gene that is a measure of the extent it appears differentially expressed between the random classes determined by the random permutation. The genes are ordered by their P-values computed for the random permutation (genes with smallest P-values at the top of the list). For each potential P-value threshold, the program records the number of genes in the list. This process is repeated for a large number of random permutations. Consequently, for any P-value threshold, we can compute the distribution of the number of genes that would have P-values smaller than that threshold for random permutations can be computed. That is the distribution of the number of false discoveries, since genes that are significant for random permutations are false discoveries. The test statistics used are random variance t-statistics for each miRNA (Wright and Simon, 2003).

Alterations were considered statistically significant if their FDR was <0.05 and displayed a fold change between the two groups of at least 1.5-fold.

Hierarchical Clustering and generation of heatmaps

Hierarchical Cluster analysis (average linkage) was performed and heatmaps were generated using the Genesis software package (http://genome.tugraz.at/genesisclient/genesisclient_description.shtml).

Quantitative real-time PCR

RNA isolations and quantitative real-time PCRs (qRT-PCRs) were carried out as recently described (Freischmidt et al., 2013) using miScript Primer Assays (Qiagen) as miRNA specific forward primers. Resulting exponential processes (Ct-values) were converted to linear comparisons relative to the control group and normalized to spiked-in miR-39-3p of Caenorhabditis elegans (Cel-miR-39-3p) using the 2^(-ΔΔCt) method. Error bars representing the standard error of the mean (SEM) were also calculated by 2^(-ΔΔCt) conversion (Livak and Schmittgen, 2001). Comparisons of groups were performed using
the two-tailed Student’s t-test. P-values \( \leq 0.05 \) were considered statistically significant (*\( P \leq 0.05 \); **\( P \leq 0.01 \); ***\( P \leq 0.001 \)).

**Identification of sequence motifs in miRNAs**

To discover any miRNA sequence motifs that would be able to characterize and discriminate specific miRNAs from selected background sets, we used the DREME tool from the MEME suite (Bailey, 2011). With the algorithm, we focused on finding short (four to eight nucleotides) sequence motifs that are over-represented in the dysregulated miRNAs in patients with familial ALS and mutation carriers, with a significant relative enrichment of the motif between the positive and the background set as computed by DREME using Fisher’s exact test. Calculated P-values were corrected for multiple testing by multiplication with the number of candidate motifs considered. The background set of sequences consisted of all mature human miRNAs present on the Affymetrix™ miRNA 3.0 array (1733 sequences) excluding the dysregulated miRNAs.

**Results**

**A prominent miRNA signature in patients with familial amyotrophic lateral sclerosis**

We obtained a profile of serum miRNAs using the Affymetrix™ miRNA 3.0 arrays, which measures the abundance of 1733 mature human miRNAs. Initially serum samples from nine patients with familial ALS and 10 age-matched control individuals of Caucasian origin were compared. The patient cohort comprised six patients carrying SOD1 mutations [two with an I104F mutation (uncle and niece), one with a R115G mutation, one with an E133K mutation and two brothers with a homozygous D90A mutation], one ALS patient with a FUS mutation (R521H) and two unrelated patients with a Southern blot-confirmed hexanucleotide repeat expansion in C9orf72 (see Supplementary Table 1 for detailed patient characteristics). Due to limited serum volumes available, individual patient samples had to be pooled in three instances for one miRNA array to achieve robust signal intensities (Supplementary Table 1). The control cohort was unrelated to individuals of the patient cohort. A false positive increase of serum miRNAs due to haemolysis has been described in other instances (Kirschner et al., 2011, 2013; Cheng et al., 2013) and altered fragility of ALS blood cells has been suggested previously (Ronnevi and Conradi, 1984). Similar to the findings of Ronnevi and Conradi (1984), spectrophotometric analysis of individual serum samples indeed revealed a significant increase in haemolysis in our familial ALS patient cohort (Supplementary Fig. 1A). We thus focused our analysis specifically on miRNAs that are downregulated in ALS and therefore even less likely to be the result of increased haemolysis. In addition, all samples were collected and prepared in the same centre according to the same standard operation procedures for all groups included. Importantly, all array measurements of this study were run in one batch.

