Effects of Pre-analytic Variables on Circulating MicroRNAs Using a CCSG Biorepository

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What are MicroRNAs?

- Small RNA molecules (~21 nt)
- Found in almost every species
- Highly evolutionarily conserved
- Regulate gene expression

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Human MicroRNAs

- Currently there are 1,527 human microRNAs annotated in miRBase (Release 18.0, Nov. 2011)
- On average, each microRNA has 200 targets
- Over 60% of all human protein encoding genes are regulated by microRNAs
- Important to all biological functions and pathways

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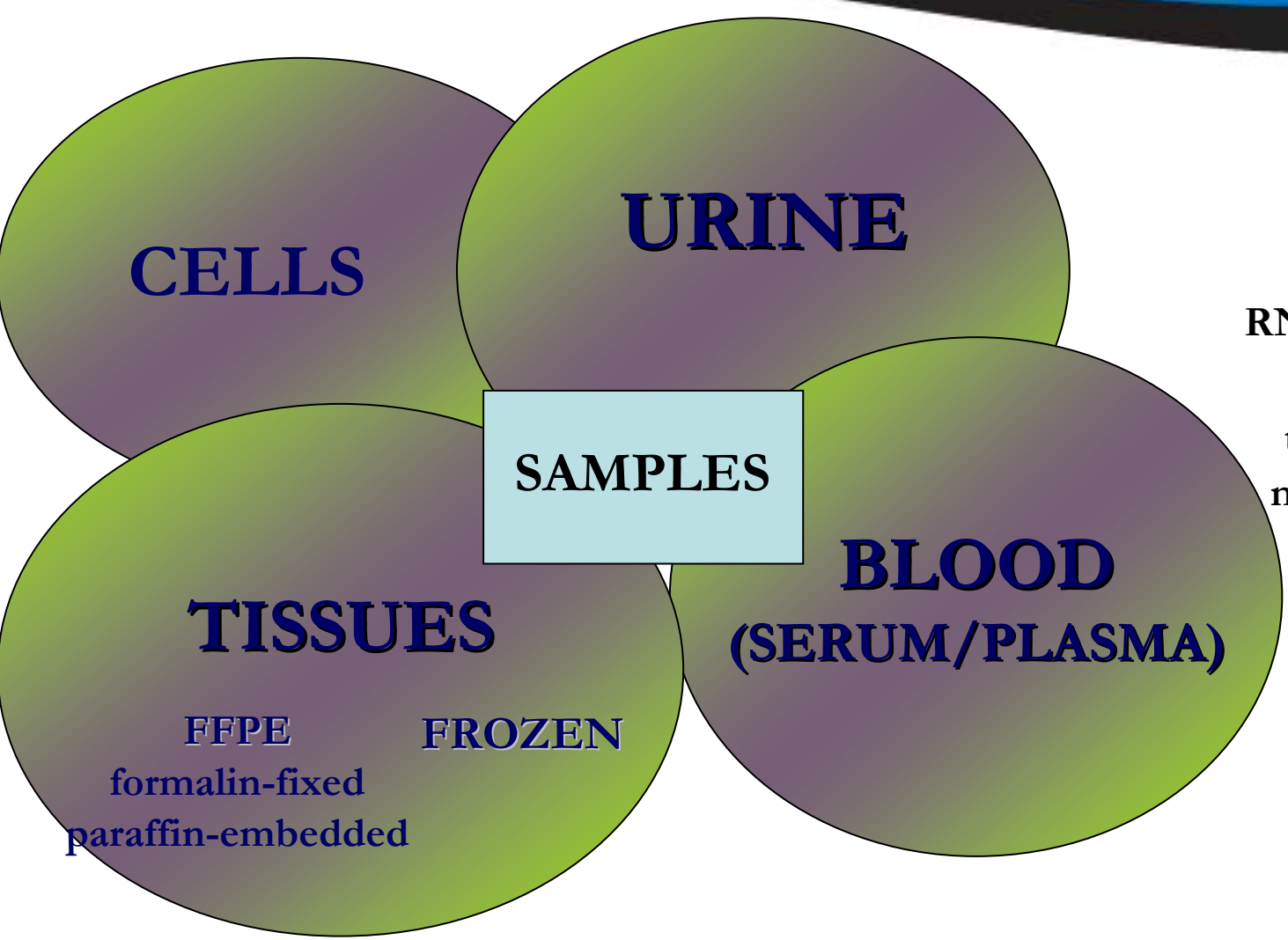
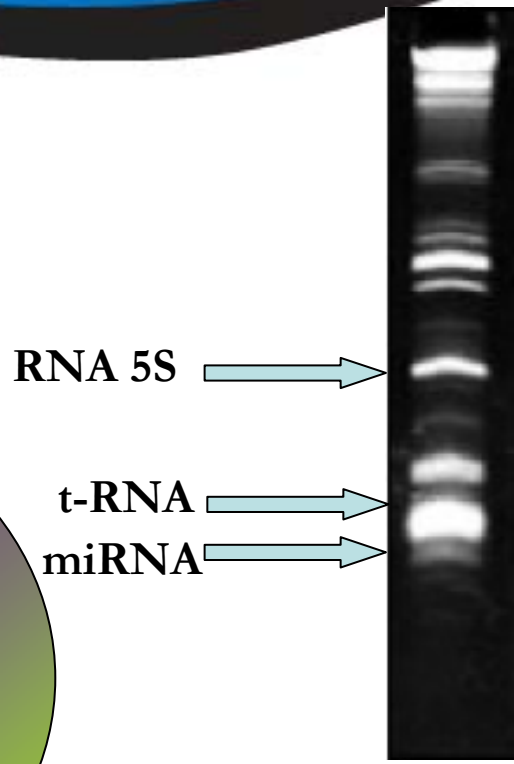
MicroRNAs and Cancer

- microRNA may play a role as tumor suppressor genes (down regulated in cancer) or oncogenes (up regulated in cancer)
- microRNA disregulation is involved in initiation, progression, and resistance to therapy of human cancers
- microRNA as promising biomarkers of cancers

MicroRNAs Detected in:

