MicroRNA’s in chronic hepatitis B and C virus infection
van der Ree, M.H.

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CHAPTER 2

MIRAVIRSEN DOSING IN CHRONIC HEPATITIS C PATIENTS RESULTS IN DECREASED MICRORNA-122 LEVEL WITHOUT AFFECTING OTHER MICRORNA’S IN PLASMA

Meike H. van der Ree, Adriaan J. van der Meer, Ad C. van Nuenen, Joep de Bruijne, Søren Ottosen, Harry L. Janssen, Neeltje A. Kootstra §, Hendrik W. Reesink §

§ Both authors contributed equally to this work

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SUMMARY

Background: MicroRNA-122 (miR-122) is an important host factor for hepatitis C virus replication. Administration of miravirsen, an anti-miR-122 oligonucleotide, resulted in a dose dependent and prolonged decrease in HCV RNA levels in chronic hepatitis C patients.

Aim: To assess the plasma level of various miRNAs in patients dosed with miravirsen.

Methods: We included 16 of 36 chronic hepatitis C patients who received five injections of either 3 mg/kg (n = 4), 5 mg/kg (n = 4), 7 mg/kg (n = 4) miravirsen or placebo (n = 4) over a 4-week period in a double-blind, randomised phase 2a study. Plasma levels of 179 miRNAs were determined by qPCR and compared between patients dosed with miravirsen or placebo.

Results: Median plasma miR-122 level at baseline in patients receiving miravirsen was $3.9 \times 10^3$ compared to $1.3 \times 10^4$ copies/4 μl in placebo-dosed patients ($P=0.68$). At week 1, 4, 6 and 10/12, patients dosed with miravirsen had respectively a median 72-fold, 174-fold, 1109-fold and 552-fold lower expression of miR-122 than at baseline ($P=0.001$, as compared to patients receiving placebo). At week 4 of dosing, miRNA-profiling demonstrated a significant lower expression of miR-210 and miR-532-5p compared to baseline (3.0 and 4.7-fold lower respectively). However, subsequent longitudinal analysis showed no significant differences in miR-210 and miR-532-5p plasma levels throughout the study period.

Conclusions: We demonstrated a substantial and prolonged decrease in plasma miR-122 levels in patients dosed with miravirsen. Plasma levels of other miRNAs were not significantly affected by antagonising miR-122.
INTRODUCTION

MicroRNAs (miRNAs) are small (~22 nucleotides), noncoding RNA molecules involved in various cellular processes. MiRNAs regulate gene expression at a posttranscriptional level by binding to the 3’ untranslated region (UTR) of target messenger RNA (mRNA). The role of miRNAs has been extensively studied since their discovery approximately 20 years ago. MiRNAs are now recognised as important players in the pathogenesis of a number of liver diseases, and are promising biomarkers and targets for therapeutic intervention.

Hepatitis C virus (HCV) is an envelope, single-stranded RNA virus of the family Flaviviridae. The HCV genome contains a single open reading frame with a length of 9.6-kb which is translated into a polyprotein of about 3000 amino acids. The HCV genome is flanked by the 5’ and 3’ UTR which are important for the translation and replication of the viral RNA. It is known that HCV uses host cellular miRNAs during its replicative cycle and is able to modulate the expression of cellular miRNAs to favour viral persistence. MicroRNA-122 (miR-122) is a liver-specific miRNA that binds to the 5’ UTR of the HCV genome to promote HCV RNA stability and accumulation. The miR-122-HCV complex protects the HCV genome from degradation by cellular exonucleases Xrn1/Xrn2, and is believed to prevent the induction of an innate immune response. MiR-122 is also involved in the regulation of lipid metabolism and may act as a tumour suppressor.

