SUPPLEMENTARY INFORMATION FILE

Effects of pathogen reduction systems on platelet microRNAs, mRNAs, activation, and function

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SUPPLEMENTARY METHODS

Platelet isolation, count, and volume

A platelet-rich plasma (PRP) was obtained by centrifugation of a platelet concentrate (PC) sample at 1,000 g for 10 min, followed by filtration through leukocyte depletion filters (Pall Corporation). The platelet suspension was subsequently incubated with CD45 antibody coupled to magnetic microbeads (EasySep, StemCell Technologies) for negative selection of PC platelets. Highly purified platelets were counted, aliquoted, harvested by centrifugation at 1,000 g for 10 min, frozen in a dry-iced ethanol bath, and stored at -80°C prior to protein and RNA extraction, as described previously.[1,2] Platelet count and mean platelet volume (MPV) in PCs were measured on a Sysmex XE-5000 Analyzer (Sysmex, Norderstedt, Germany).

qPCR analyses of selected platelet microRNAs

Platelets were isolated from PRP samples collected from the PCs on days 1, 4 and 7 after treatment (n=10 for each treatment group), and total RNA extracted. qPCR analyses were performed using TaqMan miRNA Expression Assays on 96-well plates (Life Technologies), containing dried primers and probes for 11 selected platelet microRNAs. One negative control, ath-MIR159a (expressed in *Arabidopsis thaliana*), and the U6 snRNA reference control, were

also included in each 96-well plate (Supplementary Table S1). Fifty ng of cDNA were used for each qPCR reaction in a total volume of 20 µl.

Relative quantification of microRNA expression was performed by comparing the expression level of each target gene to that of one reference gene (the U6 small nuclear RNA [snRNA]) by the ΔΔCt model.[3] The U6 snRNA was the only of 4 possible reference genes considered (U6 snRNA, RNU24, RNU44, and RNU48) that met 4 a-priori criteria for use in data normalization (Supplementary Figure S1), namely being: (i) a non-microRNA internal reference gene, (ii) a small RNA, (iii) abundantly expressed in platelets, and (iv) stably expressed across our samples [4]. The other 3 genes were either weakly or not stably expressed in the platelet samples that we tested (Supplementary Figure S1A) [4].

qPCR analyses of platelet *BCL2L1*, *CLU* and *BCL2* mRNAs

Platelets were isolated from PRP samples collected from the PCs at 1.5 and 20 h after treatment (n=6 for controls and n=4 for each treatment group). Following platelet total RNA extraction, we quantified the absolute number of copies of the pro-survival *BCL2L1* and B-cell CLL/lymphoma 2 (*BCL2*) mRNAs, and of the anti-apoptotic Clusterin (*CLU*) mRNA, the three of which are strongly associated with platelet survival.[5] For reverse transcription of these mRNAs, High Capacity cDNA Reverse Transcription Kit was utilized, as recommended by the manufacturer (Life Technologies). Absolute quantification of mRNA was performed using an eight-point standard curve in duplicates on a StepOnePlus qPCR apparatus (Life Technologies).

Each qPCR reaction contained 0.1 µM of each forward and reverse primer, 2× SYBR Select Master Mix (Life Technologies) and nuclease-free water to a final volume of 20 µl. The primers used for amplification of *BCL2L1* (forward: GGGCATTCAGTGACCTGACA; reverse:

TCCCGGAAGAGTTCATTCACTAC), *CLU* (forward: CAGGCCATGGACATCCACTT; reverse: CGCCTTCTCGTATGAATTCTGTT) and BLC2 (forward: GGGATGCCTTTGTGGAACTG; reverse: GGGCCAAACTGAGCAGAGTCT) were designed by PrimerExpress Software (Life Technologies) and generated amplicons of 91, 70 and 90 nt, respectively.

Platelet microRNA biosynthetic capacity

Briefly, 50 μg of cleared platelet protein extracts (supernatant of a 10-min centrifugation at 10,000 g; S10) were incubated with 5' ^{32}P-labeled human let-7a-3 pre-microRNA (5'-UGAGGUAGUAGGUUG UAUAGUUUGGGGCUCUGCCCUGCUAUGGGAUAACUAUACAAUCUACUGUCUUUCC -3'; miRBase accession number MI0000062) in assay buffer at 37°C for 30 min. The reaction was stopped by adding 0.5 mg/ml proteinase K (Ambion) and incubating at 50°C for 20 min. After a phenol/chloroform extraction step, the RNA products were precipitated with ethanol, resuspended in water, resolved by denaturing PAGE 12%, and analyzed by autoradiography and densitometric analyses.[1,2]

