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TITLE: Plasma Small Extracellular RNA as a Biomarker of Autoimmune Flairs and Beta Cell Death in Type 1 Diabetes Using Frequent Prospective Peri-Onset Sampling

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6. AUTHOR(S)

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

We seek to discover small RNA biomarkers of autoimmune activity and/or beta cell damage in type 1 diabetes. Pilot studies showed that heparinized plasma failed analyses, but that EDTA and citrated plasma did well, so 353 appropriate plasma samples (average 11 per subject prospectively collected every 1 to 3 months) from 32 high risk (MAB) or newly diabetic children and adolescents were collected, and the first 94 analyzed, for circulating small regulatory RNAs. 92 of 94 resulting cDNA libraries gave adequate numbers of miRNA mapped reads, but QC using spiked RNA internal standards showed abnormally high small RNA levels in 8 mildly hemolyzed plasma samples, leaving 84 of 94 with analyzable data. Equal numbers of EDTA and citrated plasma were analyzed successfully. Over the next period we will complete series on another 6 subjects, sequence the remaining 260+70=340 samples, and analyze the data for patterns of disease association with small RNA molecules in the prediabetic, perionset, and immediate post onset period. These patterns may identify biomarker small RNA predictive of autoimmune flares or beta cell loss, including predicting impending clinical onset.

15. SUBJECT TERMS

MAB -multiple antibodies
RNA-ribonucleic acid
miRNA-micro inhibitory RNA

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	18	19b. TELEPHONE NUMBER *(include area code)*

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1. INTRODUCTION:

Type 1 diabetes is an autoimmune disease attacking the beta cells in the pancreatic islets. Islet antibodies reveal pre-onset autoimmunity and ultimate prognosis, but not timing of clinical onset. The pancreas is medically inaccessible and peripheral blood biomarkers are needed to time disease flares leading up to diabetes onset. We sampled 32 high risk (MAB) or newly diabetic children every 1-2 months for a median of 11 sequential samples, measuring islet antibodies, glycemia, and pancreatic insulin reserve. We now seek to sequence these samples for extracellular small RNA signaling molecules to detect biomarkers correlated to human immune cell autoimmune activity and/or beta cell death, to provide improved peripheral blood biomarkers of this process.

2. KEYWORDS:

MAB -multiple antibodies
RNA-ribonucleic acid
miRNA-micro inhibitory RNA
cDNA- Complimentary DNA
T1D- type 1 diabetes

3. ACCOMPLISHMENTS:

What were the major goals of the project?

1) Continue monthly sample collection/characterization on 20 MAB or T1D individuals through and beyond any observed T1D onset, or for one year after onset, respectively, or for an additional 6 to 12 months beyond sampling already existing at the start of the study. The goal completion was 12 months, which will be 8/31/19. We will have completed sampling on 14 of the 20 children (**70%**) by that date and the others are expected to be completed within an additional 2 months.

2) Purify exRNA from existing and new prospective and cross sectional plasma samples (all collected by an identical protocol), prepare cDNA libraries and perform RNA-seq of the short RNAs. Identify RNAs by bioinformatics approaches. The goal completion was 12 months, which will be 8/31/19. We have a total of 353 samples currently under analysis, of which 120 have had libraries sequenced during the reporting period (**33%**). We expect ~67 additional samples to be added to the 353 existing for a total of 420 samples. We expect sequencing to be completed on all samples within the next 3 months.

3) Analyze data for differential levels of RNA marking the year just before T1D onset and the year just after T1D onset, to identify candidate biomarkers predicting conversion from MAB to T1D. Analyze sequential samples for time-dependent variation correlated with disease progression. This goal was planned to be completed in the second year of the project (beyond the time period of the current report) and has not yet been completed (**0%**).

What was accomplished under these goals?

We first performed additional quality control checks of our methods, and found to our surprise that heparinized plasma did not work for RNA analysis but that EDTA plasma and citrated plasma both worked well (included in appendix). We then developed additional plans to draw and collect sequential series of EDTA or citrated plasma within our existing studies of MAB-positive prediabetics. Collection of full sequential series of these samples, as proposed for the project, are nearly complete, although this will be about 2 months after that in the SOW timeline. We have also accomplished RNA purification, cDNA library construction, sequencing, and initial small RNA analysis on ~120 samples (example of result included in appendix), with the remainder now in analysis or soon to be in analysis, which is now estimated to be completed approximately 3 months after that in the SOW timeline. Analysis of the relationship of specific small RNAs to prediabetes progression through onset and in the first year after onset was planned in the SOW to occur in the 2nd year of the proposal. Therefore, this is not complete but is on schedule based on the SOW.

