TechNote TN-03 May 2018

Title: Circulating platelet-derived microRNAs (thrombomiRs) are non-invasive biomarkers for measuring the effects of P2Y12 anti-platelet therapy.

Background

Platelets are a rich source of microRNAs. They inherit their microRNA content from precursor cells and possess a fully functional machinery for post-transcriptional regulation [1]. There is compelling evidence that, compared to other cell types and tissues, certain microRNAs are highly enriched in platelets [2]. The stimulation of platelets via different receptors and agonists results in the secretion of these microRNA from platelets via extracellular vesicles (EVs) into the blood (plasma). Therefore, the activation as well as pharmacologic inhibition of platelets can result in an increase or decrease of plasma platelet-derived microRNA levels, respectively (Figure 1).

We have designed and developed the thrombomiR™ RT-qPCR[1] kit to facilitate standardized quantification of 10 platelet-derived microRNAs (*thrombomiRs*) and 6 quality controls in human plasma samples. This kit is based on a three-step protocol, consisting of i) RNA extraction, ii) cDNA synthesis, and iii) PCR amplification.

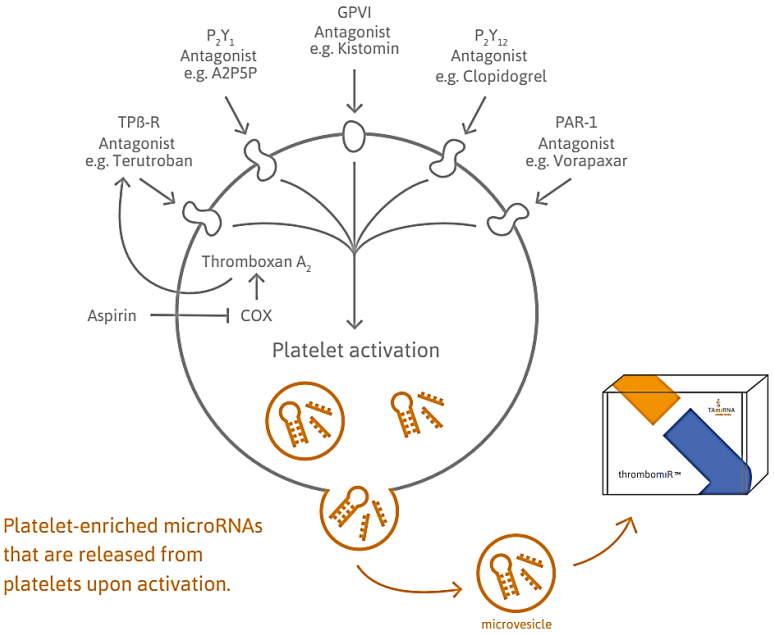


Figure 1: Concept of microRNA release from platelets in response to activating stimuli. Platelet microRNAs are released from platelets upon activation. This release is independent of the activation pathway (e.g. ADP, collagen, etc.). MicroRNAs are protected from degradation in serum/plasma due to vesicular encapsulation, and can be detected using an RT-qPCR based platform (the thrombomiR™ kit.

[1] Reverse-transcription quantitative PCR

There is accumulating evidence that thrombomiRs can be applied as biomarkers for:

- Non-invasive analysis of platelet reactivity.
- Monitoring of the effects of anti-platelet therapy.
- Platelet function and the risk of bleeding.
- Risk assessment of adverse cardiovascular events.
- All of this is possible based on analysis of stored (deep frozen) plasma samples.

In this technical note, we demonstrate the application of the thrombomiR™ kit to determine the effects of anti-platelet treatment using either clopidogrel or ticagrelor on plasma and serum levels of thrombomiRs in a cohort of young and healthy individuals.

Study design

30 healthy and treatment-naive volunteers were randomized to placebo, clopidogrel (Plavix), and ticagrelor (Brillique) treatment. Platelet-poor plasma was collected at baseline and after a 7-day course of therapy. Plasma samples from 5 randomly selected subjects per group were pooled, resulting in 4 pools per group (2 at baseline, 2 at follow-up), and 12 pools in total (see Figure 2).