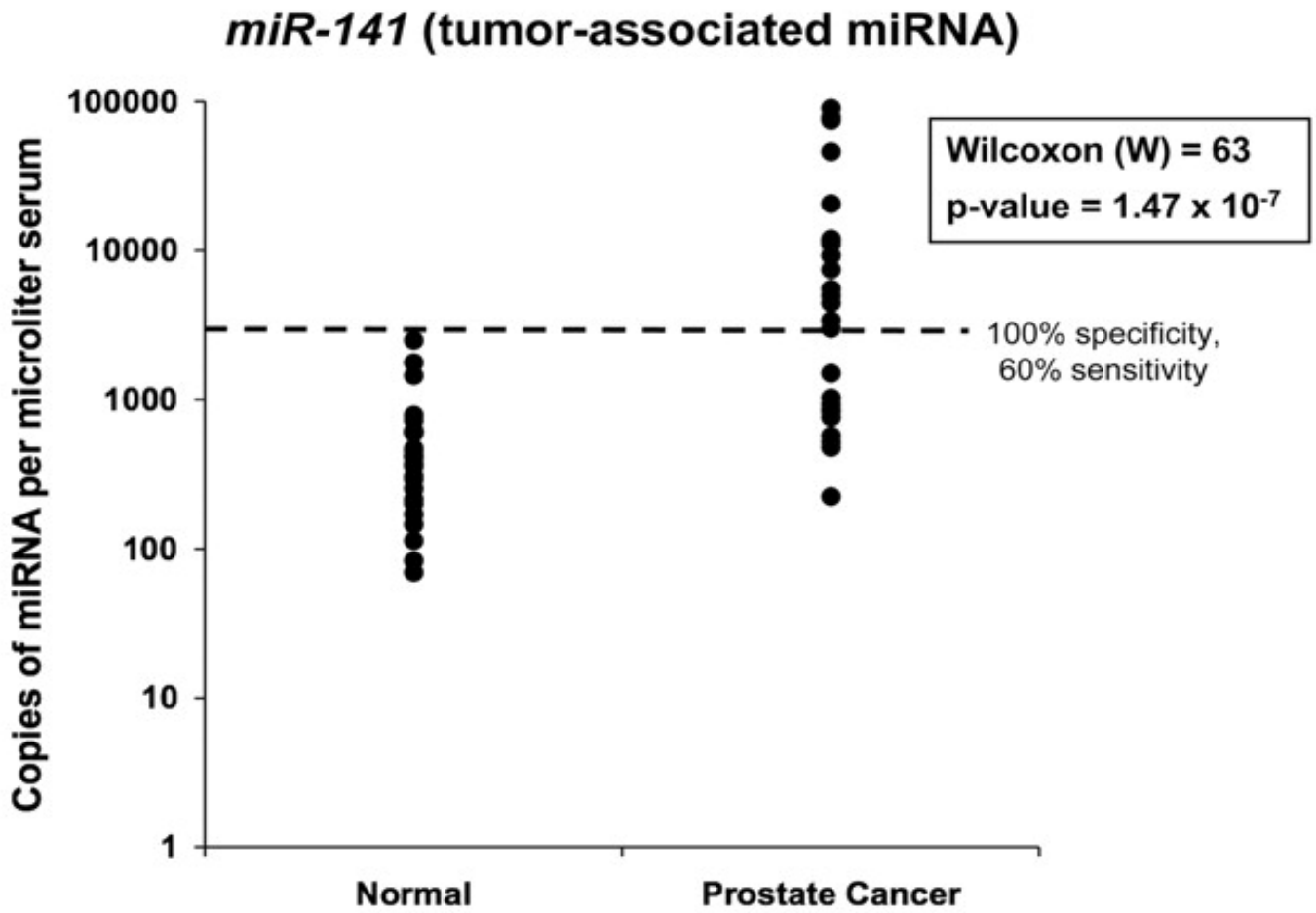




Circulating microRNAs and Human Cancer

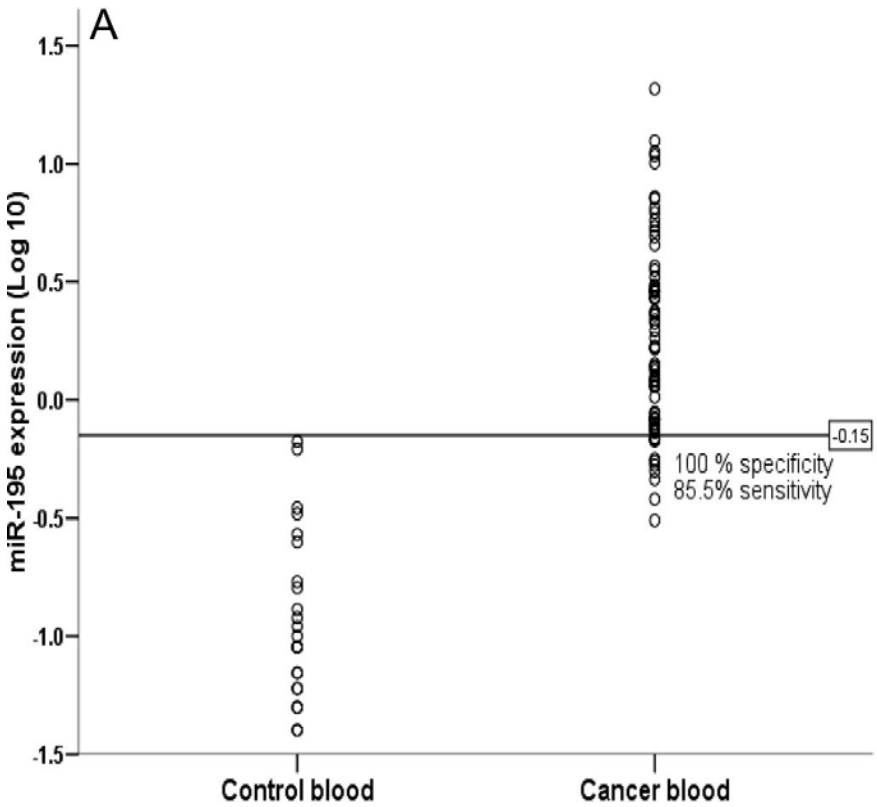
- Over 100 studies have been done in a variety of cancers, including colorectal, prostate, ovarian, breast, lymphoma, etc.
- Biospecimens include: serum, plasma, and whole blood.
- A few microRNA based biomarkers have been reported:
  - miR-141 in prostate cancer
  - miR-195 and let-7a in breast cancer
  - miR-155, miR-210 and miR-21 in lymphoma, etc