Platelet microRNA functional capacity

Briefly, 50 μg of cleared platelet protein extracts (supernatant of a 1-h centrifugation at 100,000 g; S100) were incubated with the ^{32}P-labeled RNA sensor (10,000 cpm) in assay buffer containing 20 mM Hepes, 50 mM potassium acetate, 2.5 mM $MgCl_2$, 1 mM ATP, 0.2 mM GTP, 1 mM DTT, 2.5% (v/v) Superase•In, 0.18% (v/v) Triton X-100, pH 7.6 at 30°C for 30 min. The reaction was stopped by adding 0.5 mg/ml proteinase K (Ambion) and incubating at 50°C for 20 min. After a phenol/chloroform extraction step, the RNA products were precipitated, resuspended

in water, separated by 8% denaturing PAGE, and analyzed by autoradiography and densitometric analyses.[1,2]

Platelet activation tests

Briefly, platelets were diluted with HEPES-Tyrode's buffer supplemented with 0.1% bovine serum albumin to a density of $5x10^5$ platelets per test. Platelets were initially incubated with buffer or, in the case of the positive control, 1 U/ml thrombin (Sigma) for 15 min at room temperature. Subsequently, all samples were stained for 20 min at room temperature in the dark with fluorochrome-conjugated antibodies against CD41a (Becton Dickinson GmbH, Heidelberg) to identify platelets, and against CD62p (Becton Dickinson GmbH) to detect activated platelets. Events (10,000 per sample) were recorded using a LSR II(TM) flow cytometer (Becton Dickinson GmbH), and the data were analyzed with FACSDiva(TM) software (BD). CD41a-positive events associated with anti-CD62p fluorescence intensities exceeding those seen in platelets from the Control group samples were interpreted as activated platelets.[6]

Platelet function tests

Platelet aggregation assays were performed by a single experienced medical technologist and monitored on an APACT-4 aggregometer (Labitec, Arensburg, Germany). Platelets were stimulated by adding 25 µl of ADP (Progen, Heidelberg, Germany) or thrombin-receptor activating peptide (TRAP; Bachem, Bubendorf, Switzerland) to 225 µl of PRP to obtain final concentrations of 20 µM ADP and 40 µM of TRAP, respectively. Twenty µl of collagen fibrils from equine tendon (Nycomed, Linz, Austria) were added to 230 µl of PRP to achieve a final concentration of 4 µg/L. The platelet count of the PRP was not adjusted. Aggregation was recorded for 7 min. The difference in light transmission on a linear scale between PRP and

platelet-poor plasma (PPP; prepared by centrifugation of each PC at 3,500 g for 10 min) was set to 100% to calculate the maximum change in light transmission induced by platelet aggregation. Any disaggregation was also recorded.

Statistical analysis

qPCR data were analyzed with DataAssist software (LifeTechnologies) for relative qPCR quantification. For each of the 11 microRNAs tested, fold changes were calculated, showing how many times the level of each microRNA was changed, by each treatment (Additive Solution, Irradiation, Mirasol, and Intercept), at each time point (day 1, 4, or 7 of storage), compared with its level in the Control group at the same time point. Therefore, a difference was considered significant when the two-sided p value remained < 0.05 after correction for 132 comparisons (4 treatments times 11 microRNAs times 3 time points).

The number of copies of each of the 3 anti-apoptotic mRNAs was compared across the 5 groups (Control, Additive Solution, Irradiation, Mirasol, Intercept) at 1.5 and 20 h post-treatment, by Kruskal-Wallis analysis of variance (ANOVA). If $p<0.05$ for a difference across all 5 groups, each of the 4 treatments (Additive Solution, Irradiation, Mirasol, and Intercept) was compared with the Control group by a Mann-Whitney test with correction for multiple comparisons.

Data from Dicer and RISC activity assays, platelet activation and aggregation tests, platelet volume and platelet count in PCs, were compared across 5 groups at each time point (day 1, 4, or 7 of storage) by non-parametric ANOVA, followed by a Mann-Whitney test.

