Circulating exosomal microRNAs in acquired aplastic anemia and myelodysplastic syndromes

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 doi:10.3324/haematol.2017.182824

Received: October 20, 2017.

Accepted: April 18, 2018.

Pre-published: April 19, 2018.

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Supplemental Methods

Exosome extraction

Isolation of exosomes from plasma was carried out using the PureExo Exosome Isolation kit (101Bio, Palo Alto, CA, USA). Briefly, after thawing at room temperature (RT), 200 μL of plasma and 800 μL of Sample Buffer were dispensed into each of four 1.5 ml tubes and kept on ice. In a separate tube, Solutions A, B, and C were mixed according to manufacturer's instructions and distributed 350 μL per tube containing diluted plasma. Subsequently, samples were vigorously inverted, incubated on ice for 20 min, and centrifuged at 5000 RPM for 5 min using a benchtop centrifuge (Centrifuge 5424; Eppendorf, Hauppauge, NY, USA). After removing top transparent layers, pellets were centrifuged again at 5000 RPM for 3 min. Then, top and bottom transparent layers were removed and pellets were centrifuged again. After repeat of this step one more time, pellets were air-dried for 10 min at RT and subsequently resuspended in 150 μL of sterile PBS (Lonza, Walkersville, MD, USA). Samples were then vigorously mixed and shaken at 1400 RPM for 3 min twice, followed by centrifugation at 7400 RPM for 5 min. Clear supernatant was transferred to the PureExo column and subjected to centrifugation at 3600 RPM for 5 min. Flow-through containing exosomes were collected and stored at -80°C until use.

RNA extraction and cDNA synthesis

To obtain high quality RNA, a beads-based RNA purification protocol was performed using the Direct-zol™ -96 MagBead RNA kit (Zymo Research, Irvine, CA, USA). Manufacturer's instructions were optimized as follows: 125 μL of exosomes in PBS was transferred into a 1.5 mL tube, mixed with 300 μL of Trizol-based N1 Solution (from the Exosomal RNA extraction kit, 101Bio), and incubated at RT for 5 min, followed by addition of 300 μL of Direct-zol™ Binding Buffer and then vortexing. Each sample was then incubated for 20 min at 750 RPM by

adding 10 μL of MagBinding Beads and treated with DNase I. After washing steps, eluted RNA was quantified using the Nanodrop ND-100 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Extracted RNA (mean, 14.98±3.7 ng/μL; range, 7.4-25.1 ng/μL) was subjected to cDNA synthesis with the miScript® II RT kit (Qiagen, Hilden, Germany). The protocol was optimized as follows: 8 μL of 5x miScript HiSpec Buffer, 4 μL of 10x miScript Nucleics Mix, 4 μL of miScript Reverse Transcriptase Mix, and 24 μL of template RNA (final volume, 40 μL) were gently mixed and centrifuged. Samples were incubated at +37°C for 60 min and then at +95°C for 5 min. Undiluted cDNA was stored at -20°C until use.

MiRNA profiling

Initial screening of 372 miRNAs and 12 miRNA controls was performed in the discovery set using the miScript® miRNA PCR Array Human Serum & Plasma 384HC array (MIHS-106ZE, Qiagen) and the miScript® SYBR® Green PCR kit (Qiagen). The real-time thermal cycler 7900HT Fast Real-Time PCR System with 384-Well Block Module (Applied Biosystems, Thermo Fisher Scientific) equipped with SDS 2.3 software was programmed according to manufacturer's instructions. For validation, Custom miScript miRNA PCR Array (CMIHS02531E, Qiagen) was designed including 42 targeted candidate miRNAs and six miRNA controls listed in the Qiagen miRNA Array system. As internal controls, reverse transcription control (miRTC) and positive PCR control (PPC) were included in the plates. Ct values of the PPC should be 19±2, and Ct values of miRTC were examined using the following formula: $\Delta Ct = (AVG\ Ct^{miRTC} - 1.1) - AVG\ Ct^{PPC}$. If values were ≤7, no apparent reaction inhibition was occurred and data were included in the analysis. For normalization, a snoRNA, SNORD61, was chosen and three miRNAs homogeneously expressed in the discovery set were included (miRNA-339-3p, miRNA-211-5p, and miRNA-30c-5p) as previously reported.

Subsequently, a geometric mean of SNORD61 and miRNA-211-5p miRNAs within each group (HC, SAA, and MDS patients) was used for normalization. Each sample was run in duplicate.

Extraction and concentration of exosomal proteins

Protein concentration measurement was performed to confirm the presence of extracted exosomes from plasma samples, as previously described.[31] Protein extraction from exosomes was carried out using the Exosomal RNA and Protein extraction kit (101Bio) by mixing 70 μL of a sample and 120 μL of exosomal protein lysis buffer (101Bio). Clear supernatant was store at -80°C until use. Protein concentration was assessed with the Micro BCA™ Protein Assay kit (Thermo Fisher Scientific). Absorbance was measured at 562 nm by the VICTOR[3] 1420 multilabel plates reader (PerkinElmer, Waltham, MA, USA). Each sample was run in duplicate. Extracted proteins (mean, 9175.5$\pm$4293.6 μg/mL) were stored at -80°C for further studies.

