miRNAs as Biomarkers for Drug-Induced Liver Injury and as Predictors in Clinical Trials

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<table>
<thead>
<tr>
<th>Disclosures for Herbert L. Bonkovsky</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Research Support/P.I.</strong></td>
<td>NIH contracts UO1-DK06193; U54 DK083909--Drug-Induced Liver Injury Network; Porphyrias Consortium; Alnylam Inc; Gilead Sciences</td>
</tr>
<tr>
<td><strong>Employee</strong></td>
<td>Wake Forest Univ Health Sciences Center</td>
</tr>
<tr>
<td><strong>Consultant</strong></td>
<td>Alnylam, Blue, Clinuvel, Mitsubishi-Tanabe, NA, Moderna, Recordati, Stoke</td>
</tr>
<tr>
<td><strong>Major Stockholder</strong></td>
<td>Oh, how I wish!!</td>
</tr>
<tr>
<td><strong>Speakers’ Bureaus</strong></td>
<td>None</td>
</tr>
<tr>
<td><strong>Scientific Advisory Boards</strong></td>
<td>American Porphyria Foundation; Iron Disorders Institute</td>
</tr>
</tbody>
</table>
Topics to be Covered

• Overview of miRNAs
• Central role of miR-122 in liver
• miR-122 as early marker of Apap DILI
• Profiles of miRNAs in acute DILI
• miRNAs as predictors in clinical trials
• Summary/ Conclusions/ Future Work
Overview of miRNAs

- Small, non-coding RNAs---~22-25 nt
- Broad effects modulate gene & protein expression
- Main effects are to decrease mRNAs
- Actions are mainly
  - translational repression
  - mRNA cleavage
- Multiplicity of targets
- Fine-tuning of gene expression
- Stable in blood--protected in exosomes, binding to Ago2 & HDL
MicroRNAs (miRNAs)
Topics to be Covered

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• Central role of miR-122 in liver
• miR-122 as early marker of Apap DILI
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miR-122

- Conserved among vertebrates
- ~72% of the total miRNA population in hepatocytes.
- Encoded at single locus on chromosome 18
  - Transcription regulated by HNF4a, Rev-ErbA [Circadian]
- Best-known function of hepatic miR-122 is to regulate lipid and cholesterol metabolism—decr cholesterol.
- miR-122 binds to 5’ UTR of HCV RNA, stimulates viral protein expression and promotes viral replication.
  - miR-122 blockers [Miravirsen] have anti-HCV effects
- Mice depleted of miR-122 progressively develop steatohepatitis, fibrosis and hepatocellular cancer.
Structure of miR-122 Precursor
[highly conserved among vertebrates]
Topics to be Covered

• Overview of miRNAs
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Newer Biomarkers to Predict Apap-Induced Acute Liver Injury

- 129 subjects, 51 M, 78 F, age 34 y
  - Single excess Apap ingestion
  - Apap blood level felt to require iv NAC Rx
- Blood obtained at 1st presentation—8 h post ingestion—median Apap = 120 mg/L
- Plasma separated and stored -80 C
- Assays
  - miR-122/let-7d
  - CK18—necr + apoptosis
Correlations of Biomarkers With Serum ALT

A. Presentation of miR-122 (Let-7 normalised) vs. Peak ALT activity (U/I)

B. Presentation of HMGB1 (ng/ml) vs. Peak ALT activity (U/I)

C. Presentation of apoptosis K18 (U/I) vs. Peak ALT activity (U/I)

D. Presentation of necrosis K18 (U/I) vs. Peak ALT activity (U/I)

E. Presentation of LDH activity (U/I) vs. Peak ALT activity (U/I)
Correlations of Biomarkers With Peak INR

(A) Presentation miR122 (Let-7a normalised) vs Peak INR

(B) Presentation HMGB1 (ng/ml) vs Peak INR

(C) Presentation apoptosis K18 (UI) vs Peak INR

(D) Presentation necrosis K18 vs Peak INR

(E) Presentation LDH activity (UI) vs Peak INR
Focus on 63 with 1st Blood Samples within 8 h of Apap Ingestion