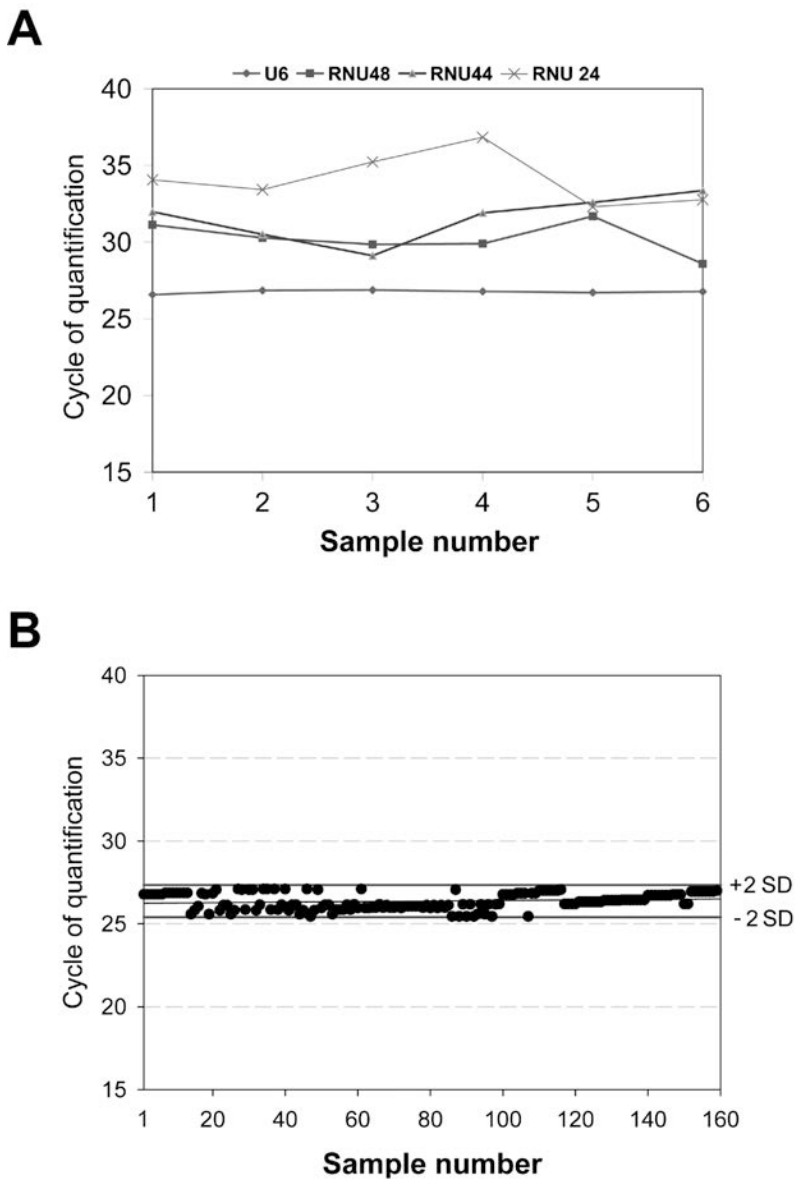
Finally, for each treatment and each of the 11 microRNAs, the existence of a linear correlation between the relative change in the percentage of activated platelets and the microRNA fold change was tested by a general linear model using SAS 9.3 software. Day of storage (1, 4, and 7) was introduced as an independent variable into the model.

SUPPLEMENTARY TABLE

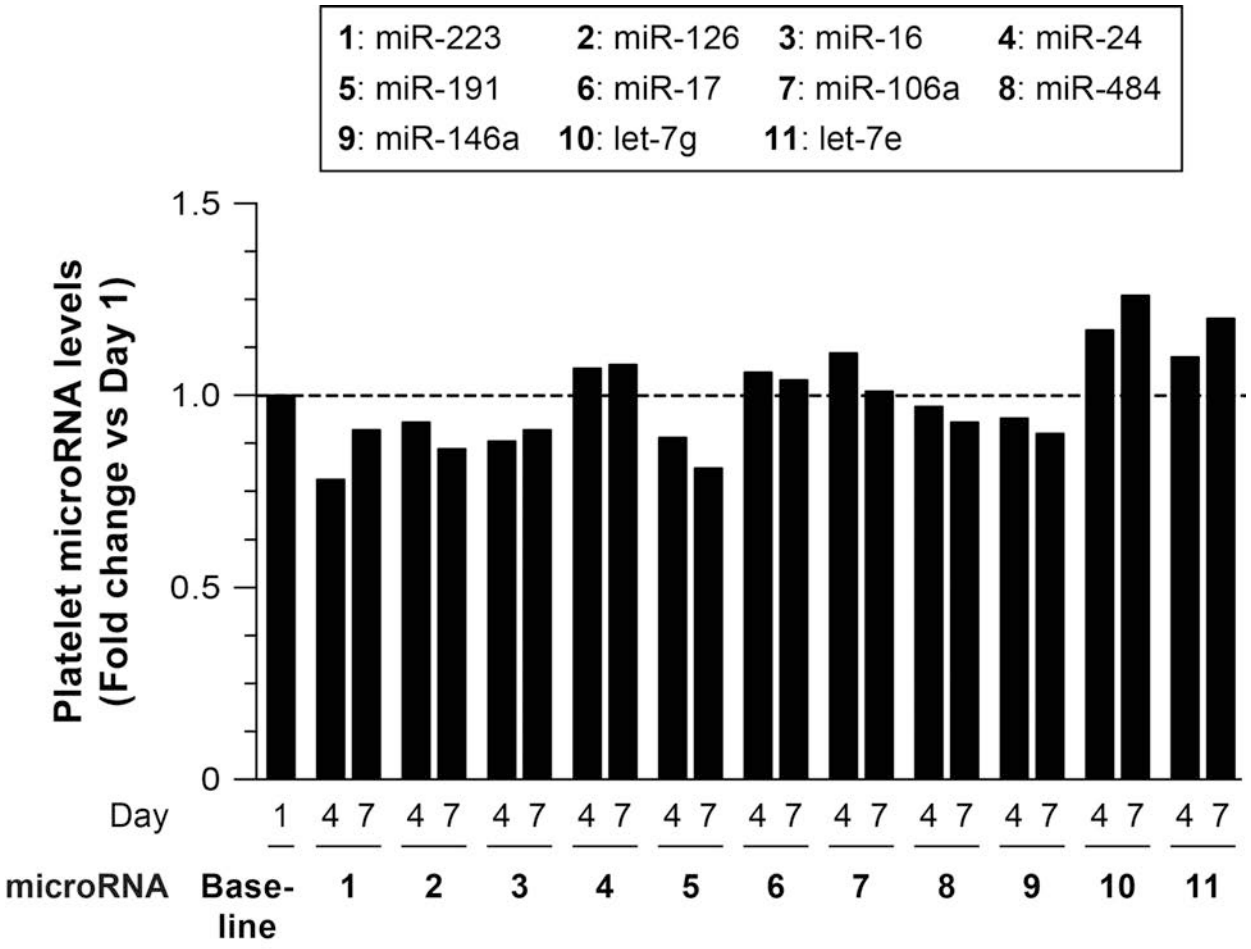
Supplementary Table S1. Accession number and sequence of the RNAs analyzed by qPCR.