Nanoparticle tracking analysis, immunoblotting, and transmission electron microscopy

To confirm the presence of exosomes in the flow-through, particle size measurement, CD63 expression, and transmission electron microscopy (TEM) were performed as previously described [*Osteikoetxea X, Németh A, Sódar BW, Vukman KV, Buzás EI.J. Extracellular vesicles in cardiovascular disease: are they Jedi or Sith? Physiol. 2016;594(11):2881-2894*]. Exosomal protein measurement was assayed colorimetrically by the VICTOR[3] 1420 multilabel plates reader (PerkinElmer), while the NanoSight NS300, an instrument for Nanoparticle Tracking Analysis (NTA) (Malvern Instruments Ltd, Malvern, Worcestershire, UK), was utilized to characterize nanoparticle sizes (*Online Supplementary Figure S1A-C*). Immunoblot was performed using an exosome-specific anti-CD63 antibody (1:50 dilution) (clone, TS63; Thermo Fisher Scientific) and β-actin as a loading control (clone, N-21; Thermo Fisher Scientific)

(*Online Supplementary Figure S1D*). Extracted exosomes and undiluted plasma from 3 healthy controls, and serum albumin, were subjected to protein extraction, as described above. Undiluted plasma was the positive control for the presence of exosome-specific CD63, and serum albumin the negative control. For each sample, 60 μg of protein were loaded onto a Novex WedgeWell 4-12% Tris-Glycine Gel (Thermo Fisher Scientific), and transferred onto a PVDF membrane using the iBlot 2 Dry Blotting System (Thermo Fisher Scientific). Immunoblot was performed with iBind™ Western Device (Thermo Fisher Scientific), according to manufacturer's instructions. Samples for TEM were purified exosomes (EXOP-145A-1; System Biosciences, Palo Alto, CA), and extracted exosomes and undiluted plasma from the same SAA patient (*Online Supplementary Figure S1E-H*). Samples were placed on 400-mesh formvar- and carbon-coated grids (Electron Microscopy Sciences, Hatfield PA) for five min, and excess sample absorbed with filter paper. The grids were rinsed in water three times and stained with 2% w/v uranyl acetate solution for five minutes. Images were obtained using a JEOL JEM 1200EX transmission electron microscope (JEOL USA, Peabody MA) equipped with an AMT XR-60 digital camera (Advanced Microscopy Techniques Corporation, Woburn MA), at the Electron Microscopy Core Facility, NHLBI.

RNase-treated exosomes and miRNA profiling

To confirm that RNA was confined within exosomes, 100 μl of extracted exosomes were treated with 4 μl of RNase A (0.4 μg/μl) (Roche, Basel, Switzerland) for 5 min at RT, and reaction terminated with 8 μl of Protector RNase Inhibitor (40 U/μl) (Roche) [*Valadi H, Ekström K, Bossios A, Sjöstrand M, Lee JJ, Lötvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol. 2007;9(6):654-659*]. Samples were centrifuged at 7400 RPM for 5 min, and clear supernatant

transferred to the PureExo column for centrifugation at 3600 RPM for 5 min. Flow-through containing exosomes were collected, and RNA extraction and miRNA profiling were performed as described above. Correlation analysis between exosomes from the same donor with or without RNase A treatment was carried out by Pearson analysis (*Online Supplementary Figure S1F*). In addition, data analysis was performed using the web-based tool from Qiagen, as described below, and no significant changes with or without RNase exposure between samples were detected.

Data analysis and statistics

Data were analyzed using Prism (v.7.02; GraphPad software, La Jolla, CA, USA). For data analysis of the miRNA PCR array, miScript miRNA PCR Array Data Analysis software was employed (Qiagen) (https://www.qiagen.com/it/shop/genes-and-pathways/data-analysis-center-overview-page/). miRNAs were selected if significant differences (P<0.05) among individual groups were observed by pair-wise group comparison. A 1.5-fold-change (FC) threshold was chosen as a cut-off as previously reported.[9] Ingenuity Pathway Analysis software (www.ingenuity.com, v33559992, Qiagen Bioinformatics) was used for pathway analysis. Differentially expressed exosomal miRNAs were selected for analysis using Ingenuity Knowledge Base (Genes Only) as a reference set. Additionally, the following analysis settings were included: direct and indirect relationships including endogenous chemicals, interaction and causal networks, all node types, all tissues and cell lines for human or uncategorized species, and a confidence set for experimentally observed or high (predicted) interactions. For further statistical analysis, Log_2 conversion of data was performed as previously described.[9] Receiver operating characteristic (ROC) curves and the area under the ROC curve (AUC) were used to assess sensitivity and specificity for the diagnosis of the 48 miRNAs selected for the validation

set. Unpaired (Mann Whitney test) or paired (Wilcoxon matched-pairs signed rank test) two-tailed t-tests for two group comparison and one-way analysis of variance (ANOVA) using Kruskal-Wallis test for three-group comparison were performed. Multiple comparison tests included two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli. Correlations between miRNA relative expression levels and clinical parameters were carried out using Pearson correlation analysis. A $P<0.05$ was considered statistically significant.

For pathway analysis, VENNY 2.1 (http://bioinfogp.cnb.csic.es/tools/venny/), an interactive tool for comparing lists with Venn Diagrams, was used to find common or unique miRNAs among SAA, MDS, and SAA-responder patients. Predicted targeted genes for each miRNA were identified using miRWalk 2.0 database (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/). Then, results were filtered in order to find genes exclusively or commonly targeted in SAA or MDS, and data were used for pathway analysis by IPA software.