The majority of HCV infected patients develop a chronic hepatitis C (CHC) virus infection and are at risk to develop cirrhosis, leading to clinical complications such as hepatocellular carcinoma (HCC). The aim of CHC treatment is to achieve a sustained virological response (SVR), which is associated with a reduced occurrence of liver failure and HCC, and with prolonged overall survival. The recent development and registration of many highly potent direct-acting anti-viral drugs (DAA) have resulted in high SVR rates following HCV treatment. Most DAA’s target viral proteins, such as NS3 protease inhibitors, NS5B polymerase inhibitors and NS5A replication inhibitors whereas other drugs target host factors that are essential for HCV replication, such as miR-122. In 2011-2012, a phase 2a study was performed in CHC patients infected with HCV genotype 1 who received 5-weekly injections of miravirsen, a locked nucleic acid-modified phosphorothioate oligonucleotide targeting miR-122, which resulted in a prolonged and dose-dependent decrease in HCV RNA levels. HCV RNA levels became undetectable in five patients, but levels of the virus eventually rebounded in all patients. A C3U nucleotide change was observed in the HCV 5’UTR in patients with a viral rebound after miravirsen dosing. Interestingly, a wide variability in patient responses to miravirsen was observed suggesting that host and/or viral factors may influence treatment response. It is unknown how many miR-122 copies are required for HCV replication and whether miravirsen is able to sequester all hepatic miR-122. Furthermore, it is unknown how long the inhibition of miR-122 is
preserved after miravirsen dosing. In addition, the direct or indirect effect of miR-122 inhibition on the expression level of other miRNAs has never been established.

Therefore, the primary objective of this study was to assess changes in plasma miR-122 levels in CHC patients before, during and after miravirsen dosing. The secondary objective was to assess if inhibiting miR-122 alters the expression levels of other miRNAs.

MATERIALS AND METHODS

Study population
We have used plasma samples of CHC patients who participated in a previous multicenter, double-blind, phase 2a study in which patients were randomised in a 3:1 ratio to receive either miravirsen (3, 5 or 7 mg/kg) or placebo (ClinicalTrials.gov number, NCT01200420).24 These plasma samples were only collected in patients who were recruited at one of the Dutch study sites (Academic Medical Center, Amsterdam or Erasmus Medical Center, Rotterdam) (n = 17). Of these 17 patients, the 16 patients included in this study received 3 mg/kg (n = 4), 5 mg/kg (n = 4) or 7 mg/kg (n = 4) miravirsen, or placebo (n = 4). One placebo-dosed patient was excluded, to achieve an equal number of patients in each dose group. All patients were treatment naïve, and infected with HCV genotype 1. Miravirsen (or placebo) was administered subcutaneously in 5-weekly doses over a 29-day period. After miravirsen dosing, patients returned for follow-up visits over a period of 14 weeks. The additional blood samples were collected at baseline, week 1, week 4, week 6 and week 10 or 12. The study was carried out in compliance with the protocol, the principles laid down in the Declaration of Helsinki, in accordance with the ICH Harmonised Tripartite Guideline for Good Clinical Practice and the local national laws governing the conduct of clinical research studies.

Biochemical and virological tests
Several biochemical and virological analyses were conducted in the phase 2a study, including measurement of alanine aminotransferase (ALT) and HCV RNA levels. HCV RNA levels were measured using the Abbott Real-Time HCV assay, with a reported lower limit of detection and quantification of 12 IU per mL.

Current study design
This study comprised three steps: (i) Absolute and relative quantification of plasma miR-122 levels in CHC patients dosed with miravirsen (n = 12) and placebo (n = 4) at baseline, week 1, 4, 6 and 10/12; (ii) Assessment if antagonising miR-122 results in changes in expression levels of other miRNAs. Therefore, a miRNA profile was determined to evaluate the difference in plasma miRNA levels at week 4 compared to baseline in CHC patients dosed with miravirsen or placebo (fold change level); (3) Analysis of
the differential expression of miRNAs identified in the miRNA-profile in longitudinally obtained plasma samples from patients dosed with miravirsen or placebo. MiRNAs were selected for the longitudinal analysis if they fulfilled the following criteria; a fold change ≥2 or ≤0.5 (log$_{10}$ ≥ 0.301 or ≤-0.301) in patients dosed with miravirsen and a fold change level between 0.5 and 2 (log$_{10}$-0.301 to 0.301) in placebo dosed patients, and a significant difference (P < 0.05) in fold change level between miravirsen and placebo dosed patients.