What opportunities for training and professional development has the project provided?

Nothing to report.

How were the results disseminated to communities of interest?

Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?

1) Continue monthly sample collection/characterization on the remaining 6 MAB or T1D individuals through and beyond any observed T1D onset, or for one year after onset, respectively, This is expected to be completed within an additional 2 months.

2) Purify exRNA from existing and new prospective and cross sectional plasma samples (all collected by an identical protocol), prepare cDNA libraries and perform RNA-seq of the short RNAs. Identify RNAs by bioinformatics approaches. We will analyze the remaining two-thirds of the 420 total samples. We expect sequencing to be completed on all samples in the next 3 months.

3) Analyze data for differential levels of RNA marking the year just before T1D onset and the year just after T1D onset, to identify candidate biomarkers predicting conversion from MAB to T1D. Analyze sequential samples for time-dependent variation correlated with disease progression. This goal will be accomplished for all individuals studied and all samples analyzed, during the next reporting period, with particular input by Drs Galas and Hagopian.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Nothing to report yet.

What was the impact on other disciplines?

Nothing to report yet.

What was the impact on technology transfer?

Nothing to report yet.

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Prior published studies by the Galas lab showed that plasma was far superior to serum due to release of nonspecific small RNA from cells during clotting necessary to produce serum. We had therefore collected plasma for the purpose of the current study goals. However, we performed 3 pilot studies which showed, respectively, that: 1) the heparin plasma samples we had collected were not suited to cDNA library construction, although EDTA plasma was excellent for these analyses, 2) heparinase treatment failed to recover RNA analyzability, 3) citrated plasma worked nearly as well as EDTA (included in the appendix). We therefore had to switch to different sample collection tubes, delaying the project somewhat. We are now on track to collect all intended samples with only ~2 month delay from planned dates and sequencing to be completed soon thereafter. We also determined by quality control of our results that hemolyzed plasma had too much release cellular small RNA and a small proportion (~10%) of our collected samples that had been sequenced may not be usable for analysis, a situation not unusual for this type of analysis. However, since we have an average of 11 samples per individual, we expect that most subjects will have ≥10 analyzable samples in their series, which is expected to still permit robust discovery of associations between small RNA levels and disease progression (i.e. islet destruction) before or after diabetes diagnosis.

Actual or anticipated problems or delays and actions or plans to resolve them

This is covered in the answer immediately above this question.

Changes that had a significant impact on expenditures

We will analyze somewhat fewer samples from subjects at risk for diabetes or with new onset diabetes. A greater number of subjects will be analyzed (38 rather than 32 proposed) but the number of samples per subject now averages 11 and not the greater number we previously proposed. However, we have also fully analyzed an extra 70 samples in three pilot studies to determine a solution to our heparin problem, which was thereby successfully resolved. The total number of all samples analyzed for RNAseq will exceed 500 so does not materially impact the planned expenditures.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, select agents

Some anonymized, sequential diabetes samples (citrated) from our IRB-approved sample repository were included in the current project's RNA analysis. Our local IRB ruled that such anonymized samples do not require any protocol change nor additional approval for this use.

Significant changes in use or care of human subjects.

None

Significant changes in use or care of vertebrate animals

None

Significant changes in use of biohazards and/or select agents.

None

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

Nothing to report yet.

Books or other non-periodical, one-time publications.

Nothing to report yet.

Other publications, conference papers and presentations.

Nothing to report yet.

- **Website(s) or other Internet site(s)**

Nothing to report yet.

- **Technologies or techniques**

Nothing to report yet.

- **Inventions, patent applications, and/or licenses**

Nothing to report yet.