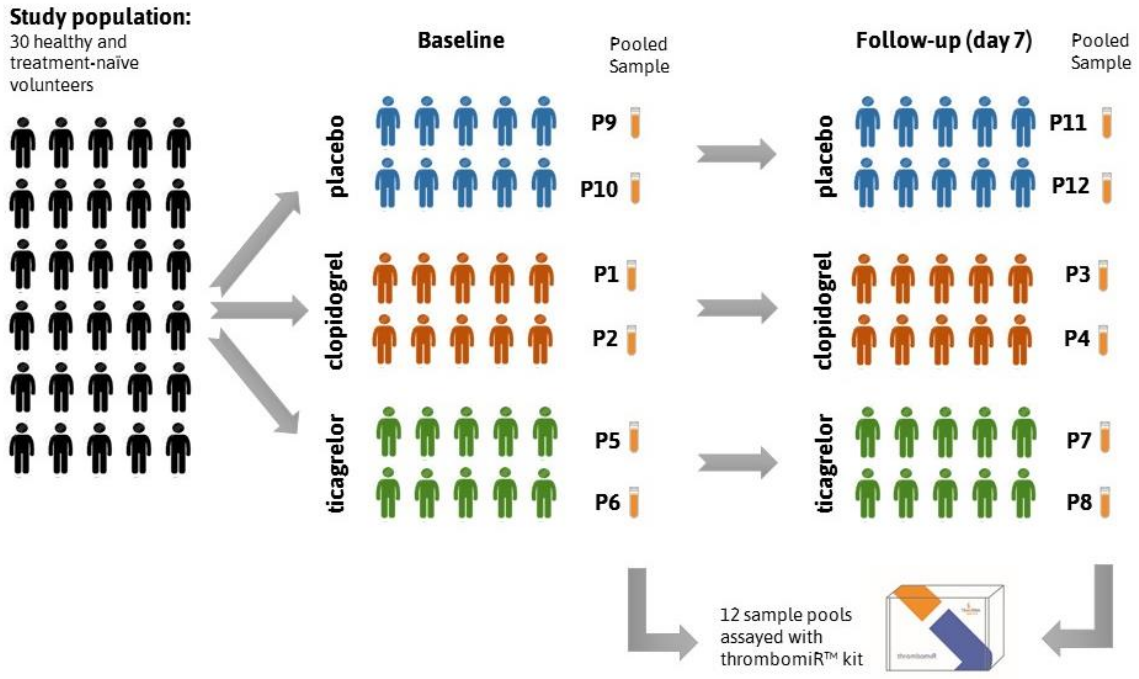


Figure 2. Study Design. 30 healthy individuals were randomized to three groups: clopidogrel, ticagrelor or placebo treatment (n=10 per group). Samples were taken at baseline as well as after a 7-day course of treatment. For analysis, 5 samples per group were pooled and used for RNA extraction. Thus, a total of 12 samples were used for the analysis with the thrombomiR™ kit.

Ten platelet-derived microRNAs (thrombomiRs) and one control microRNA (miR-122, liver-derived) were assayed in all pools using the thrombomiR™ kit (see Table 1).

Table 1 – Ten platelet-derived microRNAs can be analyzed using the thrombomiR™ kit. miR-122 serves as a liver-specific control that is not present in platelets. Three spike-in controls and two hemolysis controls (not listed in the table) complement this selection. Platelet-enrichment is based on results presented by Kaudewitz et al. in 2016 [3].

microRNA	platelet enrichment *	myocardial infarction **	T2D compli-cations **	tissue / cellar origin
hsa-miR-126-3p	+++	•	•	platelets & endothelium
hsa-miR-223-3p	+++	•	•	platelets & hematopoietic cells
hsa-miR-191-5p	+++			ubiquitous
hsa-miR-24-3p	++			platelets & epithelial cells
hsa-miR-21-5p	++			platelets
hsa-miR-28-3p	++		•	platelets & hematopoietic cells
hsa-miR-320a	++		•	ubiquitous
hsa-miR-150-5p	+			platelets & hematopoietic cells
hsa-miR-197-3p	+			platelets
hsa-miR-27b-3p	+		•	ubiquitous
hsa-miR-122-5p ***	–			liver

* Kaudewitz D et al 2016 ** platelet associated disorders *** negative control

Results

1. Quality Control of samples

Circulating microRNA analysis requires stringent quality control of RNA extraction, reverse transcription and qPCR efficiency. The thrombomiR™ RT-qPCR kit enables quality control by using synthetic spike-in controls that are 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 (Figure 3).

Spike-in based quality control is essential to

- Obtain a complete understanding of the analytical variability in the data.
- Identify potential outliers due to failed RNA extraction (UniSp4), reverse transcription (cel-miR-39) or PCR amplification (UniSp3).
- Choose an appropriate normalization strategy.

