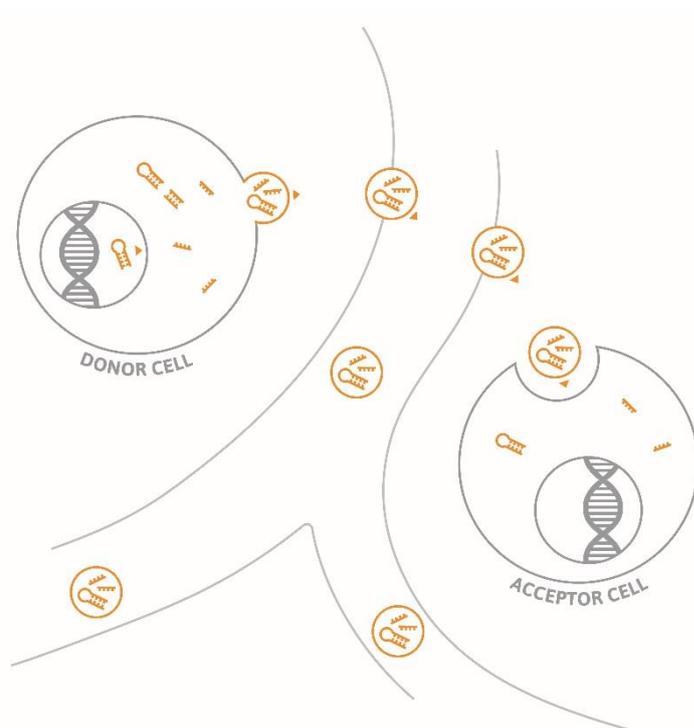


## TechNote TN-01:

# The impact of sample type (serum and EDTA-plasma) and platelet contamination on osteomiR detection

## Background

MicroRNAs are small non-coding RNAs that are 18-24 nucleotides in length and regulate gene expression by either suppressing translation or inducing mRNA degradation. Cells release a fraction of their microRNAs into the extracellular space. Extracellular (or circulating) microRNAs are protected from degradation by encapsulation in vesicles or through association with RNA binding proteins, and can be taken up by acceptor cells (figure 1). Circulating microRNAs can be found in plasma, serum as well as other biofluids. Circulating microRNAs show great potential as non-invasive biomarkers for various diseases, because microRNA expression or function is significantly altered in many disease states, including cancer, inflammatory diseases, metabolic disorders or osteoporosis.



**Figure 1 – Circulating microRNAs.**

MicroRNAs regulate gene expression and thereby contributed to control cell and tissue function. MicroRNAs are also secreted from cells (donor) transported in the circulation, and taken up by other cells at close or distant locations. Circulating microRNAs in cell-free blood can be used as biomarkers to diagnose or predict the presence, onset and progression of human disease.

The osteomiR™ RUO-kit allows the detection and quantification of selected microRNA biomarker candidates for fracture-risk in postmenopausal and diabetic osteoporosis. These microRNAs have been selected on the basis of three case/control studies conducted in patients with postmenopausal or secondary types of osteoporosis [1–4].

## Study Aim

MicroRNA analysis is influenced by pre-analytical variations [5]. **An important factor can be the choice of cell-free blood sample, such as serum or plasma:**

- **Serum** describes the cell-free blood component, which is obtained after activation of platelets and coagulation of blood cells. During activation platelets release proteins as well as RNA from their granules to enable the binding of other blood cells to form an insoluble clot.
- **Plasma** describes the cell-free blood component before coagulation of blood cells. In order to generate plasma, platelet activation must be inhibited through the addition of inhibiting agents such as EDTA, citrate or heparin. Plasma can be separated into two different types depending on the centrifugation parameters:
  - platelet rich plasma (PRP): single centrifugation step at lower speed. Contains residual platelets
  - platelet poor plasma (PPP): two centrifugation steps for complete depletion of platelets

Inadvertent platelet contamination of presumably cell-free plasma samples can be a major confounder. Platelets are small, anucleated blood cells which are derived from fragmentation of precursor cells in the bone marrow and play an important role in haemostasis and thrombus formation. According to latest research, platelets are rich in microRNAs shed from their precursor cells. Platelet contamination or insufficient inhibition can become a significant bias of circulating microRNA experiments.

In this study, we prepared serum, PPP, and PRP in parallel from three independent healthy donors and investigated the impact of sample type on the detection of bone biomarkers using the osteomiR™ workflow. In addition three platelet specific microRNAs (miR-21-5p, miR-24-3p, miR-150-5p) and a marker to determine liver toxicity in human (miR-122-5p) were analyzed in all samples [6,7].