miRBase Accession Number	Gene ID/ Reference Number	RNA Species	Mature Sequence (5' → 3')
MIMAT0000280	hsa-miR-223	MicroRNA - Major strand	UGUCAGUUUGUCAAAUACCCCA
MIMAT0000445	hsa-miR-126	MicroRNA - Major strand	UCGUACCGUGAGUAAUAAUGCG
MIMAT0000069	hsa-miR-16	MicroRNA - Major strand	UAGCAGCACGUAAAUAUUGGCG
MIMAT0000080	hsa-miR-24	MicroRNA - Major strand	UGGCUCAGUUCAGCAGGAACAG
MIMAT0000440	hsa-miR-191	MicroRNA - Major strand	CAACGGAAUCCCAAAAGCAGCUG
MIMAT0000070	hsa-miR-17	MicroRNA - Major strand	CAAAGUGCUUACAGUGCAGGUAG
MIMAT0000103	hsa-miR-106a	MicroRNA - Major strand	AAAAGUGCUUACAGUGCAGGUAG
MIMAT0002174	hsa-miR-484	MicroRNA - Major strand	UCAGGCUCAGUCCCCUCCCGAU
MIMAT0000449	hsa-miR-146a	MicroRNA - Major strand	UGAGAACUGAAUUCCAUGGGUU
MIMAT0000414	hsa-let-7g	MicroRNA - Major strand	UGAGGUAGUAGUUUGUACAGUU
MIMAT0000066	hsa-let-7e	MicroRNA - Major strand	UGAGGUAGGAGGUUGUAUAGUU
-	U6 snRNA	Small nucleolar RNA - Normalization control	GUGCUCGCUUCGGCAGCACAUA UACUAAAAUUGGAACGAUACAGA GAAGAUUAGCAUGGCCCCUGCG CAAGGAUGACACGCAAAUUCGUG AAGCGUUCCAUAUUUU
MIMAT0000177	ath-miR-159a	MicroRNA from *Arabidopsis thaliana* - Negative control	UUUGGAUUGAAGGGAGCUCUA