In addition, the thrombomiR™ test returns data on the effects of hemolysis on the analyzed samples by determining the ratio between miR-23a-3p and miR-451a. This ratio has been repeatedly shown to be indicative of red blood cell lysis in serum and plasma [4,5].

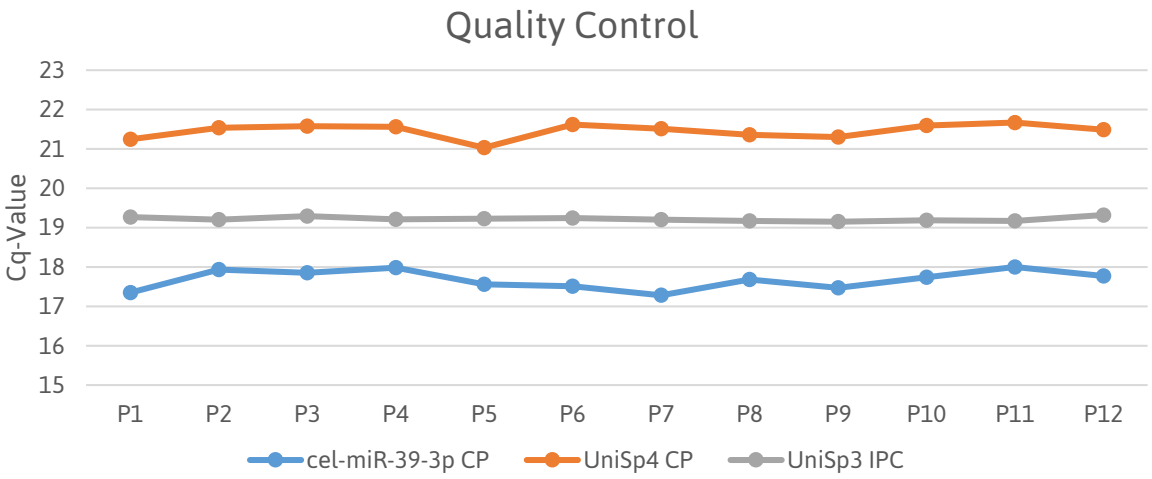


Figure 3 – Spike-In quality controls: controls show very low technical variation in the analysis and not outliers due to failed RNA extraction, reverse transcription or PCR. Standard deviation for RNA spike-in was 0.25 cycles.

2. Anti-platelet therapy results in an overall reduction in plasma levels of thrombomiRs.

The average relative change (%) between 7-day follow-up and baseline measurements for placebo, clopidogrel and ticagrelor were calculated using data from 10 thrombomiRs (Figure 4). The thrombomiR™ kit detected a significant and even decrease in plasma thrombomiR levels in one clopidogrel group (R1, 47% average reduction) and both ticagrelor groups (45% and 32% reduction, respectively). As expected, no significant changes in thrombomiR levels were observed in placebo treated groups (blue) as well as one group of clopidogrel treated individuals (R2, red).

