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miR-122: more than just your average leakage biomarker

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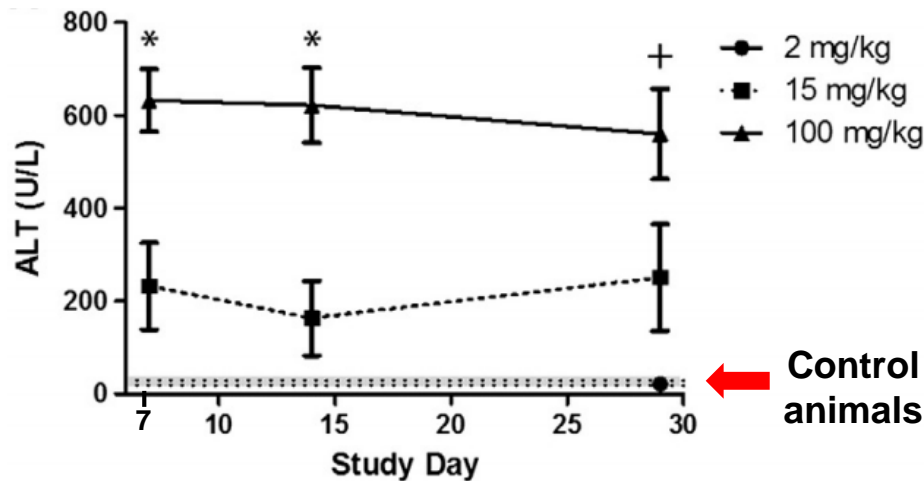
UNC Eshelman School of Pharmacy

Shortcomings of Traditional Biomarkers of Drug-Induced Liver Injury (DILI)

- Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are not specific to the liver. Elevations can be related to:
 - Muscle injury (Pettersson J, Hindorf U, *et al. Br J Clin Pharmacol.* 2008;65:253-9)
 - Changes in diet (Purkins L, Love ER, *et al. Br J Clin Pharmacol.* 2004;57:122-208)
- These biomarkers are passively released in response to injury, therefore changes are not observed until injury has already occurred.
- Traditional biomarkers including ALT do not necessarily reflect the injury present concurrently in the liver.

Elevated ALT Does Not Necessarily Reflect Ongoing Hepatocellular Damage

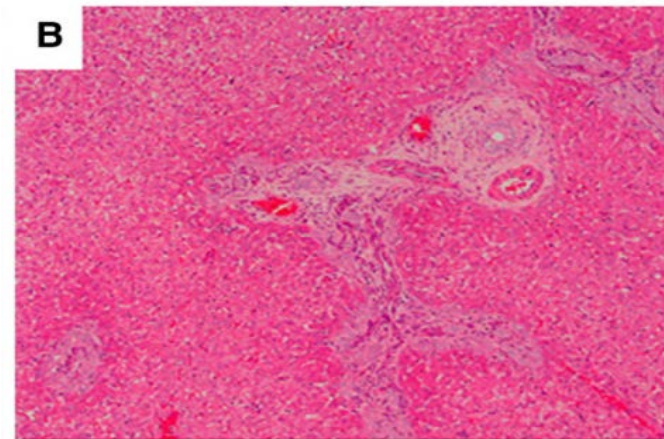
Beagles were given daily oral doses of proprietary compound NP260 for 28 days. Interim blood samples were collected for serum and necropsy was done on Day 29 to assess histological changes.



*p<0.05

Harrill AH, Eaddy JS, et al. *Toxicol Appl Pharmacol*. 2014; 277: 131-7

Day 29; 100 mg/kg; 20X



Persistent ALT elevations were observed in the absence of hepatocellular necrosis.

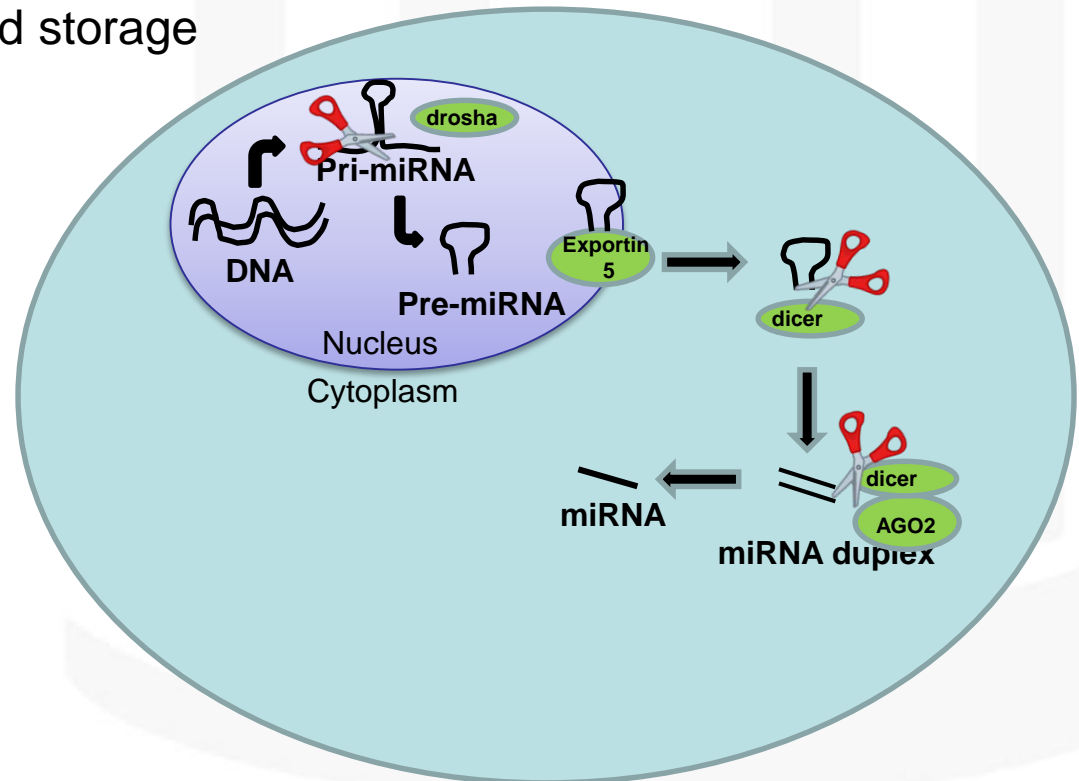
There is a need for new DILI biomarkers!



Advantages of miRNAs as Biomarkers

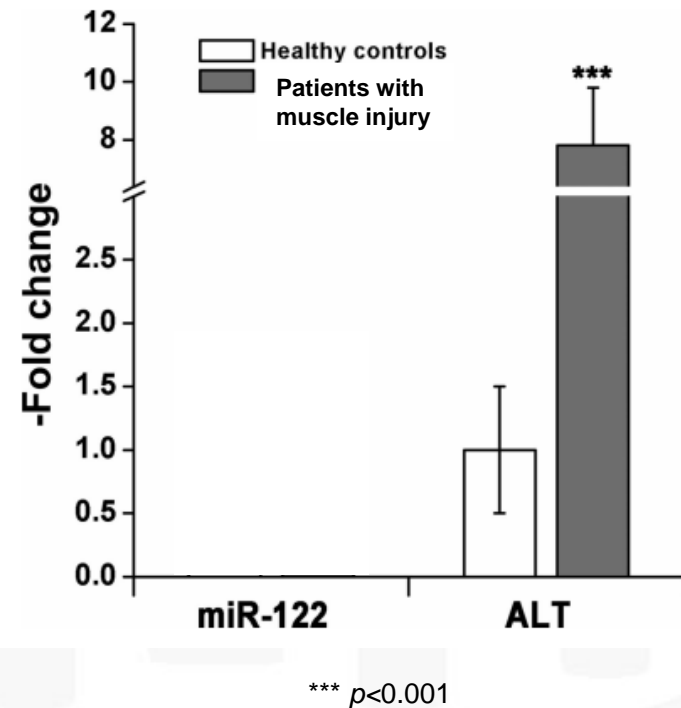
- miRNAs are:
 - Small ~22 nucleotide non-coding RNA species that post-transcriptionally regulate mRNA expression through complete or partial binding
 - Found in many different bodily fluids including blood and urine
 - highly conserved across species
 - Stable against digestion by RNAases, repeated freeze/thaw cycles, various temperatures and prolonged storage

Are there any miRNAs that may serve as valuable DILI biomarkers?



miR-122 is a Liver-Specific miRNA

- miRNA sequencing studies have identified miR-122 as highly specific to the liver of multiple species.
 - Mice (Landgraf P, Rusu M. *et al. Cell.* 2007;129:1401-14)
 - Rats (Smith A, Calley J, *et al. BMC Genomics.* 2016;17:694)
 - Beagles (Koenig EM, Fisher C, *et al. BMC Genomics.* 2016;17:694)
 - Humans (Landgraf P, Rusu M. *et al. Cell.* 2007;129:1401-14)
- miR-122 is also highly abundant in the liver, accounting for up to ~70% of hepatic miRNAs.
 - (Lagos-Quintana M, Rauhut R, *et al. Curr Biol.* 2002;12:735-9)
- Functions of miR-122 include regulation of hepatocyte differentiation, cholesterol metabolism, and enables hepatitis C virus infection.
- Unlike ALT, miR-122 does not become elevated in response to muscle injury.