- 11 [17%] developed serum ALT > 3 x ULN [> 150].
- Median plasma Apap concns not signif different.
- Plasma Apap extrapolated to 4 h post ingestion not significantly different.
- Newer biomarkers, especially miR-122, HMGB1, & K18 necrosis were significantly higher early in those who eventually developed Apap DILI.
Newer Biomarkers, especially miR-122, HMGB1, and CK18 necrosis, are High Before ALT Levels Rise
ROC Curves support Potential for newer Biomarkers to Predict Apap DILI

A

Sensitivity

1.00

0.75

0.50

0.25

0.00

1 - Specificity

0.00

0.25

0.50

0.75

1.00

miR-122
AUC 0.93
P < 0.0001
SENS 0.83

B

Sensitivity

1.00

0.75

0.50

0.25

0.00

1 - Specificity

0.00

0.25

0.50

0.75

1.00

HMGB1
AUC 0.97
P < 0.0001
SENS 0.91

C

Sensitivity

1.00

0.75

0.50

0.25

0.00

1 - Specificity

0.00

0.25

0.50

0.75

1.00

Apoptosis K18
AUC 0.77
P = 0.0009
SENS 0.21

D

Sensitivity

1.00

0.75

0.50

0.25

0.00

1 - Specificity

0.00

0.25

0.50

0.75

1.00

Necrosis K18
AUC 0.94
P < 0.0001
SENS 0.90

E

Sensitivity

1.00

0.75

0.50

0.25

0.00

1 - Specificity

0.00

0.25

0.50

0.75

1.00

GLDH
AUC 0.80
P = 0.0003
SENS 0.19

F

Sensitivity

1.00

0.75

0.50

0.25

0.00

1 - Specificity

0.00

0.25

0.50

0.75

1.00

ALT
AUC 0.54
P = 0.059
SENS 0.09

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Summary/Conclusions

- miR-122, HMGB1, CK 18-necrosis more sensitive than ALT at early identification of Apap DILI.
- Perhaps, newer biomarkers may allow confident exclusion of Apap DILI and avoidance of hospitalization, administration of iv NAC.
- Still, serum ALT, AST are readily available, whereas miR-122, HMGB, CK 18-necrosis are not.
- Critical values for Apap—or other drugs, other causes of acute liver injury-- still need definition.
miRNA Profiling in Apap Hepatotoxicity & Non-Apap Hepatox

- Royal Infirmary Edinburgh
- Age- & sex-matched subjects with \([n=27]\) & without Apap acute liver injury \([\text{peak serum ALT} > 3 \times \text{ULN} (> 150)]\)
- Scots LT Unit--RIE--Non-Apap ALF*, \(n = 5\)
- Separate validation cohort, \(n = 81\)
- Assess 1809 miRNAs [Mirbase 18]
  - *AIH, Primary graft non-fct, small for size, malignancy, clarithromycin DILI

Nat Sci Rept 2015; 5: 15501

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Main Results

- 75/1809 miRNAs increased > 3-fold
- 46/1809 decreased > 3-fold
- Largest increases: miR-122, miR-855, miR-151a
- Highest rank for increase miR-382
- Unaffected by acute kidney injury
- Similar changes in acute liver injury of diverse other causes
Volcano Plot of miRNA Data
Comparing Apap-Tox vs Apap-no Tox
Box & Whisker Plot showing 15 highest Fold Increase & 5 Highest Fold Decrease miRNAs [log scale]
Random Forest Statistics

Top 16 miRNAs – Apap-Tox vs Apap – No tox

miR-382 best; miR-122 ranks 11th
ROC Curve of Top 16 miRNAs from Training Set

*Distinguish Apap-Tox vs Apap-No Tox*

AUC = 0.96 (0.92-1.01)