- **Other Products**

Nothing to report yet.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name	Project Role	Researcher Identifier (e.g. ORCID ID)	Nearest person month worked	Contribution to project
William Hagopian	PD/PI	3-2979-0475	1	*Dr. Hagopian designed and oversees the entire project, including sample collection, RNA measurement, and final analysis and reporting of results*
David Galas	Co-Investigator	1-8248-8617	1	*Dr. Galas designed the RNA measurement and analysis portions of the project the data analysis, and the reporting of results.*
Alton Etheridge	Senior Scientist	2-6216-5869	3	*Dr. Etheridge performs the RNA purification, library construction and sequencing, quality control and interpretation of the data.*
Ashley Akramoff	Clinical Coordinator	none	2	*Ms. Akramoff coordinated and performed sample collection from research subjects.*
Claire Crouch	Sr. Clinical Coordinator	none	5	*Ms. Crouch coordinated and performed sample collection from research subjects.*
Jennifer Skidmore	Clinical Coordinator	none	1	*Ms. Skidmore coordinated and performed sample collection from research subjects.*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

See next page

HAGOPIAN, WILLIAM – Other Support Changes
Title: The Environmental Determinants of Diabetes in Youth (TEDDY) Consortium– Washington Clinical Center
Supporting Agency: NIH/NIDDK U01 DK063829
Change: Performance period ended 3/30/2018

Title: The Environmental Determinants of Diabetes in Youth (TEDDY) Consortium– Washington Clinical Center Bridge Funding
Supporting Agency: University of South Florida Board of Trustees/ NIH/NIDDK subaward
Change: Subcontract ran 4/1/18 to 8/31/2018 and has now ended.

Title: The Environmental Determinants of Diabetes in Youth (TEDDY) Consortium– Washington Clinical Center
Supporting Agency: NIH/NIDDK U01 DK063829
Change: Grant was awarded and started 9/1/2018.

Title: TEDDY Post-Onset Study
Supporting Agency: Juvenile Diabetes Research Foundation
Change: Performance period ended 3/31/2019

Title: Developing a Combined Genetic/Phenotypic Risk Score (CRS) For Precise Diabetes Typology across Diverse Ethnicities In SEARCH
(subcontract, PI D'Agostino)
Supporting Agency: NIH/NIDDK UC4 DK108173 supplement subaward from Wake Forest
Change: Revised end date of 6/30/2020.

Title: 9828-4150 Trial- Anti-IL21 and Liraglutide Combination Therapy in New Onset Type 1 Diabetes
Supporting Agency: PNRI under contract from Novo-Nordisk Inc
Change: Performance period ended 4/30/2019

Title: Combined data analysis of prospective diabetes trials by IBM Watson
Supporting Agency: Juvenile Diabetes Research Foundation
Change: A $58,000 supplemental award started 11/1/2018 and ends 10/31/2019.

Title: A systematic dynamic model to understand progression and heterogeneity of Type 1 Diabetes. (subcontract, PI Huang)
Supporting Agency: Juvenile Diabetes Research Foundation
Change: Awarded 10/1/2017 ends 9/30/2019.

Title: Optimization and Multiplexing of high performance LIPS assays for T1D antibodies. (subcontract, PI Piemonti)
Supporting Agency: Juvenile Diabetes Research Foundation
Change: Awarded 11/1/2017 ends 10/31/2019.

GALAS, DAVID – Other Support Changes
Title: exRNA Data Management Research Repository (DMRR)
Supporting Agency: Baylor College of Medicine/NIH subaward
Change: Performance period extended to 7/31/2019 through a no cost extension.

Title: Reference Profiles of Extracellular RNA in 4 Body Fluids of Healthy Humans
Supporting Agency: NIH U01 HL126496
Change: Performance period extended to 1/31/2019 through a no cost extension.