For prediction analysis, miRWalk 2.0 was employed. Putative target genes were found by choosing the position 2 as start position of miRNA seed and a minimum seed length of 6 nucleotides, as previously described [*Ellwanger DC, Büttner FA, Mewes HW, Stümpflen V. The sufficient minimal set of miRNA seed types. Bioinformatics. 2011;27(10):1346-1350.*]. The promoter region (2 kb), 3' UTR, coding sequence (CDS), and 5' UTR of known genes were checked in miRWalk 2.0, RNA22, miRanda, and Targetscan databases.

Supplementary Tables

Supplementary Table S1. Characteristics of donors of the discovery and validation sets.

Supplementary Table S2. Custom miScript miRNA PCR Array (CMIHS02531E) for the validation set

Supplementary Table S3. Statistical analysis of the validation set data three-group comparison one-way ANOVA and receiver operating characteristic (ROC) curves.

Supplementary Table S4. Pearson correlation analysis between clinical parameters and miRNA expression levels in SAA before and after therapy and MDS patients. Statistical analysis of miRNA expression in SAA patients before and after IST by paired t-test.

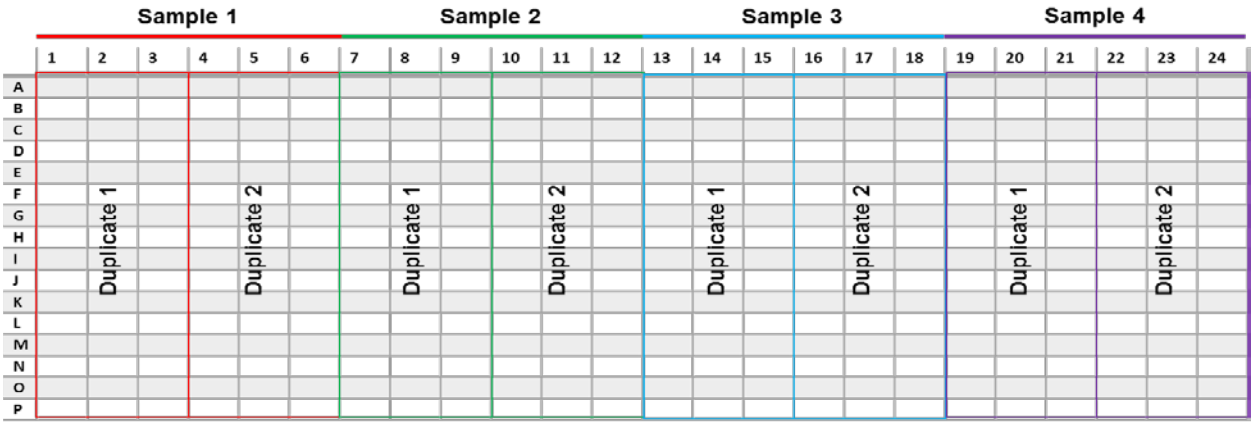
Supplementary Table S5. Canonical pathway analysis using differentially expressed miRNAs only in SAA, MDS or SAA-responders, or common in SAA and MDS.

Table S1. Characteristics of patients and healthy controls for the discovery and validation sets

	SAA	MDS	HC
Discovery set (n=42)			
No.	16	10	16
Median age, years (range)	33 (6-70)	54 (23-71)	34 (25-67)
Sex (M/F)	8/8	5/5	8/8
Disease status			-
SAA	16		
WHO			
RCUD		5	
MDS-U		2	
RCMD		1	
RARS		1	
RAEB-1		1	
IPSS			
Low		0	
Int-1		9	
Int-2		1	
BM cellularity			
Hypocellular MDS		7	
Normocellular MDS		0	
Hypercellular MDS		3	
Treatment		-	-
hATG+CsA+Eltrombopag	16		
Not evaluable	0		
Transfusion	15/16		-
Not evaluable	0	10	
Median follow-up (months)	7.5 (2.7-20.5)	-	-
Chromosome abnormalities		-	-
Del5q			
Monosomy 7/ Del of chr 7			
Del20q			
Trisomy 8	1		
Negative	15		
Validation set (n=99)			
No.	44	20	35
Median age, years (range)	34 (2-75)	49.9 (23-71)	36.5 (23-58)
Sex (M/F)	21/23	11/9	15/21
Disease status			-
SAA	44		
WHO			
RCUD		5	
MDS-U		5	
RCMD		5	
RARS		2	
RAEB-1		3	
IPSS			
Low		3	
Int-1		11	
Int-2		4	
BM cellularity			
HypoMDS		4	
Normocellular MDS		11	
Hypercellular MDS		4	
Not evaluable		1	
Treatment		-	-
hATG+CsA+Eltrombopag	9		
rATG+CsA	12		
hATG+CsA	19		
Not evaluable	4		
Transfusion	43/46	15/20	-
Not evaluable	0	3	
Median follow-up (months)	52.3 (0.3-160.2)	41.2 (0.8-128.7)	-
Chromosome abnormalities			-
Del5q	1		
Monosomy 7/ Del of chr 7	4		
Del20q	1		
Trisomy 8	0		
Negative	40	20	

SAA: severe acquired aplastic anemia; MDS: myelodysplastic syndromes; HC: healthy controls; WHO: World Health Organization; RCUD: refractory cytopenia with unilineage dysplasia; MDS-U: unclassifiable MDS; RCMD: refractory cytopenia with multilineage dysplasia; RARS: refractory anemia with ring sideroblasts; RAEB-1: refractory anemia with excess blasts; IPSS: International Prognostic Scoring System; BM: bone marrow; hATG: horse anti-thymocyte globulin; CsA: cyclosporine A; chr: chromosome; rATG: rabbit ATG.