P < 0.0001
Time Lines of Selected miRNAs vs serum ALT

49 yo man after Large Apap Overdose

miRNAs peak earlier
Summary/Conclusions

- Largest study to date of circulating miRNome in humans with Apap toxicity.
- miR-122 was highest, but others [miR-885-5p, miR-151-3p] were relatively increased as much or more or ranked higher by random Forest plot [miR-382-5p] to discriminate.
- miRNAs bound to Ago2 in plasma.
- Kidney injury associated to decreased miRNAs.
- No selectivity found for diverse etiologies of ALI.
Topics to be Covered

• Overview of miRNAs
• Central role of miR-122 in liver
• miR-122 as early marker of Apap DILI
• Profiles of miRNAs in acute DILI
• miRNAs as predictors in clinical trials
• Summary/ Conclusions/ Future Work
Study Design

- Acute non-Apap, idiosyncratic DILI—78 subjects, enrolled within 14 d of DILI onset
- Controls—40 normal volunteer blood donors without known liver disease; normal H/H, ALT
- Perform profiles of miRNAs in sera
- Summarize data and perform initial comparisons with salient clinical and other lab data

Liv Intl 2017; 37:757
Study Design

Acute DILI
N=78

Death within 6 mo of onset
N=9

Did not complete 6 mo f/u
N=37

Completed 6-month follow-up
N=32

1 death at 315 days

Normal controls
N=40

0 liver transplant

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Methods

- mRNA and miRNAs isolated from 200 uL of serum using miRNeasy Mini Kit (Qiagen, Valencia, CA). Samples ligated to biotinylated signal molecules using the FlashTag™ Biotin HSR RNA labeling Kit (Genisphere, LLC, Hatfield, PA, USA).
- Enzyme Linked Oligosorbent Assay (ELOSA) performed for QC to verify labeling prior to array hybridization.
- Samples hybridized to GeneChip® 3.0 miRNA microarrays (Affymetrix, Santa Clara, CA, USA). This chip contains 1733 probes for human mature miRNAs + 1658 probes for human pre-miRNAs [3391 total].
- Probe-level signal intensities [Arbitrary fluorescent units/ 200uL] analyzed using Partek Genomics Suite (Partek, St. Louis, MO, USA).
- Robust multichip averaging used for background correction, quantile normalization and probeset summarization.
- Statistical differences calculated by two-way ANOVA analysis with suitable adjustments [Benjamini-Hochberg] to minimize false discovery rate (FDR).
Main Results--Summary

- 8/1733 miRNAs were significantly increased in acute DILI vs normal controls
- 3/1733 miRNAs were significantly decreased in acute DILI vs normal controls
- 3/1733 miRNAs were significantly higher at DILI baseline than at DILI 6 month follow-up
- Overall: there were NOT many differences between normal controls and acute DILI and few between acute DILI and DILI at 6 months. No differences in pre-miRNAs.
miRNAs Higher in Acute DILI than in Normal Controls [adjusted]

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Fold increase over Ctrl</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-122</td>
<td>18.81</td>
<td>$1.22 \times 10^{-11}$</td>
</tr>
<tr>
<td>miR-4532</td>
<td>8.16</td>
<td>$1.97 \times 10^{-9}$</td>
</tr>
<tr>
<td>miR-4484</td>
<td>4.68</td>
<td>$1.17 \times 10^{-4}$</td>
</tr>
<tr>
<td>miR-4463</td>
<td>4.46</td>
<td>$3.43 \times 10^{-5}$</td>
</tr>
<tr>
<td>miR-4270</td>
<td>3.01</td>
<td>$1.29 \times 10^{-4}$</td>
</tr>
<tr>
<td>miR-1246</td>
<td>2.88</td>
<td>$5.34 \times 10^{-5}$</td>
</tr>
<tr>
<td>miR-4433</td>
<td>2.67</td>
<td>$2.03 \times 10^{-4}$</td>
</tr>
<tr>
<td>miR-4767</td>
<td>1.52</td>
<td>$7.93 \times 10^{-5}$</td>
</tr>
<tr>
<td>miRNA</td>
<td>Ratio DILI/Control</td>
<td>P value</td>
</tr>
<tr>
<td>-----------</td>
<td>--------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>miR-455-3p</td>
<td>0.387</td>
<td>$2.46 \times 10^{-7}$</td>
</tr>
<tr>
<td>miR-1281</td>
<td>0.348</td>
<td>$1.66 \times 10^{-6}$</td>
</tr>
<tr>
<td>miR-4274</td>
<td>0.576</td>
<td>$6.61 \times 10^{-6}$</td>
</tr>
</tbody>
</table>
# Lack of Effect of Time from DILI Onset on Serum miRNA Levels