Detection of Human Prostate Cancer by Serum *miR-141*

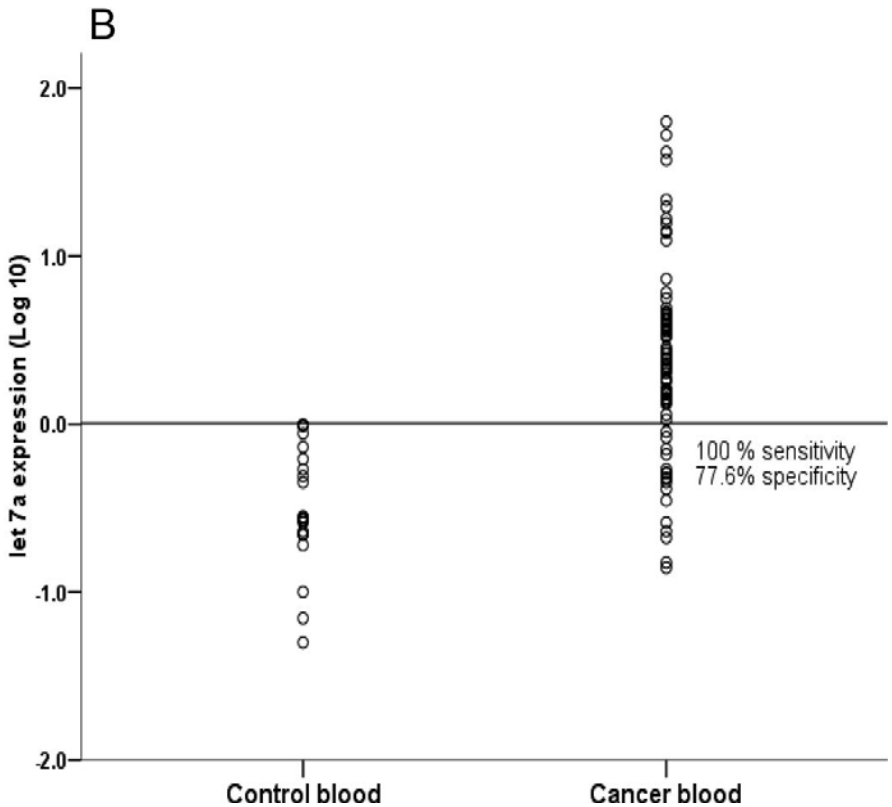


Mitchell P S et al. PNAS 2008

Blood *miR-195* and *let-7a* in Breast Cancer



miR-195



Let-7a

Heneghan, et al, Annual of Surgery, 2010

The Results are Inconsistent across the Studies

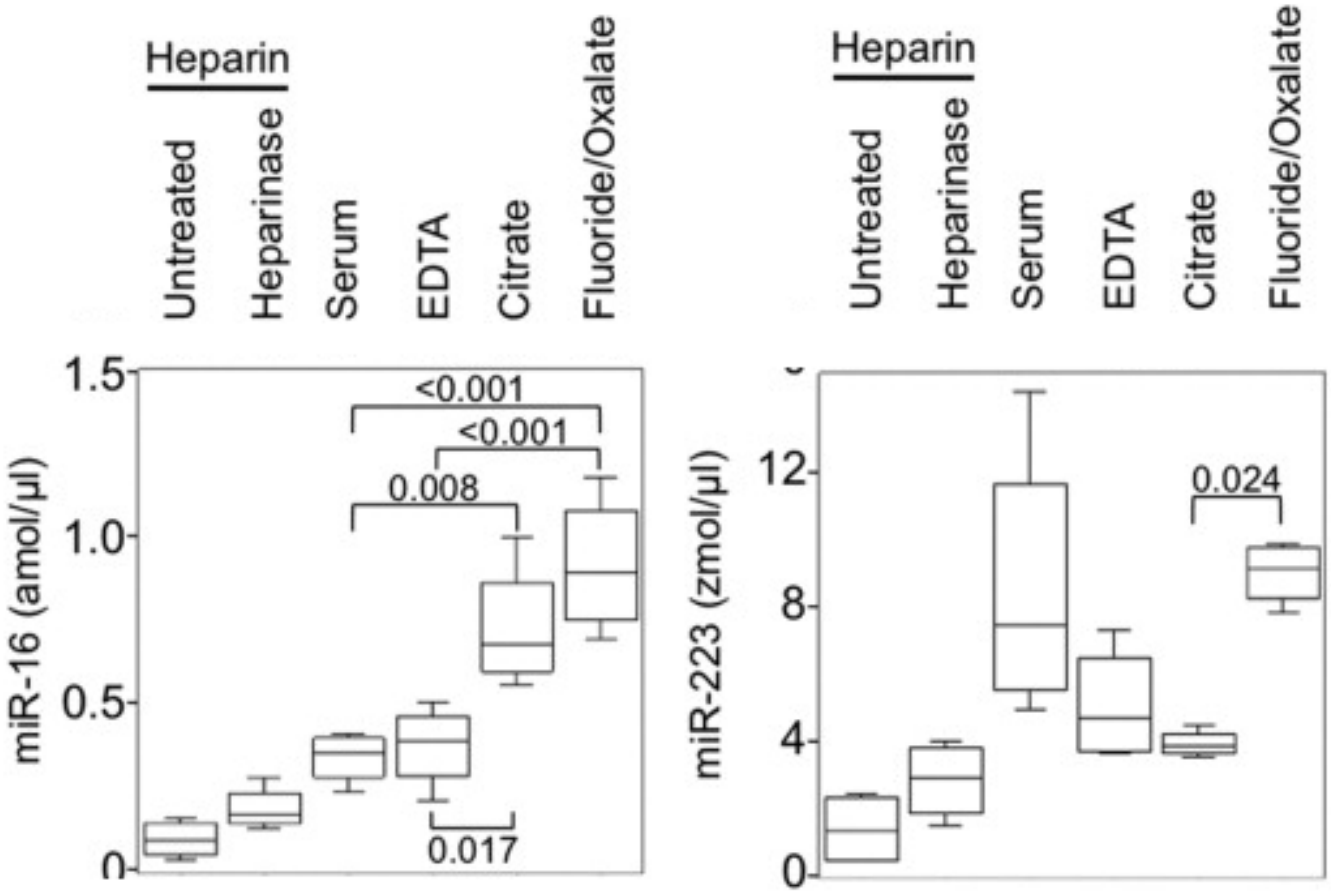
The inconsistency might be due to:

- Unclear biological mechanisms
- Variations in study design and execution
- Variations among the cancer patient population
- Tumor heterogeneity
- Variations in biospecimens, from sample procurement to final analyses

Challenges from Biospecimen Perspective

- Types of biospecimens: serum, plasma or whole blood, etc
- Reliable internal controls to normalize the data
- The effects of pre-analytic variables on circulating microRNAs

Levels of Circulating MicroRNAs Are Affected by Types of Biospecimens



Kim, et al, J Mol Diagn, 2012

Pilot study: Freeze/Thaw and Plasma MicroRNA

Study design

- Number of freeze/thaw: 1 vs 2
- Compare number of microRNAs that can be detected in plasma
- Compare expression of selected microRNAs used as internal controls in literatures (*miR-15* and *miR-16*)

Results

- 1 cycle: 137 microRNAs were detected
- 2 cycles: 117 microRNAs were detected
- The expression of *miR-15* was reduced 15% (1 cycle vs 2 cycles)
- The expression of *miR-16* was reduced 20% (1 cycle vs 2 cycles)

Conclusions

- The number of freeze/thaw affects circulating microRNA expression
- Biospecimen research in circulating microRNA is needed

Milestones of The Project

I. *Discover a panel of "housekeeping" circulating microRNAs which can be used as internal controls.*

II. *Development of circulating microRNA QC tools by studying the effects of pre-analytic variables on the internal control microRNAs identified in Milestone 1.*

Scheme of Milestone I

- Perform microRNA profiling in 40 plasma samples to identify a panel of circulating microRNAs based on the following criteria:
  - *occur in all tested samples*
  - *expression levels are not significantly different between cancer cases and controls*
  - *show little inter-individual variations among cases and controls*

- Further evaluated individually in an additional 200 plasma samples from 100 cancer cases and 100 healthy controls using quantitative real-time PCR based analysis

- In parallel, we repeated microRNA profiling and validation analysis in the same study subjects as above, but using whole blood samples collected using PAXgene Blood RNA System

DataBank and BioRepository

- DataBank and BioRepository (DBBR) of Roswell Park Cancer Institute (RPCI) has been used to
  - *recruit study subjects*
  - *obtain high quality biospecimens*
  - *collect epidemiological/clinical data*
- Standard Operation Procedure (SOP) has been developed and strictly followed
- Blood collection, sample processing, and storage are completed in one hour

Cases and Controls in Microarray Profiling

20 Cases

- 8 prostate cancer patients (men) and 12 breast cancer patients (women)
- Average age of cancer diagnosis: 56 years old
- All Caucasians

20 Controls

- Matched with cases on age, gender and ethnicity

MicroRNA Profiling Analysis

- Exiqon MicroRNA Ready-to-use PCR array was used
- 742 human microRNAs and 6 reference RNAs
- Spike RNAs were used to normalize plate-plate variations

Data Analysis Procedure

748 miRNAs profiled in 40 whole bloods using Exiqon PCR array

209 miRNAs with Ct value <38 in all 40 whole blood samples

52 miRNAs with FC<1.5 & FDR>0.1 in all three case vs. control comparisons.

1) All group: 20 vs.20
2) Breast group: 12 vs. 12
3) Prostate group: 8 vs. 8

3 miRNAs whose CV rank and FC rank are consistently among the top 10.