We observed a significant downregulation of 30 mature miRNAs in patients with familial ALS compared to controls after correction for multiple testing (false discovery rate (FDR) \( \leq 0.05 \); Table 1). These alterations were surprisingly homogenous across samples with different ALS genes mutated. MiRNA profiles of patients with a FUS or C9orf72 mutation were in some instances even more similar to SOD1 mutant patients than the SOD1 mutant patients among each other, suggesting a possible mutation-independent dysregulation of serum miRNAs (Fig. 1).

To validate our results in an independent cohort with an alternative method we performed a qRT-PCR quantification of the four miRNAs with the lowest FDR values in serum samples of 13 additional patients with familial ALS (five with a SOD1 mutation and eight carrying a hexanucleotide repeat expansion in C9orf72; none of the patients was related to patients of our initial screening cohort) compared to 13 additional age matched controls (Supplementary Table 2). In accordance with the miRNA array results from the discovery cohort we could confirm the highly significant downregulation of all four miRNAs (Fig. 2A). To determine if downregulation of those four miRNAs might be a general characteristic of ALS we determined their relative abundance also in a cohort of 14 patients with sporadic ALS compared with 14 matched healthy controls (Supplementary Table 3) by qRT-PCR. Although a generally higher variability was observed in sporadic ALS compared to familial ALS patients, significant downregulation of three of the four miRNAs could be confirmed in sporadic ALS while one miRNA (miR-1915-3p) showed a strong trend (\( P = 0.061 \); Fig. 2B).

**Significantly altered miRNA fingerprints in presymptomatic amyotrophic lateral sclerosis mutation carriers**

The broad and surprisingly distinct miRNA fingerprint detected in the familial ALS patient samples above encouraged us to further validate the circulating miRNA signature and to search for evidence of earliest molecular pathology in a cohort of presymptomatic mutation carriers at a stage when secondary alterations, e.g. due to immobility or ALS-specific medication, are absent.

We included 18 asymptomatic mutation carriers with mutations in SOD1 \( n = 4 \); three family members with a R115G mutation (sister and two children of the familial ALS patient with a R115G mutation), one with an E100K mutation, PFN1 \( n = 5 \); all with T109M mutation from the same family (four siblings and their aunt) or a C9orf72 hexanucleotide expansion \( n = 8 \); from four different families of which two are related to the familial ALS patients with a C9orf72 hexanucleotide expansion] and eight age-matched healthy controls. Due to limited serum volumes available, patient samples had to be pooled in four cases for the miRNA arrays (see Supplementary Table 4 for detailed sample characteristics).

Seventeen of these pre-manifest individuals were completely free of neurological symptoms. In addition, we analysed one proband (‘unknown’ genotype in Supplementary Table 4) whose father and brother were affected by clinically definite ALS. The
The causative gene could not be determined in this family yet (negative results for mutations in SOD1, C9orf72, FUS, TARDBP, OPTN, PFN1, UBQLN2 by Sanger sequencing). The absence of any manifest or latent paresis precluded the diagnosis of ALS at this point. However, as this individual had generalized fasciculations and EMG abnormalities fully compatible with chronic and acute denervation in three body regions, we included this proband as the 18th subject in the mutation carrier group, assuming that he is highly likely to represent a ‘very late pre-manifest’ mutation carrier in transition to ALS.

Similar to symptomatic genetic ALS patients, a significant increase in haemolysis was evident in serum samples of the ALS mutation carrier cohort compared to the healthy controls (Supplementary Fig. 1B) and hence we again restricted our analysis to downregulated miRNAs. We observed a defined miRNA signature in ALS mutation carriers. After correction for multiple testing (FDR ≤ 0.05), 24 mature miRNAs were found to be significantly downregulated (Table 1). Similar to our observation in ALS patients, the downregulation of miRNAs in mutation carriers seemed to be widely independent from the affected gene, providing a distinct general miRNA signature in ALS mutation carriers (Fig. 3).