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: Not applicable

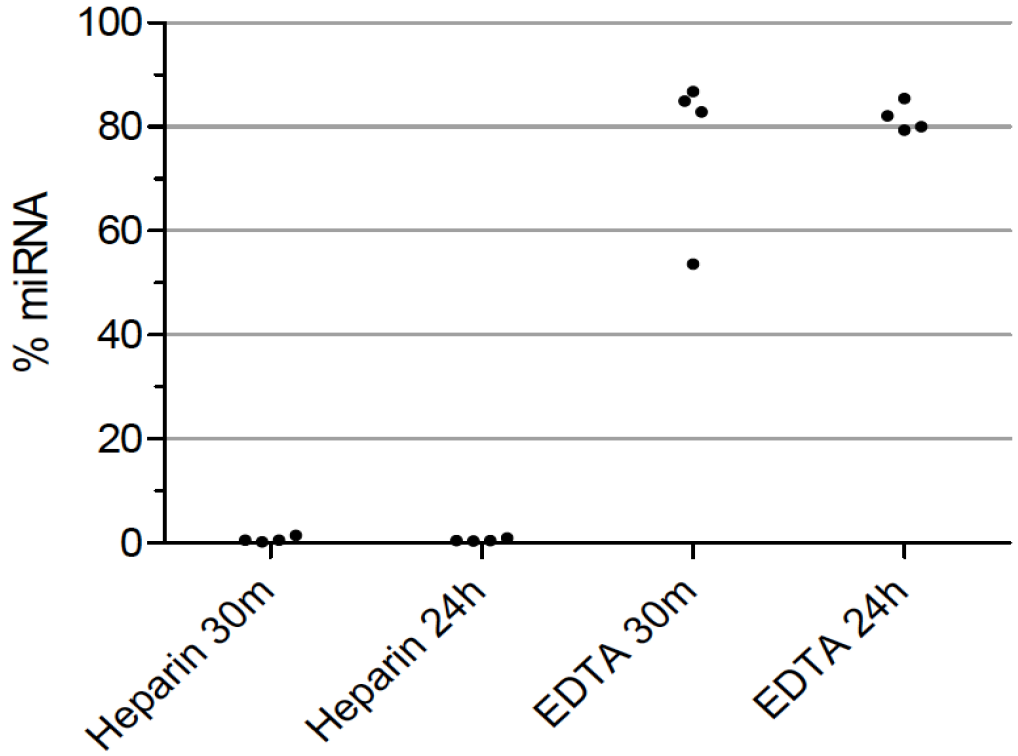
QUAD CHARTS: Not applicable

9. APPENDICES: Attached

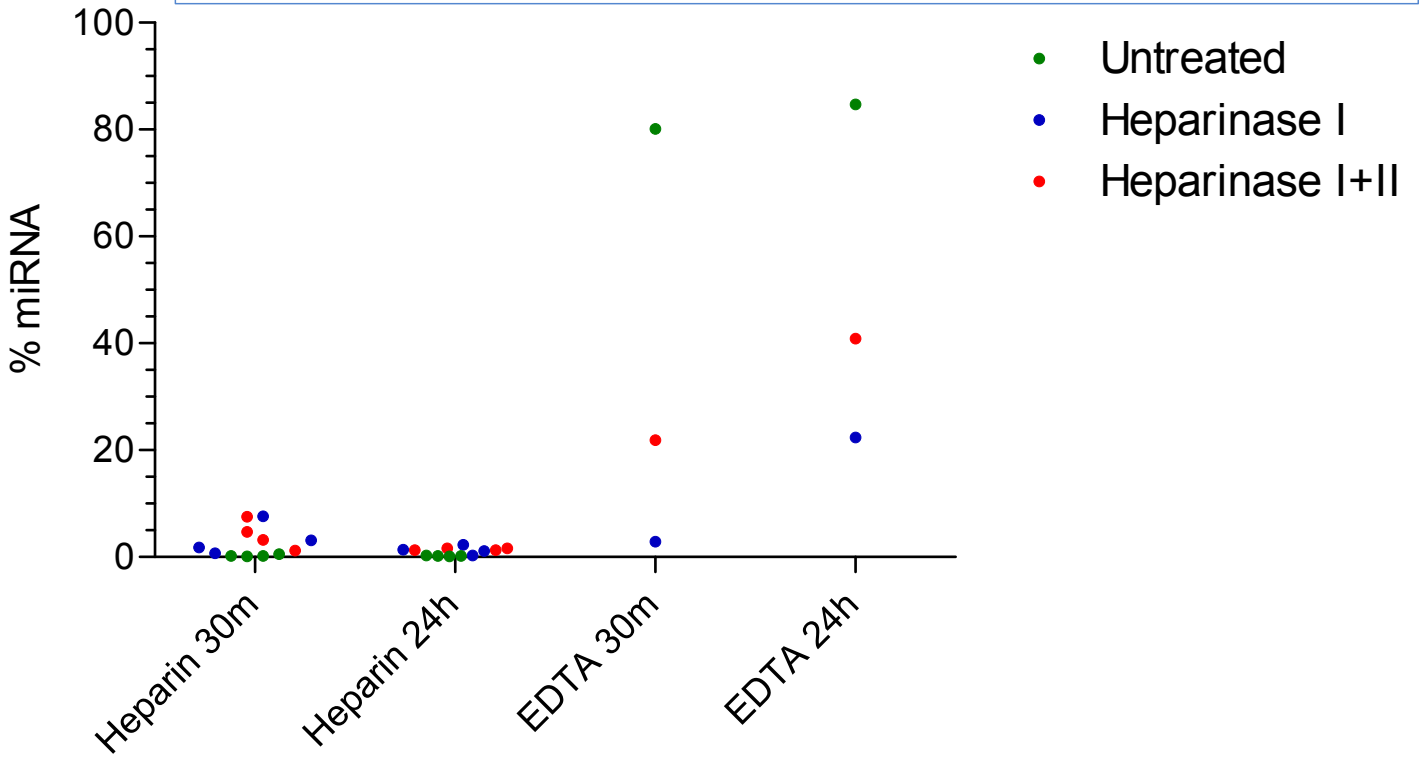
PR171235- Plasma small extracellular RNA as a biomarker of autoimmune flares and beta cell death in type 1 diabetes using frequent prospective peri-onset sampling.