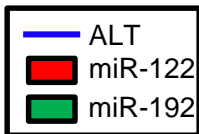
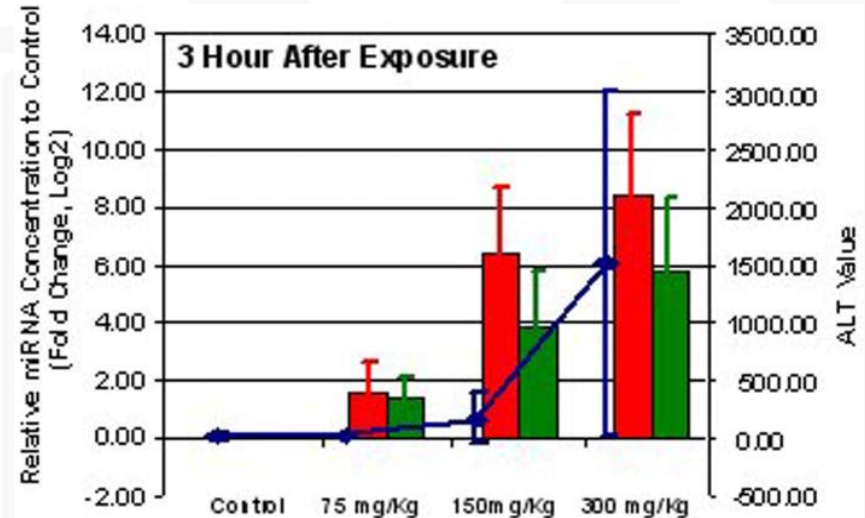
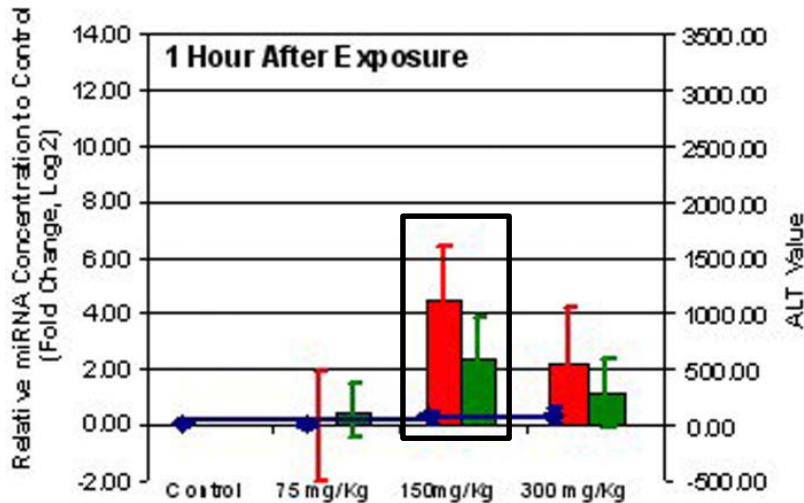


Great, but do miR-122 changes occur in response to DILI?



miR-122 is a Sensitive Pre-Clinical DILI Biomarker

Mice were given a single *i.p.* dose of APAP at multiple dose concentrations



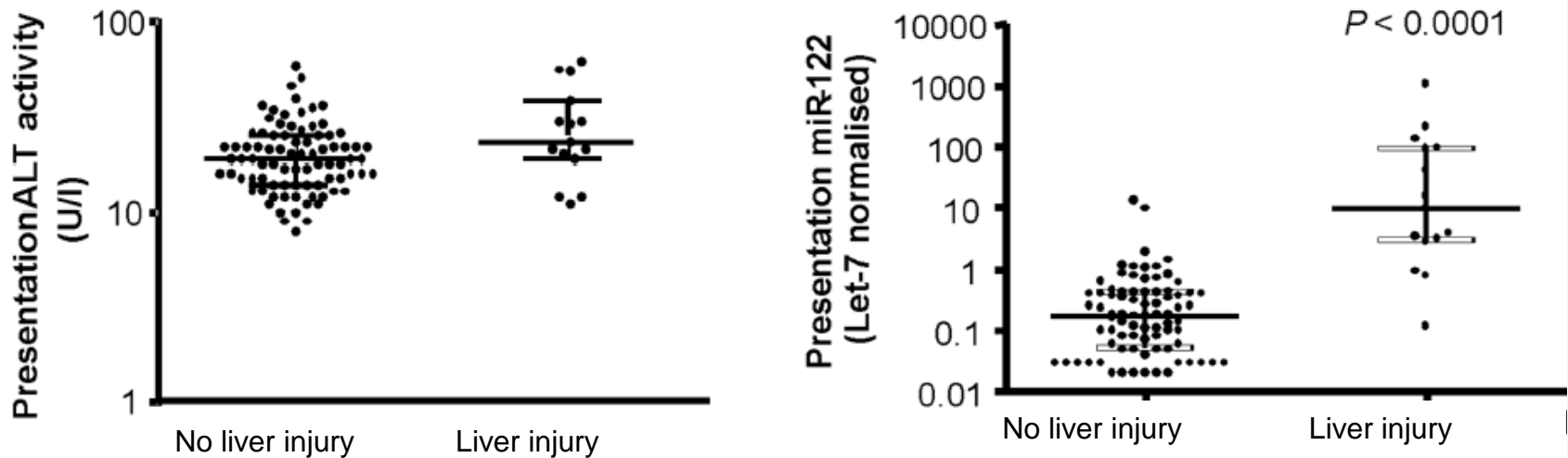
Wang K, Zhang S, *et al.* PNAS. 2009; 106: 4402-7

A clear elevation of miR-122 is observed at 150 mg/kg in mice 1h post-APAP administration. At 3h post-dose, a dose dependent elevation in miR-122 is observed similar to ALT.

Is the utility of miR-122 as a DILI biomarker translatable to humans?

miR-122 is a Sensitive Clinical DILI Biomarker

ALT and miR-122 values were quantified in serum samples collected at presentation (prior to liver injury) in patients with APAP over dose. Patient data was divided by those who never developed liver injury and those that eventually did.



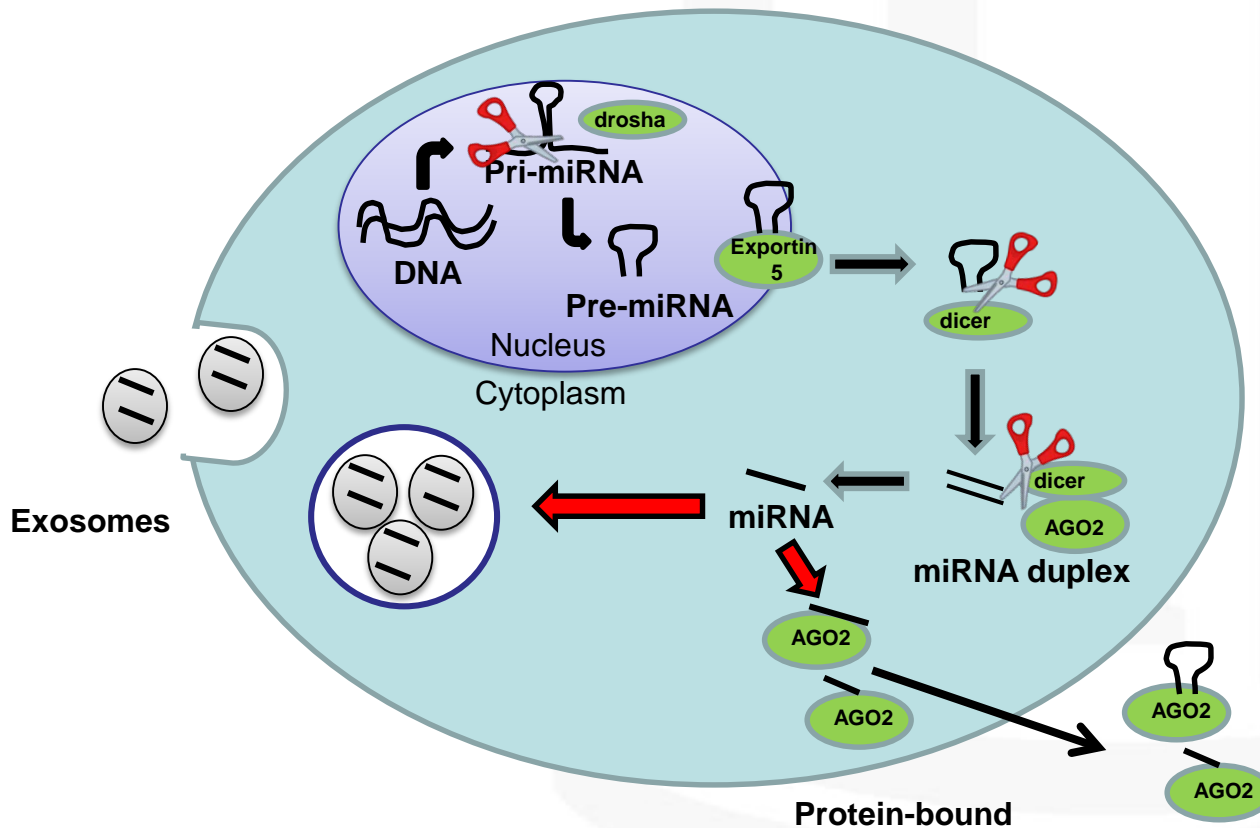
Antoine DJ, Dear JW, et al. *Hepatology*. 2013; 58: 777-87

miR-122 appears to be a very sensitive and translatable DILI biomarker.

Is miR-122, like ALT, a passive leakage biomarker?

miRNA Packaging: Protein vs. Exosomes

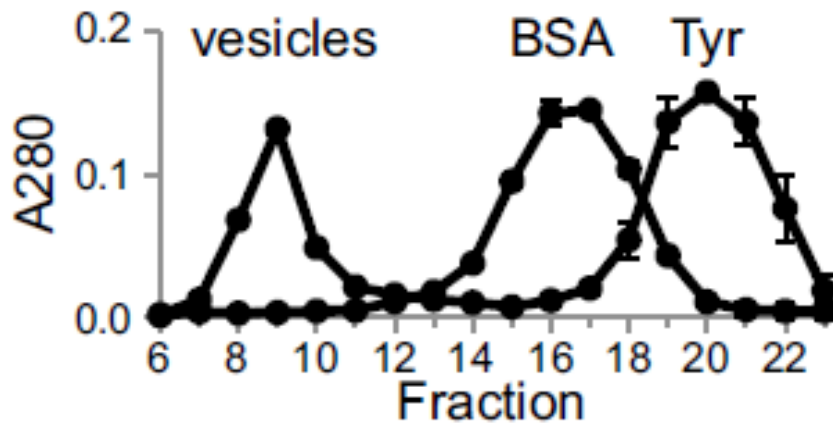
- Stability of miRNAs is thought to be related to packaging within biofluids.
 - Protein-bound (ex. Argonaute 2, AGO2)
 - Extracellular vesicles (ex. exosomes)



miR-122 Is Primarily Protein-Bound in Blood of Healthy Humans

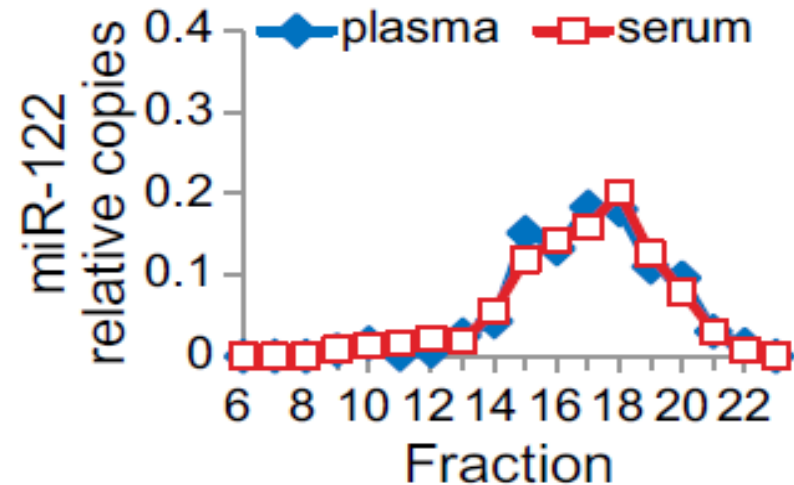
Size-exclusion chromatography and qPCR was utilized to determine the primary packaging source of miRNA in blood supernatant in normal humans (n=3)

Standards



Arroyo JD, Chevillet JR, *et al.* PNAS. 2011;108:5003-8

miR-122



miR-122 was found to elute in the protein-bound fraction of serum/plasma collected from healthy volunteers, suggesting passive leakage during hepatocyte turnover similar to ALT.