Most remarkably, the miRNA profiles of our preclinical mutation carrier cohort strikingly overlapped with the miRNA profile in familial ALS patients: Of the 24 miRNAs downregulated at the adjusted significance level in mutation carriers, 22 (91.7%) were also significantly regulated in patients with familial ALS and were always changed in the same direction (Fig. 4A). Biometric analysis further substantiated the consistency and strong overlap between both groups: The set of miRNAs significantly downregulated in preclinical mutation carriers was sufficient to completely separate patients with familial ALS from controls by an unbiased hierarchical cluster analysis (Fig. 4B).

With regard to the majority of the affected miRNAs the degree of miRNA downregulation was stronger in the symptomatic than in the pre-manifest mutation carriers (Fig. 5). This observation suggests a progressive nature of miRNA alteration in individuals carrying an ALS gene mutation.

### Table 1 MiRNAs significantly downregulated in serum of patients with familial ALS and presymptomatic ALS mutation carriers compared with healthy control subjects using Affymetrix™ miRNA 3.0 arrays

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Fold-change Familial ALS</th>
<th>FDR</th>
<th>Fold-change ALS mutation carriers</th>
<th>FDR</th>
</tr>
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<td>&lt;1 × 10^{-7}</td>
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<td>0.0065</td>
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n.a. = not significantly altered in the corresponding cohort.
Figure 1 MiRNAs significantly downregulated in patients with familial ALS compared with age-matched healthy control subjects (after correction for multiple testing). Heatmap shows regulation of respective miRNAs according to the colour coding (bottom). Columns are ordered according to similarity (hierarchical cluster analysis at the top). Age and gender, as well as the respective mutations are indicated at the top. C indicates healthy control and the asterisk (*) indicates pooled samples and in these cases the age corresponds to the mean age of the respective individuals.
Downregulated miRNAs in patients with familial ALS and mutation carriers contain a common sequence motif

Recent evidence suggests that certain miRNA consensus sequence motifs result in binding to specific RNA binding proteins, which are involved in miRNA biogenesis (Buratti et al., 2010) and localization, e.g. exosomal targeting (Villarroya-Beltri et al., 2013). We hypothesized that the observed reduction of specific serum miRNAs in ALS might be due to a global, ALS-related dysfunction of RNA binding protein(s), which are usually ubiquitously expressed [e.g. heterogeneous nuclear ribonucleoproteins (Kamma et al., 1995); Expression Atlas (Petryszak et al., 2014; http://www.ebi.ac.uk/gxa)]. Using the DREME tool (see ‘Materials and methods’ section) we could independently identify two motifs highly enriched in the downregulated miRNAs in patients with familial ALS and ALS mutation carriers. The most significant GDCGG motif (with D being G, A or U; see Fig. 6A) is present in the majority of downregulated mature miRNAs in patients and carriers, but hardly contained in the sequences used as ‘background’ set (i.e. all mature human miRNAs on the array that were not downregulated; Table 2). The second motif, SGGC (with S being G or C), is also significantly enriched in downregulated miRNAs but far more common in the background set than the first motif (Fig. 6B; Table 2). Interestingly, the GDCGG consensus sequence motif is not randomly embedded in miRNA sequences but almost always found downstream of the miRNA seed region responsible for miRNA target specificity (usually nucleotides 2–7 relative to the 5′-end of the miRNA), or near the 3′-end. We thus present a potential RNA binding motif highly significantly enriched in miRNAs downregulated in ALS and pre-manifest mutation carriers, which supports a specific mechanism common for most miRNAs found to be downregulated.

Discussion

We performed a comprehensive characterization of circulating miRNA fingerprints in patients with familial ALS and asymptomatic mutation carriers. Increasing evidence points to ALS as a systemic disease. For example, changes in connective tissue (Kolde et al., 1996), immune system (Butovsky et al., 2012) or metabolic disturbances (Dupuis et al., 2004; Lawton et al., 2012) have been shown. Moreover, all established ALS genes seem to be expressed outside the CNS (McCord and Fridovich, 1969; Aman et al., 1996; Petryszak et al., 2014 (http://www.ebi.ac.uk/gxa)). It is thus plausible that our findings mirror pathological effects of ALS disease mutations or other pathogenic events in the periphery and represent a surrogate for sensing pathomolecular alterations even in asymptomatic mutation carriers.