## Material and Methods

Peripheral blood from 3 healthy male volunteer donors was collected after overnight fasting using a 21 G needle and serum and EDTA plasma collection tubes (VACUETTE® TUBE 4 ml Z Serum Separator Clot Activator Cat# 454067, VACUETTE® TUBE 4.5 ml K3E K3EDTA Cat# 454223, Greiner Bio-one).

Serum preparation: Blood was collected in red cap serum tubes and centrifuged at 2500 x g for 10 min after 30 min clotting time at room temperature. The supernatant was collected and aliquoted in 1.5 ml low nucleic acid binding tubes using 225 µl and stored at -80°C.

Plasma preparation: EDTA plasma samples were collected either at 4°C or at room temperature, allowing an incubation time of at least 30 min. For the PRP fraction, the plasma tubes were centrifuged at 2000 x g for 10 min. The whole plasma above the buffy coat was collected and transferred to 1.5 ml low nucleic acid binding tubes and stored at -80°C. The PPP preparation procedure consisted of two centrifugations steps either at 4°C or room temperature. In order to obtain PPP, the plasma tubes were centrifuged at 2000 x g for 10 min and the supernatant transferred to a 1.5 ml tube, followed by another centrifugation step at 10.000 x g for 10 min. 225 µl aliquots were prepared and stored in low bind tubes at -80°C.

MicroRNA qPCR was performed according to the protocol for the osteomiR™ RUO workflow.

## Results

### 1. Quality Control of samples

The data quality for each sample was monitored using synthetic spike-in controls provided with the kit: RNA-isolation efficiency (UniSp4), cDNA synthesis efficiency (cel-miR-39-3p) and PCR amplification (UniSp3) can be monitored to detect the presence of enzyme inhibitors and ensure equal purification efficiencies. Hemolysis was controlled by the ratio of miR-23a-3p and miR-451a-5p and optical density measurement at 414 nm (absorbance peak of the free haemoglobin).

**Table 1- Quality Control of samples.** Serum 1 was identified as an outlier due to hemolysis ratio > 7 and OD 414 nm > 0.3. All other samples exhibit uniform Cq values for the spike-ins

Sample Type	Serum	PPP	PPP	PRP	PRP
Temperature	RT	RT	4°C	RT	4°C
Donor 1	✗	✓	✓	✓	✓
Donor 2	✓	✓	✓	✓	✓
Donor 3	✓	✓	✓	✓	✓

### 2. Circulating microRNA variation – principal component analysis

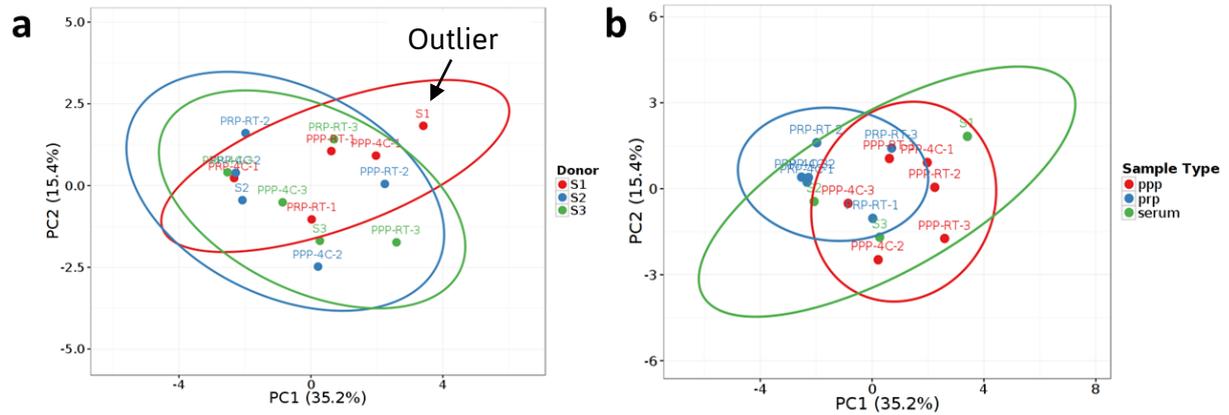
Normalized Cq-values were subject to exploratory data analysis using ClustVis, a web tool allowing principal component and heatmap clustering analysis of multivariate dataset using different R packages<sup>1</sup>.

Figure 2 illustrates the variance in osteomiR levels based on donor variability (a) and sample type (b). The results show that sample type impacts the levels of osteomiRs more than donor variation, especially in the context of PPP vs PRP. In addition, the low quality sample “S1” is a clear outlier. This shows that sample type and sample quality impact osteomiR detection.