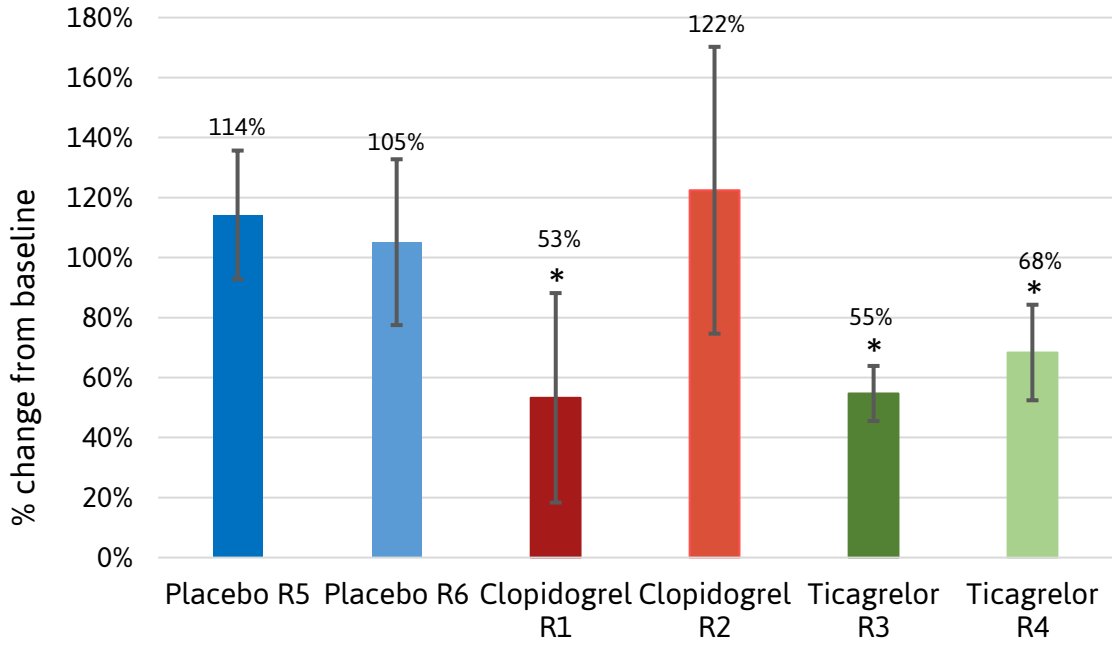


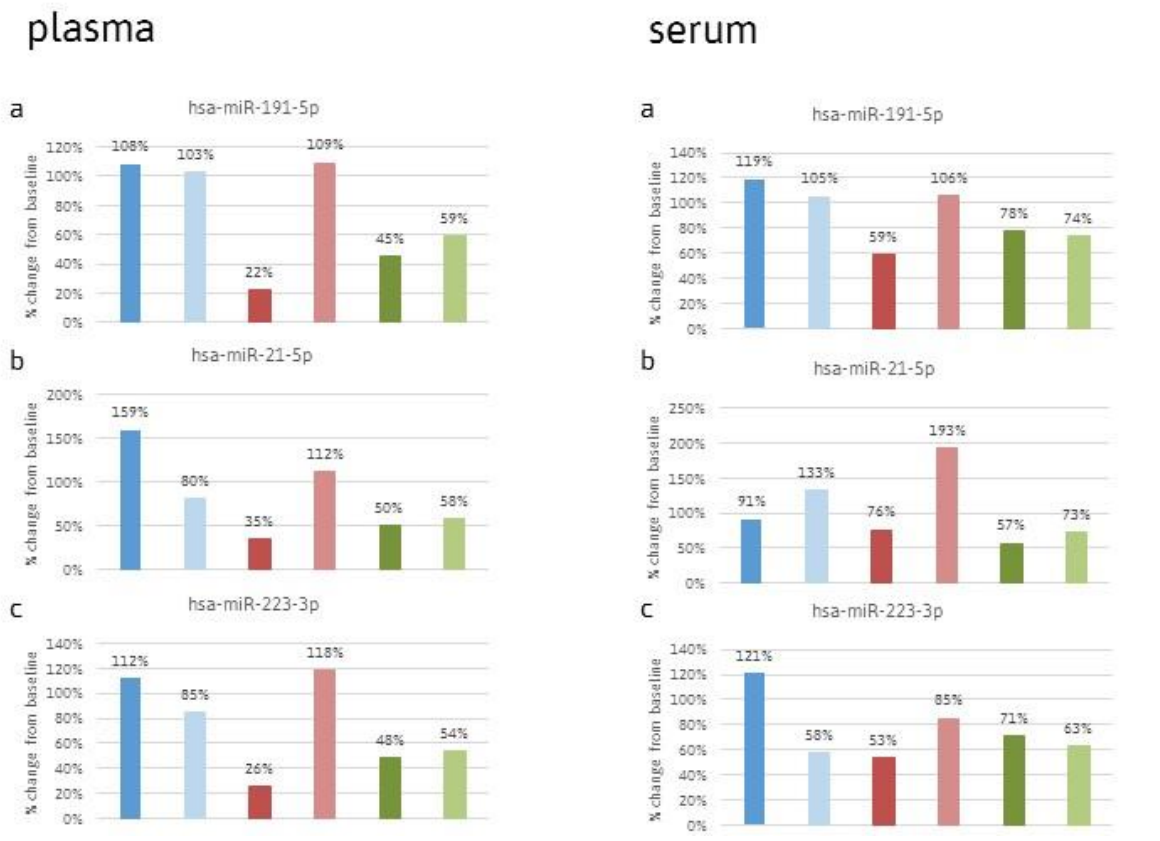
Figure 4 – The global effect of anti-platelet therapy on plasma thrombomiR levels. Bar charts represent the average relative reduction observed for all ten thrombomiRs levels following a 7-day course of anti-platelet therapy with placebo, clopidogrel, or ticagrelor. ANOVA and Tukey post hoc test for pairwise comparisons against Placebo. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

This observation is of relevance, because in contrast to ticagrelor, clopidogrel is a pro-drug that requires activation to the active drug compound by liver enzymes. There is evidence that specific livery enzyme variants are required for efficient activation, and that activation can fail in critically ill patients [6]. Therefore, our observation that only one group of patients treated with clopidogrel shows a significant reduction in thrombomiR levels, while both ticagrelor groups experienced a significant thrombomiR decline in plasma might be related to inadequate treatment response.