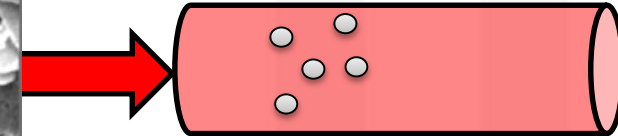
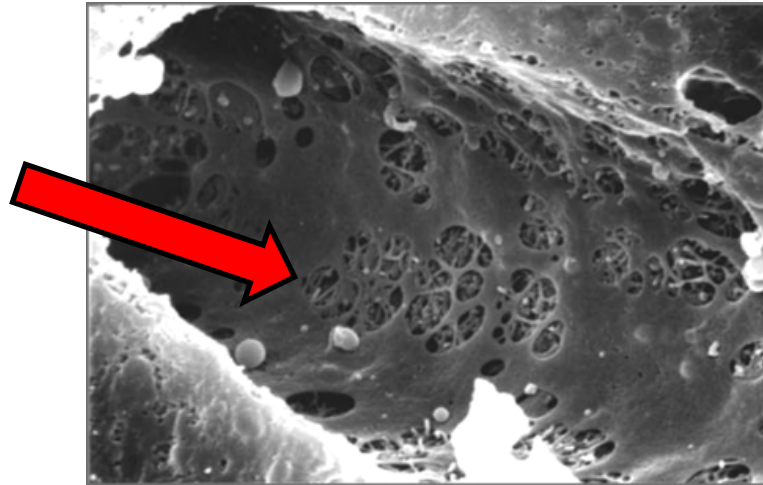
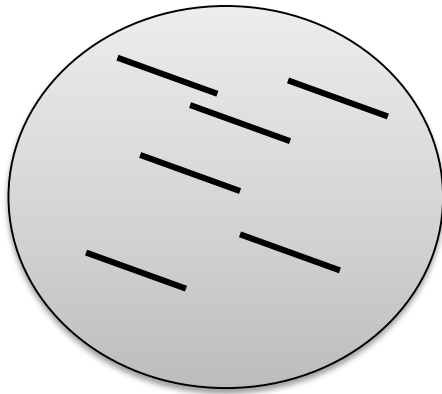
Can miR-122 be released actively into circulation as well?

Can miR-122 be Released Actively into Blood?

Exosomes: 40-100 nm

Liver Fenestrations: 150-175 nm

The diameter of sinusoidal fenestrations make it possible for exosomes to reach circulation without overt injury

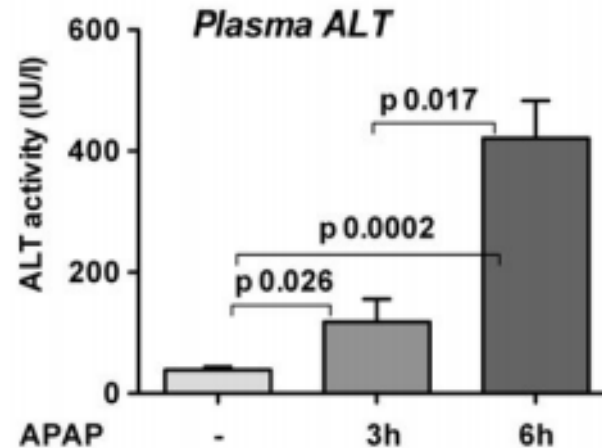
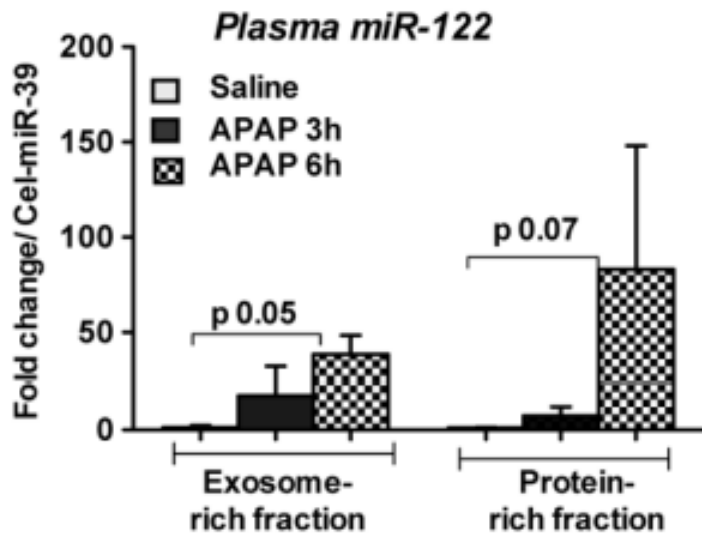


Vollmar B. and Menger MD. *Physiol Rev.* 2009;89:1269-1339

Does DILI induce active release of miR-122 in exosomes?

Exosomal miR-122 is Released into Circulation in Mice Following APAP Administration

Mice were given a lethal dose of APAP (500 mg/kg) and sacrificed 3 or 6h post-dose. Levels of miR-122 were quantified in exosomes and in the protein-rich fraction of plasma.



Injury already evident at earliest timepoint examined!!

Bala S, Petrasek J, et al. *Hepatology*. 2012;56:1946-1957

Although miR-122 was primarily in the protein-rich fraction of plasma, exosomal miR-122 was released following injury, as well.

Is miR-122 released actively prior to the onset of injury?

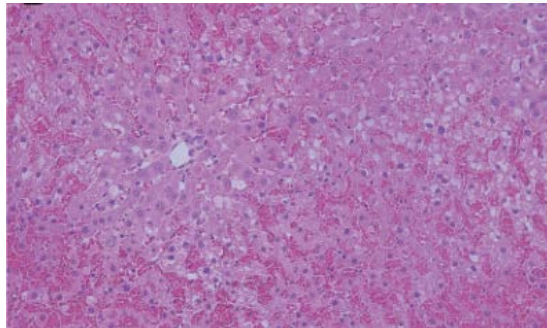
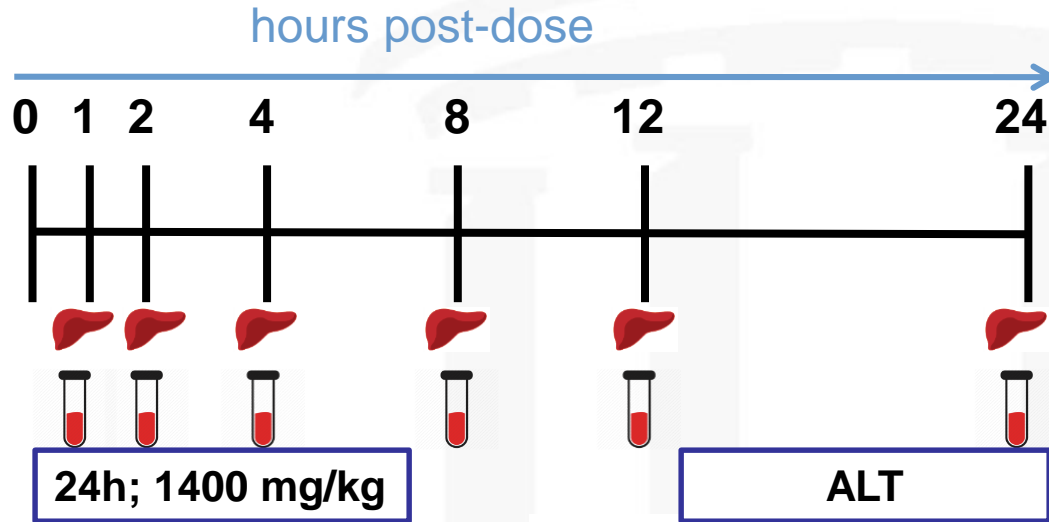
DILI in Rats Administered APAP