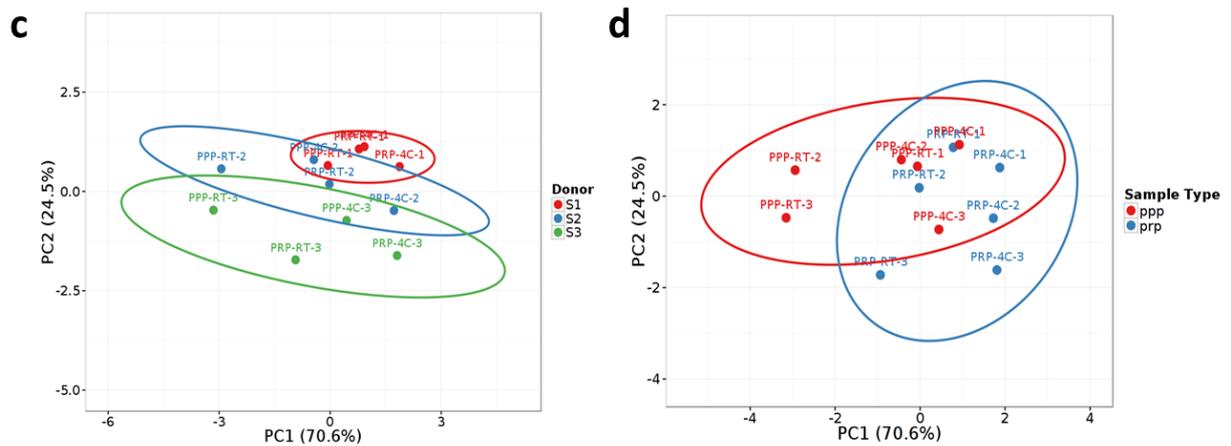
The same analysis was performed for known platelet-enriched microRNAs miR-150-5p, miR-21-5p and miR-24-3p (Fig 2c and d). As expected, the samples cluster according to sample type (PPP vs PRP).

<sup>1</sup> Metsalu T, Vilo J. ClustVis: A web tool for visualizing clustering of multivariate data using Principal Component Analysis and heatmap. *Nucleic Acids Res* 2015;43:W566–70.

OsteomiRs



Platelet miRNAs



**Figure 2 – Principal Component Analysis (PCA) of osteomiRs versus platelet specific microRNAs.** PCA analysis revealed donor-independent microRNA profiles for the osteomiRs (a) and demonstrates that the sample type highly affects the microRNA expression levels (b). Platelet-enriched microRNAs were found to be different between three healthy donors (c), and expectedly between PPP and PRP (d).

3. Differential expression analysis

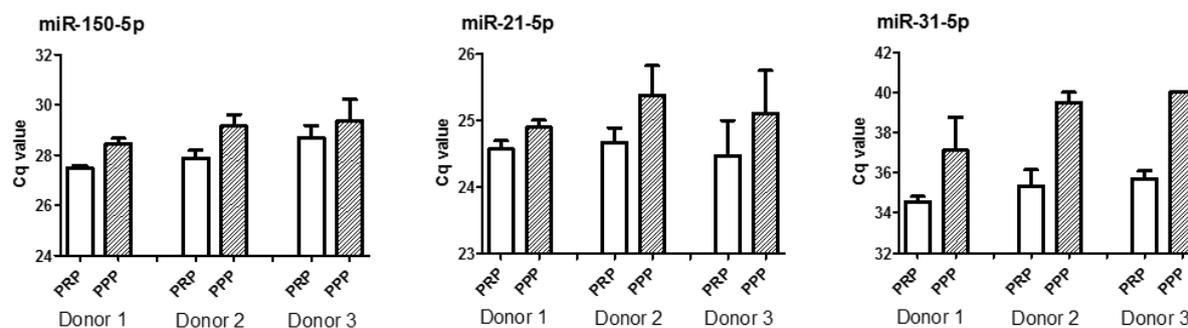
A paired two-tailed t-test was used to test for differences between PRP and PPP samples for each donor. The resulting p-values were adjusted for multiple testing using Benjamini Hochbergs method for false-discovery rate calculation (Table 1). Most microRNA levels were found to be higher in PRP vs PPP, which could be due to the contamination with intracellular microRNAs. Two of three tested platelet-enriched microRNAs (miR-150-5p and miR-21-5p) were found to be significantly up-regulated in PRP vs PPP. The third platelet miRNA, miR-24-3p, was ranked #4 (BH-adjusted p-value) but not significantly regulated. Liver-specific miR-122 was not found to be differentially regulated between PRP and PPP, and ranked on last place.