Table S2. Custom miScript miRNA PCR Array (CMIHS02531E) for the validation set



Position	miRBase Accession No.	Mature miRNA ID
A1, A4, A7, A10, A13, A16, A19, A22	MIMAT0015085	hsa-miR-3200-3p
B1, B4, B7, B10, B13, B16, B19, B22	MIMAT0000226	hsa-miR-196a-5p
C1, C4, C7, C10, C13, C16, C19, C22	MIMAT0004488	hsa-miR-15a-3p
D1, D4, D7, D10, D13, D16, D19, D22	MIMAT0004502	hsa-miR-28-3p
E1, E4, E7, E10, E13, E16, E19, E22	MIMAT0005878	hsa-miR-1287-5p
F1, F4, F7, F10, F13, F16, F19, F22	MIMAT0000280	hsa-miR-223-3p
G1, G4, G7, G10, G13, G16, G19, G22	MIMAT0000081	hsa-miR-25-3p
H1, H4, H7, H10, H13, H16, H19, H22	MIMAT0000692	hsa-miR-30e-5p
I1, I4, I7, I10, I13, I16, I19, I22	MIMAT0002888	hsa-miR-532-5p
J1, J4, J7, J10, J13, J17, J19, J22	MIMAT0001080	hsa-miR-196b-5p
K1, K4, K7, K10, K13, K16, K19, K22	MIMAT0000770	hsa-miR-133b
L1, L4, L7, L10, L13, L16, L19, L22	MIMAT0004500	hsa-miR-26b-3p
M1, M4, M7, M10, M13, M16, M19, M22	MIMAT0000101	hsa-miR-103a-3p
N1, N4, N7, N10, N13, N16, N19, N22	MIMAT0000072	hsa-miR-18a-5p
O1, O4, O7, O10, O13, O16, O19, O22	MIMAT0000063	hsa-let-7b-5p
P1, P4, P7, P10, P13, P16, P19, P22	MIMAT0000435	hsa-miR-143-3p
A2, A5, A8, A11, A14, A17, A20, A23	MIMAT0000762	hsa-miR-324-3p
B2, B5, B8, B11, B14, B17, B20, B23	MIMAT0001340	hsa-miR-423-3p
C2, C5, C8, C11, C14, C17, C20, C23	MIMAT0005825	hsa-miR-1180-3p
D2, D5, D8, D11, D14, D17, D20, D23	MIMAT0000427	hsa-miR-133a-3p
E2, E5, E8, E11, E14, E17, E20, E23	MIMAT0017987	hsa-miR-3610
F2, F5, F8, F11, F14, F17, F20, F23	MIMAT0019774	hsa-miR-4687-5p
G2, G5, G8, G11, G14, G17, G20, G23	MIMAT0005899	hsa-miR-1247-5p
H2, H5, H8, H11, H14, H17, H20, H23	MIMAT0004673	hsa-miR-29c-5p
I2, I5, I8, I11, I14, I17, I20, I23	MIMAT0004819	hsa-miR-671-3p
J2, J5, J8, J11, J14, J17, J20, J23	MIMAT0019074	hsa-miR-378i
K2, K5, K8, K11, K14, K17, K20, K23	MIMAT0000444	hsa-miR-126-5p
L2, L5, L8, L11, L14, L17, L20, L23	MIMAT0005939	hsa-miR-1281
M2, M5, M8, M11, M14, M17, M20, M23	MIMAT0005911	hsa-miR-1260a
N2, N5, N8, N11, N14, N17, N20, N23	MIMAT0019715	hsa-miR-4651
O2, O5, O8, O11, O14, O17, O20, O23	MIMAT0017392	hsa-miR-3200-5p
P2, P5, P8, P11, P14, P17, P20, P23	MIMAT0006790	hsa-miR-675-3p
A3, A6, A9, A12, A15, A18, A21, A24	MIMAT0003239	hsa-miR-574-3p
B3, B6, B9, B12, B15, B18, B21, B24	MIMAT0000680	hsa-miR-106b-5p
C3, C6, C9, C12, C15, C18, C21, C24	MIMAT0015049	hsa-miR-1193
D3, D6, D9, D12, D15, D18, D21, D24	MIMAT0000737	hsa-miR-382-5p
E3, E6, E9, E12, E15, E18, E21, E24	MIMAT0000074	hsa-miR-19b-3p
F3, F6, F9, F12, F15, F18, F21, F24	MIMAT0016893	hsa-miR-4267
G3, G6, G9, G12, G15, G18, G21, G24	MIMAT0018065	hsa-miR-3646
H3, H6, H9, H21, H15, H18, H21, H24	MIMAT0004808	hsa-miR-625-3p
I3, I6, I9, I12, I15, I18, I21, I24	MIMAT0005573	hsa-miR-1225-3p
J3, J6, J9, J12, J15, J18, J21, J24	MIMAT0016906	hsa-miR-4274
K3, K6, K9, K12, K15, K18, K21, K24	SA_miRNA_005	miRTC
L3, L6, L9, L12, L15, L18, L21, L24	SA_00104	PPC
M3, M6, M9, M12, M15, M18, M21, M24	MIMAT0004702	hsa-miR-339-3p
N3, N6, N9, N12, N15, N18, N21, N24	MIMAT0000268	hsa-miR-211-5p
O3, O6, O9, O12, O15, O18, O21, O24	MIMAT0000244	hsa-miR-30c-5p
P3, P6, P9, P12, P15, P18, P21, P24		SNORD61

Supplementary Figure 1. Giudice V. et al.