RNA extraction, cDNA synthesis and qPCR
Total RNA was extracted from 200 μl plasma using the miRCURY RNA isolation kit (Exiqon, Vedbæk, Denmark) according to the manufacturer’s protocol. Synthetic cel-miR-39 (Qiagen, Hilden, Germany) was spiked into the lysis buffer to check RNA isolation efficiency. RNA samples were stored at –80 °C. cDNA was synthesised using microRNA cDNA synthesis kit (Quanta BioSciences, Gaithersburg, MD, USA) and miRCURY LNA Universal RT cDNA synthesis Kit (Exiqon, Denmark) (Data S1). An individual miR-122 qPCR assay was used to determine plasma miR-122 levels (Data S1). A serum/plasma focus microRNA qPCR panel (Exiqon, Denmark) was used to determine the expression level of 179 different miRNAs that are known to be found in plasma (Data S1).

Data analysis and statistics
The amplification curves of miRNAs were analysed using the Lightcycler 480 software (Roche Diagnostics, Ltd, Rotkreuz, Switzerland) for the quantification of cycles (Cq) and for the melting curve analysis. All assays were inspected for distinct melting curves. Data were pre-processed using GenEx pro software (MultiD Analyses, Goteborg, Sweden) (Data S1). Suitable reference genes were found by applying the NormFinder and GeNorm algorithms on the miRNA profile panel results. All miRNAs were normalised using the ΔCq method (ΔCq = Cq miRNA of interest – Cq reference miRNA). The normalised individual data points are presented as the log$_{10}$ 2$^\Delta$Cq (Table S2). The difference in the expression level of miRNAs between baseline and either week 1, 4, 6 or 10/12 was calculated with the comparative Cq-method (=$2^{ΔCq}$ (Cq miRNA of interest – Cq reference) baseline) and expressed as the fold change level. Formulas and interpretation of the delta Cq and comparative delta Cq method are described in Table S2.

We tested for difference in plasma miR-122 levels between treatment groups using the Mann–Whitney U-test, and one-way ANOVA with post-test for linear trend using SPSS (IBM SPSS Statistics for Windows, Version 22.0, Armonk, NY, USA.) and GraphPad Software, La Jolla, CA. (version 5). Correlations were analysed using the Spearman’s rank correlation coefficient using SPSS (version 22.0). We tested for difference in miRNA profiling data between treatment groups using t-test and corrected for multiple testing (Benjamini–Hochberg method). Unsupervised hierarchical clustering was performed with GenePattern software.
RESULTS
Patient characteristics
This study included 16 patients of whom 12 patients received various doses of miravirsen and 4 received placebo \(^2\). Baseline characteristics levels were similar in the different study groups (Table 1).

### Absolute plasma miR-122 levels at baseline and after miravirsen dosing
The median plasma miR-122 level at baseline in patients receiving miravirsen was \(3.9 \times 10^3\) compared to \(1.3 \times 10^4\) copies/\(4 \mu l\) in placebo treated patients (\(P = 0.68\)) (Figure 1). One week after the first dose, the median plasma miR-122 level was significantly lower in patients dosed with miravirsen than in those who received placebo, respectively \(3.1 \times 10^1\) versus \(1.1 \times 10^4\) copies/\(4 \mu l\) (\(P = 0.001\)) (Figure 1). Median plasma levels of miR-122 remained low in patients administered with miravirsen compared to those administered with placebo throughout the study period (\(P = 0.001\)) (Figure 1).