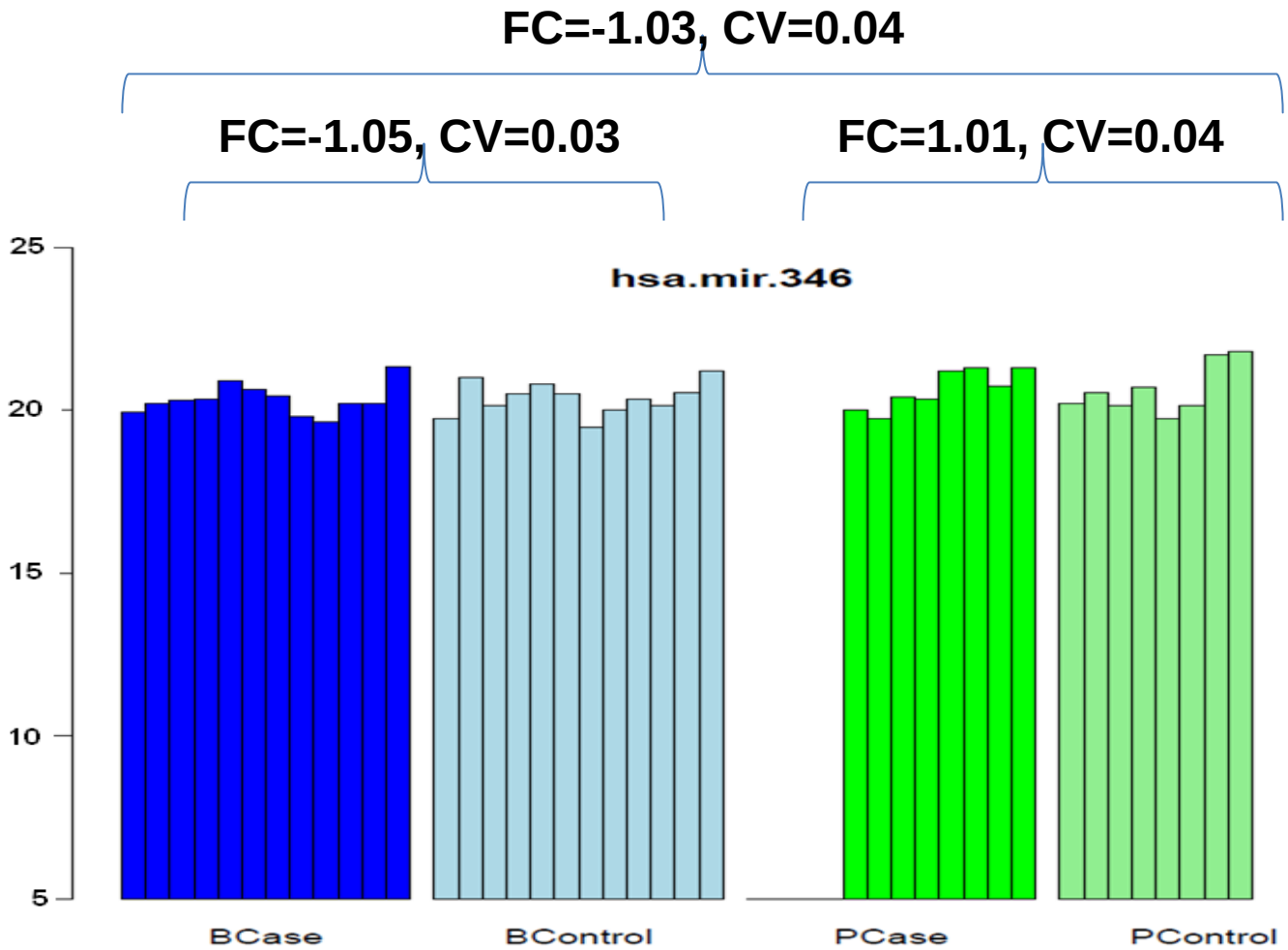
miR-134, miR-346 and miR-934

Step 1: identify miRNAs expressed in all biospecimens

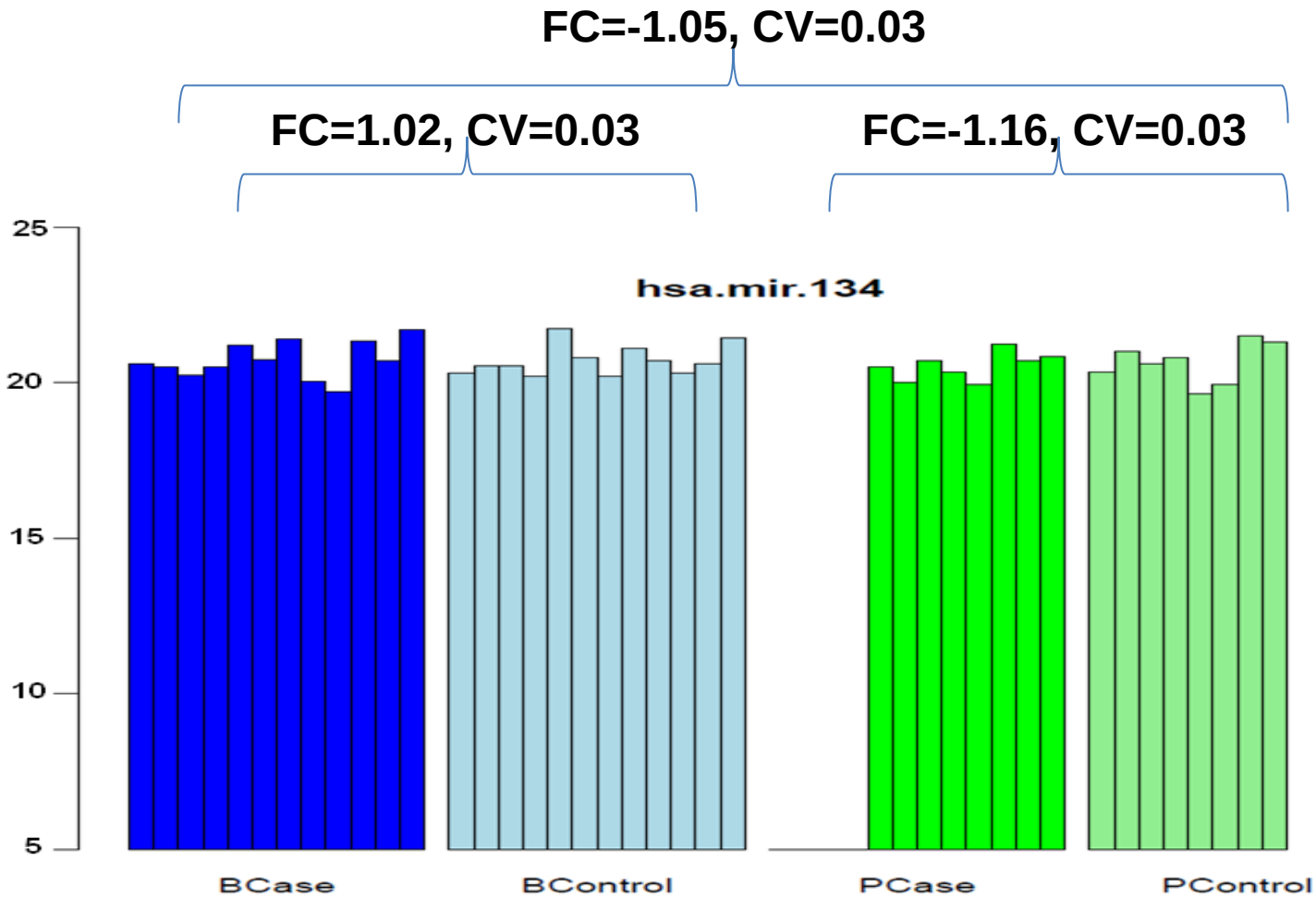
Step 2: identify miRNAs without significant expression changes

Step 3: rank miRNAs based on a composite score of FC (fold change) and CV (correlation of variation)