3. Platelet-derived microRNAs are significantly reduced in plasma but not serum following a one-week course of mono anti-platelet therapy.

Further to the analysis of the global (average) changes in thrombomiR levels, individual changes in thrombomiR were analyzed in both plasma and serum, which were collected simultaneously at baseline and follow-up on day 7. The relative changes of circulating microRNAs with highest enrichment in platelets are shown in figure 5: miR-126-3p, miR-191-5p, miR-197-5p, miR-21-5p, miR-223-3p, and miR-24-3p.

ThrombomiRs with the strongest reduction as a consequence of anti-platelet therapy (> 50% decline in at least 2/4 treatment groups) and compared to placebo were miR-191-5p, miR-197-3p, miR-21-5p, and miR-223-3p. For those microRNAs, the reduction in platelet-poor plasma was stronger compared to that in serum (figure 5 a-d). Circulating levels of microRNAs with weaker enrichment in platelets, specifically miR-150-5p, which is present in leukocytes and reflects inflammatory processes and miR-320a, which is a pro-angiogenic factor, did not show a strong decline in any of the four treatment groups (data not shown)



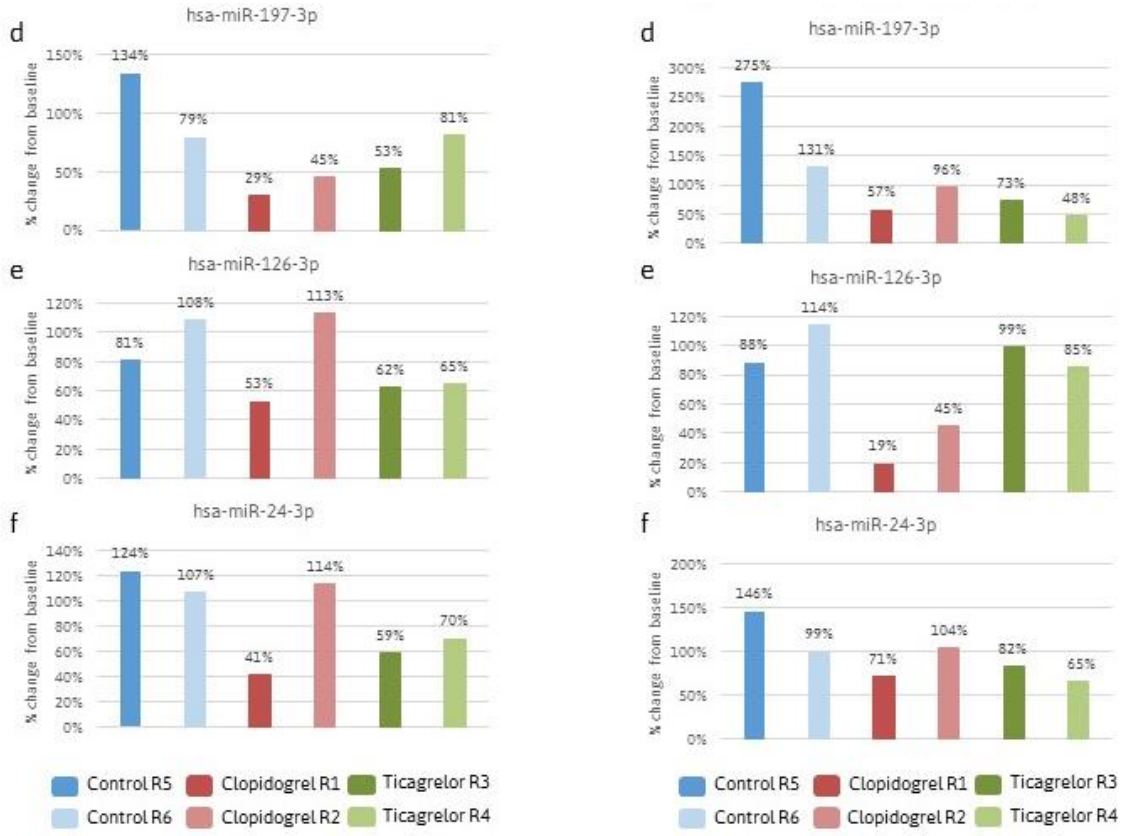


Figure 5 – Relative changes in thrombomiRs levels in plasma and serum. ThrombomiRs were measured before and after a 7-day course of anti-platelet therapy (placebo, clopidogrel and ticagrelor). MicroRNAs are presented in alphabetical order.

4. Circulating levels of miR-122 increase after a one-week course of clopidogrel treatment, while remaining constant in ticagrelor treated individuals.

