A development and analysis pipeline for microRNA biomarker signatures in molecular diagnostics based on circulating EVs

Michael W. Pfaffl et al.

Dominik Buschmann, Benedikt Kirchner, Stefanie Hermann, Agnes Meidert, Melanie Borrmann, Anja Lindemann, Florian Brandes, Marlene Reithmair, Alex Hildebrandt, Veronika Mussack, Gustav Schelling

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Michael W. Pfaffl
Animal Physiology & Immunology Weihenstephan
TUM - School of Life Sciences Weihenstephan, 85354 Freising, Germany
Michael.Pfaffl@tum.de

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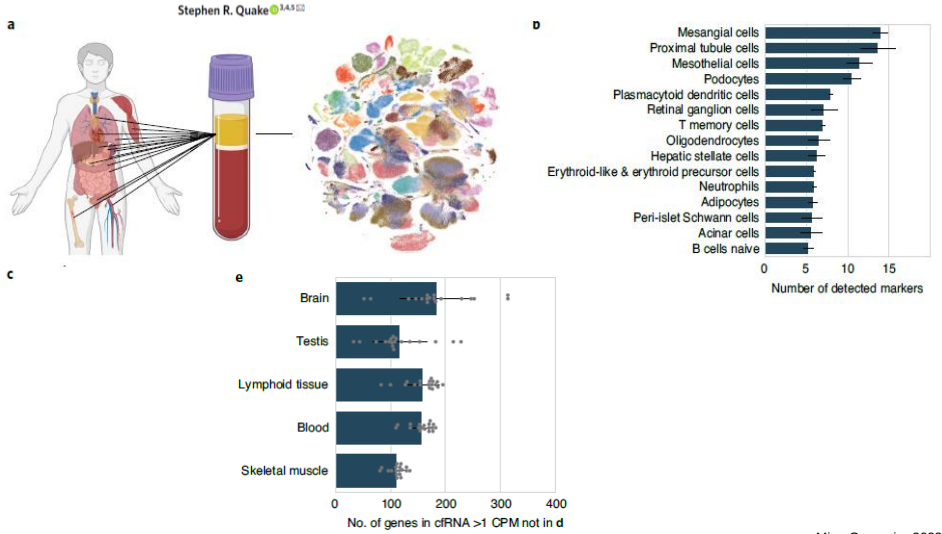
nature biotechnology
BRIEF COMMUNICATION
https://doi.org/10.1038/s41587-021-01188-9
Check for updates

OPEN

Cell types of origin of the cell-free transcriptome

Sevahn K. Vorperian[1,2], Mira N. Moufarrej[3], Tabula Sapiens Consortium* and Stephen R. Quake[3,4,5]



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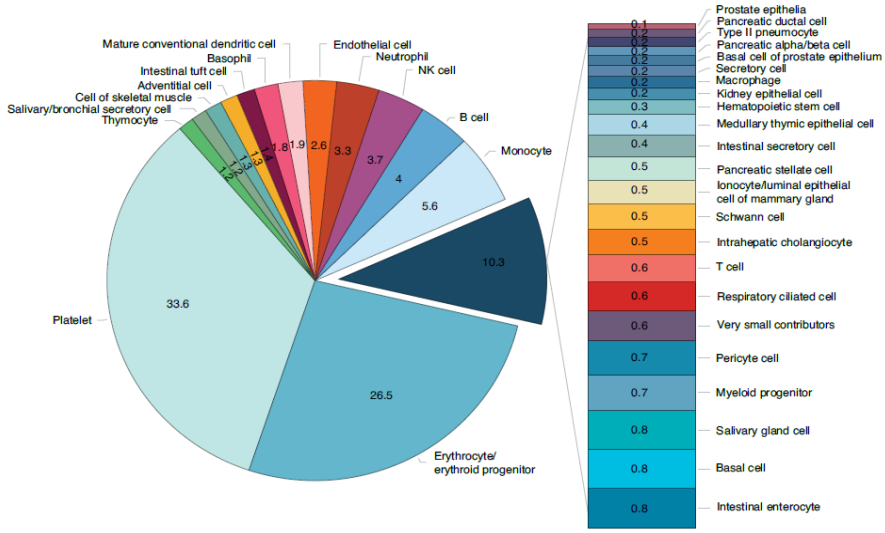
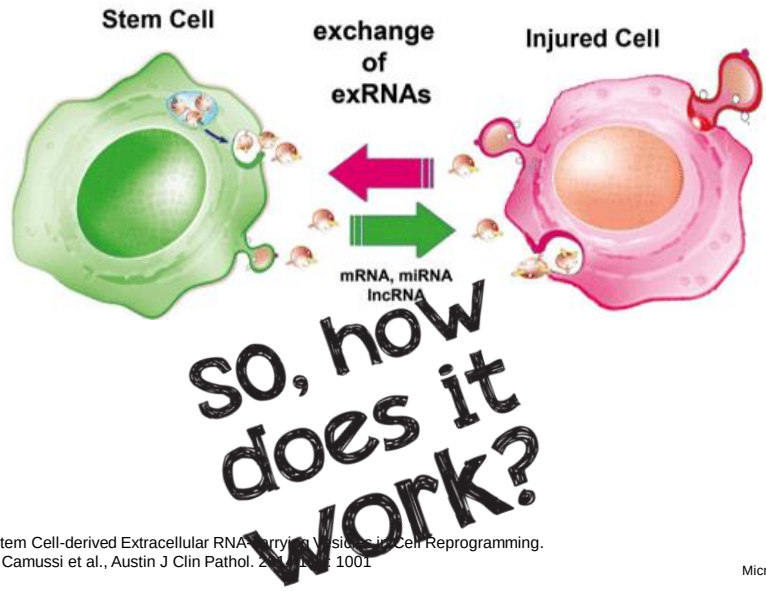