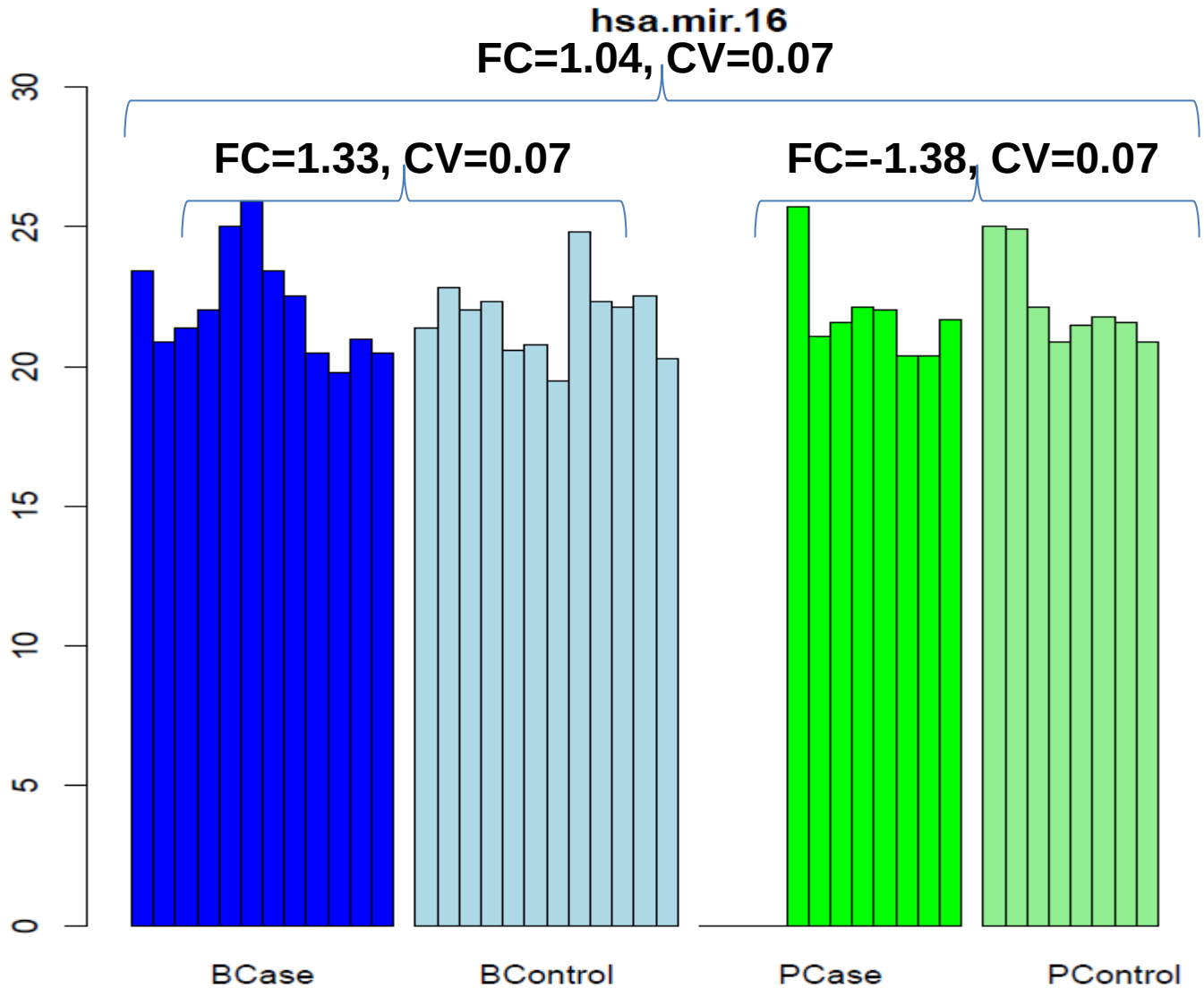
Whole Blood miR-346 Levels Across The Study Subjects



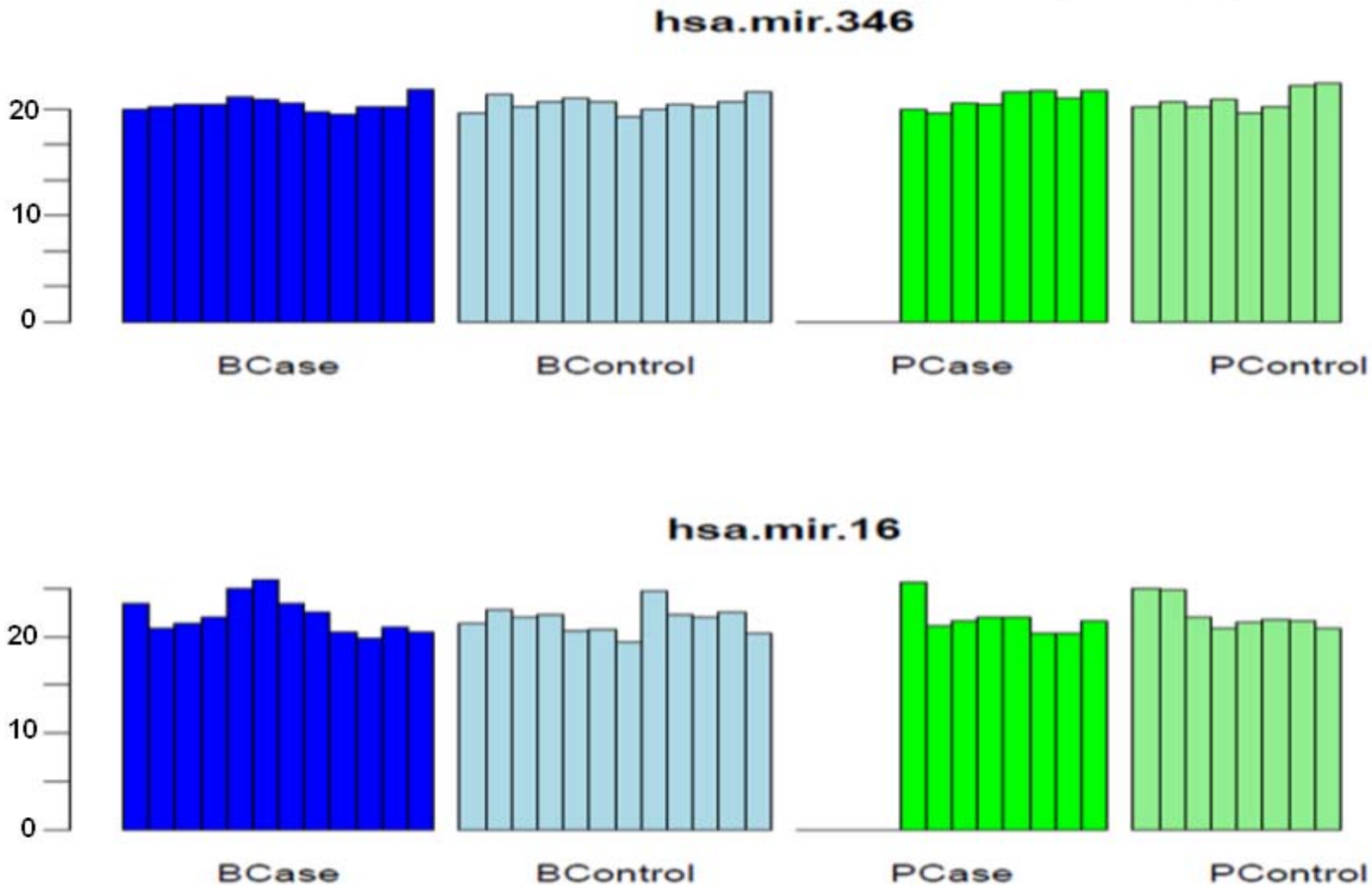
Whole Blood miR-134 Levels Across The Study Subjects