Our data demonstrate a surprisingly early occurrence of miRNA fingerprints associated with highly penetrant ALS mutations, even decades before the likely onset of disease. Considering that earlier commencement of neuroprotective treatment is plausibly more effective and that, according to our results, molecular pathophysiology precedes the onset of disease for many years, our findings strongly argue in favour of evaluating presymptomatic treatment options in ALS. The early, subclinical onset of molecular pathology provides also a possible explanation for the failure to substantially attenuate disease progression by disease-modifying treatment attempts in ALS to date.

Another prominent finding of our study are the highly homogenous miRNA profiles in genetically-defined familial ALS patients and pre-manifest mutation carriers. Moreover, 91.7% of the miRNAs downregulated in the mutation carriers overlapped with altered miRNAs in familial ALS. This was seen irrespective of the underlying genetic alteration as most of the patients with familial ALS carried a SOD1 mutation and the majority of asymptomatic mutation carriers carried a C9orf72 repeat expansion. Downregulation of the selected four miRNAs that were most significantly altered in patients with familial ALS could also be shown in patients with sporadic ALS. Therefore, common pathogenic denominators connecting defects in several different ALS genes and even sporadic ALS may be hypothesized. There is increasing evidence supporting this view. For example, miRNA targets and downstream events of FUS and TDP-43 partially overlap (Kawahara and Mieda-Sato, 2012; Morlando et al., 2012), and
TDP-43 pathology is observed not only in patients with TARDBP mutations but also in patients with sporadic ALS (Mackenzie et al., 2007). Moreover, recent reports indicate direct molecular interaction between different known ALS-related proteins, e.g. SMN1/FUS (Yamazaki et al., 2012), TDP-43/ATXN2 (Elden et al., 2010) or PFN1/TDP-43 (Wu et al., 2012), suggesting convergence of different ALS genes on common pathways. In line with this idea, few pathophysiological principles have emerged and link different ALS genes to common functional pathways, e.g. impairment of RNA metabolism or disturbance of cytoskeletal regulation (Cooper-Knock et al., 2012).

The majority of miRNAs found to be downregulated in this study has been described only very recently. Consequently, there are only few published studies on their expression or even function. Limited data suggest, for example, links to known apoptosis pathways: glycogen synthase kinase 3 alpha (GSK3A) is an experimentally validated target of miR-149-3p (Jin et al., 2011) and apoptosis regulator BCL2 is a validated target of miR-1915-3p (Xu et al., 2013). This would be in agreement with the higher level of GSK3A/B in the spinal cord of patients with sporadic ALS (Hu et al., 2003) and the upregulation of BCL2A1 in motor neurons of the SOD1-G93A transgenic mice described previously (Crosio et al., 2006).

Figure 3 MiRNA signature in the serum of preclinical ALS mutation carriers. Serum miRNA levels of first-degree relatives of patients with familial ALS carrying the respective pathogenic mutation were compared to age-matched controls without ALS mutations using miRNA arrays. The heatmap shows changes of downregulated miRNAs according to the colour coding (bottom). Columns are ordered according to hierarchical clustering displayed on the top of heatmaps. For indicators of age, gender and respective mutations, see the legend for Fig. 1.
Figure 4  Serum miRNA profiles of patients with familial ALS (fALS) and presymptomatic mutation carriers are highly similar. (A) Venn diagram visualizing the large overlap of significantly downregulated miRNAs between patients with familial ALS and ALS mutation carriers.
An unbiased ingenuity pathway core analysis (Ingenuity Systems, Inc.), which took into account all miRNAs significantly downregulated (FDR ≤ 0.05) in the patients with familial ALS, preclinical mutation carriers or the respective overlapping miRNAs revealed an enrichment in the functional networks ‘Cell Death and Survival, Cell Cycle, Cardiac Necrosis/Cell Death’ (patients with familial ALS), ‘Carbohydrate Metabolism, Molecular Transport, Small Molecule Biochemistry’ (mutation carriers) and ‘Cell-To-Cell Signaling and Interaction, Nervous System Development and Function, Psychological Disorders’ (miRNAs overlapping between familial ALS and mutation carriers; Supplementary Table 5). Although these networks are interestingly related to, for example, ‘cell death’ or ‘nervous system development’, such results have to be interpreted with great caution, also because the functions of extracellular miRNAs, if any, might differ from that of intracelluar miRNAs.