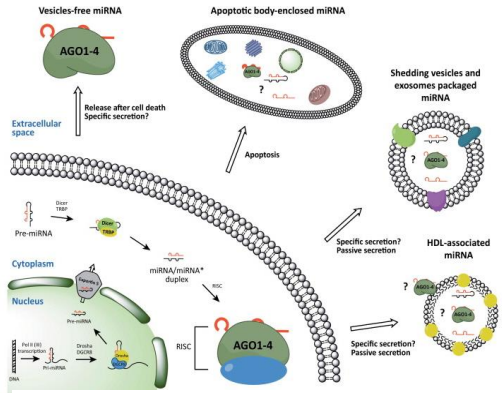
Cell-to-Cell Communication via exRNA



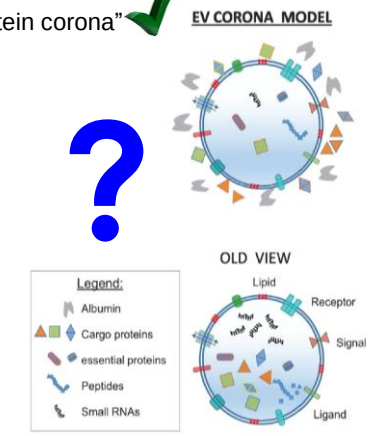
Role of Stem Cell-derived Extracellular RNA Carrying Vesicles in Cell Reprogramming.
Giovanni Camussi et al., Austin J Clin Pathol. 2[illegible] 1001

State of exRNAs in Biofluids

- Intrinsically stable in blood, urine, swabs, milk, saliva, … … body liquids
- Circulating exRNAs are “bound to proteins”
- Circulating exRNAs are “protected inside vesicles”
- Circulating exRNAs are “associated to the EV protein corona”

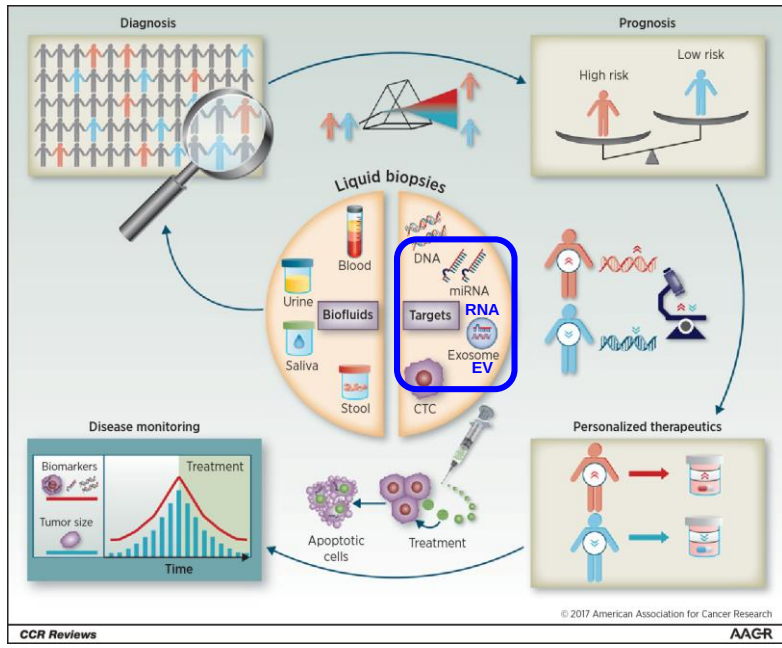


Turchinovich et al., Trends in Biochemical Sciences (2012)

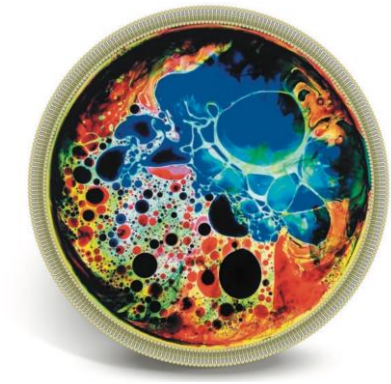


Wolf et al., Journal of Extracellular Vesicles (2022)

Liquid Biopsy – circulating exRNA – Biomarker Signature



A multitude of intra-cellular bodies and vesicles



www.nature.com

Which blood compartment is the "best" for RNA biomarker discovery?

- Full-blood (e.g. Paxgene)
- White blood cells (WBC)
- Cell-free compartment (e.g. cfRNA, cfDNA)
- Extracellular vesicles (EV)

Which type of EV is suitable for biomarker development?

- Exosomes
- Microvesicles
- Ectosomes
- Exomers (… the newest & smallest type)
- Apoptotic Bodies
- … all Extracellular Vesicles (EVs)

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Why Extracellular vesicles?