Whole Blood miR-16 Levels Across The Study Subjects



Comparison Between miR-346 and miR-16 Across The Study Subjects

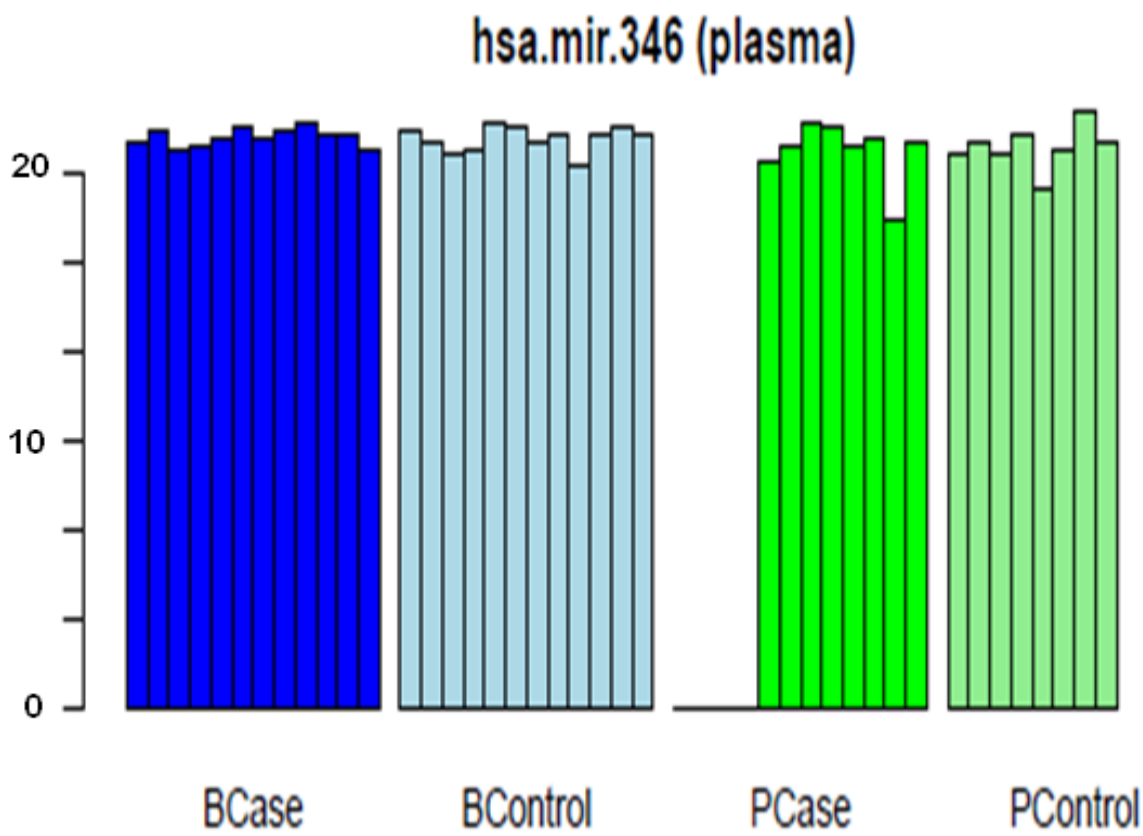


Plasma miR-346 Levels Across The Study Subjects

FC=1.01, CV=0.03

FC=1.07, CV=0.04

FC=-1.08, CV=0.03



Validation Analysis

Cases (100)

- 56 breast cancer, 36 prostate cancer, 3 colon cancer, and 5 lung cancer cases
- 91 Caucasians, 6 Blacks, and 3 Hispanics
- 62 women and 38 men
- Mean age of cancer diagnosis: 57 years old

Controls (100)

- 93 Caucasians, 6 Blacks, and 1 Hispanics
- 80 women and 20 men
- Mean age of enrollment: 56 years old

Molecular and data analysis

- MiScript PCR System (Qiagen) is used to quantify each selected microRNAs
- Selected microRNAs include: *miR-346*, *miR-134*, *miR-934*, *miR-16*, *miR-421*, *miR-222*, *miR-1207*, *miR-339*, *miR-505*, *miR-183*, *miR-374b*, *miR-1260*, *miR-345*, and *miR-323*.
- Linear regression analysis was applied.

microRNA Internal Controls in Whole Bloods

microRNAs	Overall fold changes/CV	Breast cancer fold changes/CV	Prostate Cancer fold change/CV
miR-346	1.05/0.07	1.08/0.09	1.06/0.11
miR-134	1.09/0.11	1.08/0.09	1.12/0.12
miR-934	1.07/0.06	1.07/0.09	1.08/0.08
miR-16	1.05/0.06	1.08/0.10	1.06/0.09
miR-421	1.13/0.11	1.12/0.14	1.14/0.15
miR-222	1.14/0.16	1.13/0.12	1.18/0.17
miR-1207	1.21/0.21	1.24/0.30	1.20/0.19
miR-339	1.25/0.26	1.24/0.34	1.23/0.18
miR-505	1.32/0.30	1.29/0.28	1.24/0.36
miR-183	1.29/0.26	1.31/0.40	1.30/0.26
miR-374b	1.31/0.17	1.29/0.21	1.29/0.31

microRNA Internal Controls in Whole Bloods

microRNAs	Overall fold changes/CV	Breast cancer fold changes/CV	Prostate Cancer fold change/CV
miR-346	1.05/0.07	1.08/0.09	1.06/0.11
miR-134	1.09/0.11	1.08/0.09	1.12/0.12
miR-934	1.07/0.06	1.07/0.09	1.08/0.08
miR-16	1.05/0.06	1.08/0.10	1.06/0.09
miR-421	1.13/0.11	1.12/0.14	1.14/0.15
miR-222	1.14/0.16	1.13/0.12	1.18/0.17
miR-1207	1.21/0.21	1.24/0.30	1.20/0.19
miR-339	1.25/0.26	1.24/0.34	1.23/0.18
miR-505	1.32/0.30	1.29/0.28	1.24/0.36
miR-183	1.29/0.26	1.31/0.40	1.30/0.26
miR-374b	1.31/0.17	1.29/0.21	1.29/0.31

microRNA Internal Controls in Plasmas

microRNAs	Overall fold changes/CV	Breast cancer fold changes/CV	Prostate Cancer fold change/CV
miR-346	1.30/0.26	1.29/0.26	1.26/0.31
miR-134	1.19/0.31	1.24/0.21	1.22/0.22
miR-934	1.27/0.25	1.29/0.19	1.38/0.29
miR-16	1.15/0.12	1.19/0.17	1.16/0.13

Conclusions for Milestone 1

- We have identified several internal control candidates for whole blood based circulating microRNA analysis, including miR-346, miR-134, miR-934 and miR-16.
- miR-16 is the only microRNA which shows relatively consistent expression in plasma samples.

Milestone 2

Development of the circulating microRNA QC tools by studying the effects of the pre-analytic variables on the "housekeeping" microRNAs identified in Milestone 1.