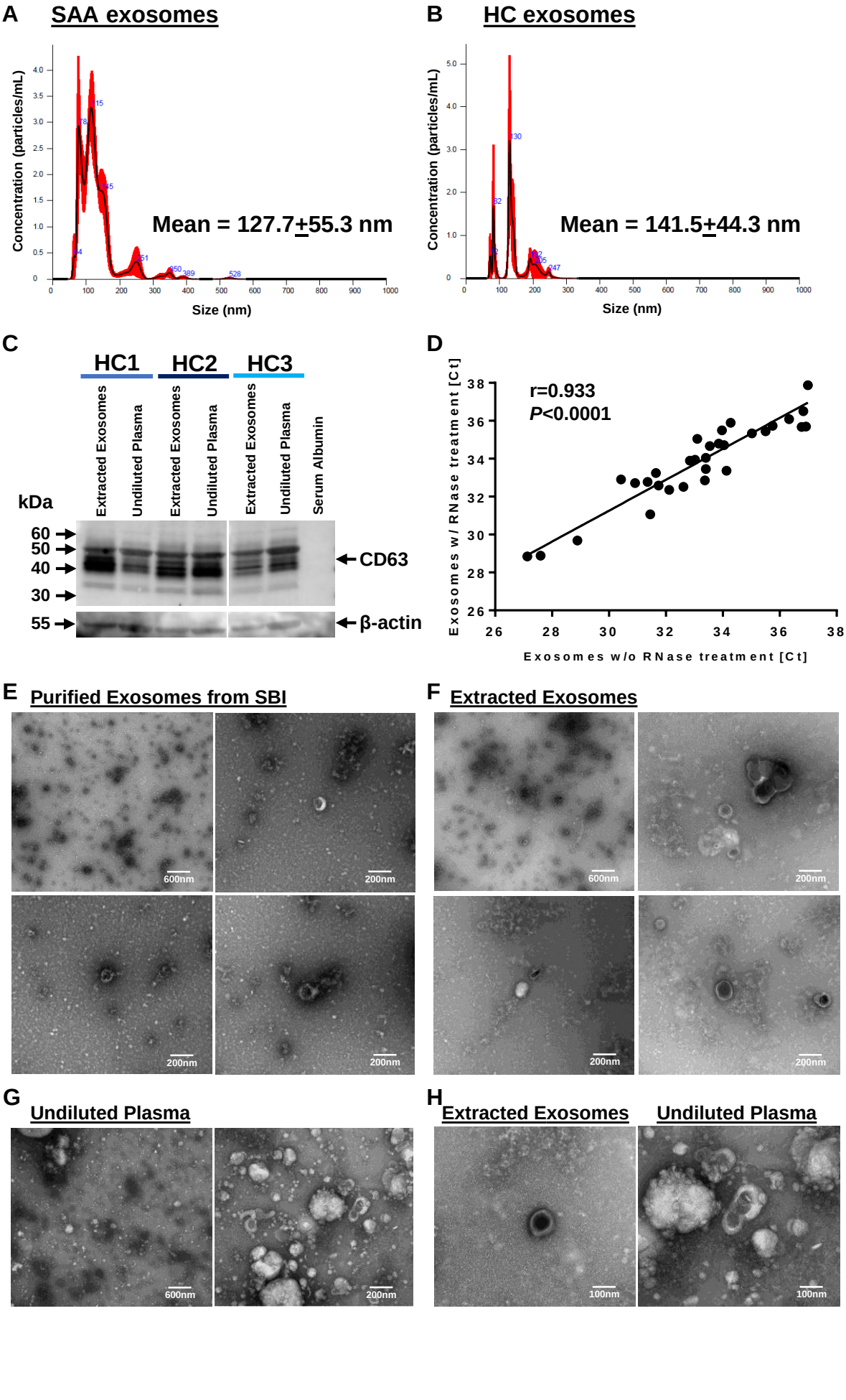


Figure S1. Size measurement, immunoblotting, and transmission electron microscopy (TEM) of exosomes. To confirm the presence of exosomes in the flow-through, particle size measurement and CD63 detection were performed. Nanoparticle characterization was carried out using the NanoSight NS300 (Malvern Instruments Ltd). Diluted enriched exosomes (1:100) from severe aplastic anemia (SAA) patients (A), and healthy controls (HC) (B). (C) Immunoblot was performed in three HC using exosome-specific anti-CD63 antibody (1:50 dilution) (clone, TS63; Thermo Fisher Scientific) and β-actin as a loading control (clone, N-21; Thermo Fisher Scientific). (D) Extracted exosomes were treated with (w/) or without (w/o) RNase A (0.4 μg/μl) and miRNA profiling performed using custom miScript miRNA PCR array. All miRNAs are shown for one representative sample. Shown are representative images of purified exosomes (E) (EXOP-145A-1; System Biosciences, SBI), and extracted exosomes (F) and undiluted plasma (G) from the same SAA patient. (H) Higher magnification images (100nm) for extracted exosomes and undiluted plasma. TEM images were acquired using JEOL JEM 1200EX transmission electron microscope at the Electron Microscopy Core Facility, NHLBI (scale bars indicate in each figure).