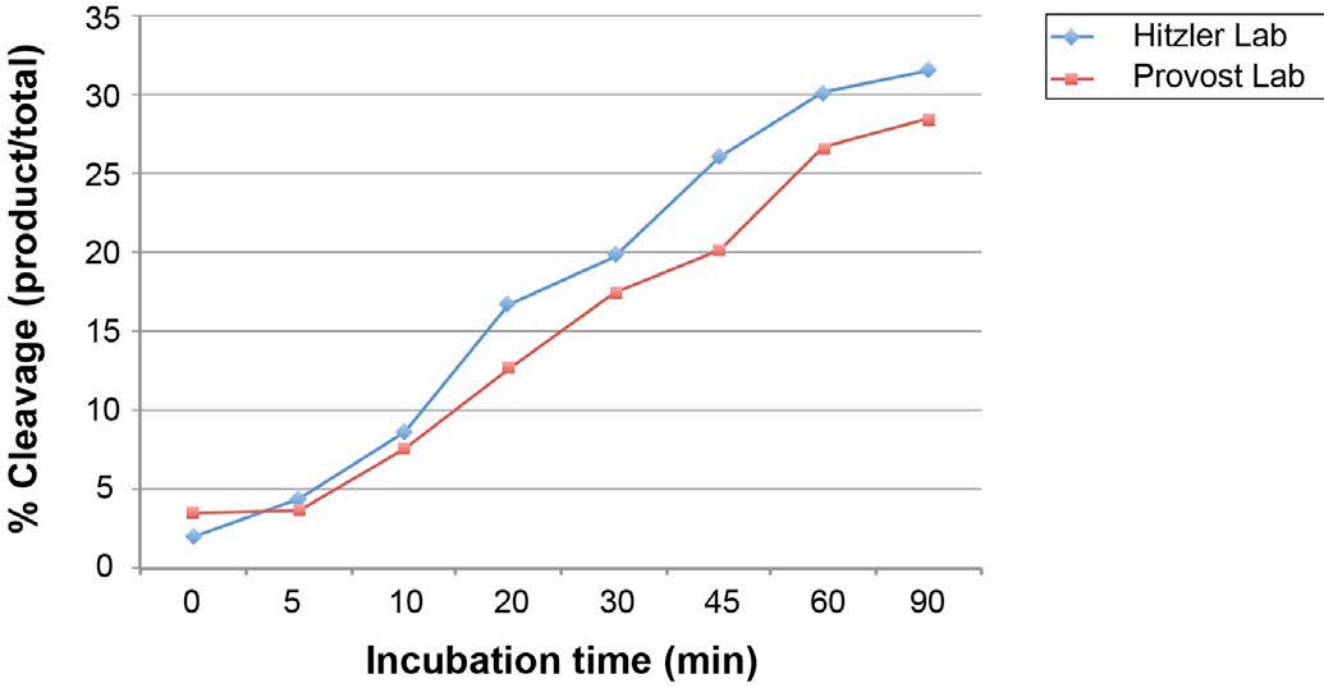
SUPPLEMENTARY FIGURES



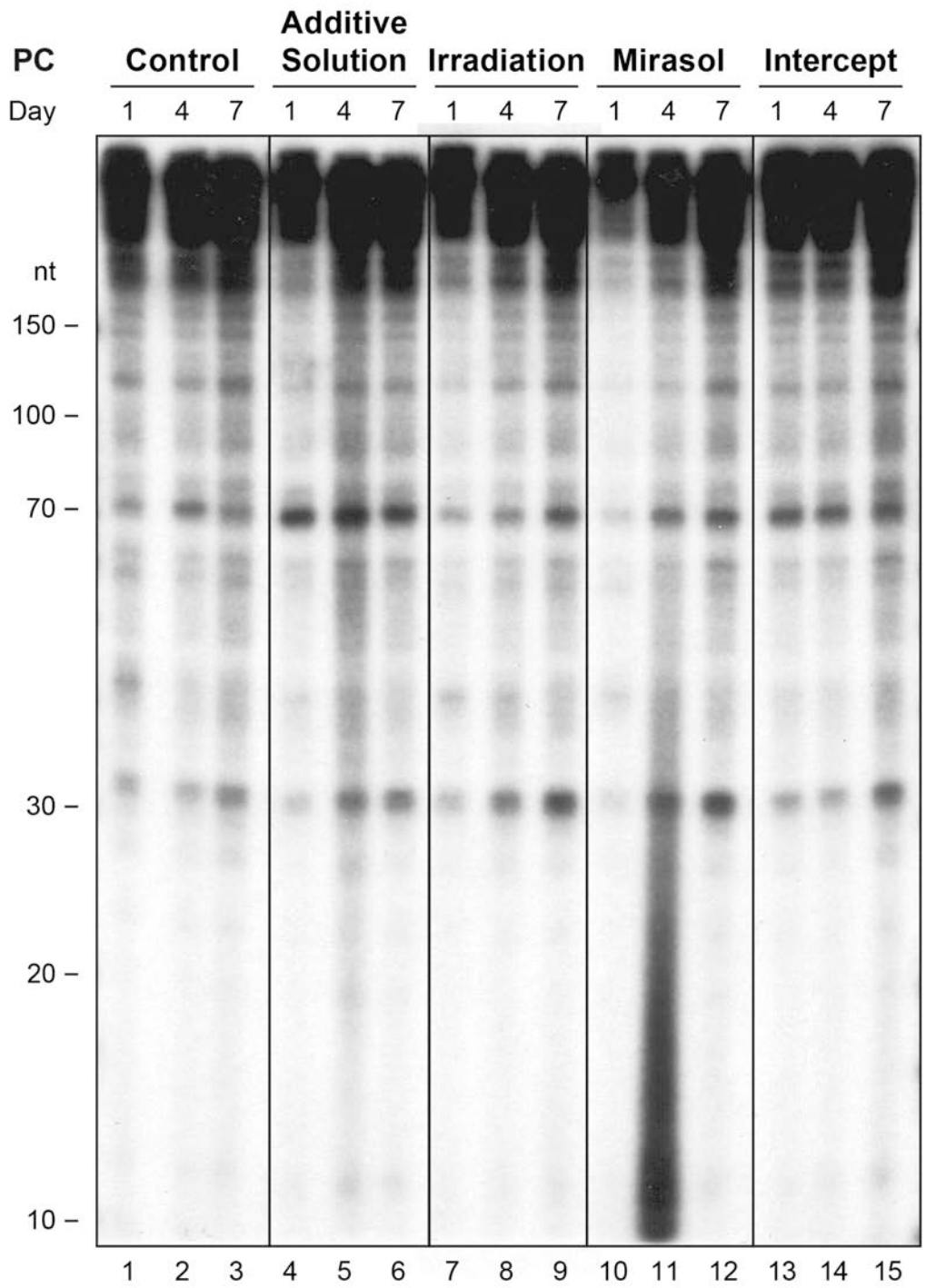
Supplementary Figure S1. Selection of U6 snRNA as a normalization control for platelet microRNA levels quantified by qPCR. (A) qPCR monitoring of the levels of the small nuclear RNA (snRNA) U6, and of the small nucleolar RNAs RNU48, RNU44, and RNU24 in human platelets isolated from 6 healthy individuals. U6 snRNA showed the highest abundance and lowest degree of inter-individual variation, in terms of fluctuations in the cycle of quantification (cycle threshold; Ct) among the samples tested. (B) U6 RNA levels were quantified by qPCR analysis of 159 samples of stored platelets from the 5 groups under study. The Ct values obtained remained within 2 standard deviations (SDs) of the mean. NormFinder software-based analysis confirmed that U6 RNA was the most stably expressed snRNA analyzed, thereby supporting the rationale for using U6 RNA as a normalization control for the relative quantification of platelet microRNA levels.