Blood compartment-specific microRNA Biomarkers
for early disease detection

Cells ⇔ Serum ⇔ EV / Exosomes

Michael W. Pfaffl
Animal Physiology & Immunology Weihenstephan
TUM - School of Life Sciences Weihenstephan, 85354 Freising, Germany
Michael.Pfaffl@TUM.de

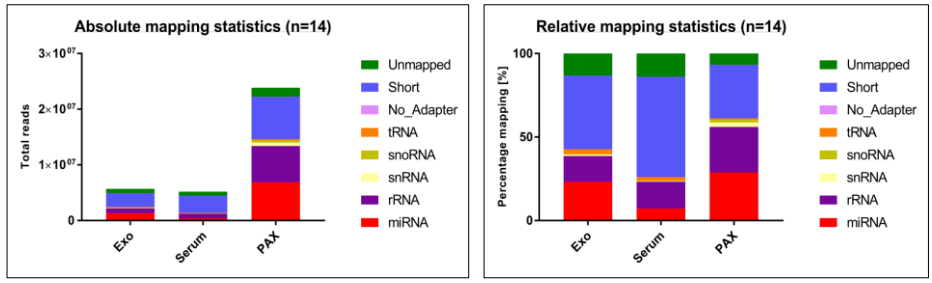
Cellular and extracellular miRNAs in different blood compartments - a feasibility study

Exosome/EV associated RNA (1 ml)

Serum RNA (1 ml)

PAXgene = whole blood cellular RNA (1 ml)

Absolute and relative mapping statistics of small-RNAs



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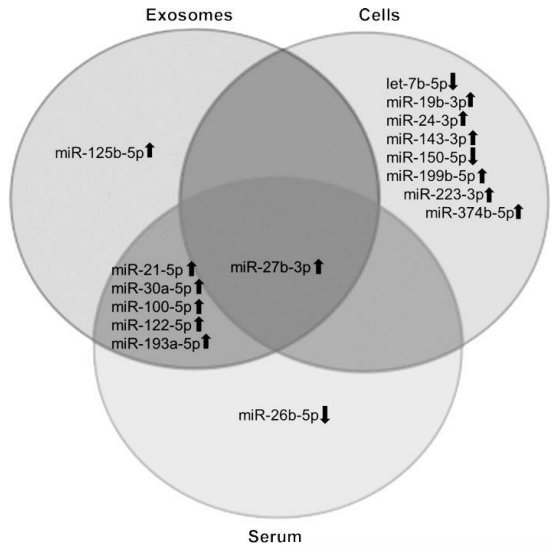
Cellular and extracellular miRNAs are blood compartment-specific diagnostic targets in sepsis



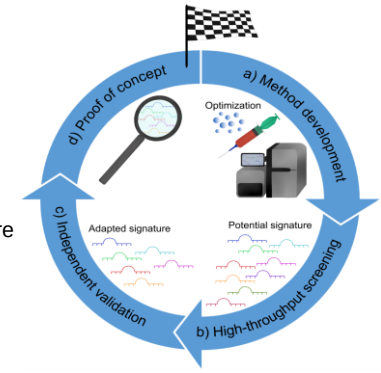
Reithmair et al. J Cell Mol Med. 2017(10): 2403-2411

Journal of Cellular and Molecular Medicine Open Access

Advantages of EV associated miRNA?

Advantage of EV miRNAs:

- EVs are central part of liquid biopsy => “non-invasive”
- miRNA are short molecule => “stable marker”
- miRNA in EV => „EV protected marker”
- miRNA at EV => „protected by EV corona”
- miRNA expression changes => allows early evaluation of cellular and physiological changes
- Combination of multiple miRNAs => biomarker signature

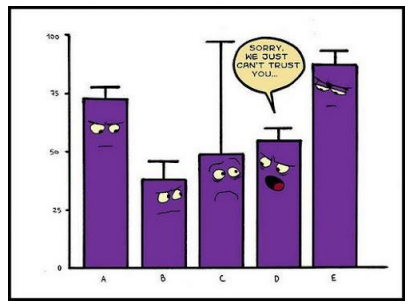


Advantage of a Biomarker Signature:

- **Biomarker signature = specific information**
  => Physiological or pathological indicator
  => Directly related to a certain disease, a cancer stage/type
- **Signal amplification via RT-qPCR** => easy and early detection
- RT-qPCR allows reliable quantification a **very low abundance level**
- Standardization via MIQE and MISEV
  => Precise, reproducible and fast evaluation in an objective manner

Extracellular vesicle derived microRNA biomarkers -- Goals and Pitfalls.
Stefanie Hermann et al., Trillium Extracellular Vesicles 2020 1(1): 42-47

High need for standardization in the quantitative workflow



- … in experimental design
- … in tissue sampling
- … in EV isolation
- … in EV characterization
- … in RNA extraction
- … in RNA sequencing (biomarker screening)
- … in RT-qPCR (biomarker confirmation)
- … in Data Analysis & Bioinformatics

MISEV Guidelines

- **Jan Lötvall et al., J Extracellular Vesicles 2014 (3): 26913**
  Minimal experimental requirements for definition of extracellular vesicles and their functions: A position statement from the International Society for Extracellular Vesicles (MISEV 2014 guidelines)
- **Kenneth W. Witwer et al., J Extracellular Vesicles 2017 6(1): 1396823**
  Updating the MISEV minimal requirements for extracellular vesicle studies: building bridges to reproducibility.
- **Clotilde Théry, Kenneth W. Witwer et al., J Extracellular Vesicles 2018 7(1): 1535750**
  Minimal Information for Studies of Extracellular Vesicles 2018 (MISEV 2018): A position statement of the International Society for Extracellular Vesicles and update of the MISEV 2014 guidelines
- **MISEV 2023 Update will be submitted very soon to JEV !**

MISEV Checklist with six main headings:

1) **Nomenclature:**
   - Mandatory
   - Optional
2) **Collection & Pre-procession:**
   - Tissue culture conditioned medium
   - Biofluids or Tissues
   - Storage and Recovery
3) **EV Separation & Concentration:**
   - Specify category of the chosen EV separation/concentration method
   - Experimental details of the method
4) **Characterization:**
   - Quantification
   - Global Characterization
   - Single EV Characterization
5) **Functional Studies**
6) **Reporting**