Finally, it was observed that miR-122-5p plasma and serum levels showed an increase in both clopidogrel treated groups (178% and 213%) between follow-up on day 7 and corresponding baseline levels, while the relative increase in both ticagrelor groups was relatively weak (Figure 6). miR-122-5p transcription is highly activated in liver tissue, while absent in most other cell types. In liver, miR-122 is known to regulate a number of genes associated to fatty acid and cholesterol synthesis [7]. Changes in circulating levels of miR-122 are known to be associated with lipid levels an informative of liver injury as well as the risk of new-onset metabolic diseases [8].

Therefore, a potential explanation for the increase in miR-122 in clopidogrel groups is the liver-dependent activation of the pro-drug to form the active drug. In comparison, no changes in miR-122 were observed in case of the already activated drug ticagrelor. These results indicate, that the liver-dependent activation of clopidogrel might exert metabolic burden on hepatocytes, resulting in an increased secretion of miR-122-5p.

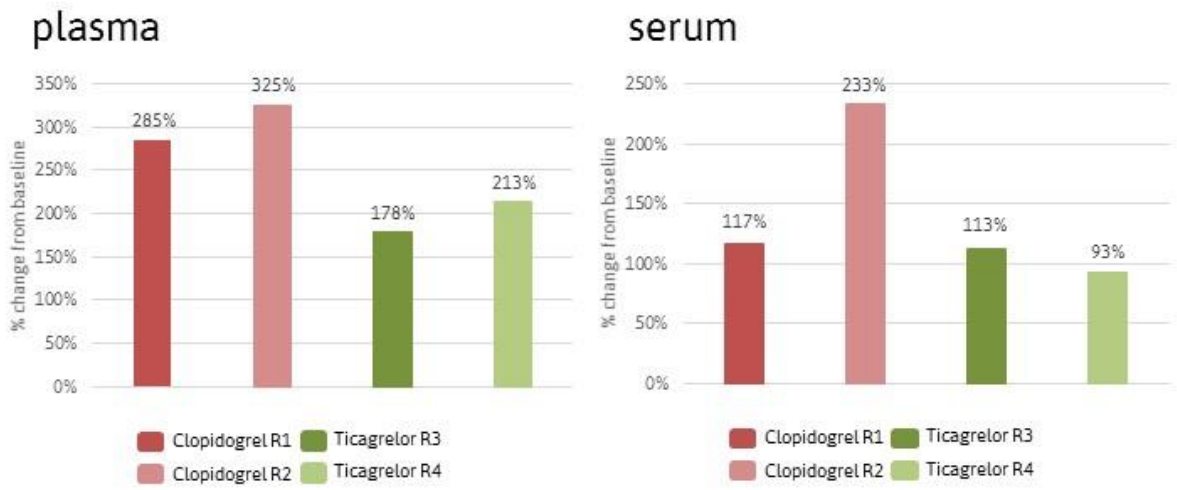


Figure 6 – Relative changes in miR-122-5p levels in plasma and serum.

Summary

The thrombomiR™ kit provides an easy-to-use RT-qPCR protocol that includes all reagents required for standardized analysis of ten platelet-derived microRNAs in human plasma or serum. Here, we have demonstrated the application of the kit to quantify the effect of two types of P2Y12 mediated anti-platelet therapies on thrombomiRs™ levels in platelet-poor plasma and serum. We demonstrate that ticagrelor results in an even and significant reduction of thrombomiRs™ levels after 7-days. We further show that the thrombomiRs™ response to clopidogrel treatment is more heterogeneous, and might result in an enhanced liver metabolism compared to ticagrelor treatment.

Acknowledgements

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References

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