### Fold change in plasma miR-122 levels upon miravirsen treatment
One week after receiving the first dose of miravirsen, plasma miR-122 levels were 72-fold lower than at baseline in patients dosed with miravirsen, as compared to a 1.1-fold higher miR-122 plasma level in placebo dosed patients (\(P = 0.001\)). Patients dosed with miravirsen had a median 174-fold, 1109-fold and 552-fold lower expression of miR-122 compared to baseline at week 4, 6, and 10/12, compared to a 1.5-fold higher, 5.8-fold

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**Table 1. Baseline patient characteristics**

<table>
<thead>
<tr>
<th></th>
<th>Miravirsen</th>
<th>Placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 mg/kg</td>
<td>5 mg/kg</td>
</tr>
<tr>
<td>Patients, n</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Median age, years (range)</td>
<td>56 (46 - 66)</td>
<td>49 (33 - 65)</td>
</tr>
<tr>
<td>Male/Female</td>
<td>2/2</td>
<td>3/1</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Asian</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HCV RNA – (\log_{10}) IU/ml</td>
<td>6.3 ± 0.6</td>
<td>6.2 ± 0.3</td>
</tr>
<tr>
<td>HCV subtype</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1a</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>1b</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>1a/3a</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ALT level – U/L</td>
<td>70 ± 38</td>
<td>77 ± 18</td>
</tr>
<tr>
<td>Plasma miR-122 level – (\log_{10}) copies/(4 \mu l)</td>
<td>3.5 (2.7 - 3.7)</td>
<td>3.6 (2.7 - 4.3)</td>
</tr>
</tbody>
</table>

Data are given as median (minimum–maximum), as mean ± SD or as number. ALT, alanine aminotransferase.
lower and 3.1-fold lower expression of miR-122 in patients dosed with placebo, P-values all P = 0.001. There was a significant linear trend for fold change in plasma miR-122 levels and miravirsen dose at week 1, 4, 6 and 10/12 (P < 0.001) (Figure 2A). All patients receiving miravirsen showed an initial decrease in plasma miR-122 levels (Figure 2). At week 10/12, all patients dosed with 3 and 5 mg/kg miravirsen had stable or increasing relative plasma miR-122 levels from nadir, whereas relative plasma miR-122 levels of 3/4 patients dosed with 7 mg/kg were still decreasing (Figure 2B).

Figure 1. Absolute plasma miR-122 level. Absolute plasma levels of miR-122 in patients dosed with miravirsen compared to placebo. The median absolute level of miR-122 at baseline was similar between patients dosed with placebo and miravirsen. At week 1, 4, 6, and 10/12 median absolute miR-122 plasma levels were lower in patients dosed with miravirsen, as compared to placebo. The boxes represent the median and range, and Mann-Whitney-U test was used to compare the different treatment groups. LLOD is lower limit of detection.
Association of plasma miR-122 levels with clinical parameters
The mean HCV RNA level at baseline for patients dosed with miravirsen was 6.1 log_{10} IU/mL, and the viral load decreased significantly to 5.3 at week 4 (P = 0.01), 4.8 (P = 0.005) at week 6 and 4.7 (P = 0.008) log_{10} IU/mL at week 10/12. At baseline, there was no correlation between absolute plasma miR-122 and HCV RNA levels (R = 0.29, p = 0.28) (Figure 3A). No correlation was found between the change in HCV RNA level and fold change in plasma miR-122 level at week 4 (R = 0.42, P = 0.10) (Figure 3B).

Mean ALT levels at baseline were similar in patients dosed with miravirsen and placebo (Table 1), and showed no significant correlation with absolute plasma miR-122 levels (Figure 3C). ALT levels decreased to 44 U/L at week 4 and onwards in anti-miR-122 dosed patients compared to baseline (P = 0.008). At week 4, fold change in plasma miR-122 level showed a significant correlation with the changes in ALT levels (log_{10} U/L) (R = 0.57, P = 0.02) (Figure 3D).