Supplement to report for the first year- July 31, 2019

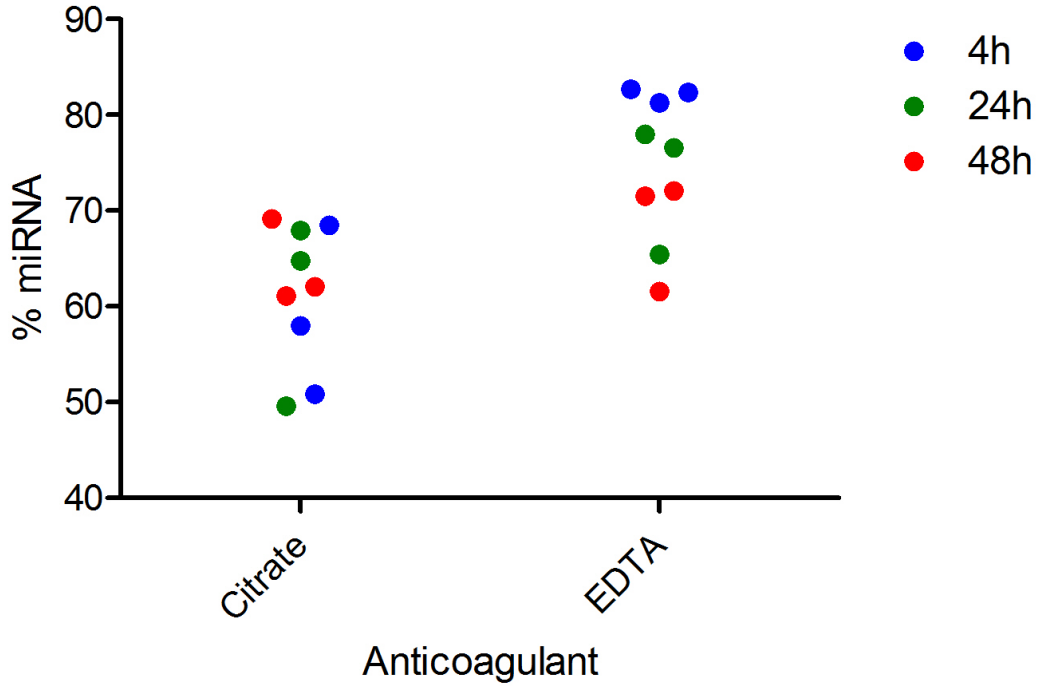
First Pilot Study, comparing plasma samples in Heparin vs EDTA anticoagulant, and comparing samples frozen 0.5 hr or 24 hr after draw. Heparinized plasma does not work, but samples drawn in EDTA are stable over time up to 24 hrs.