APAP

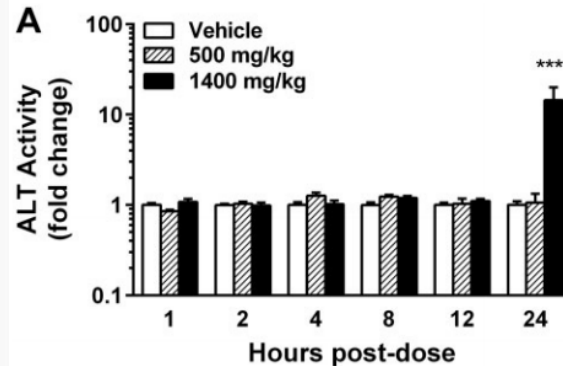


Doses

Vehicle: 0 mg/kg
Low: 500 mg/kg
High: 1400 mg/kg



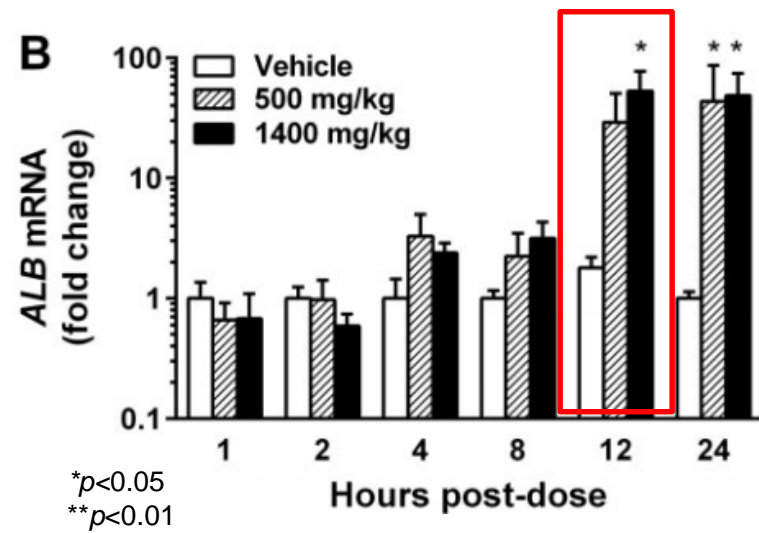
Holman NS, Mosedale M, et al. *Tox Sci.* 2016;151:365-75



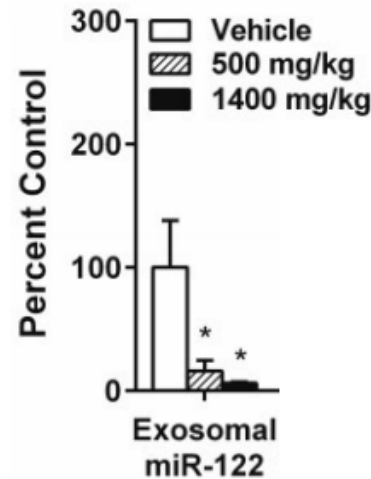
Hepatocellular injury occurred only at 24h in rats administered the high dose of APAP.

Were exosomal changes evident prior to the onset of overt injury

Selective Packaging of Exosomal miR-122 in Rats Administered APAP

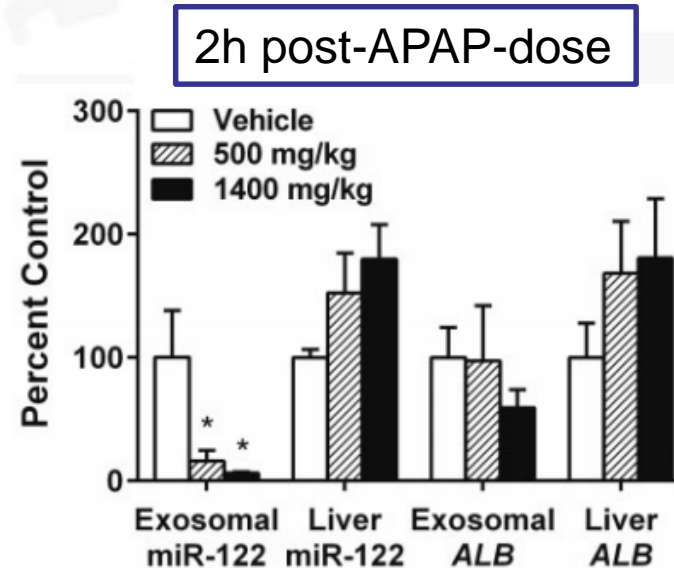
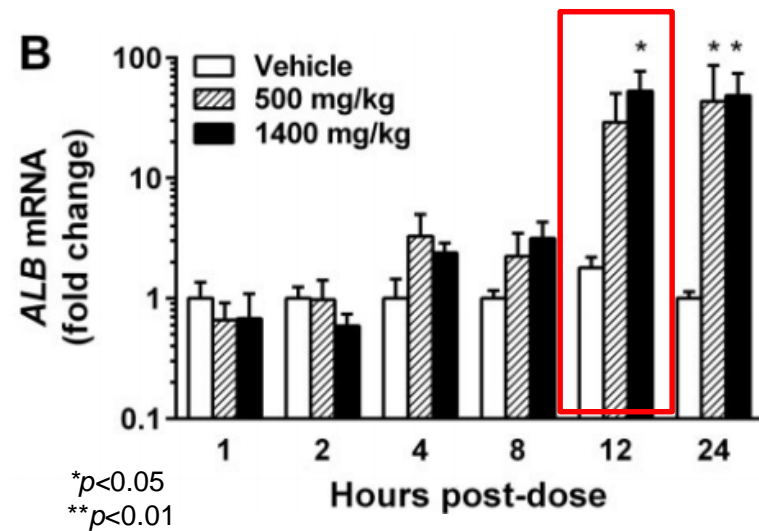


2h post-APAP-dose





Selective Packaging of Exosomal miR-122 in Rats Administered APAP

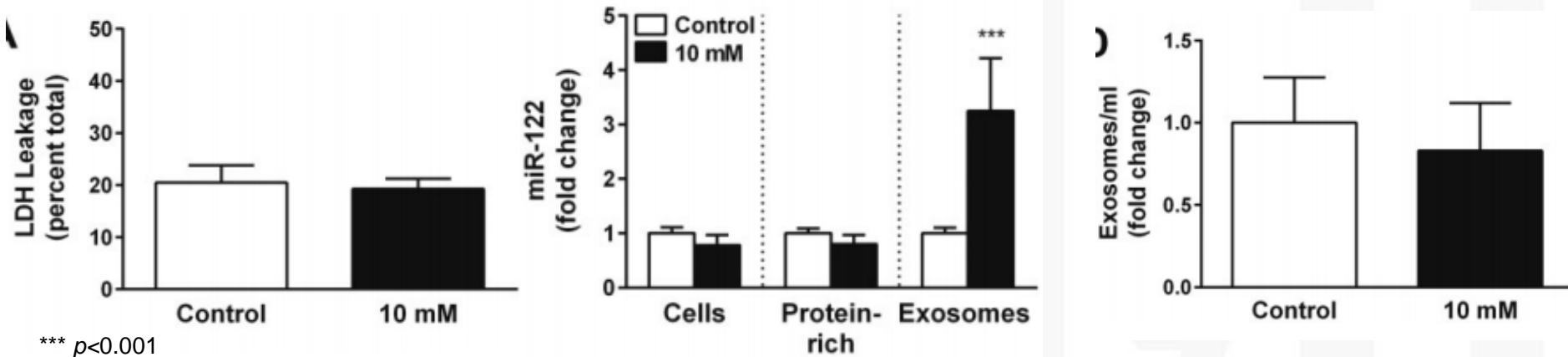


Exosomal *ALB* was elevated in plasma at 12h post-dose, prior to the onset of overt cellular injury. miR-122 was significantly reduced in exosomes 2h post-dose, suggesting retention by the liver and selective packaging into exosomes.

Is there evidence for selective packaging of miR-122 in humans?

Packaging of Exosomal miR-122 in Primary Human Hepatocytes Exposed to APAP

Exosomal release from hepatocytes was explored *in vitro* in primary human hepatocytes treated with sub-toxic APAP exposure for 24h

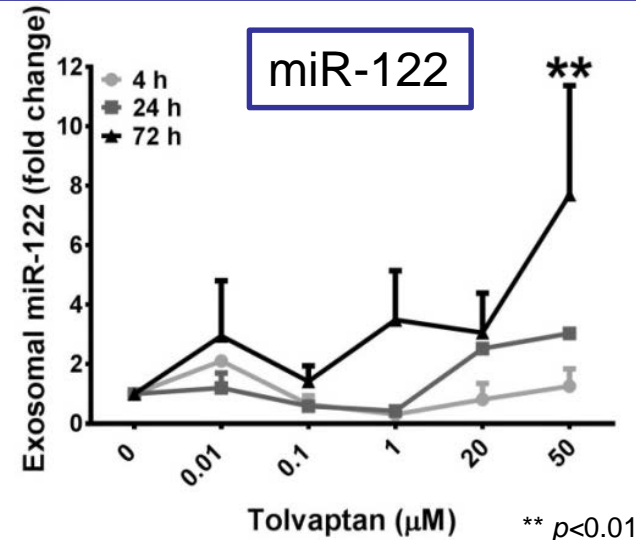
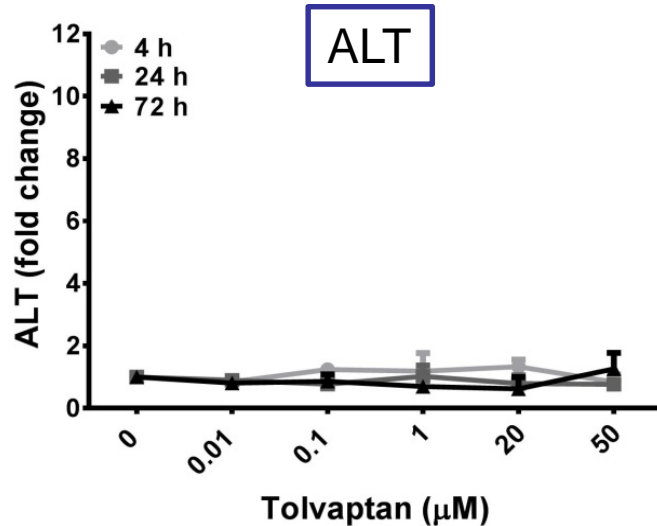


In primary human hepatocytes exposed to a sub-toxic dose of APAP, elevations of exosomal miR-122, but not protein-rich miR-122, were observed. This difference occurred without an alteration in exosome number, suggesting selective packaging of miR-122.

Are exosomal miR-122 alterations also observed following exposure to idiosyncratic-inducing DILI compounds?