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Establishment of a MISEV compliant EV workflow

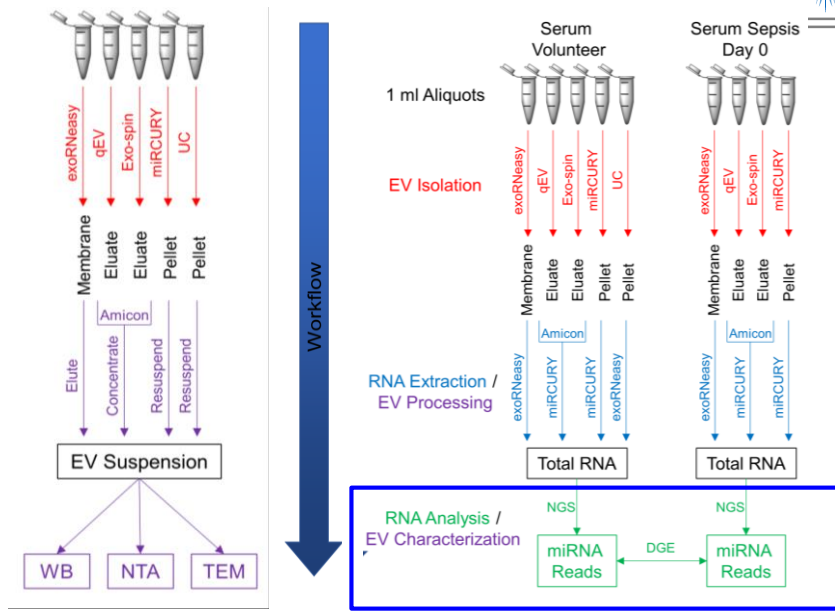
=> Optimization of EV Isolation Methods from Serum

Goals: - fast and reproducible
- reliable in “daily clinical practice”
- suitable for microRNA based Biomarker Discovery

Michael W. Pfaffl
Animal Physiology & Immunology Weihenstephan
TUM - School of Life Sciences Weihenstephan, 85354 Freising, Germany
Michael.Pfaffl@TUM.de

1) EV Characterization

2) Kit Comparison in Serum



Buschmann et al., J Extracell Vesicles. 2018 7(1): 1481321

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1st main cluster

Exo-Spin

qEV

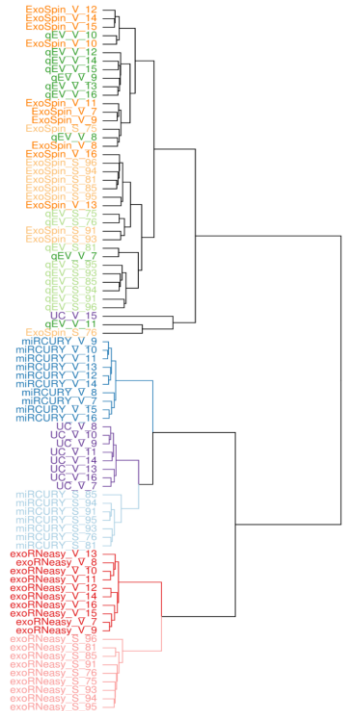
2nd main cluster

miRCURY

diff. UC

miRCURY

exoRNeasy



Conclusion:

Yes, … there is a high significant impact of the EV isolation method on the microRNA signature?

HCA based on all miRNA reads

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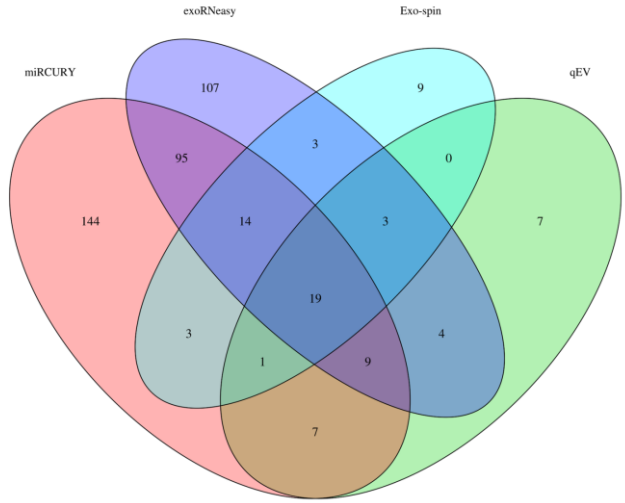
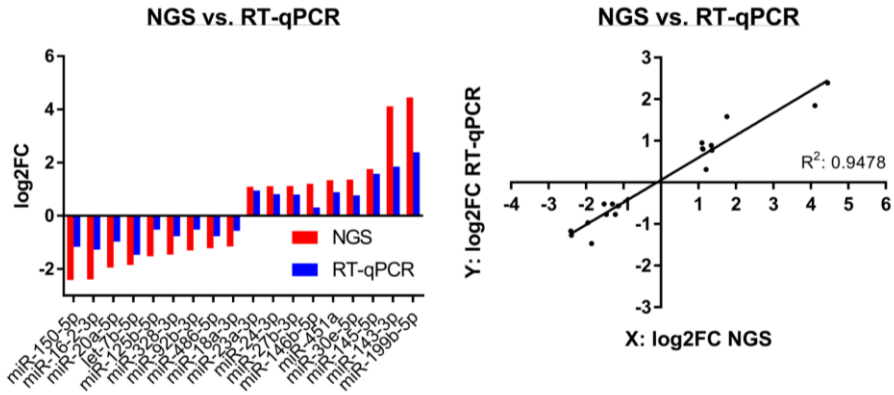
Significant regulated microRNAs