MiRNA profiling: change in plasma miRNA levels upon miravirsen dosing
At baseline and week 4, plasma levels of 179 miRNAs were analysed of which 13 miRNAs were eliminated from further analyses because of a too low number of samples with valid data (Figure 4A). Among the remaining 166 miRNAs, we identified 19 miRNAs with a fold change level ≥2 or ≤0.5 in patients dosed with miravirsen and a fold change level between 0.5 and 2 in placebo-dosed patients (Figure 4A). Unsupervised hierarchical clustering using these 19 miRNAs clustered all patients dosed with miravirsen or placebo in separate groups (Figure 4B). Next to miR-122, two miRNAs (miR-210 and miR-532-5p) were differentially expressed between placebo and miravirsen dosed patients with a P < 0.05, and were selected for longitudinal analysis (Figure 4A, 4B).

At baseline, the plasma level of miR-122 and miR-210 were comparable between miravirsen and placebo dosed patients, with a mean delta Cq value of respectively 0.80 versus 0.64 (P = 0.58) and -0.79 vs. -0.80 (P = 0.98) (Table 2 and Figure S1). The mean delta Cq value of miR-532-5p at baseline was higher in patients who received miravirsen compared to placebo, respectively 3.65 vs. 2.70, P = 0.03 (Table 2 and Figure S1). At week 4, patients dosed with miravirsen had a mean 3.0-fold lower expression of miR-210 than at baseline, as compared with a 1.5-fold higher expression in placebo dosed patients (P = 0.007) (Table 2). MiR-532-5p levels were 4.7-fold lower compared to baseline, whilst placebo-dosed patients showed a mean 1.2-fold increase in plasma miR-532-5p levels (P = 0.02) (Table 2).

Longitudinal analysis of miRNAs identified in miRNA-profiling
Plasma levels of miR-210 and miR-532-5p were measured at baseline, week 1, 4, 6 and 10/12 in an individual RT-qPCR assay. In patients dosed with miravirsen, plasma miR-210
Figure 2. Fold change in plasma miR-122 level compared to baseline. (A) Fold change in relative plasma miR-122 levels per dose group per week, compared to baseline. The median fold decrease in miR-122 level at week 1, 4, 6 and 10/12 was lower in patients dosed with miravirsen, as compared to placebo. A significant linear trend, indicated by the black line, was found for fold change in plasma miR-122 levels and miravirsen dose. The boxes represent the range. * P <0.05; ** P <0.01; *** P <0.001. *** above the black line indicates a significant linear trend with P = 0.001. Mann-Whitney-U test and one-way ANOVA with a post test for linear trend was used to compare different study groups. (B) Individual plots of fold change levels of plasma miR-122 in all patients.
Figure 3. Correlation plasma miR-122 with HCV RNA and ALT level. Correlation between baseline absolute plasma level of miR-122 and (A) HCV RNA level, and (B) ALT level. Correlation between week 4 fold change miR-122 levels and (C) HCV RNA level, and (D) ALT levels. The correlation coefficient (R) is calculated using the Spearman’s correlation test. The 95% confidence interval is shown.

levels showed a mean 1.5-fold increase at week 1 (P = 0.69), 1.5-decrease at week 4 (P = 0.12), 2.6-fold decrease at week 6 (P = 0.79) and 1.1-fold decrease at week 10/12 (P = 0.48), as compared to placebo (Table 2 and Figure 5A). In patients dosed with miravirsen vs. placebo, plasma miR-532-5p levels showed a median 2.0-fold versus 1.0-fold increase between baseline and week 1 (P = 0.55), 1.4-fold vs. 1.7-fold increase at week 4 (P = 0.84), 7.6-fold vs. 3.0-fold decrease at week 6 (P = 0.42) and 3.2-fold vs. 3.1-fold decrease at week 10/12 (P = 0.98) (Table 2 and Figure 5B).