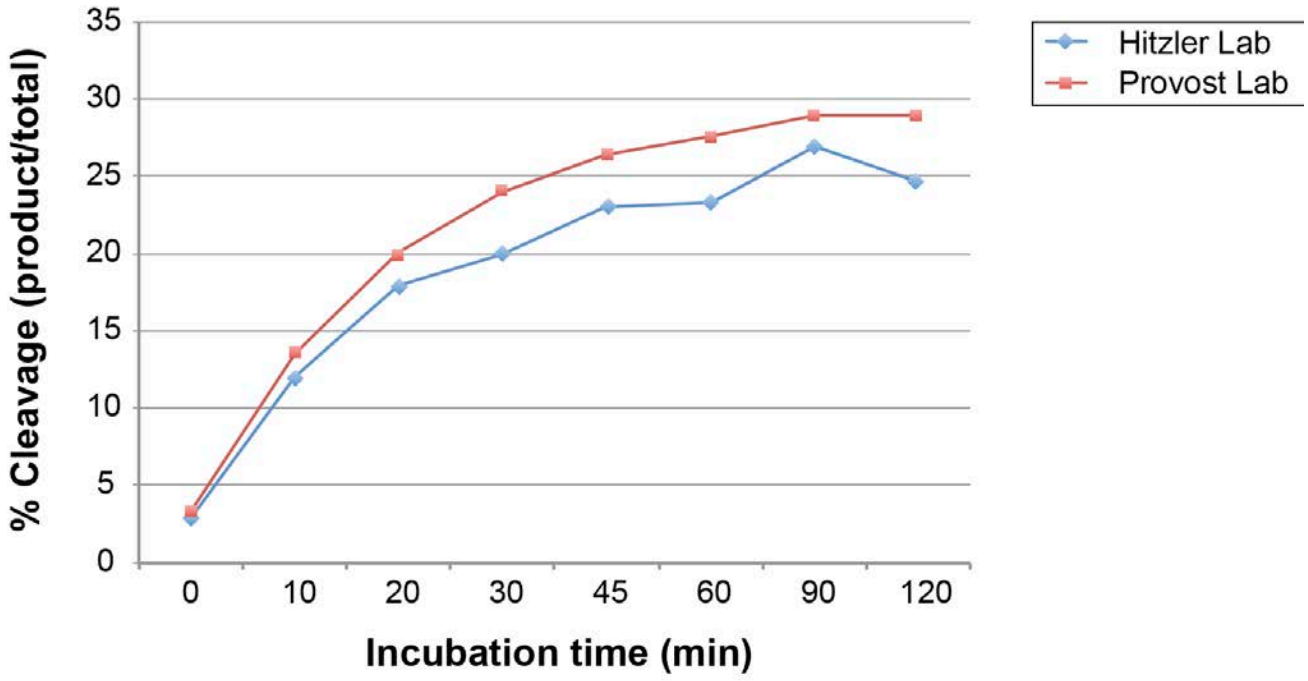
Supplementary Figure S2. Platelet microRNA levels remained stable between Day 1 and Day 7 of storage in control samples. The figure shows the median fold change of the Day 4/Day 1 and Day 7/Day 1 ratios for each individual platelet microRNA level in control samples on Day 4 or Day 7 of storage, compared to their level in controls on Day 1 of storage (n=10). No significant difference in microRNA levels was observed between Day 4 and Day 1, as well as betwen Day 7 and Day 1 of storage.