Tolvaptan Increases Exosomal miR-122 Release in Primary Human Hepatocytes

- Tolvaptan is a vasopressin V₂-receptor antagonist which caused idiosyncratic DILI in clinical trials in patients with autosomal dominant polycystic kidney disease.
- Hepatocytes from 3 adult donors were exposed to tolvaptan continuously for 4, 24, 72h



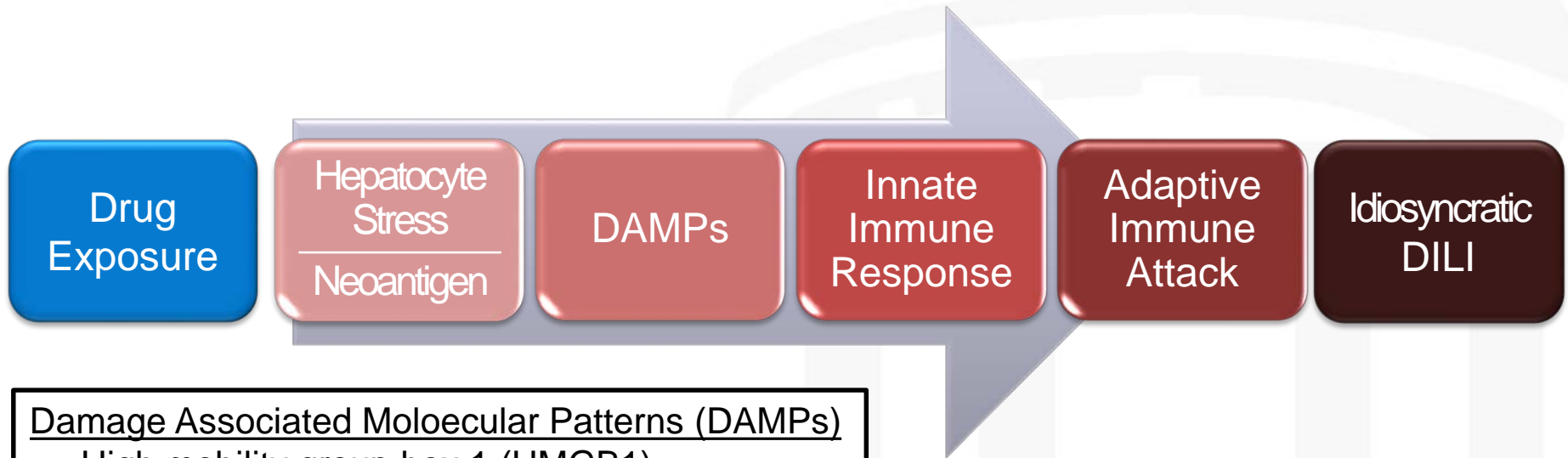
Mosedale M, Eaddy JS, *et al.* *The Toxicologist*. 2016. Abstract 3175

Exposure to tolvaptan resulted in significantly increased exosomal miR-122 at 72h, in the absence ALT elevations in primary human hepatocytes. A trending increase was observed after exposure to tolvaptan for 24h.

Does exosomal miR-122 released from the liver have a functional consequence?



Idiosyncratic DILI Proposed Mechanism



Damage Associated Molecular Patterns (DAMPs)

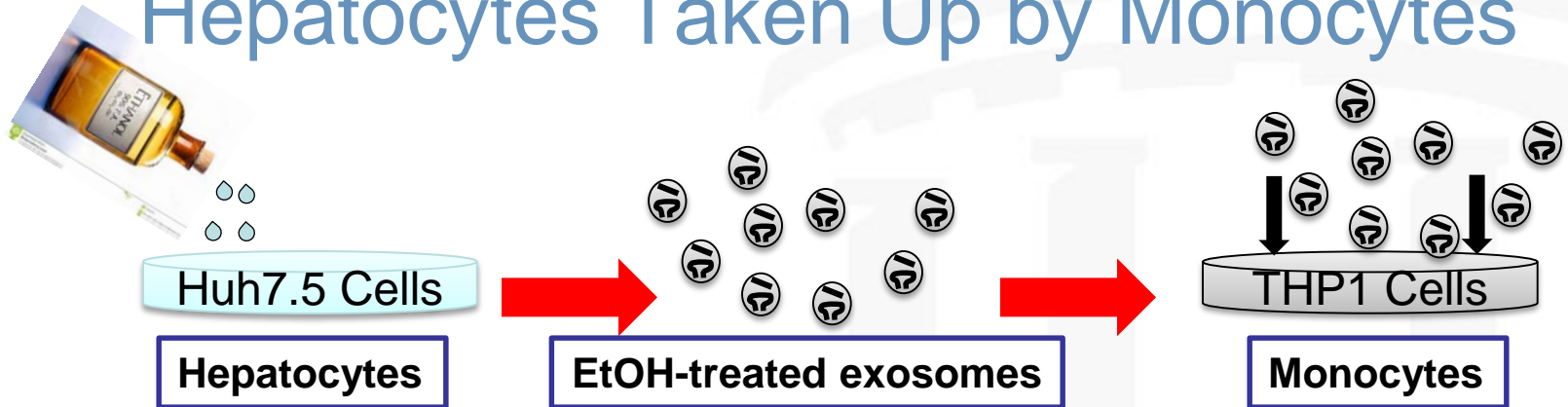
- High mobility group box 1 (HMGB1)
- Heat shock proteins (HSPs)
- Mitochondrial DNA

Adapted from Mosedale M. and Watkins PB. *Clin Pharmacol Ther.* 2017;101:469-80

Can exosomal miR-122 be a DAMP?



Exosomes from EtOH-treated Human Hepatocytes Taken Up by Monocytes



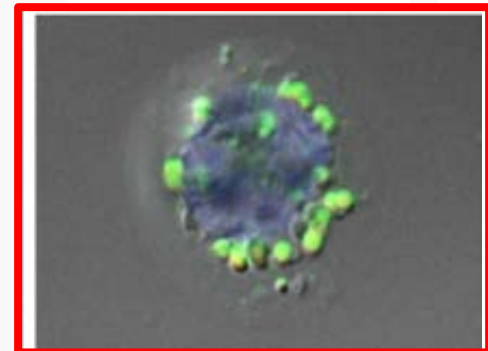
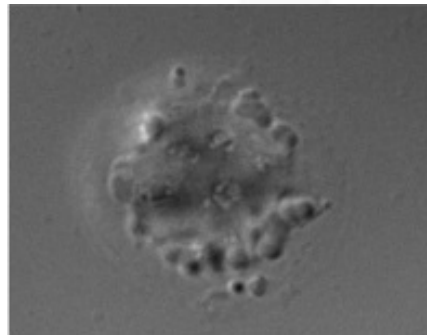
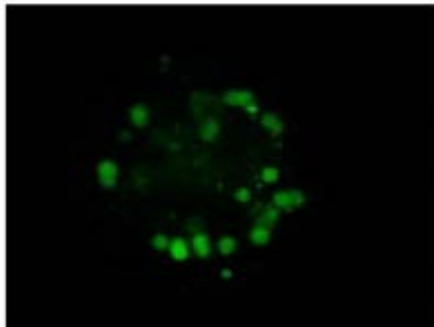
PKH67

DAPI

Exosomes

Cytoplasm

Merge



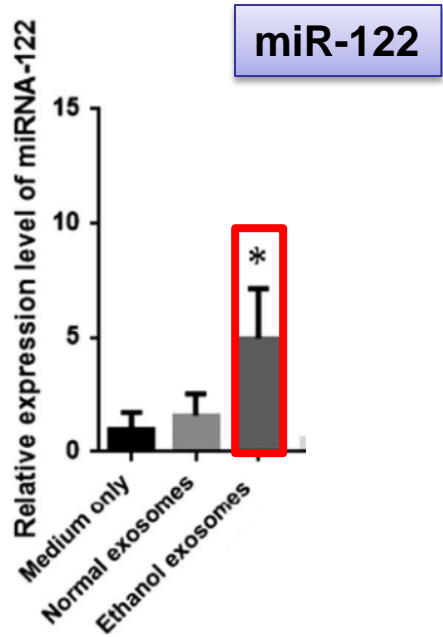
Momen-Haravi F, Bala S, *et al.* *Sci Rep.* 2015;5:1-16

Exosomes collected from EtOH-treated hepatocytes are taken up by monocytes.

Can uptake of exosomal miR-122 prime monocytes for activation?

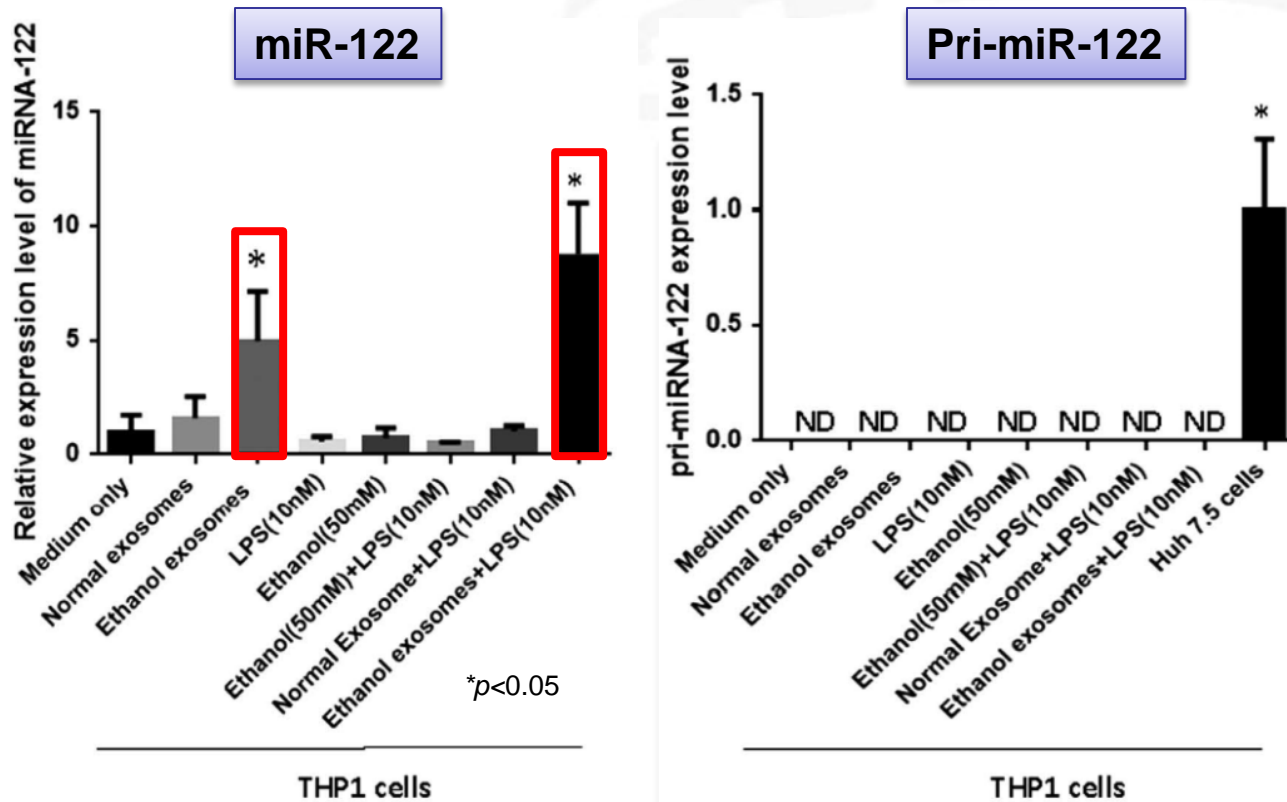


Mature miR-122 Elevated In Monocytes Treated With Alcohol-Induced Exosomes from Hepatocytes