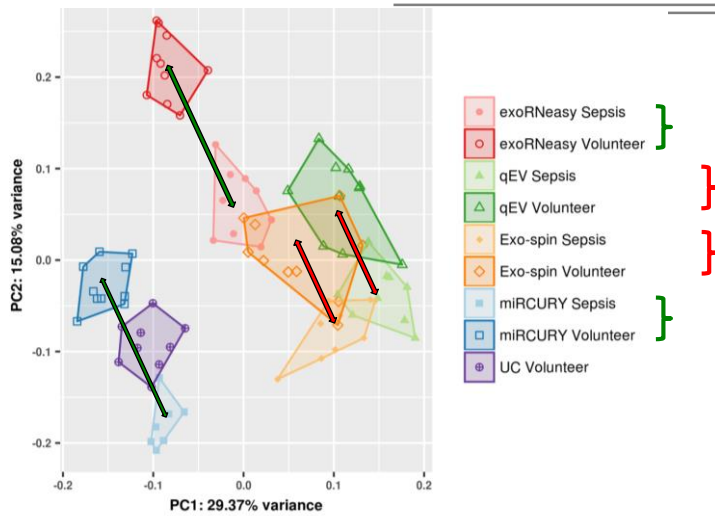


Supplemental Figure 3 Differential expression of miRNAs in EVs isolated by commercial methods. Results from DESeq2 were not filtered for expression level, log2 fold change or statistical significance.

RT-qPCR Validation of NGS candidate microRNAs



PCA of 200 most significant different canonical microRNAs



Evaluation of serum extracellular vesicle isolation methods for profiling miRNAs by Next-Generation Sequencing

Dominik Buschmann, Benedikt Kirchner, Stefanie Hermann, Melanie Märte, Christine Wurmser, Florian Brandes, Stefan Kotschote, Michael Bonin, Ortrud K. Steinlein, Michael W. Pfaffl, Gustav Schelling, and Marlene Reithmair

Journal of Extracellular Vesicles

J Extracell Vesicles. 2018 7(1): 1481321

microRNA @ EV based decision making:

=> 1st patient cohort => learning cohort

=> classification of sepsis patient groups

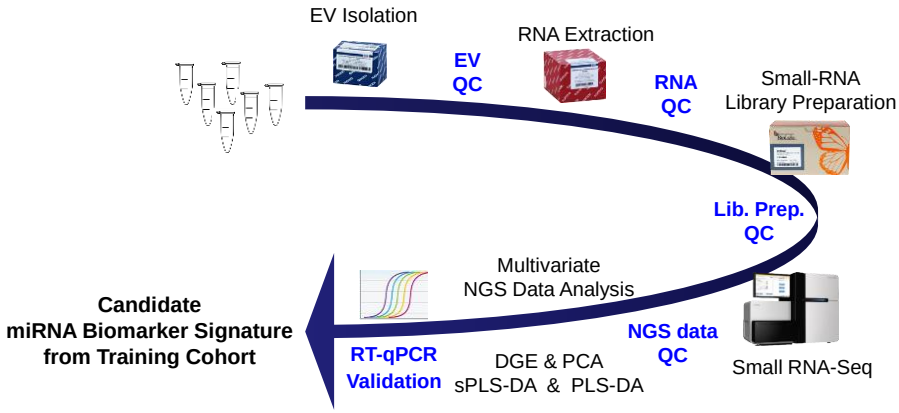
=> physiological & functional validation

=> 2nd patient cohort => validation cohort

Michael W. Pfaffl
Animal Physiology & Immunology Weihenstephan
TUM - School of Life Sciences Weihenstephan, 85354 Freising, Germany
Michael.Pfaffl@TUM.de

Standardized development & analysis pipeline

1st Patient Cohort = Training Set (n=67)
=> Development of Candidate Biomarker Signature



2nd Patient Cohort = Validation Set (n=75)
=> Confirmation of Candidate Biomarker Signature

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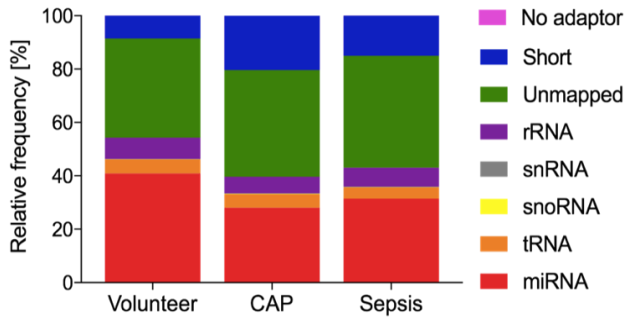
Small-RNA Seq mapping

Inclusion criteria for biological samples:

> 1 Mio. total microRNA reads / sample

> 15% microRNAs



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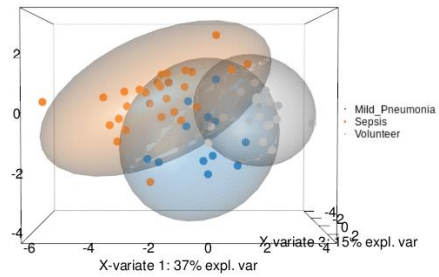
PLS-DA: reduction to 3 patient groups
CAP – Sepsis/ARDS/Severe-Pneumonia – Volunteer

- **Filtering: baseMean ≥ 500**
- **4 components with maximum 5 features eac**
- **PLS-DA**

