SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

Bone Marrow Monocyte Isolation and M1 or M2 Differentiation

Bone marrow (BM) cells were collected from femurs and tibias of WT mice (C57BL/6J, 000664, Jackson Laboratory) by flushing them with buffer (PBS with 2% FBS and 1mM EDTA) using a 25-gauge needle. From the resulting BM cell suspension, the EasySep Mouse Monocyte Isolation Kit (StemCell Technologies, 19861) was used to isolate BM-derived monocytes, which were cultured for 7 days in RPMI medium (Thermo Fisher Scientific 11875093) supplemented with 20% FBS (Life Technologies, 10437028), 1% L-glutamine (Thermo Fisher Scientific, 25030-081), 1% Penicillin-Streptomycin (Thermo Fisher Scientific, 15140122) and 100ng/mL mouse CSF (Thermo Fisher Scientific,14898380). On day 7 post-isolation, cells were polarized to M1 or M2 macrophages by culturing them with either 100ng/mL LPS (Sigma-Aldrich, L7895) and 20ng/mL IFNγ (BioLegend, 575304) for M1 or 20ng/mL IL-4 (PeproTech, 214-14) for M2. For cellular RNA isolation, the traditional Trizol-chloroform method (1) was followed, while the exoRNeasy kit (Qiagen 77044) was used for EV-RNA isolation from culture media. mRNA reverse transcription was performed by using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, 43-688-13; 25°C-10', 37°C-2h, 85°C-5', 12°C-∞), while the miRCURY LNA RT Kit (Qiagen, 339340; 42°C-1h, 95°C-5', 4°C-∞) was used for miRNA. qRT-PCRs were performed in a QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher, 4485694) using the Kapa SYBR® FAST qPCR Kit (Kapa Biosystems, KK4621) for mRNA and the PCR Master Mix (Exiqon, 203420-01) for miRNA. The primer sequences used for determining signature genes of each macrophage subset and therefore polarization efficacy were the following; tnfa F: 5'-CCACCACGCTCTTCTGTCTAC-3', R: 5'-

TTGGGAACTTCTCATCCCTTT-3’; nos2 F: 5’- TGCGCCTTTGCTCATGACATCGA-3’, R: 5’-ATGGATGCTGCTGAGGGCTCTGTT-3’; il1b F: 5’-GCTGTGGCAGCTACCTGTGTCTT-3’, R: 5’-GGGAACGTCACACACCAGCAGGT-3’; il6 F: 5’-CTGCAAGAGACTTCCATCCAG-3’, R: 5’-AGTGGTATAGACAGGTCTGTTGG-3’; ccl2 F: 5’-GAAGGAATGGGTCCAGACATAC-3’, R: 5’-TCACACTGGTCACTCCTACA-3’; arg1 F: 5’-CTCCAAGCCAAAGTCCTTAGAG-3’, R: 5’-GGAGCTGTCATTAGGGACATCA-3’; actb F: 5’-GTGACGTTGACATCCGTAAAGA-3’, R: 5’-GCCGGACTCATCGTACTCC-3’. The miRNA primers were purchased from Qiagen: hsa-miR-150-5p miRCURY LNA miRNA PCR Assay (YP00204660), hsa-miR-29a-3p miRCURY LNA miRNA PCR Assay (YP00204698), hsa-miR-30a-5p miRCURY LNA miRNA PCR Assay (YP00205695) and 5S rRNA miRCURY LNA miRNA PCR Assay (YP00203906).

SUPPLEMENTAL RESULTS

Supplemental Table 1: Association between miRNAs and SCD

miRNA	Odds Ratio for Tertile 3 vs Tertile 1 with 95% CI	p-value trend*
miR-150-5p	2.03 (1.12 – 3.67)	0.02
miR-29a-3p	1.93 (1.07 – 3.50)	0.02
miR-30a-5p	0.55 (0.31 – 0.97)	0.04
miR-30d-5p	0.65 (0.35 – 1.21)	0.17
miR-21-5p	0.68 (0.37 – 1.26)	0.24
miR-106b-5p	0.73 (0.42 – 1.30)	0.25
miR-194-5p	0.71 (0.40 – 1.27)	0.25
miR-22-3p	1.39 (0.76 – 2.54)	0.27
miR-151a-3p	0.72 (0.39 – 1.32)	0.28
miR-29c-3p	1.39 (0.76 – 2.53)	0.28
miR-320a	1.27 (0.70 – 2.29)	0.41
miR-146a-5p	1.22 (0.68 – 2.18)	0.48
miR-146b-5p	1.20 (0.67 – 2.16)	0.5
miR-423-5p	1.17 (0.64 – 2.12)	0.53
miR-378c	1.21 (0.65 – 2.27)	0.54
miR-100-5p	0.87 (0.49 – 1.54)	0.6
miR-223-3p	0.95 (0.54 – 1.67)	0.83
miR-92a-3p	0.96 (0.48 – 1.91)	0.92