Supplementary Figure 2. Giudice V. et al.

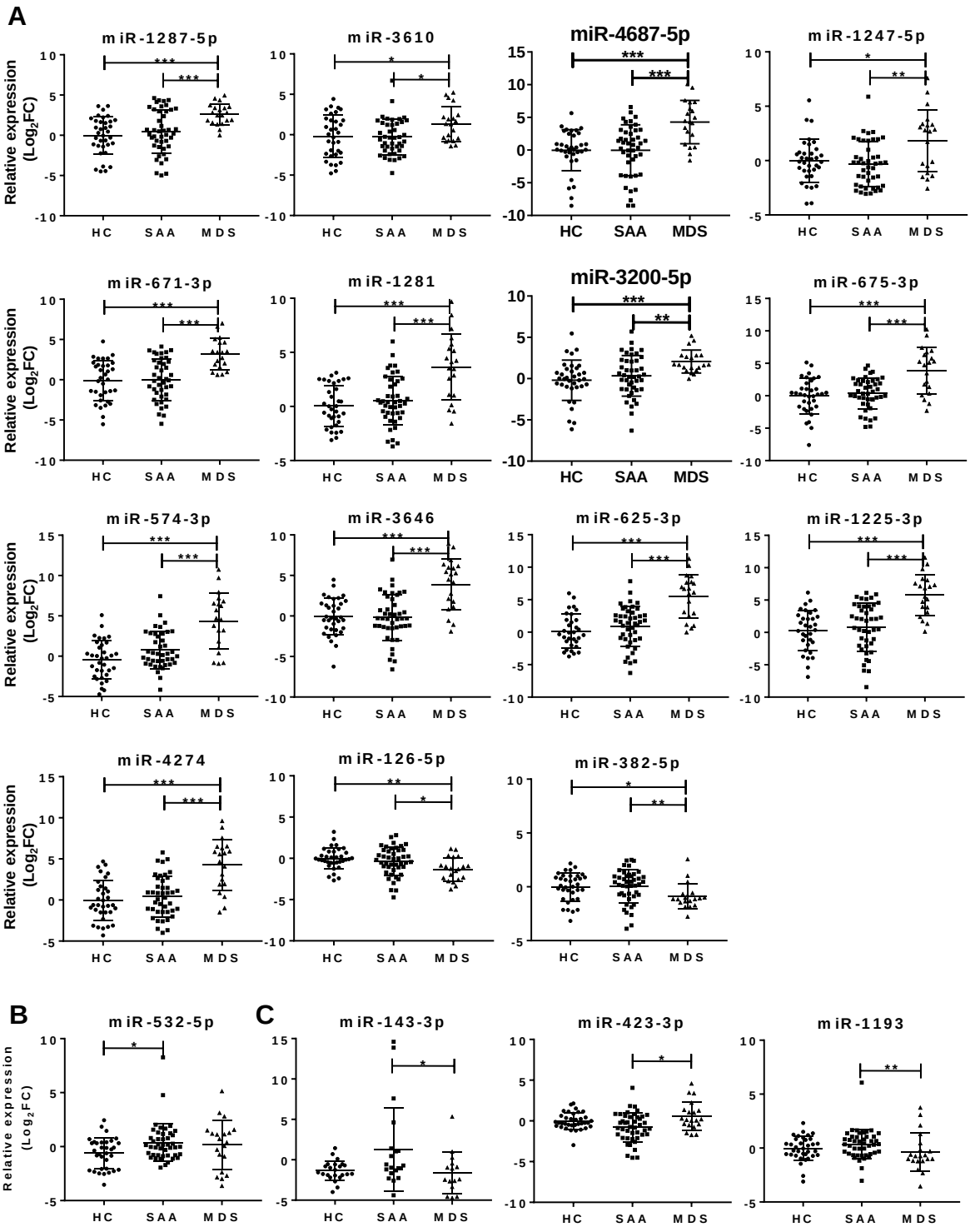
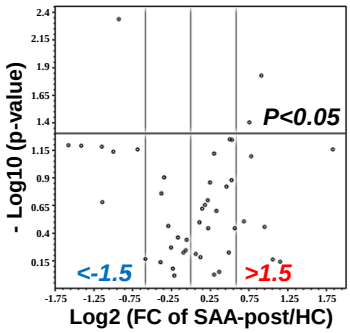


Figure S2. Comparison of the remaining miRNAs in the validation set. Relative expression levels were calculated as Log_2FC and shown for each group [SAA, MDS, and healthy controls (HC)]. (A) Differentially expressed miRNAs in MDS compared to SAA patients and HC. (B) miR-532-5p was found differentially expressed in SAA compared to HC and MDS. (C) miRNAs differentially expressed between SAA and MDS patients. Data are shown as mean±SD. One-way ANOVA with Kruskal-Wallis and the two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli tests were used for comparison between groups. $P<0.05$ was considered statistically significant.