Hardly any of the miRNAs downregulated in patients with familial ALS or mutation carriers has been implicated in neurodegeneration so far. From a total of 32 downregulated miRNAs in familial ALS patients and mutation carriers, only three were above the detection limit in a study that identified 180 significantly dysregulated miRNAs in blood samples of patients with Alzheimer’s disease by next-generation sequencing (Leidinger et al., 2013); while miR-3960 and miR-4508 were not significantly altered in Alzheimer’s disease, miR-371b-5p was significantly upregulated. Although a comparison of miRNA profiles obtained from different technical platforms has to be taken with caution, these data suggest that miRNA profiles revealed in our study might be specific for ALS as far as it can be judged at this stage. However, dysregulation of miR-1915-3p, miR-3185, miR-1469, miR-2861, miR-638, miR-3940-5p, miR-3196, miR-149-3p, miR-455-3p and miR-1825 have been detected in various types of cancer (Sand et al., 2012; Long et al., 2013; Sun et al., 2013; Wang et al., 2013; Ma et al., 2014).

We identified a highly enriched consensus sequence motif in downregulated miRNAs, which provides support for a specific mechanism common for most miRNAs found to be downregulated in ALS serum. It is tempting to raise the hypothesis that loss-of-function of one or few RNA binding proteins may be responsible for decreased miRNA biogenesis in cells or reduced serum export of specific miRNAs. In line with this hypothesis, Villarroya-Beltri et al. (2013) demonstrated HNRNPA2B1 binding and subsequent HNRNPA2B1-dependent exosomal targeting of miRNAs in lymphocytes. Hence, using a similar strategy as Villarroya-Beltri et al. the highly significant GDCGG-motif identified in downregulated miRNAs in our study may be the future identification of potential RNA binding protein(s) possibly altered in ALS. Alternatively, a common transcriptional misregulation could also be responsible for the reduced abundance of groups of miRNAs. However, regarding the chromosomal arrangement of downregulated miRNAs (Supplementary Table 6), only miR-2861 and miR-3960 are ‘clustered’ miRNAs, i.e. miRNA genes that are located <10 kbp apart from each other and thus likely to be simultaneously transcribed (e.g. Hu et al., 2011). Moreover, some of the downregulated miRNAs are clustered with miRNAs that were not altered in ALS serum. Additionally, miRNA genes often overlap with protein coding genes (‘host genes’; Supplementary Table 6). If transcriptional misregulation is responsible for downregulation of miRNAs, the host genes should be downregulated, too. Review of the literature published to date did not reveal any of the host genes of downregulated miRNAs reported by us to be downregulated in ALS. Taken together, we consider a parallel impairment of transcription of our altered miRNAs to be the less likely reason for their downregulation.