<table>
<thead>
<tr>
<th>miR</th>
<th>1-7 d after onset</th>
<th>8-14 d after onset</th>
<th>Diff in Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>-122</td>
<td>8.01</td>
<td>7.67</td>
<td>-0.34</td>
</tr>
<tr>
<td>-4532</td>
<td>5.45</td>
<td>5.22</td>
<td>-0.23</td>
</tr>
<tr>
<td>-4484</td>
<td>7.56</td>
<td>8.15</td>
<td>+0.59</td>
</tr>
<tr>
<td>-4463</td>
<td>4.96</td>
<td>4.93</td>
<td>-0.03</td>
</tr>
<tr>
<td>-4270</td>
<td>3.62</td>
<td>3.60</td>
<td>-0.02</td>
</tr>
<tr>
<td>-1246</td>
<td>2.98</td>
<td>2.66</td>
<td>-0.32</td>
</tr>
<tr>
<td>-4433</td>
<td>3.15</td>
<td>3.05</td>
<td>-0.10</td>
</tr>
<tr>
<td>-4767</td>
<td>2.34</td>
<td>2.28</td>
<td>-0.06</td>
</tr>
</tbody>
</table>

Mean values. Arbitrary FU. None of the differences is significant.
Correlation with Serum Aminotransferase Levels

*miRNAs Increased above Controls*

<table>
<thead>
<tr>
<th>AminoT’ase</th>
<th>Statistic</th>
<th>miR-122</th>
<th>miR-4532</th>
<th>miR-4463</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT onset</td>
<td>r</td>
<td>0.361</td>
<td>0.307</td>
<td>0.378</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.0017</td>
<td>0.0082</td>
<td>0.001</td>
</tr>
<tr>
<td>ALT peak</td>
<td>r</td>
<td>0.319</td>
<td>0.311</td>
<td>0.284</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.005</td>
<td>0.0062</td>
<td>0.013</td>
</tr>
<tr>
<td>AST onset</td>
<td>r</td>
<td>0.227</td>
<td>0.352</td>
<td>0.261</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.050</td>
<td>0.002</td>
<td>0.024</td>
</tr>
<tr>
<td>AST peak</td>
<td>r</td>
<td>0.232</td>
<td>0.302</td>
<td>0.202</td>
</tr>
<tr>
<td></td>
<td>p</td>
<td>0.044</td>
<td>0.0013</td>
<td>0.080</td>
</tr>
</tbody>
</table>

Similar results also for miR-4270, -1246, -4433, -4767
Correlation with Serum Aminotransferase Levels