DISCUSSION

This is the first study to assess changes in plasma miRNA levels in CHC patients dosed with an anti-miR-122 oligonucleotide. We demonstrate a substantial and prolonged decrease in plasma miR-122 levels in patients dosed with miravirsen. Furthermore, plasma levels of other miRNAs were shown to be variable in both patients dosed with placebo and
Table 2. Mean delta Cq values of miRNA-profiling and longitudinal analysis of miR-122, miR-210 and miR-532-5p levels in miravirsen and placebo dosed patients

<table>
<thead>
<tr>
<th></th>
<th>Miravirsen</th>
<th>Placebo</th>
<th>P-value (Benjamini-Hochberg)</th>
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<tr>
<td>Patients, n</td>
<td>12</td>
<td>4</td>
<td>N.A.</td>
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<tr>
<td>miR-122 profiling</td>
<td></td>
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<tr>
<td>Baseline (log₁₀ 2⁻ΔCq)</td>
<td>0.80 ± 0.42</td>
<td>0.64 ± 0.65</td>
<td>0.58</td>
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<tr>
<td>Week 4 (log₁₀ 2⁻ΔCq)</td>
<td>-2.64 ± 0.48</td>
<td>0.74 ± 0.49</td>
<td>7.94E-09</td>
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<tr>
<td>FC week 4 (log₁₀)</td>
<td>-3.44 ± 0.53</td>
<td>0.09 ± 0.21</td>
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<td>miR-210 profiling</td>
<td></td>
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<tr>
<td>Baseline (log₁₀ 2⁻ΔCq)</td>
<td>-0.79 ± 0.25</td>
<td>-0.80 ± 0.19</td>
<td>0.98</td>
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<tr>
<td>Week 4 (log₁₀ 2⁻ΔCq)</td>
<td>-1.26 ± 0.25</td>
<td>-0.63 ± 0.11</td>
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<tr>
<td>FC week 4 (log₁₀)</td>
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<td>0.17 ± 0.18</td>
<td>0.007</td>
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<td>Longitudinal analysis</td>
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<tr>
<td>FC week 1 (log₁₀)</td>
<td>0.18 ± 0.66</td>
<td>0.99 ± 1.34</td>
<td>0.69</td>
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<tr>
<td>FC week 4 (log₁₀)</td>
<td>-0.17 ± 0.52</td>
<td>1.05 ± 1.40</td>
<td>0.12</td>
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<td>FC week 6 (log₁₀)</td>
<td>-0.41 ± 0.72</td>
<td>0.34 ± 1.89</td>
<td>0.79</td>
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<td>FC week 10/12 (log₁₀)</td>
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<td>0.46 ± 1.90</td>
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<td>miR-532-5p profiling</td>
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<tr>
<td>Baseline (log₁₀ 2⁻ΔCq)</td>
<td>-1.10 ± 0.18</td>
<td>-0.81 ± 0.25</td>
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<td>Week 4 (log₁₀ 2⁻ΔCq)</td>
<td>-1.77 ± 0.59</td>
<td>-0.73 ± 0.41</td>
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<td>FC week 4 (log₁₀)</td>
<td>-0.67 ± 0.55</td>
<td>0.08 ± 0.25</td>
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<tr>
<td>FC week 1 (log₁₀)</td>
<td>0.30 ± 0.84</td>
<td>0.01 ± 0.72</td>
<td>0.55</td>
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<tr>
<td>FC week 4 (log₁₀)</td>
<td>0.16 ± 0.71</td>
<td>0.24 ± 0.42</td>
<td>0.84</td>
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<tr>
<td>FC week 6 (log₁₀)</td>
<td>-0.89 ± 0.83</td>
<td>-0.48 ± 0.83</td>
<td>0.42</td>
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<td>FC week 10/12 (log₁₀)</td>
<td>-0.51 ± 1.07</td>
<td>-0.49 ± 1.27</td>
<td>0.98</td>
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</table>

Data are given as mean values ± SD. FS, fold change. N.A., not applicable.

Miravirsen during the study period, and were not significantly affected by the inhibition of mir-122.