A SAA post- treatment *vs* HC



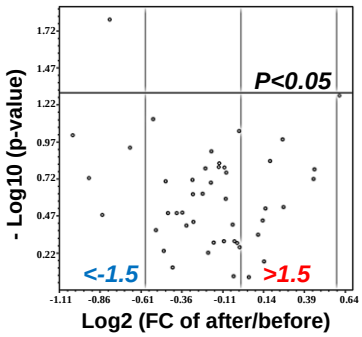
miRNAs enriched in SAA

Mature ID	FR	*P* value
miR-196a-5p	1.69	0.040
miR-196b-5p	1.88	0.015

miRNAs enriched in HC

Mature ID	FR	*P* value
miR-1260a	-1.91	0.005

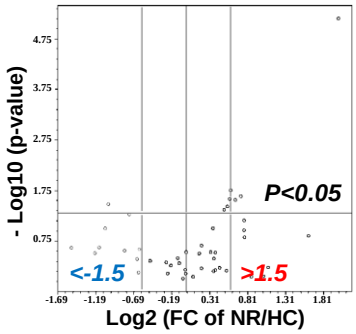
B SAA post- treatment *vs* SAA diagnosis



miRNAs enriched in SAA at diagnosis

Mature ID	FR	*P* value
miR-1287-5p	-1.75	0.016

C SAA-non-responders *vs* HC



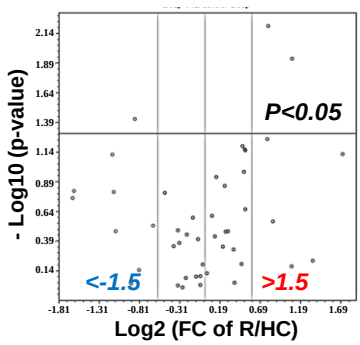
miRNAs enriched in NR

Mature ID	FR	*P* value
miR-196a-5p	1.65	0.023
miR-196b-5p	1.57	0.028
miR-143-3p	1.50	0.018
miR-378i	4.03	<0.0001

miRNAs enriched in HC

Mature ID	FR	*P* value
miR-1260a	-2.03	0.033

D SAA-responders *vs* HC



miRNAs enriched in R

Mature ID	FR	*P* value
miR-196b-5p	2.114	0.012
miR-26b-3p	1.726	0.006

miRNAs enriched in HC

Mature ID	FR	*P* value
miR-1260a	-1.83	0.038

Figure S3. Differentially expressed exosomal miRNAs after treatment. By using principal component analysis, volcano plots of 48 exosomal miRNA expression levels are shown when SAA post-treatment patients were compared to healthy controls (HC; A) or SAA patients at diagnosis (B). Furthermore, SAA-non-responder (NR; C) and SAA-responders (R; D) of SAA patients were also compared to HC. These results are shown using volcano plots and tables as described in Figure 1. For each comparison, miRNAs with ±1.5 FR and $P<0.05$ are displayed in correspondent tables.