=> 12 microRNAs

miR-181b-5p
miR-182-5p
miR-186-5p
miR-193a-5p
miR-199a-3p=miR-199b-3p
miR-21-5p
miR-215-5p
miR-30e-5p
miR-340-5p
miR-425-5p
miR-93-5p
miR-941



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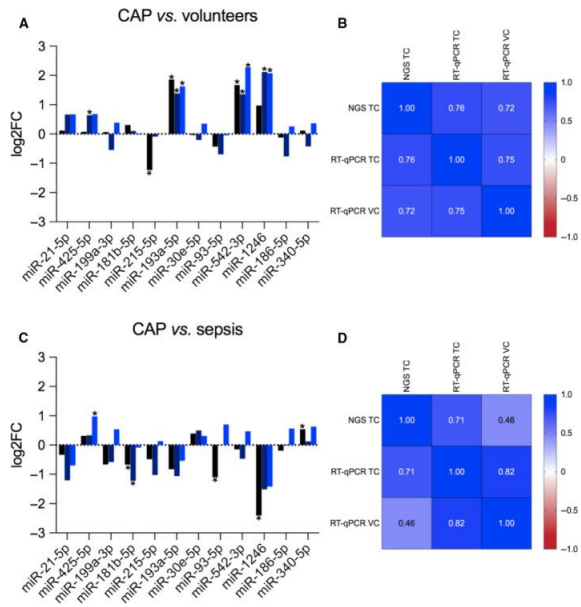
RT-qPCR confirmation using 12 microRNAs
training cohort = 67 patients; validation cohort = 75 patients



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Validity of the 12 microRNA Biomarker Signature
(3 patient groups)

Training Cohort
n = 67 patients

Training Set Accuracy 92.4% – 99.6%

Validation Cohort
n = 75 patients

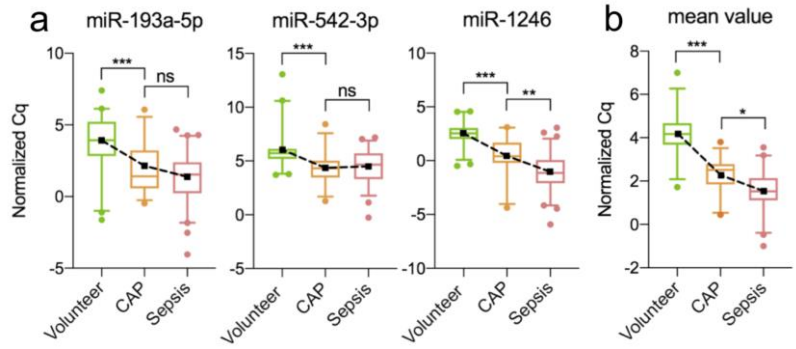
Prediction Accuracy @ day 1 ICU

- **CAP** **83.3%** Sepsis 11.1% Vol 5.6%
- **Sepsis** **78.4%** CAP 21.6% Vol 0.0%
- Volunteer 55.0% Sepsis 30.0% CAP 5.0%
- Over all groups 73.4%

S. Hermann, F. Brandes, B. Kirchner, D. Buschmann, M. Borrmann, M. Klein, S. Kotschote, M. Bonin, M. Reithmair, I. Kaufmann, G. Schelling, MW. Pfaffl (2020)
Diagnostic potential of circulating cell-free microRNAs for community-acquired pneumonia and pneumonia-related sepsis.
Journal of Cellular and Molecular Medicine 2020; 24(20): 12054-12064

High significant regulated marker miRNAs

- Combination of training and validation cohort n = 152
- Validated and quantified by RT-qPCR
- Marker miRNA for CAP: **miR-193a-5p, miR-542-3p, miR-1246**
- Increased risk marker miRNA to develop sepsis: **miR-1246**



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Diagnostic potential of circulating cell-free microRNAs for community-acquired pneumonia and pneumonia-related sepsis.
Journal of Cellular and Molecular Medicine 2020; 24(20): 12054-12064

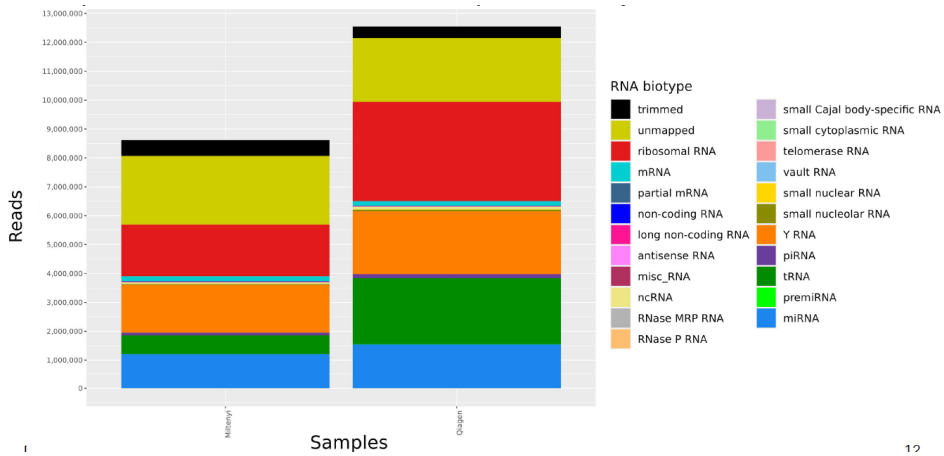
Development of bioinformatical pipelines to standardize quantitative transcriptomics