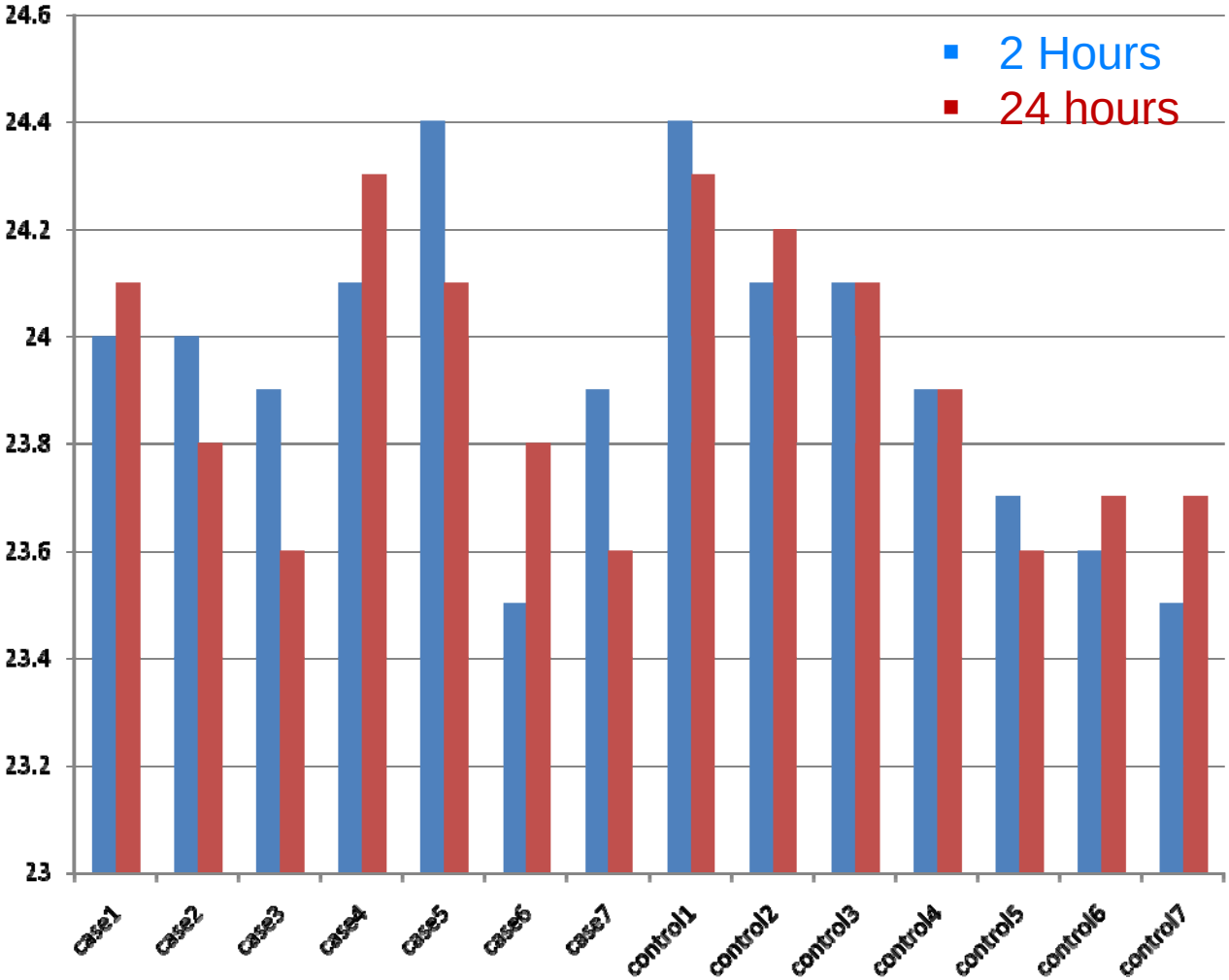
Objective 1 in Milestone 2

- **Processing delay time**
  - Plasma: No delay vs 24 hours delay
  - Whole blood: 2 hours vs 24 hours delay

- **Recruitment**
  - 7 cases (completed)
  - 7 controls (completed)

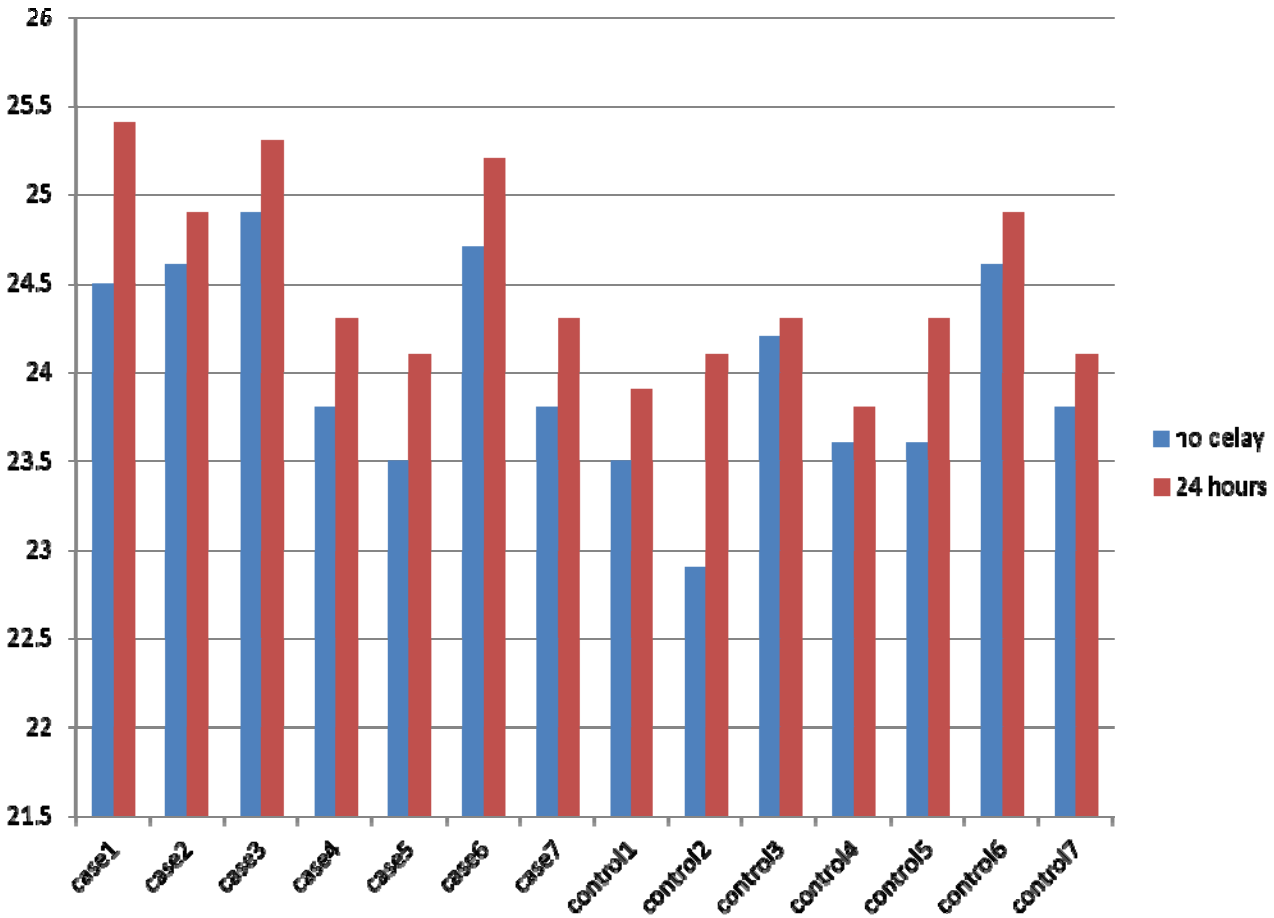
- **Selection of microRNAs**
  - Whole bloods: miR-346, miR-134 and miR-934
  - Plasma: miR-16

Whole bloods: 2 vs 24 hours



- No significant changes in miR-346 levels between 2 hrs and 24 hrs delay
- P=0.892
- Similar trends for miR-134 and miR-934

Plasmas: no delay vs 24 hours



- Systematical decrease miR-16 levels between no delay and 24 hrs delay
- P=0.004
- Processing time delay affects quantity of circulating microRNAs in plasma