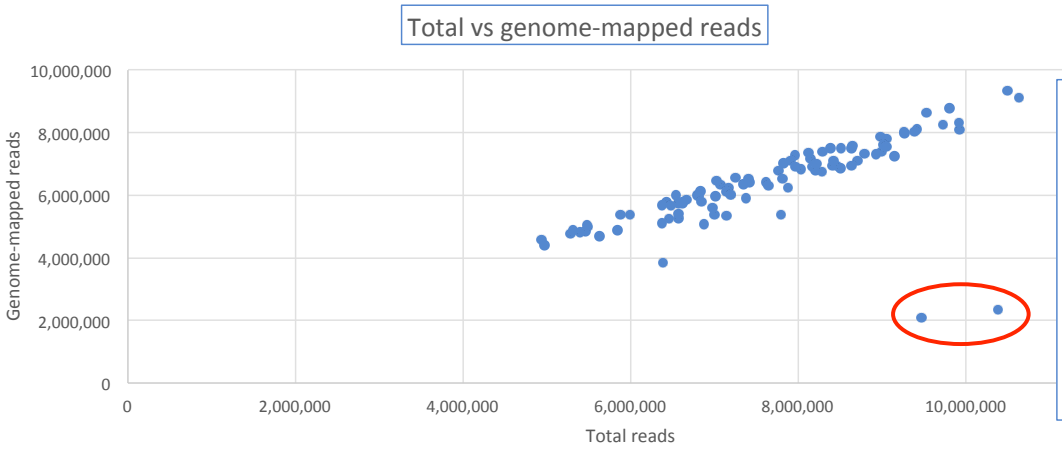


Second Pilot Study, attempting to recover ability to use heparinized plasma samples after 2 kinds of heparinase treatment. Results of this plus other data from this pilot suggests that heparinase treatment restores the ability to create cDNA libraries, but also decreases RNA recovery.