MiR-122 is the most abundant hepatocellular miRNA, with ~66,000 copies per cell, and is required for efficient HCV replication. MiR-122 positively affects HCV RNA stability, translation and replication, but the exact mechanism involved is not fully understood. Interestingly, earlier studies showed that serum and hepatic miR-122 levels, and serum miR-122 and HCV RNA levels are not associated. In addition, it is unknown how many miR-122 copies are required for efficient HCV replication. In this study, we show that all
Figure 4. MiRNA profiling results. (A) Flowchart of analysis and selection of miRNAs. (B) Hierarchical clustering of patients and a selection of miRNAs with fold change ≥ 2 or ≤ 0.5 in patients dosed with miravirsen and fold change level between 0.5 and 2 in placebo-treated patients (n=19). The color scale illustrates the relative expression of a miRNA across all samples: yellow color represents a high expression, blue color represents low expression. Unsupervised clustering clustered patients dosed with miravirsen and placebo in separate groups. *P < 0.05 between study groups.
patients dosed with miravirsen had a significant reduction in plasma miR-122 levels, and that miR-122 levels became undetectable in some patients. However, this was not always accompanied by a substantial reduction in the viral load, and there was no significant correlation between the fold change in plasma miR-122 and change in HCV RNA levels at week 4 as compared to baseline. Recently, it was demonstrated that a C3U nucleotide change in the 5’ UTR of the HCV RNA appeared in patients with virological relapse after miravirsen dosing. The C3U viral mutant had a decreased fitness and was insensitive to miravirsen, but fully susceptible to other anti-HCV agents. It was postulated that the C3U mutant renders the virus independent of miR-122, suggesting that this mutant uses an alternative mechanism to support RNA stabilisation and replication.

Figure 5. Fold change in plasma miR-210 and miR-532-5p level. Fold change in miRNA-level at week 1, 4, 6 and 10/12 compared to baseline in patients dosed with placebo or miravirsen. Longitudinal change in (A) plasma miR-210 levels and (B) plasma miR-532-5p levels in patients dosed with miravirsen compared to placebo. Figure showing mean and SEM; ns: not significant.
It has previously been shown that an elevated serum miR-122 level is a sensitive marker for inflammatory activity in the liver and that it is associated with ALT level. It was postulated that the presence of circulating miR-122 may be the result of normal hepatocyte turnover, and that increased miR-122 level are caused by hepatocyte lysis. In addition, an inverse relation between the serum miR-122 level and the stage of fibrosis was described, possibly due to a decreased number of functional hepatocytes in liver cirrhosis. However, the presence of miR-122 in plasma could also be the result of active secretion by the hepatocyte, either associated with RNA-binding proteins and lipoprotein complexes or packaging into micro-particles. In this study, we demonstrate a rapid decrease in plasma miR-122 levels in patients dosed with miravirsen. One week after the first injection, the plasma miR-122 level was significantly decreased compared to baseline, and this remained low throughout the study period. In our study, we did not find a significant correlation between plasma miR-122 and ALT level at baseline. However, there was an association between change in plasma miR-122 and ALT level 4 weeks after the first dose of miravirsen. The lower plasma miR-122 levels after miravirsen dosing could be the result of less hepatocyte injury, or a diminished active secretion of miR-122 by targeting hepatic miR-122.

MiR-122 is believed to have a tumour suppressive role and has been related to the development of HCC. In adult mice, a short-term inhibition of miR-122 using an antisense oligonucleotide for 5 weeks was well tolerated, and these mice did not develop HCC. Despite our follow-up study wherein no safety problems were observed in miravirsen-dosed patients, the exact long-term risk of HCC development needs to be elucidated. To better valuate the risk of transient miR-122 inhibition on HCC development, we assessed how long plasma miR-122 levels remained decreased after miravirsen dosing in CHC patients. We observed an increasing trend of plasma miR-122 levels until approximately 2 months (week 10/12) after the last dose of miravirsen (at week 4), in patients dosed with 3 and 5 mg/kg miravirsen. Plasma miR-122 levels of the majority of patients dosed with 7 mg/kg miravirsen were still decreasing at week 10/12, indicating a dose-dependent difference in the duration of miR-122 inhibition. Taken together, these data suggest that there is a prolonged inhibitory effect on miR-122 but that miR-122 levels are expected to return to baseline over time. The prolonged effect on miR-122 levels may be explained by the long tissue half-life or miravirsen (~30 days), and that miravirsen does not only target mature miR-122, but was shown to also suppress the biogenesis of miR-122 at the primary and precursor miRNA levels in vitro.