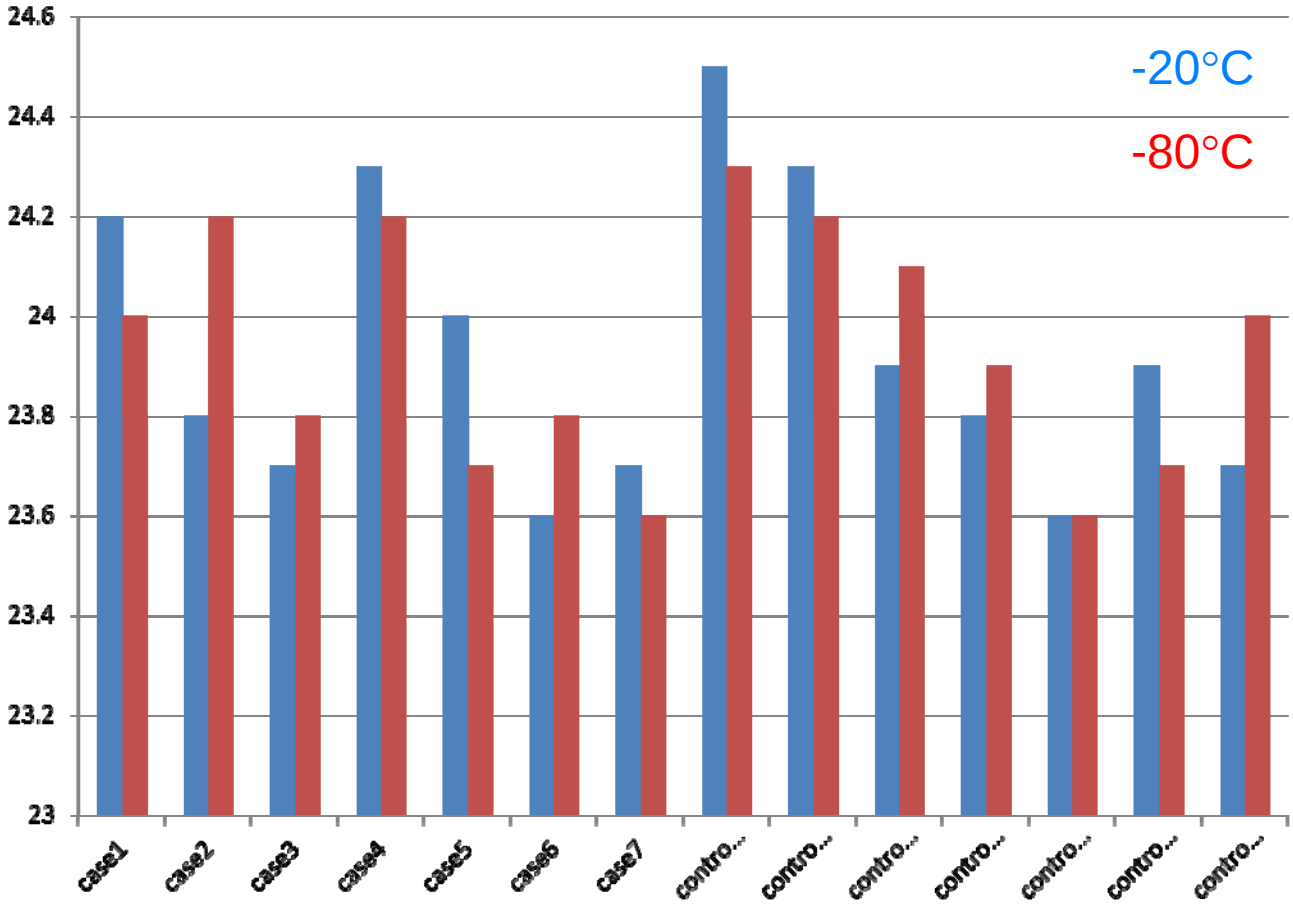
Objective 2 in Milestone 2

- **Storage conditions**
  - Plasma: cryovials at -80°C vs straws in liquid nitrogen
  - Whole blood: -20°C vs -80°C

- **Recruitment**
  - 7 cases (completed)
  - 7 controls (completed)

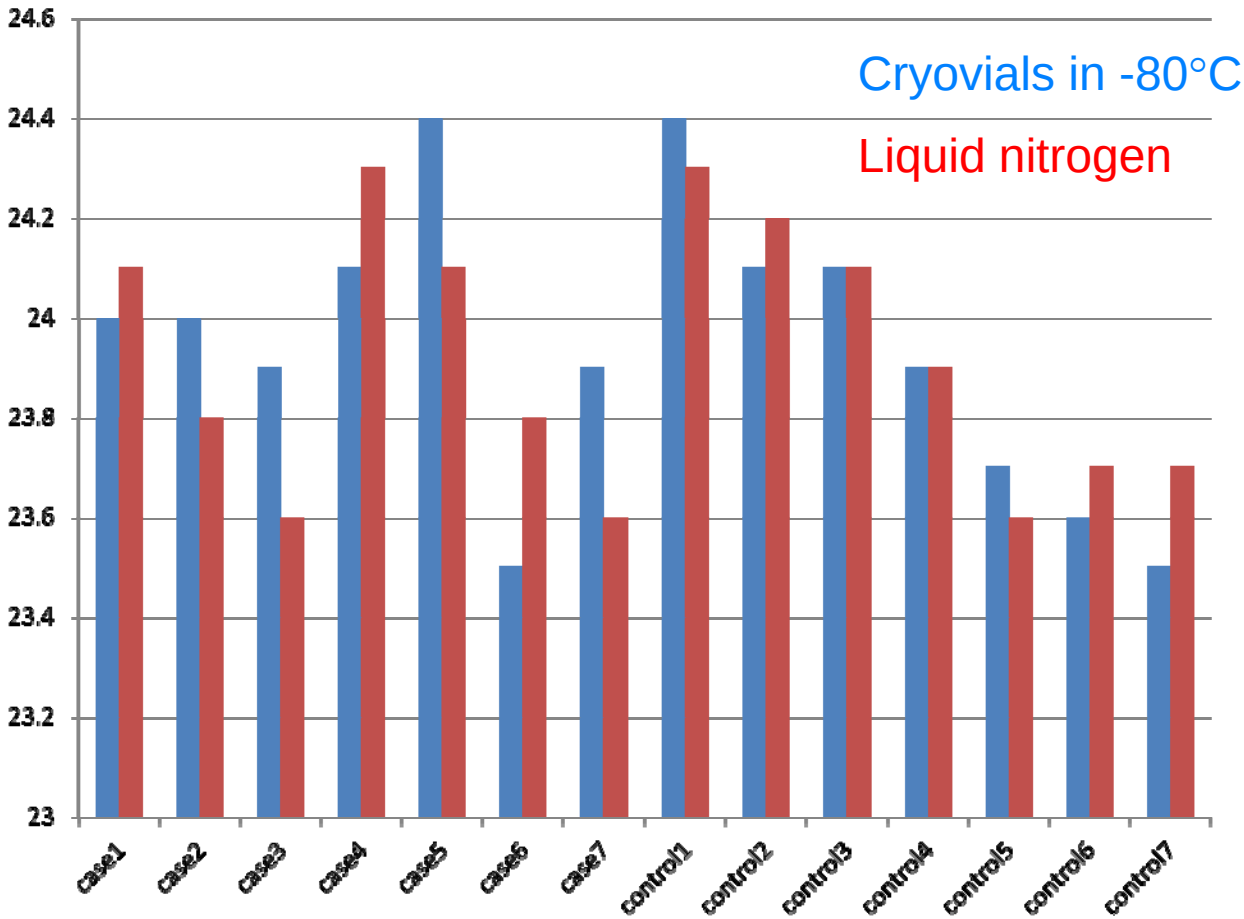
- **Selection of microRNAs**
  - Whole bloods: miR-346, miR-134 and miR-934
  - Plasma: miR-16

Whole blood: -20°C vs -80°C



- No significant changes in miR-346 levels between -20°C and -80°C
- P=0.852
- Similar trends for miR-134 and miR-934

Plasma: cryovials at -80°C vs straws in liquid nitrogen



- No significant changes in miR-16 levels between cryovials at -80°C and straw in liquid nitrogen
- P=0.792

Objective 3 in Milestone 2

- **Storage duration**
  - Plasma: 0 vs 6 months
  - Whole blood: 0 vs 6 months
- **Recruitment**
  - 7 cases (Completed)
  - 7 controls (completed)

Objective 4 in Milestone 2

- **Number of freeze-thaw**
  - Plasma: 0 vs 1, 2, and 4
  - Whole blood: 0 vs 2

- **Recruitment**
  - 7 cases (Completed)
  - 7 controls (completed)

Acknowledgement

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Thank You!