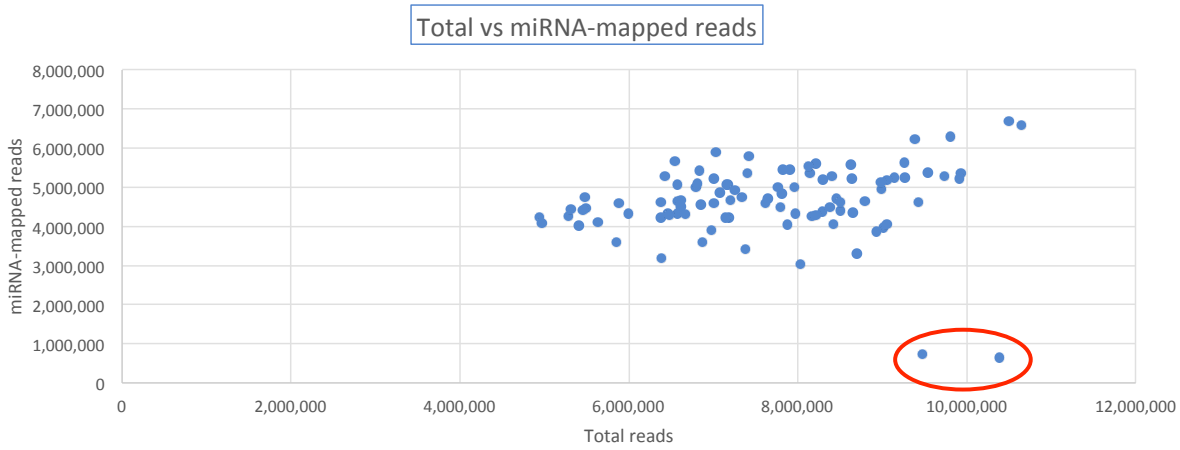


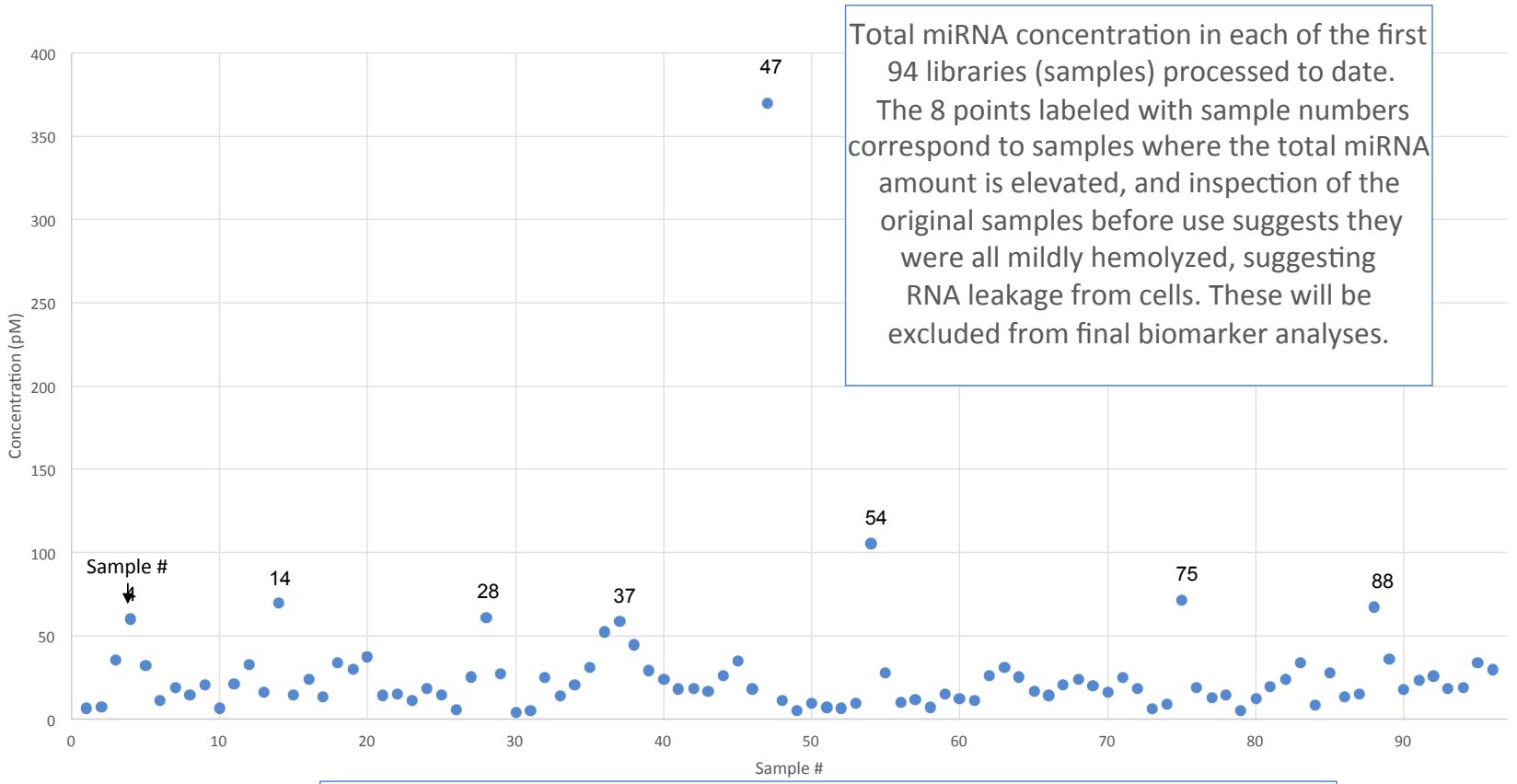
Third Pilot Study, comparing plasma samples in Citrate vs EDTA anticoagulants, and comparing samples frozen 4, 24 or 48 h after draw. Citrate as well as EDTA anticoagulants allow RNA analysis, and samples are stable up to 48h post draw.