- NGS data analysis pipeline for **small-RNA** (microRNA & tRFs & piRNA)
- NGS data analysis pipeline for **long-RNA** (mRNA & lncRNA)
- **Multivariate-analysis** (Heatmap, HCA, PCA, PLS-DA, …)
- **Physiological "in silico" validation** (IPA & pathway analysis)
  => "validation of identified biomarker candidates in the clinical context"
- **caRNAge** => comprehensive (small) RNA Seq data analysis computational pipeline
  https://www.physio.wzw.tum.de/caRNAge/
- **miREV** => select stable microRNA signature in EVs
  https://www.physio.wzw.tum.de/mirev/
- **isomirroR** => deep microRNA analysis to detect isomiRs
  https://gitlab.lrz.de/Physio/isomiRROR

- **Transcriptomic Network**
  Integrative analysis of microRNA, lncRNA, mRNA, and protein data **=> coming soon!**

Michael W. Pfaffl
Animal Physiology & Immunology Weihenstephan
TUM School of Life Sciences, Weihenstephan, 85354 Freising, Germany
Michael.Pfaffl@TUM.de

caRNAge
= comprehensive small RNA Seq data analysis



caRNAge output

Small-RNA Seq Quality Control (GC)	Differential Gene Expression (DGE)
• Detection of degradational status • Detection of sequencing errors • Detection of batch effects • Detection of outliers • Relative distribution of all RNA species • Length distribution of alignments	• Normalization according to specified RNA species • Statistical evaluation of gene expression changes of all small-RNA species • Pairwise comparisons of all specified experimental groups • Evaluation of stable reference transcripts for qPCR validation (miREV)

Pathway & Gene List Analysis	Clustering	miRNA & isomiR Analysis

• Identification of predicted and experimentally validated targets of mature miRNAs
• Overrepresentation analysis of significantly regulated transcripts:
  • Gene Ontology
  • KEGG
  • Reactome
  • Wiki-Pathways
	• PCA
	• Detection of optimal error rates
	• Sparse projection on latent squares (SPLS)
	• Classification based on biomarker profiles
		• Integrative differential transcript analysis between predominant isomiRs and parent miRNAs
		• Analysis of miRNA clusters
		• Detection of miRNA localization motifs
		• Evaluation of miRNA stability by isomiR modifications

Go deeper in your small-RNA data!

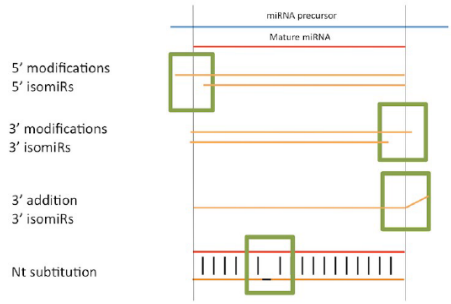
Discover new microRNA Biomarkers!

⇒ isomiR analysis (miRNA isoforms)

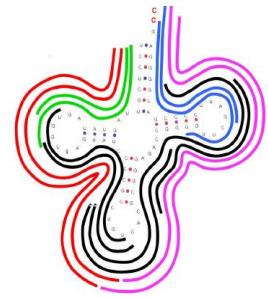
⇒ tRFs (tRNA fragments)

Michael W. Pfaffl
Animal Physiology & Immunology Weihenstephan
TUM School of Life Sciences, Weihenstephan, 85354 Freising, Germany
Michael.Pfaffl@TUM.de

Full transcriptome analysis - as part of caRNAge
e.g. isomiRs & tRFs



isomiR analysis
mature miRNAs 2,000+ vs. 26,000+ isomiRs



tRFs (tRNA derived fragments)
mature 49 tRNA families vs. 3,000+ tRFs

MicroGenomics 2023

Development of miREV

A web-based tool for EV meta-analyses

to find stable reference miRNAs

miREV – microRNA

miREV – Reference(s)

miREV – Extracellular Vesicle

miREV: An Online Database and Tool to Uncover Potential Reference RNAs and Biomarkers in Small-RNA Sequencing Data Sets from Extracellular Vesicles Enriched Samples.
Hildebrandt A, Kirchner B, Nolte-'t Hoen ENM, Pfaffl MW.
J Mol Biol. 2021 433(15): 167070

MicroGenomics 2023

miREV delivers bioinformatically validated microRNAs

miRNA	frequency	miRNA	frequency	miRNA	frequency
miR-140-5p	3/3	miR-361-3p	2/3	miR-21-5p	1/3
miR-23a-3p	3/3	miR-378a-5p	2/3	miR-210-3p	1/3
miR-30d-5p	3/3	miR-425-3p	2/3	miR-22-5p	1/3
let-7f-1-3p	2/3	miR-501-3p	2/3	miR-221-3p	1/3
let-7i-5p	2/3	miR-505-3p	2/3	miR-24-1-5p	1/3
miR-103a-3p	2/3	miR-9985	2/3	miR-26a-5p	1/3
miR-10527-5p	2/3	let-7a-3p	1/3	miR-29c-5p	1/3
miR-145-3p	2/3	let-7b-3p	1/3	miR-30c-5p	1/3
miR-148b-3p	2/3	let-7f-2-3p	1/3	miR-32-5p	1/3
miR-185-3p	2/3	miR-101-3p	1/3	miR-424-5p	1/3
miR-222-3p	2/3	miR-10a-3p	1/3	miR-499a-5p	1/3
miR-24-3p	2/3	miR-140-3p	1/3	miR-532-5p	1/3
miR-29b-3p	2/3	miR-152-3p	1/3	miR-542-3p	1/3
miR-331-5p	2/3	miR-19b-3p	1/3	miR-652-3p	1/3
miR-340-5p	2/3				