*Test for linear trend across tertiles

Supplemental Table 2:

Baseline characteristics of the individuals included in the cytokine analysis

Characteristic	Overall Cohort (N=352)	Individuals not included in the cytokine analysis (N=295)	Individuals included in the cytokine analysis (N=57)	p-value***
Age*	66 (11) years	66 (11) years	67 (11) years	0.81
Female	20%	19%	21%	0.76
Prior MI	86%	87%	81%	0.23
History of diabetes	38%	36%	46%	0.18
NYHA class ≥2	26%	26%	28%	0.73
History of hypertension	80%	80%	79%	0.86
History of atrial arrhythmia	20%	21%	14%	0.25
Family history of SCD	31%	32%	30%	0.84
Current smoker	14%	15%	11%	0.42
Non-white	8%	8%	4%	0.28
BMI**	29.6 (26.2 – 34.0)	29.9 (26.5 – 34.2)	28.8 (25.6 – 33.9)	0.24
Ejection fraction**	45 (40 – 53)	46 (40 – 53)	45 (40 – 50)	0.48
Aspirin	83%	81%	91%	0.08
Betablocker	78%	77%	86%	0.12
Statin	90%	89%	93%	0.48
Follow up years*	3.6 (1.6)	3.4 (1.6)	4.5 (1.6)	< 0.0001

*mean (standard deviation)

**median (quartile 1 – quartile 3)

*** p-value comparing individuals included and not included in the cytokine analysis

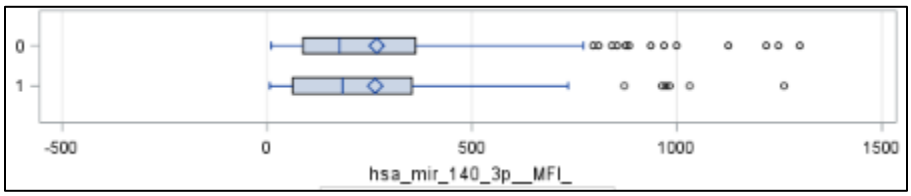
Supplemental Table 3: Spearman Correlation Coefficients of miRNAs and cytokines

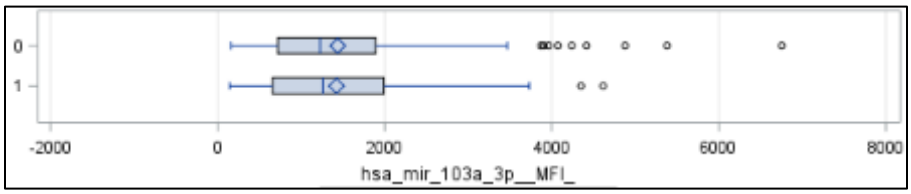
	IL-6 (n=57)	IL-10 (n=44)	TNF-α (n=57)	MCP-1 (n=57)
miR-150-5p	r = 0.10, p=0.4787	**r = -0.32, p=0.0331**	r = 0.08, p=0.5357	r = 0.14, p=0.3129
miR-29a-3p	**r = 0.32, p=0.0145**	r = -0.05, p=0.7333	r = 0.14, p=0.2898	r = 0.12, p=0.3853
miR-30a-5p	**r = 0.28, p=0.0348**	r = 0.28, p=0.0704	r = 0.22, p=0.1036	r = -0.01, p=0.9538

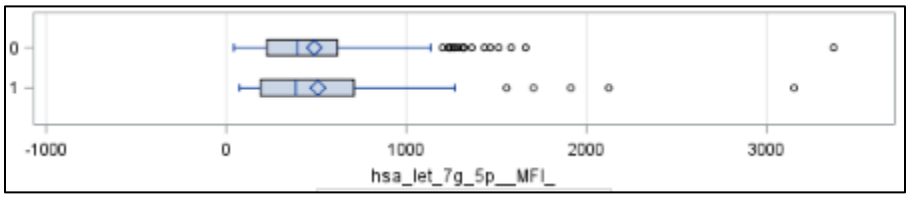
IL, interleukin; TNF, tumor necrosis factor; MCP-1, Monocyte Chemoattractant Protein-1

Supplemental Figure 1:

Distribution plots of the raw pre-normalized values for the 3 selected normalizer miRNAs: miR-140-3p, miR-103a-3p, and miR-let-7g-5p stratified by case (1) versus control (0) status