miRNAs Decreased below Controls

<table>
<thead>
<tr>
<th>Amino T’ase</th>
<th>Statistic</th>
<th>miR-455_3p</th>
<th>miR-1281</th>
<th>miR-4274</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT onset</td>
<td>r</td>
<td>-0.378</td>
<td>-0.343</td>
<td>-0.098</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.001</td>
<td>0.003</td>
<td>0.409</td>
</tr>
<tr>
<td>ALT peak</td>
<td>r</td>
<td>-0.337</td>
<td>-0.358</td>
<td>-0.106</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.003</td>
<td>0.001</td>
<td>0.362</td>
</tr>
<tr>
<td>AST onset</td>
<td>r</td>
<td>-0.299</td>
<td>-0.283</td>
<td>-0.102</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.009</td>
<td>0.014</td>
<td>0.385</td>
</tr>
<tr>
<td>AST peak</td>
<td>r</td>
<td>-0.264</td>
<td>-0.328</td>
<td>-0.069</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.021</td>
<td>0.004</td>
<td>0.554</td>
</tr>
</tbody>
</table>

2/3 miRNAs that were decreased had values correlated inversely with serum ALT, AST, suggesting greater decreases of miRNAs with higher Amino T’ase levels.
Significant Correlations of miRNA levels with Selected Categorical Variables

- Correlated with acute death [within 6 months]
  - miR-122, -4463, -4270: *Lower, not higher values*

- Correlated with death at anytime
  - miR-122, -4463, -4270, -4433, -4767: *Lower, not higher values*

- Pattern of liver injury: Different in HC vs Cholestatic vs Mixed
  - miRNAs increased: -122, -4532, -4463, -4270, -1246, -4767
  - miRNA decreased: -4274
## Selected miRNA Levels in DILI Subjects with and without Acute Death and Controls

<table>
<thead>
<tr>
<th>miRNA-122</th>
<th>DILI-acute death (n=9)</th>
<th>DILI-no acute death (n=69)</th>
<th>Controls (n=38)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (+SD)</td>
<td>5.37±2.50</td>
<td>8.18±1.83</td>
<td>3.92±1.48</td>
</tr>
<tr>
<td>Median (range)</td>
<td>6.32 (1.10-7.88)</td>
<td>8.49 (2.3-11.18)</td>
<td>4.36 (1.25-6.32)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>miRNA-4270</th>
<th>DILI-acute death (n=9)</th>
<th>DILI-no acute death (n=69)</th>
<th>Controls (n=38)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (+SD)</td>
<td>2.24±1.53</td>
<td>3.80±1.51</td>
<td>2.14±1.04</td>
</tr>
<tr>
<td>Median (range)</td>
<td>1.22 (0.99±4.84)</td>
<td>4.11 (0.79-6.60)</td>
<td>1.76 (0.83-4.62)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>miRNA-4463</th>
<th>DILI-acute death (n=9)</th>
<th>DILI-no acute death (n=69)</th>
<th>Controls (n=38)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (+SD)</td>
<td>3.07±1.67</td>
<td>5.2±1.87</td>
<td>2.92±1.05</td>
</tr>
<tr>
<td>Median (range)</td>
<td>2.43 (1.24-5.59)</td>
<td>5.67 (1.01-7.90)</td>
<td>2.79 (1.45-5.51)</td>
</tr>
</tbody>
</table>
# Model for Prediction of Outcome of Acute DILI: miR-122 + Serum Alb

<table>
<thead>
<tr>
<th>miR-122 + SA</th>
<th>Died</th>
<th>Lived</th>
<th>Totals</th>
<th>Sensit</th>
<th>Specif</th>
<th>PPV</th>
<th>NPV</th>
<th>Accur</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lower</td>
<td>8</td>
<td>13</td>
<td>21</td>
<td>100</td>
<td></td>
<td></td>
<td>100</td>
<td>62/75</td>
</tr>
<tr>
<td>Higher</td>
<td>0</td>
<td>54</td>
<td>54</td>
<td>81</td>
<td>38</td>
<td></td>
<td></td>
<td>83%</td>
</tr>
<tr>
<td>Totals</td>
<td>8</td>
<td>67</td>
<td>75</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Performance of this model is inferior to our model derived from cytokine profiles, but this model is perhaps easier, requiring only measures of miR-122 and serum albumin.**