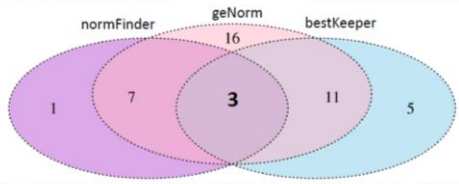


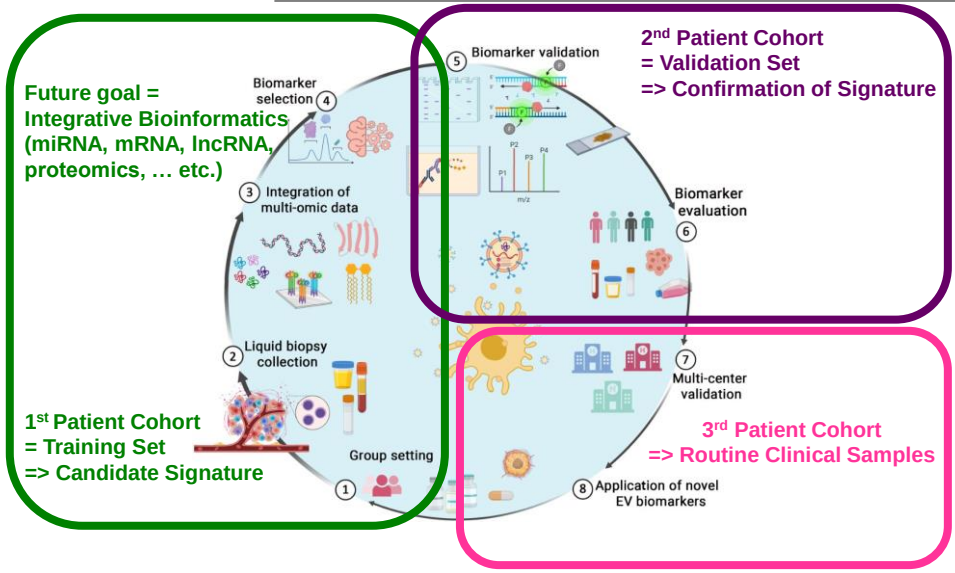
Fig. 4: Overlap of candidate miRNAs by stability measure algorithm irrespective of normalization method. Candidate miRNAs are ordered by the frequency of occurrence in any of the three stability measurement algorithms.

All data sets combined		Yuan *et al.* data set	
miRNA	frequency	miRNA	frequency
miR-148b-3p	6/6	miR-30d-5p	5/6
miR-425-3p	6/6	miR-146a-5p	3/6
miR-9985	6/6	miR-99a-5p	1/6
miR-140-5p	5/6	miR-99b-3p	1/6
miR-24-3p	5/6		
miR-29b-3p	5/6		
miR-30d-5p	5/6		
miR-10527-5p	4/6		
miR-23a-3p	4/6		
let-7f-1-3p	3/6		
miR-145-3p	3/6		
miR-340-5p	3/6		
miR-222-3p	2/6		
let-7i-5p	1/6		
miR-103a-3p	1/6		
miR-185-3p	1/6		
miR-361-3p	1/6		
miR-501-3p	1/6		
miR-505-3p	1/6		

***Table 1:** Processing of all data sets combined (left) and the Yuan et al. data set alone (right). Frequency defines in how many normalization methods miRNAs were present during the overlap analysis.*

miREV: An Online Database and Tool to Uncover Potential Reference RNAs and Biomarkers in Small-RNA Sequencing Data Sets from Extracellular Vesicles Enriched Samples.
Hildebrandt A, Kirchner B, Nolte-'t Hoen ENM, Pfaffl MW.; J Mol Biol. 2021 433(15): 167070

Circulating Biomarkers – Liquid Biopsy – exRNA



Liquid biopsy of extracellular vesicle biomarkers for prostate cancer personalized treatment decision. Meng Han et al., EVCNA 2022 (3): 3-9

Acknowledgements -- Biomarkers

Technische Universität München

Dominik Buschmann, Benedikt Kirchner, Stefanie Hermann, Veronika Mussack
Christine Wurmser (NGS@WZW)

städtisches Klinikum München NEUPERLACH

LMU KLINIKUM DER UNIVERSITÄT MÜNCHEN

Agnes Meidert, Florian Brandes
Gustav Schelling, Marlene Reithmair,
Melanie Borrmann, Anja Lindemann

IMGM LABORATORIES

Stefan Kotschote
Michael Bonin

ZIM Zentrales Innovationsprogramm Mittelstand

Bundesministerium für Wirtschaft und Energie
aufgrund eines Beschlusses des Deutschen Bundestages

Bayerische Forschungsstiftung

TUM Universitätsstiftung

Acknowledgement – caRNAge & miREV

caRNAge @ TUM

- https://www.physio.wzw.tum.de/en/caRNAge/

isomiRROR @ GitLab

- https://gitlab.lrz.de/Physio/isomiRROR

miREV @ TUM

- https://www.physio.wzw.tum.de/en/wg-prof-pfaffl/miREV/
- http://207.180.239.84:3838/miREV/

Alex Hildebrandt

Benedikt Kirchner

Esther N. Nolte-'t Hoen

Technical University of Munich

Utrecht University

Acknowledgement – our team

ISEV 2017 Annual Meeting in Toronto

GSEV Meetings: 2018 in Frankfurt & 2021 in Freiburg

ISEV 2019 Annual Meeting in Kyoto

ASEV - GSEV 2022 Autumn Meeting in Salzburg