* $p < 0.05$

Mature miR-122 Elevated In Monocytes Treated With Alcohol-Induced Exosomes from Hepatocytes



Momen-Haravi F, Bala S, et al. *Sci Rep.* 2015;5:1-16

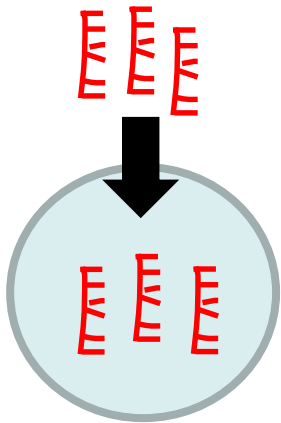
Monocytes given exosomes treated with EtOH or EtOH + LPS show elevated levels of miR-122. This is not a result of miR-122 production by monocytes.

Can exogenous miR-122 prime monocytes for activation?

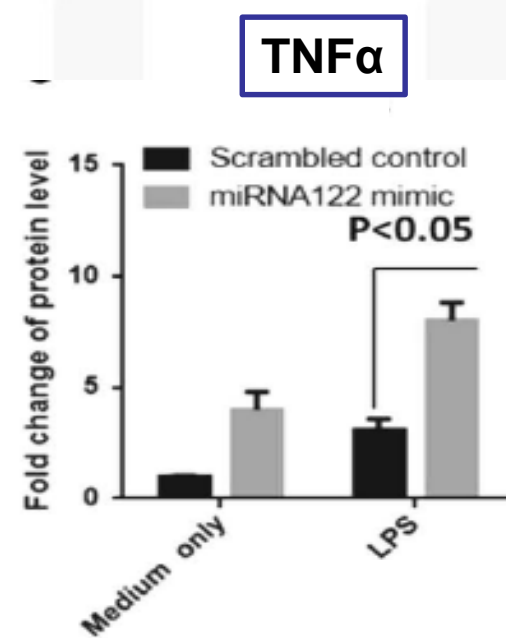
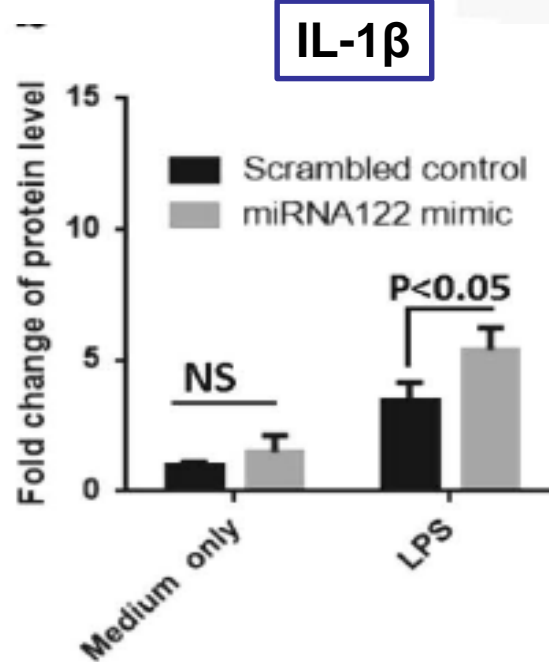
miR-122 Primes Cytokine Induction in Monocytes

To explore the effect of exosomal miR-122 in monocytes, THP1 cells were electroporated with miR-122 mimic or scrambled control and treated with LPS .

miR-122 mimic



THP1 Cell



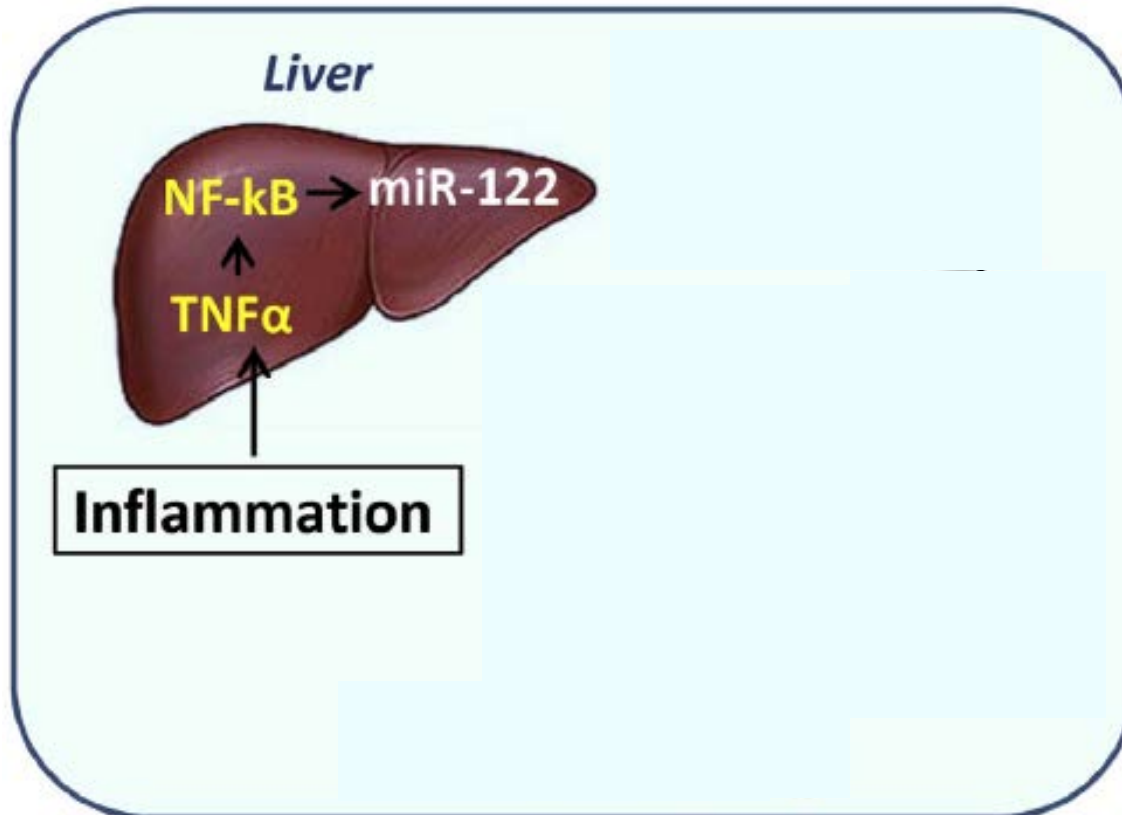
Momen-Haravi F, Bala S, et al. *Sci Rep.* 2015;5:1-16

Exogenous miR-122 increased cytokine release into the supernatant of LPS-treated THP1 cells.



Are there Effects of miR-122 Outside of the Liver?

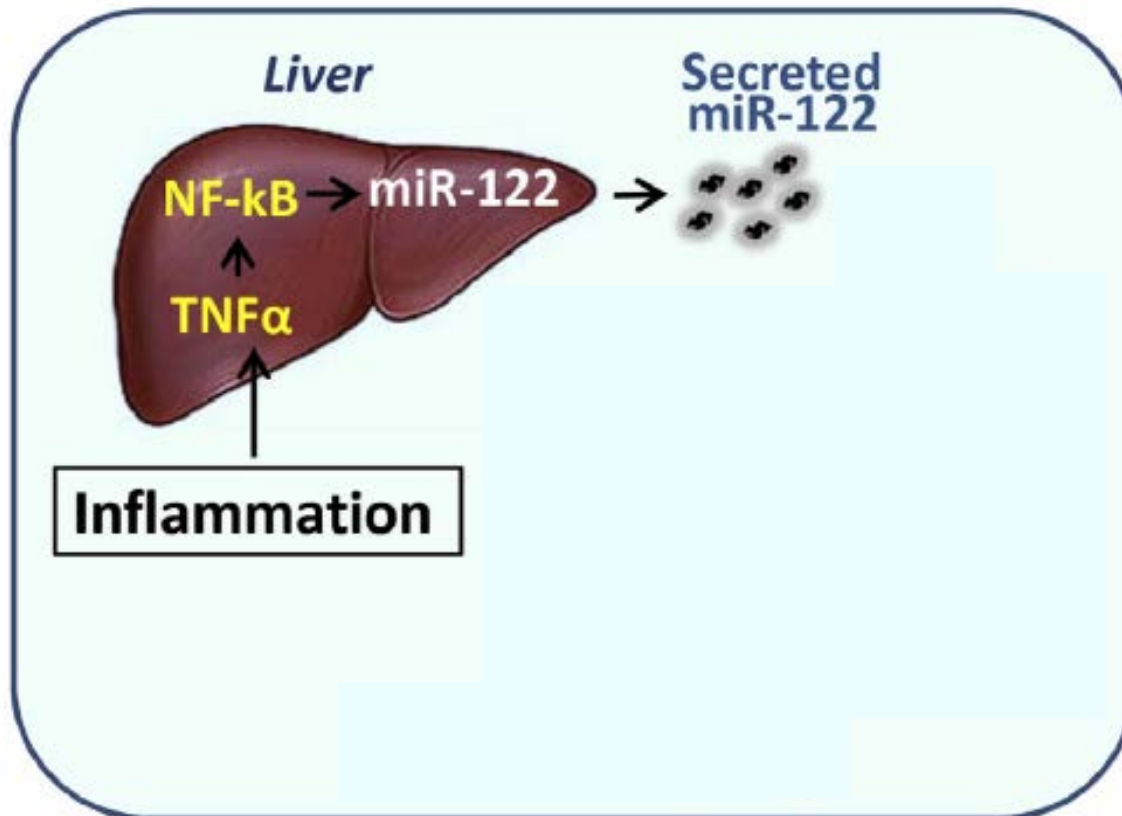
Recent *in vivo* evidence suggests yes!!! Hepatic-derived miR-122 was found to be taken up by kidney, reduce expression of erythropoietin, and promote anemia in the absence of overt injury to the liver in mice.