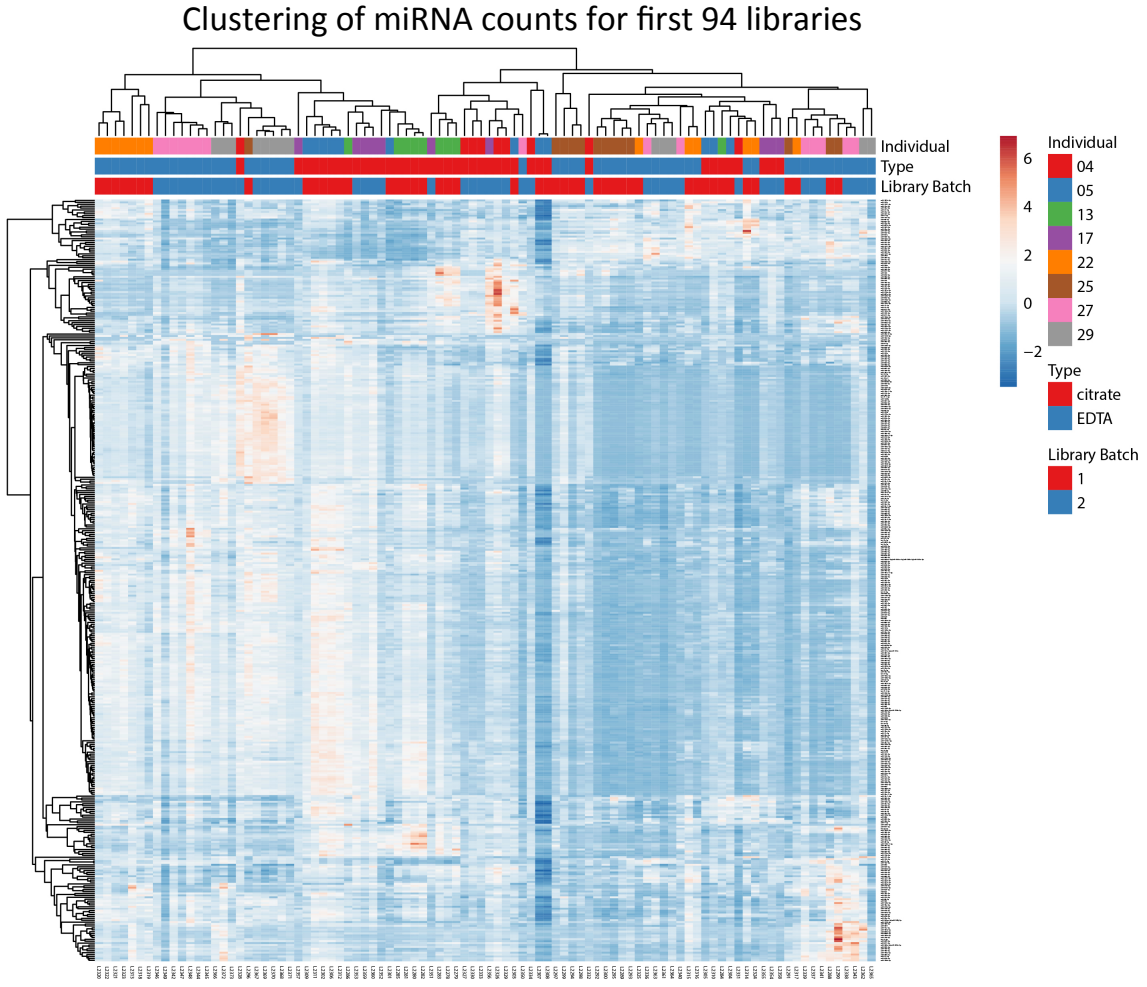


All of the 94 libraries sequenced in the main sample runs (except the 2 circled) should have a sufficient number of mapped reads for analysis.





Total miRNA concentration per sample based on RNA isolation spike-ins


Clustering of miRNA counts for first 94 libraries
Individual
Type
Library Batch
6
4
2
0
−2
Individual
04
05
13
17
22
25
27
29
Type
citrate
EDTA
Library Batch
1
2

Conclusions to date

1- Plasma using EDTA or citrate (but not heparin) anticoagulants work well for small RNA plasma analyses.

2- Samples collected within 24 to 48 hours post draw appear to be stable.

3- Inadequate sequence reads were seen in 2/94 samples, and another 8/94 have nonspecific RNA apparently due to mild hemolysis. 84/94 samples pass both quality control measures, coming equally from both EDTA and citrated plasma.

4- For the first 94 samples, an example analysis of clustering of miRNA shows interesting patterns within and between the 8 individuals included, but this just provides an example and a larger sample set will be needed to form hypotheses regarding roles of these miRNA in islet autoimmunity.