In this study, we assessed whether antagonising miR-122 alters the expression level of other miRNAs that are found in plasma. MiRNAs are involved in regulating various cell functions and homoeostasis, they function by targeting the 3’ or 5’ UTR of mRNA and thereby degrade their target or its translation. Each miRNA can bind multiple target mRNAs, and each mRNA has numerous binding sites for multiple miRNAs. Furthermore,
miRNAs are known to have a tremendous interplay with each other and expression is tightly controlled by both transcriptional and post-transcriptional regulatory systems. Next to those for miR-122, binding sites for several miRNAs have been identified in the HCV RNA genome. In contrast to the stimulatory effect of miR-122 on HCV, miR-199a, let-7b, miR-196b and miR-491 were shown to inhibit HCV replication in vitro. We showed that antagonising miR-122 solely results in reduced plasma miR-122 levels and that plasma levels of other miRNAs are not significantly affected. Furthermore, we observed substantial variations in plasma miRNA levels during the study period in both placebo and miravirsen dosed patients. Unfortunately, little is known about normal miRNA levels and their variability in humans, and especially in CHC patients. Since miRNAs are important players in regulating various cellular processes, the unchanged miRNA-profile (except miRNA-122) indicates that miravirsen targets exclusively miRNA-122, which is favourable from the perspective of safety.

A limitation of this study is the small number of patients which is due to the fact that additional blood samples were only collected in a selection of patients dosed with miravirsen. Since no liver biopsies were taken prior and after miravirsen dosing, we can only speculate about changes in hepatic miRNA levels in these patients. However, as observed with the substantial reduction in plasma miR-122 levels, we believe that important changes in tissue-specific miRNA levels will be reflected in the plasma. Nevertheless, this study provides important information on changes in plasma miRNA levels in the first cohort of patients dosed with an anti-miRNA oligonucleotide.

We have used the mean expression values of miR-93 and miR-191 for normalisation of plasma miR-122 levels. However, miR-191 has previously been described to be slightly elevated in serum of patients with CHC compared to that of healthy controls. Currently, there is no consensus which miRNA to use to establish normalisation of plasma miRNA levels. Nevertheless, we believe that our reference miRNAs are justified in this study since miR-191 was stable in our cohort and that absolute (without normalisation) and relative (with normalisation) miR-122 levels showed the same pattern.

The therapeutic field in hepatitis C treatment is changing quickly with the registration and ongoing development of several DAA's. Miravirsen is the first anti-miRNA oligonucleotide that has been administered in humans, and in particular in CHC patients. Although the development of miravirsen has ceased, a Gal-NAc conjugated anti-miR-122 oligonucleotide with a 20-fold higher potency compared to miravirsen is currently being investigated in clinical trials. This study evaluated changes in plasma miRNA levels after anti-miR-122 oligonucleotide dosing. We demonstrated that administration of various doses of miravirsen resulted in a substantial and prolonged decrease in plasma miR-122 levels and did not affect plasma levels of other miRNAs in CHC patients.
REFERENCES


49. Van Der Ree M, de Vree ML, Stelma F, Willemsen S, van der Valk M, Rietdijk S, et al. LO7: A single subcutaneous dose of 2mg/kg or 4mg/kg of RG-101, a GalNAc-conjugated oligonucleotide with antagonist activity against MIR-122, results in significant viral load reductions in chronic hepatitis C patients. J Hepatol. 2015 Apr;62:5261.
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