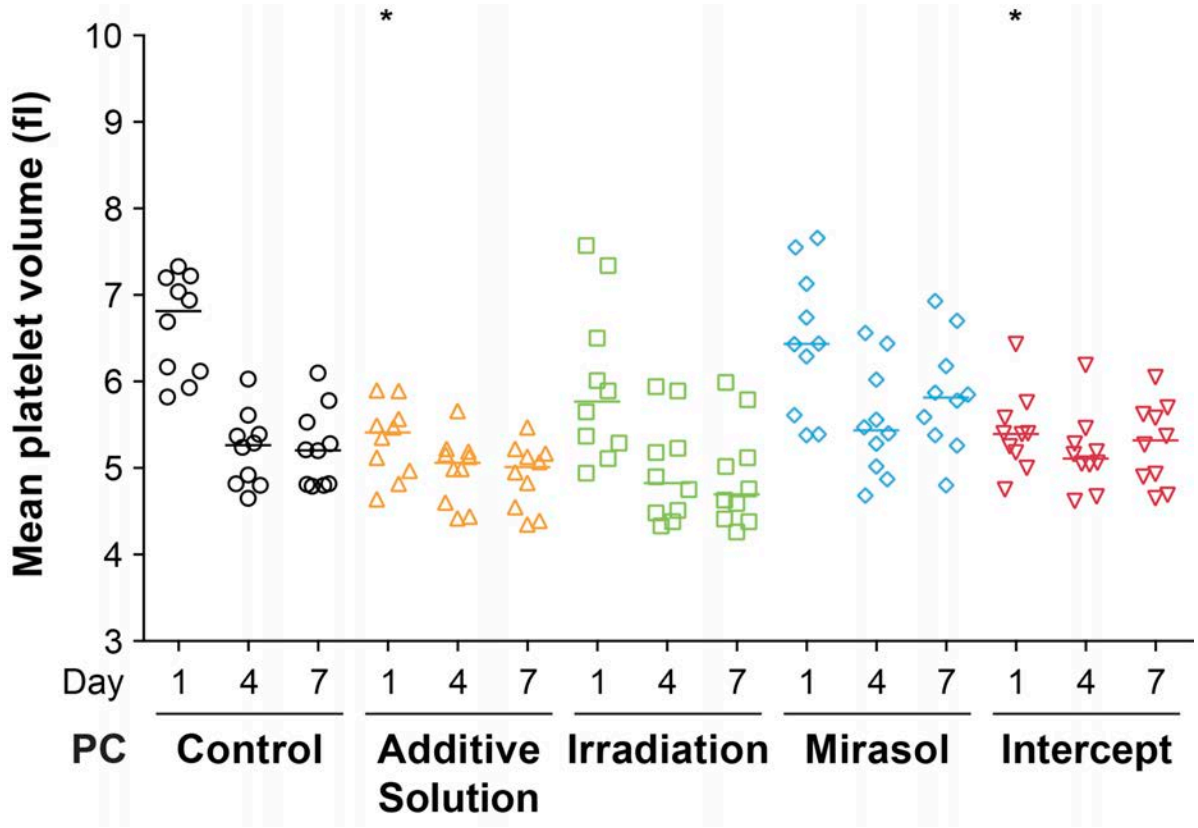
Supplementary Figure S3. Reproducibility of the Dicer activity assay using platelet protein extracts prepared from platelets isolated in the Hitzler and Provost laboratories. Platelet microRNA biogenesis was assessed in Dicer activity assays. Five prime (5') ^{32}P-labeled let-7a-3 pre-microRNA was incubated in the presence of 50 µg of S10 platelet protein extracts at 37°C for different periods of time. The reaction was stopped by adding proteinase K, followed by phenol/chloroform extraction and ethanol precipitation of RNA. The cleavage reaction was resolved by 12% denaturing PAGE and autoradiography, and the intensity of the bands was quantitated to calculate the percentage of pre-microRNA cleavage by Dicer.