Are there Effects of miR-122 Outside of the Liver?

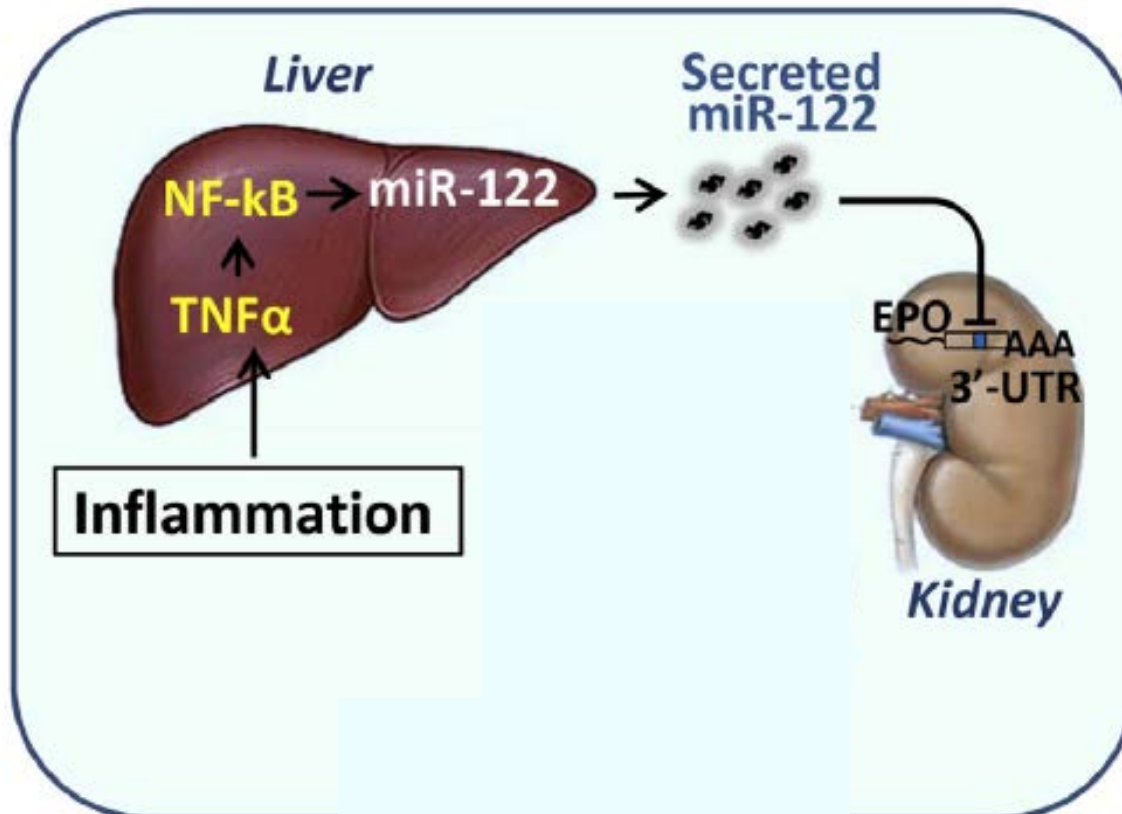
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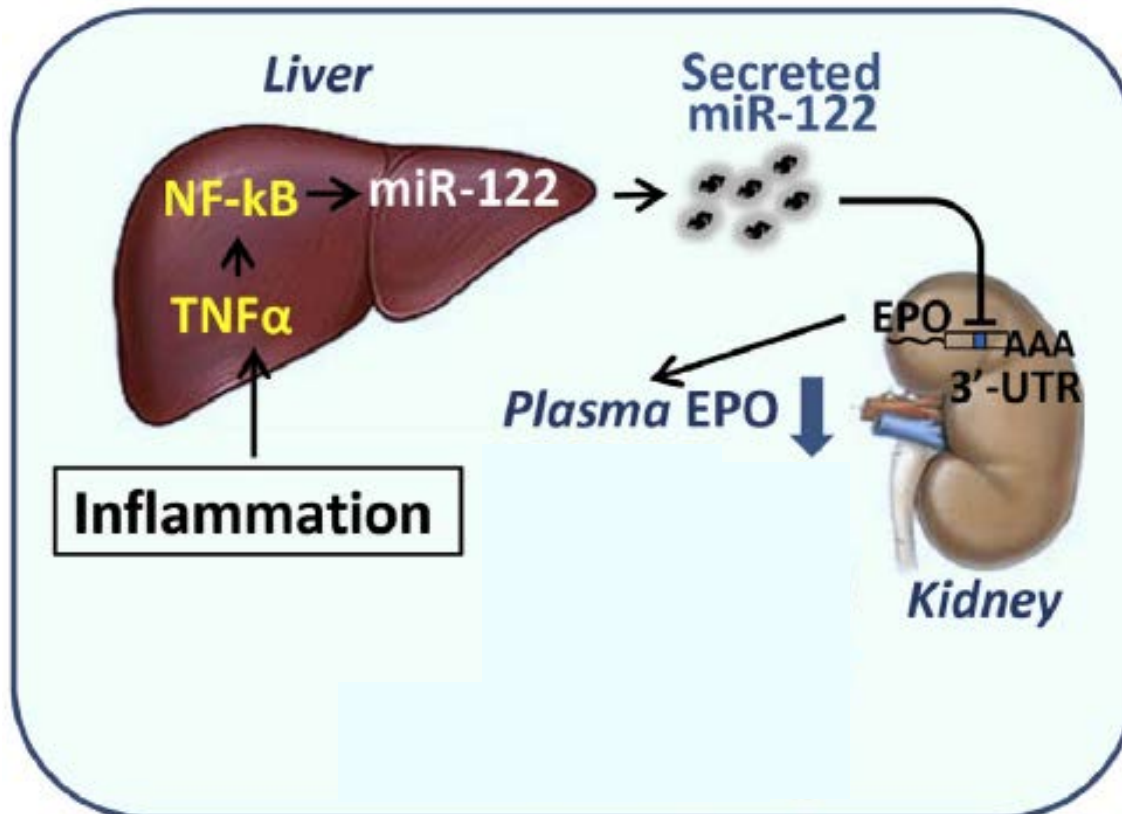
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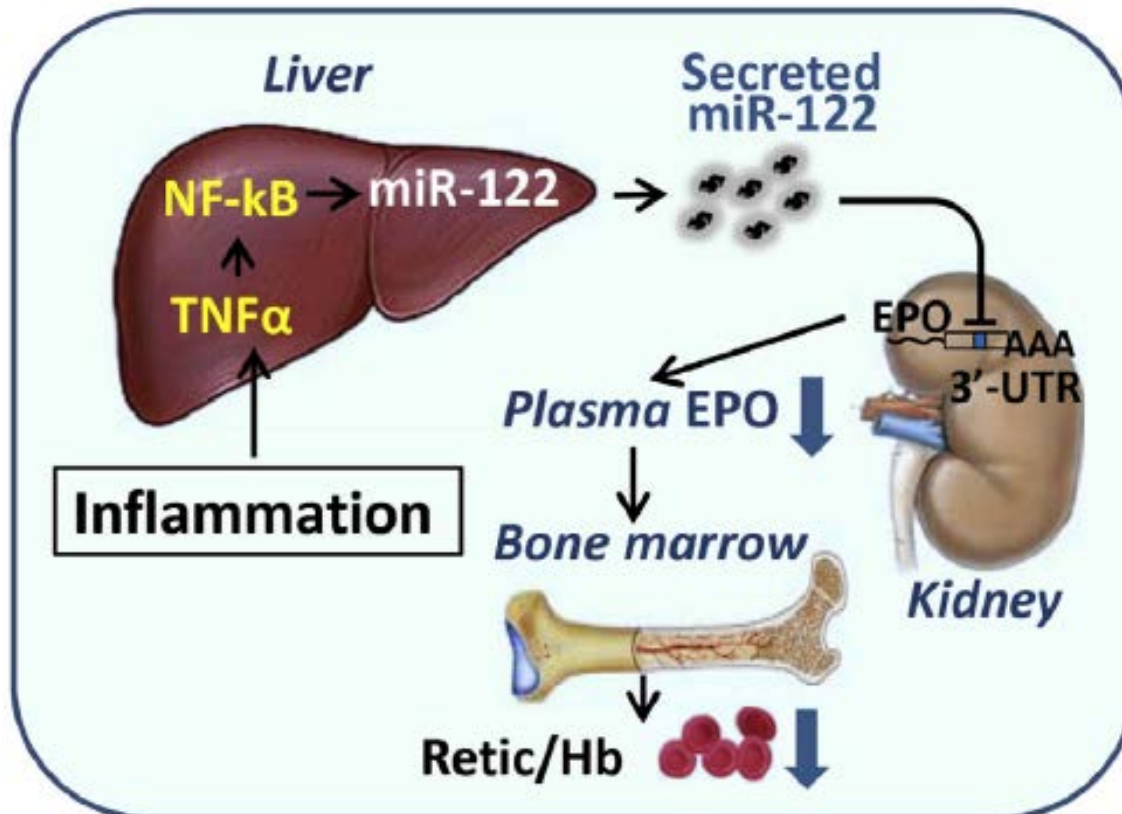
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Are there Effects of miR-122 Outside of the Liver?