<table>
<thead>
<tr>
<th>Cytok + SA</th>
<th>Died</th>
<th>Lived</th>
<th>Totals</th>
<th>Sensit</th>
<th>Specif</th>
<th>PPV</th>
<th>NPV</th>
<th>Accur</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lower</td>
<td>7</td>
<td>1</td>
<td>8</td>
<td>78%</td>
<td></td>
<td></td>
<td>97%</td>
<td>74/77</td>
</tr>
<tr>
<td>Higher</td>
<td>2</td>
<td>67</td>
<td>69</td>
<td>99%</td>
<td>88%</td>
<td></td>
<td></td>
<td>96%</td>
</tr>
<tr>
<td>Totals</td>
<td>9</td>
<td>68</td>
<td>77</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Plos 1 2013; 8: 1974*
What might miRNAs Other than miR-122 be Doing?

- Short answer—we do not know.
- Searches of Pub Med, Gene, OMIM, Genome, miRBase, Google, Wikipedia have turned up little except for miR-122.
- miR-4460 seems linked to miR-32—Increased in prostate Ca [Gene 2012; 31: 4460]
miRNA levels can readily be detected and relative values compared in sera of subjects with acute DILI.

Among a total of 3391 [1733 probes for human mature miRNAs + 1658 probes for human pre-miRNAs], most showed no significant differences in levels in serum, compared to normal controls.

8 miRNAs, led by miR-122, the predominant miRNA expressed in hepatocytes were significantly increased. These 8 were highly correlated to one another.

3 miRNA’s were significantly decreased vs normal controls.

Levels of miRNAs in serum showed little change, at least during the 1st two weeks of illness.

A model that uses only levels of serum albumin and miR-122 at onset provides satisfactory prognostic prediction of outcome, albeit less than for our model involving 4 cytokines and serum albumin.
Topics to be Covered

• Overview of miRNAs
• Central role of miR-122 in liver
• miR-122 as early marker of Apap DILI
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• miRNAs as predictors in clinical trials
• Summary/ Conclusions/ Future Work
Little published thus far—at least that I found

No evidence as yet that miRNAs are unique, offer any striking advantage over serum ALT, AST, GLDH.

miRNAs likely increase earlier, have shorter half-lives

miRNAs more expensive; take more time

Some have found miR-122 more variable than ALT
Variability in Serum miR-122

- 42 volunteers—Genentech employees; 18 F, 23 M
- No known disease; No alcohol x 48 h prior to blood draws
- 6 samples over 6-9 weeks
- Panel of 82 miRNAs studied
- Methods after Starkey-Lewis et al, Hepatology 2011; 54: Cel-39 spiked in as ext control; also normalized to 4 endogenous miRNAs
Variability in Serum miR-122

- miR-122—133-fold variability
- ~50% of variability was among subjects [inter-subj]
- ~50% variability within subject [intra-subject]
- miR variability >>> ALT variability
- ALT: ~80% among subjects
- ALT: ~20% within subject
- Genentech has put miRNAs on ‘back burner’—not better than GLDH as liver-specific biomarker
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- miRNAs as predictors in clinical trials
- Summary/ Conclusions/ Future Work
miRNAs occur in plasma and serum in various forms and are stable—protected from RNAses.

Several miRNAs, especially miR-122 [most studied], are increased in sera in acute liver injury.

miR-122, the major miRNA in hepatocytes, rises more quickly than ALT, AST in acute Apap overdose: may find role in early prediction of Apap heptox.
In acute DILI, only a limited numbers of miRNAs are significantly changed.

Relatively lower levels of miR-122 + lower levels of serum albumin predictive of adverse outcomes of acute DILI at 6 months.

Profiles of groups of miRNAs, perhaps, with those of cytokines, may prove more useful and to differentiate DILI from other etiologies of liver injury. Much more data needed!!

We are a long way from replacing serum ALT, AST, GLDH with miRNAs as biomarkers of DILI.
## Acknowledgements

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That’s all folks!

Questions?