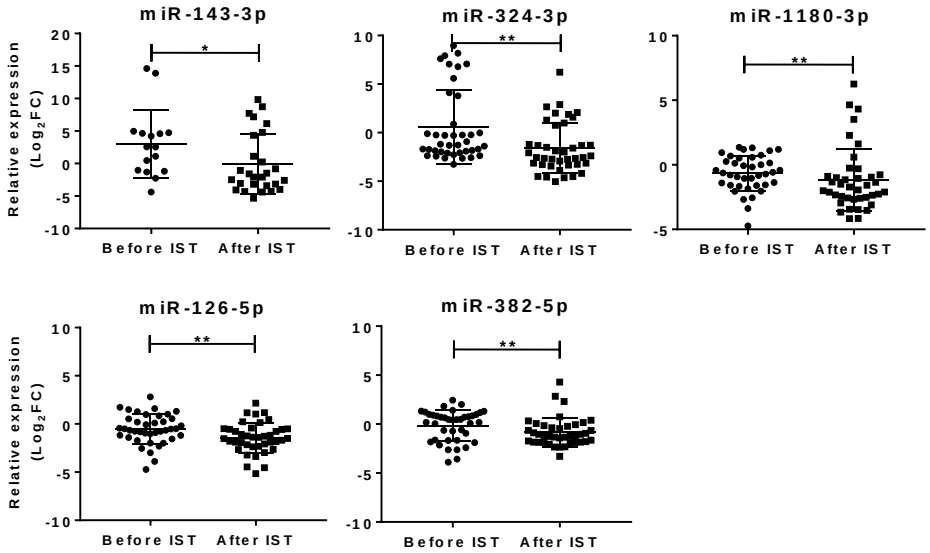
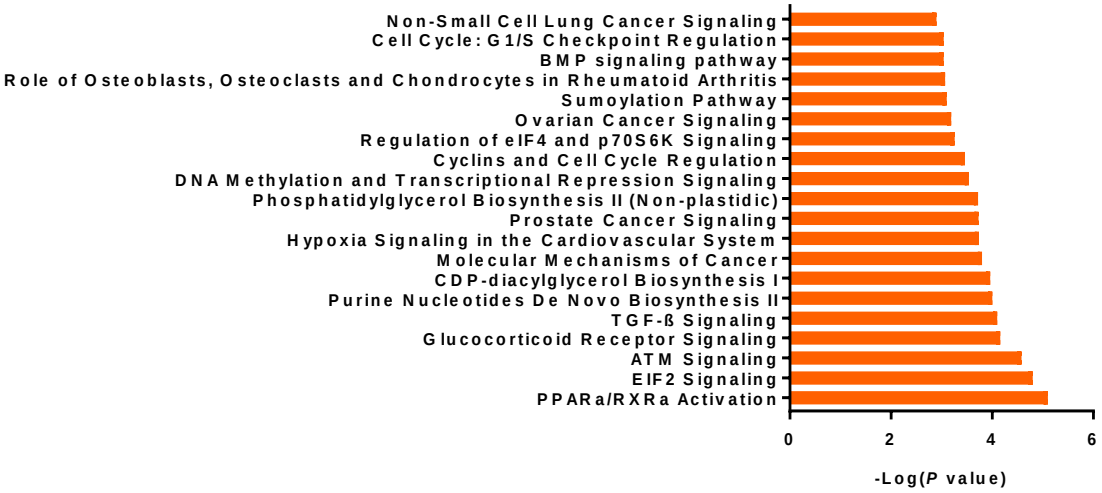
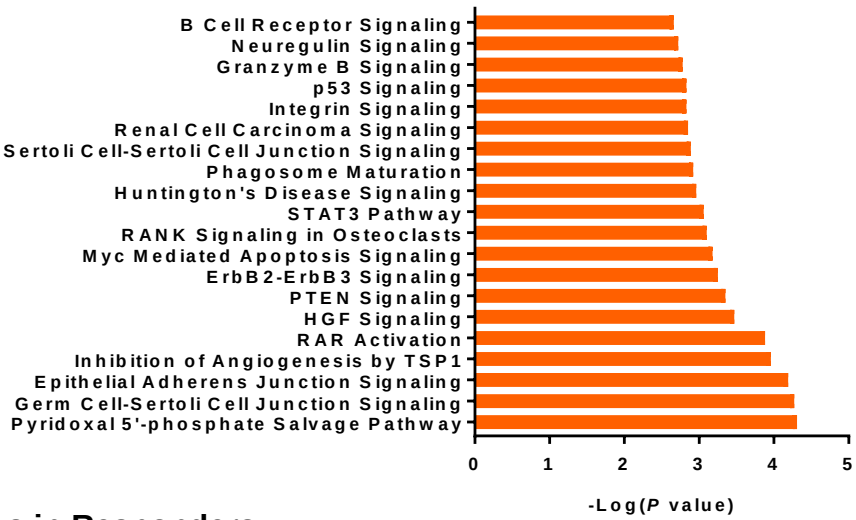


Figure S4. Comparison of miRNAs before and after immunosuppressive therapy (IST). Relative expression levels were calculated as Log_2FC for each group (before and after IST), and differentially expressed miRNAs are shown. Data are displayed as mean±SD. One-way ANOVA with the Kruskal-Wallis and a two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli tests were used for comparison between groups. $P<0.05$ was considered statistically significant.

A Top 20 pathways in SAA from common miRNAs



B Top 20 pathways in SAA and MDS



C Top 20 pathways in Responders

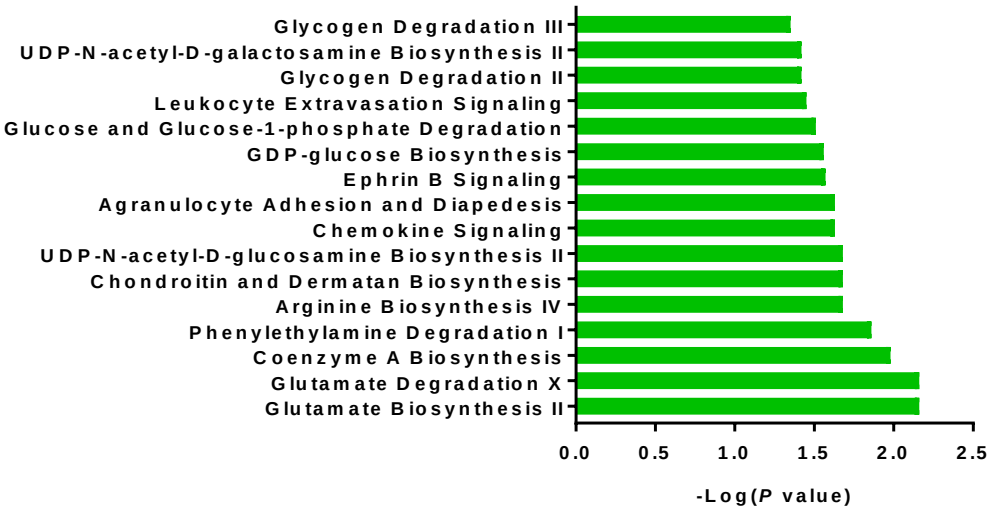


Figure S5. Pathways analysis using differentially expressed miRNAs. Employing IPA software and using predicted targeted genes, top 20 pathways are shown. Pathway analysis was performed using genes targeted by shared miRNAs but exclusively present in SAA (A) or in common between SAA and MDS (B). (C) Targeted genes by differentially expressed miRNAs in SAA-responder patients were used for pathway analysis and the top 20 pathways are reported.