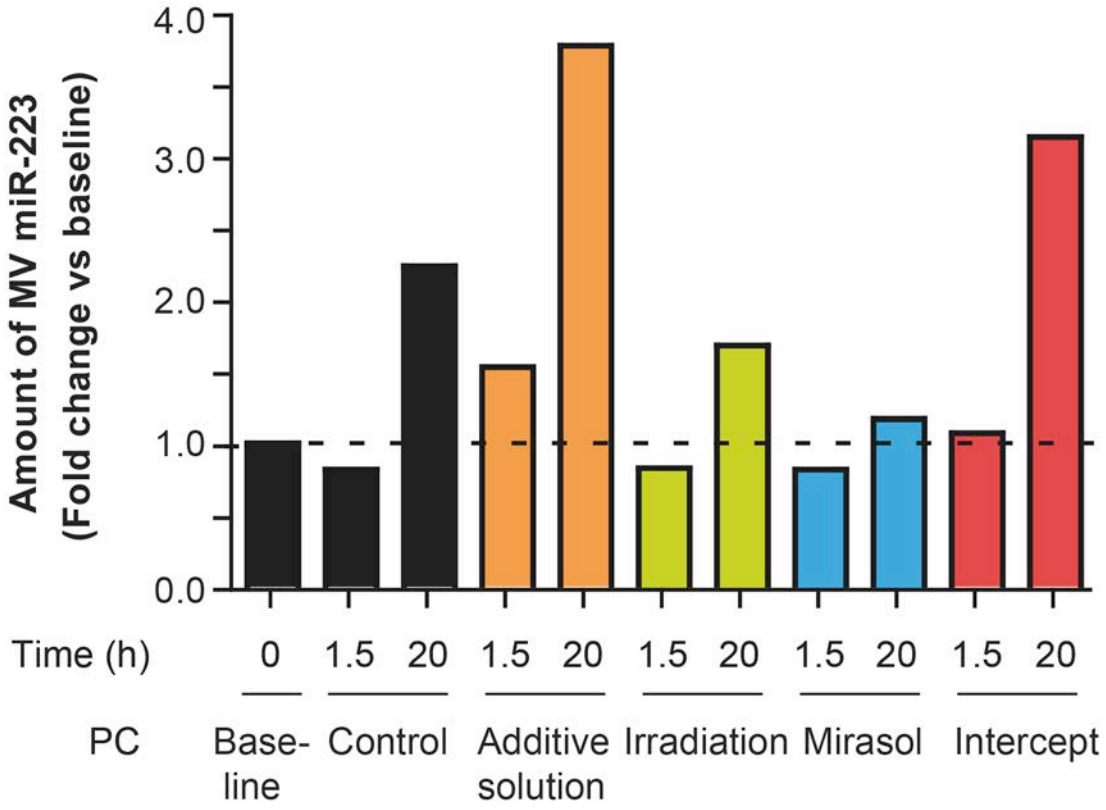
Supplementary Figure S4. Storage in Additive Solution or treatment with Irradiation, Mirasol, or Intercept does not induce formation of cross-linked adducts of endogenous platelet RNAs. The figure shows the lack of cross-linked adducts of endogenous platelet RNAs induced by PR treatments, as visualized by total RNA extraction, dephosporylation, ^{32}P labeling, and analysis by denaturing PAGE in any of the 5 groups (Control, Additive Solution, Irradiation, Mirasol, or Intercept) at any time point (day 1, 4, or 7 of storage). Compare with Figure 4, which shows the results in the absence of dephosphorylation.



Supplementary Figure S5. Reproducibility of the RISC activity assays using platelet protein extracts prepared from platelets isolated in the Hitzler and Provost Laboratories. Platelet microRNA function was assessed in RISC activity assays. Five prime (5') ^{32}P-labeled mRNA sensor perfectly complementary to miR-223 was incubated in the presence of 50 µg of S100 platelet protein extracts at 37°C for different periods of time. The reaction was stopped by addition of proteinase K, followed by phenol/chloroform extraction and ethanol precipitation of RNA. The cleavage reaction was resolved by 8% denaturing PAGE and autoradiography, and the intensity of the bands was quantitated to calculate the percentage of Ago2-mediated sensor RNA cleavage.



Supplementary Figure S6. Effect of storage in Additive Solution or treatment with Irradiation, Mirasol, or Intercept on mean platelet volume. The figure shows the mean platelet volume in samples collected from the PCs on day 1, 4, or 7 for each group (Control, Additive Solution, Irradiation, Mirasol, or Intercept). Individual data are shown along with the median (n=10 donors per group). * indicates $p < 0.05$ (i.e., a statistically significant difference between the corresponding treatment and the Control group on the same day of storage). Fl, femtoliter.



Supplementary Figure S7. Effect of storage in Additive Solution or treatment with Irradiation, Mirasol, or Intercept on the total amount of microparticular miR-223 in the supernatant fraction. The figure shows the total amount of microparticular miR-223 present in the supernatant fraction collected from a small number of PCs each group (Control, Additive Solution, Irradiation, Mirasol, or Intercept) 1.5 h and 20 h after treatment. Microparticles were isolated,[7] and the total amount of microparticular miR-223, one of the most abundant platelet microRNAs,[2,8] was analyzed by qPCR, as described previously.[7] Data are expressed as mean fold changes versus the baseline values (n=3-4 donors per treatment group; n=7 donors in the control group).

SUPPLEMENTARY REFERENCES

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