Out of 11 tested osteomiRs only miR-31-5p was found to be significantly up-regulated in PRP vs PPP, suggesting that this microRNA could be prone to analytical bias due to platelet contamination. All other osteomiRs were not found to be significantly regulated between PRP and PPP.

**Table 2 – Differential expression analysis** of 15 microRNAs of three individual healthy male donors between platelet rich plasma and platelet poor plasma, ranked according to the paired two-tailed p-values.

PRP versus PPP								
#	microRNA ID	biomarker function	Log2 FC	Linear FC	p-value	95% CI	Bonferroni adjusted p-value	Benjamini-Hochberg FDR adjusted p-value
1	miR-150-5p	platelet	0,96	1,95	0,001	1,348 to 0,5787	0,020	0,018
2	miR-21-5p	platelet	0,56	1,47	0,003	0,8149 to 0,3018	0,038	0,018
3	miR-31-5p	osteomiR	3,68	12,77	0,004	5,500 to 1,850	0,053	0,018
4	miR-24-3p	platelet	0,94	1,92	0,034	1,778 to 0,1083	0,504	0,126
5	miR-335-5p	osteomiR	2,61	6,09	0,059	5,364 to -0,1509	0,891	0,178
6	miR-550a-5p	osteomiR	2,88	7,34	0,085	6,322 to -0,5725	1,000	0,209
7	miR-29b-3p	osteomiR	0,38	1,30	0,098	0,8600 to -0,1000	1,000	0,209
8	miR-155-5p	osteomiR	2,59	6,03	0,139	6,379 to -1,192	1,000	0,247
9	miR-127-3p	osteomiR	2,16	4,45	0,148	5,397 to -1,087	1,000	0,247
10	miR-199b-5p	osteomiR	0,84	1,79	0,285	2,633 to -0,9599	1,000	0,418
11	miR-214-3p	osteomiR	-0,90	0,54	0,307	1,132 to -2,932	1,000	0,418
12	miR-203a	osteomiR	1,20	2,30	0,394	4,506 to -2,106	1,000	0,480
13	let-7b-5p	osteomiR	0,22	1,17	0,416	0,8705 to -0,4238	1,000	0,480
14	miR-188-3p	osteomiR	1,31	2,48	0,479	5,724 to -3,100	1,000	0,513
15	miR-122-5p	liver toxicity	-0,01	1,00	0,968	0,2947 to -0,3047	1,000	0,968

Figure 3 displays the microRNA levels of miR-150-5p, miR-21-5p and miR-31-5p in PRP and PPP for each donor. Although PRP levels are consistently higher than PPP levels, the trend between the three donors seems to be stable.



**Figure 3 – PRP and PPP expression levels of platelet specific miR-150-5p and miR-21-5p as well as osteomiR-31-5p** of three individual male donors. PRP exhibits higher expression levels than PPP for all donors.

## Discussion & Summary

In the present study, we have analyzed levels of 11 osteomiRs, 3 platelet-enriched miRNAs, 1 liver-specific microRNA and 3 synthetic controls in three healthy male donors using the osteomiR™ kit as well as individual microRNA assays. We aimed to determine whether the detection of osteomiRs is dependent on sample type and processing conditions.

### → **Data quality assessments are essential to detect potential outliers and improve results**

Therefore, it is important to carefully monitor the quality of each sample in respect to RNA extraction and reverse transcription efficiency. Hemolysis should be assessed using either spectrophotometric analysis (OD414) or a validated microRNA ratio [8], which is automatically calculated by the osteomiR software.

### → **osteomiR-31-5p levels could be affected by platelet contamination**

Exploratory data analysis revealed distinct circulating microRNA profiles in different sample types (PPP, PRP, serum). The presence of residual platelets in PRP leads to significantly increased levels of miR-31-5p levels compared to PPP samples. This contamination can be eliminated with an additional centrifugation step with higher speed force. During the entire study, the sample type and procedure should not be switched.

Therefore, it is of utmost importance to use standardized pre-analytical protocols throughout a clinical study and to train study nurses according to these specifications. Strict adherence to a standardized pre-analytical protocol is essential to obtain comparable qPCR-results.

We further recommend to include overnight fasting prior to blood collection. This can reduce biological variance in miRNA levels due to activity and diet. Circadian rhythm, activity and diet are known to influence the levels of circulating microRNA content in patients. In addition, hemolysis must be recorded for all samples as it can influence the expression levels of microRNAs.

We have also observed that temperature during EDTA plasma processing can reduce the technical variance of circulating microRNA analysis, which is likely due to more efficient inactivation of platelets.

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