Our study is the first showing earliest molecular changes in asymptomatic ALS mutation carriers, providing evidence that alterations of serum miRNA signatures are prominent even decades before the (estimated) disease onset in some individuals. However, our work was not designed as a classical biomarker study. We could not detect a statistically significant correlation of miRNA-levels and disease duration or ALS functional rating scale scores, which would require larger or more homogenous cohorts including, for example, individuals with mutations in only one ALS gene. Nevertheless, single peripheral miRNAs or a combined set of certain miRNAs could be the basis for the development of easily accessible ALS biomarkers at the level of group comparisons. In

**Figure 4 Continued**

(B) MiRNA signatures of asymptomatic mutation carriers predict familial ALS patients. Heatmap shows an unbiased hierarchical cluster analysis of familial ALS patients and age-matched healthy control subjects based on all miRNAs significantly downregulated in the presymptomatic mutation carrier cohort. These 24 miRNAs are sufficient to completely separate the familial ALS patients from healthy controls. For indicators of age, gender and respective mutations, see the legend for Fig. 1.
In this context it is interesting to note that alterations of miRNA profiles seem to be progressive when comparing the pre-manifest and manifest phase of the disease (Fig. 5). They might turn out to be an interesting readout parameter in the context of future clinical studies on presymptomatic disease modifying therapies. In general, the use of circulating miRNAs as biomarkers still requires establishing standard operating procedures for both collection and standardization of miRNAs in the serum, plasma or other body fluids.

Importantly, most patients with familial ALS who donated serum to this study were taking riluzole. An effect of this medication on the miRNA profile can be largely ruled out regarding the substantial proportion of downregulated miRNAs overlapping between familial ALS patients and (riluzole-naive) mutation carriers. MiRNA alterations found in the pre-manifest mutation carrier cohort are also not biased by other potential secondary changes due to disease progression or therapy. They could, therefore, eventually provide most valuable hints for the definition of earliest pathogenic events and respective novel therapeutic strategies. Therefore, whether observed alterations in miRNA serum concentrations are functionally contributing to disease or represent reactive fingerprints of disease mechanisms remains an exciting open question that needs to be addressed in future studies. Specifically the proposed reuptake of plasma or serum miRNAs in cells, their blood–brain barrier penetration and even more functional effects of circulating miRNAs in potential target cells are largely unresolved questions (Turchinovich et al., 2012; Kosaka et al., 2013). Of note, functions of extracellular miRNAs, if any, might differ from that of intracellular miRNAs. Currently it can just be hypothesized that serum miRNAs might not only reflect but also contribute, for example, to peripheral axon or synaptic pathology or regenerative failure.

In summary, our study is the first providing miRNA signatures of both patients with familial ALS and asymptomatic mutation carriers. We observed surprisingly homogenous miRNA alterations in familial ALS patients and mutation carriers as well as a consensus sequence motif common to the vast majority of downregulated miRNAs. This suggests common pathophysiological pathways instigated by different ALS genes, hypothetically converging on the dysfunction of one or few RNA binding protein(s). Our work could establish a basis for the discovery of novel therapeutic targets. Specifically the miRNAs altered in asymptomatic mutation carriers may play a role at the most upstream positions in the pathophysiological cascades of ALS. Generally, we were able to discover the earliest molecular changes in ALS mutation carriers known to date. The study thus strongly argues in favour of evaluating presymptomatic treatment options in ALS.

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Table 2  Bioinformatic sequence analysis of downregulated miRNAs in patients with familial ALS and mutation carriers using the DREME tool

<table>
<thead>
<tr>
<th>Motif</th>
<th>Motif in downregulated miRNAs</th>
<th>Motif in background</th>
<th>Corrected P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Familial ALS Carrier</td>
<td>22/24 (91.7%)</td>
<td>44/1709 (2.6%)</td>
<td>4.60 x 10^{-28}</td>
</tr>
<tr>
<td>Familial ALS Carrier</td>
<td>15/24 (62.5%)</td>
<td>230/1709 (13.5%)</td>
<td>3.00 x 10^{-5}</td>
</tr>
<tr>
<td>Familial ALS Carrier</td>
<td>19/30 (63.3%)</td>
<td>226/1703 (13.3%)</td>
<td>4.30 x 10^{-7}</td>
</tr>
<tr>
<td>Familial ALS Carrier</td>
<td>21/30 (70.0%)</td>
<td>45/1703 (2.6%)</td>
<td>8.90 x 10^{-22}</td>
</tr>
</tbody>
</table>

*D = A, G or U; **S = G or C.
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Supplementary material

Supplementary material is available at Brain online.

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