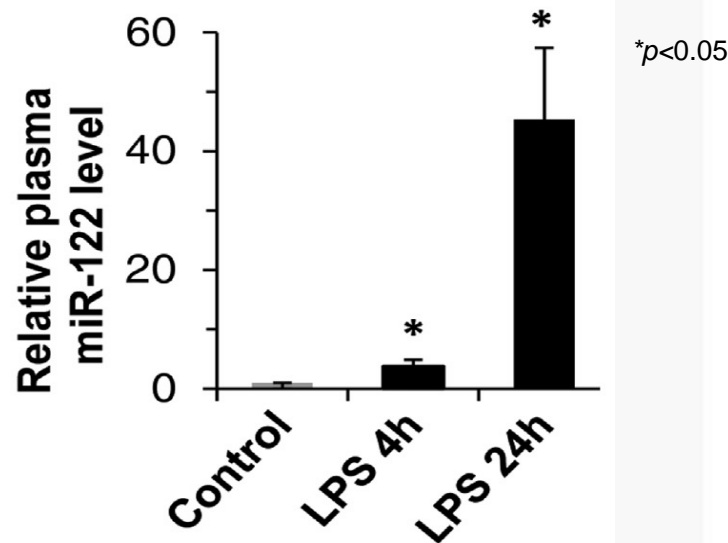
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LPS Induces Elevation of plasma miR-122 *In Vivo* in Mice

Mice were *i.p.* injected with LPS. miR-122 levels were assessed in plasma at 4h and 24h post-dose.



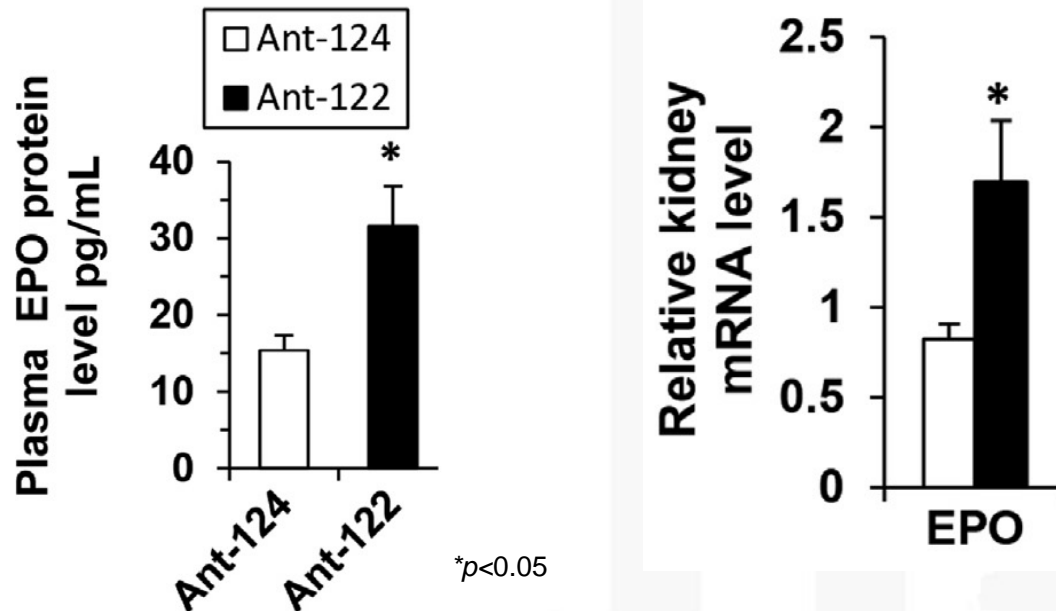
Rivkin M, Simerzin A, *et al.* *Gastro.* 2016;151:999-1010

ALT and AST levels were measured and reported to be normal, although this data was not shown. This suggests miR-122 release occurred in the absence of cellular damage (presumably through exosomal release).



miR-122 Regulates *In Vivo* Expression of Renal-Derived Erythropoietin

- A binding site for miR-122 was identified and confirmed in the erythropoietin gene (*Epo*).
- miR-122 was repressed by hydrodynamic tail vein injection of antagomiR-122 (or control antagomiR-124) and mice were subsequently injected with LPS.

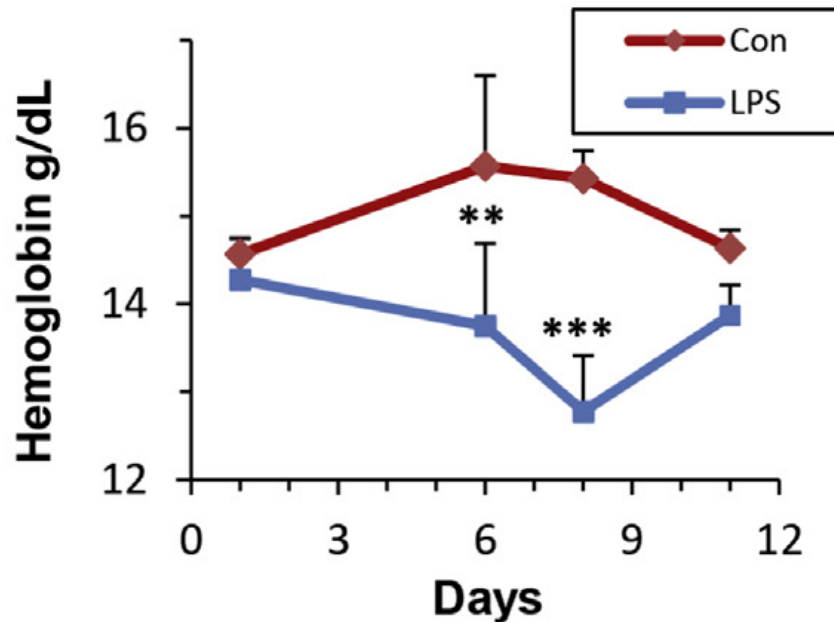


Inhibition of miR-122 resulted in elevated levels of plasma EPO and renal EPO mRNA.



LPS-Induced Anemia In Mice

Mice were injected with LPS administrations that occurred on days 0, 2, 4, and 7. Mice were sacrificed on days 1, 3, 8, and 11 and hemoglobin levels were assessed.



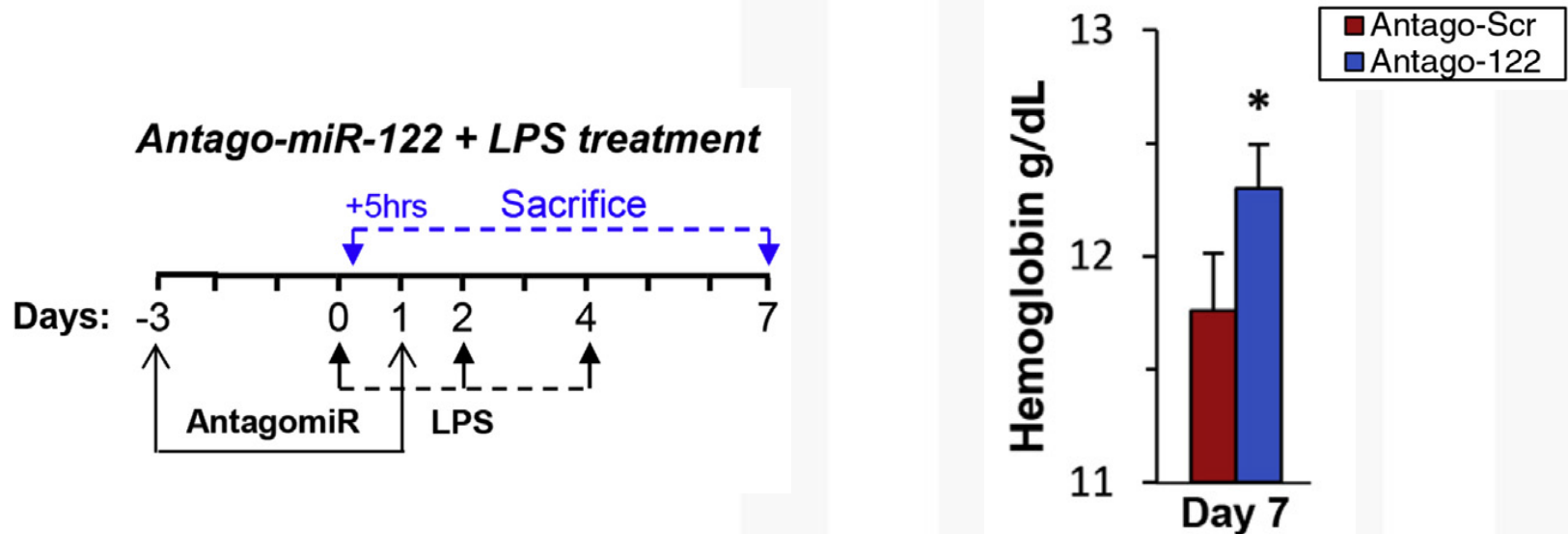
Treatment of mice with LPS results in significantly reduced hemoglobin (i.e. anemia) in blood.

Is LPS-induced anemia regulated by miR-122?



miR-122 Involvement in LPS-Induced Anemia

Mice were injected with antagomiR-122 (or control) before/during LPS administrations that occurred on days 0, 2, and 4. Mice were sacrificed 5 hours and 7 days following the initial LPS administration.



Inhibition of miR-122 resulted in significantly increased hemoglobin levels.

Proposed mechanism for anemia in patients with chronic diseases!!!



Summary

- miRNAs are attractive biomarker candidates because of species conservation, presence in biofluids, and stability.
- miR-122 is a liver-specific miRNA that is highly abundant (~70%) relative to other miRNAs in the liver.
- miR-122 is primarily found in the protein-rich fraction of plasma and serum in healthy humans.
- Following APAP DILI in rodents, miR-122 is found primarily in the protein-rich fraction of plasma, suggesting passive leakage in response to overt injury. However, APAP injury also results in elevation of exosomal miR-122 which could be released actively.



Summary

- In the absence of overt cellular injury, miR-122 can reach circulation via exosomes (40-100 nm) that are small enough to travel through fenestrations (140-175 nm) in the sinusoid of the liver.
- *In vitro* and *in vivo* pre-clinical and clinical data suggests that exosomal miR-122 is released into circulation in the absence of overt cellular injury following exposure to intrinsic and idiosyncratic DILI-promoting compounds.
- Exosomal miR-122 may be considered a DAMP that can activate an innate immune response.
- Exosomes can be taken up by monocytes and exogenous miR-122 can prime monocytes for cytokine production.
- miR-122 released into circulation can be taken up by the kidneys and regulate promote anemia by inhibiting expression of EPO.



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