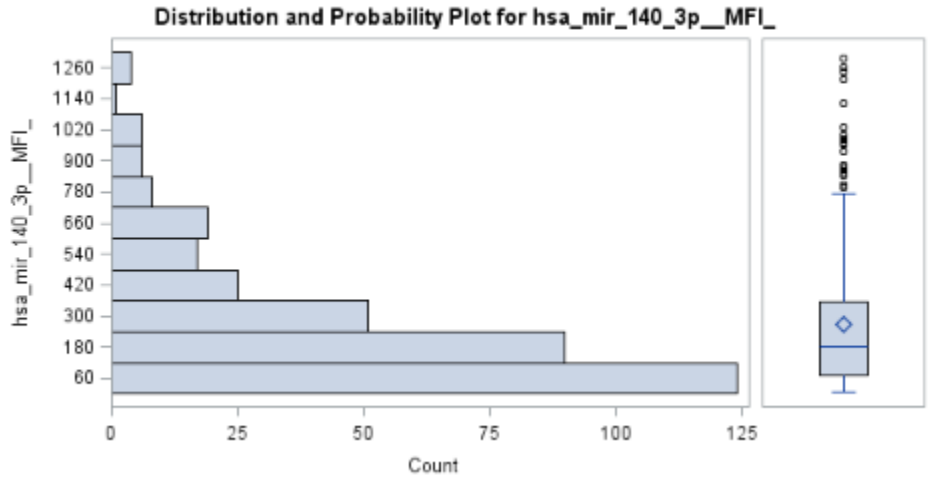


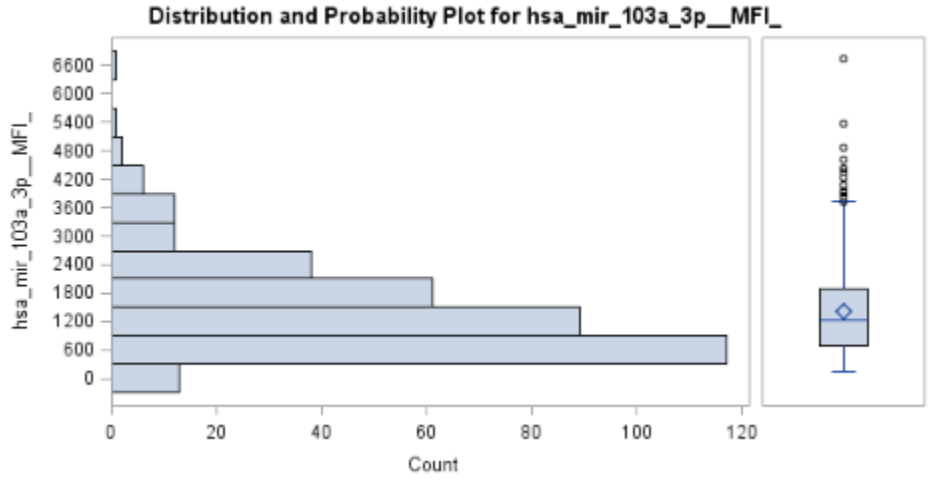


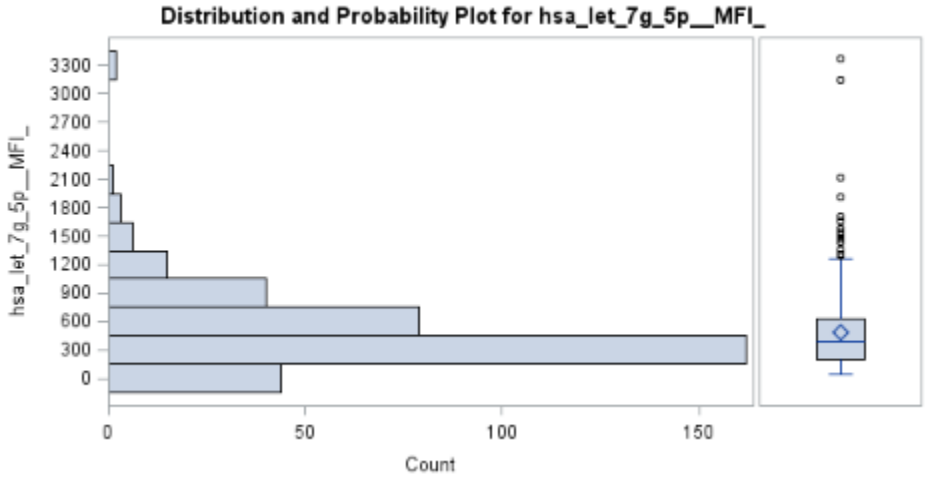


Supplemental Figure 2:

Distribution and probability plots for the 3 normalizer miRNAs (pre-normalization)

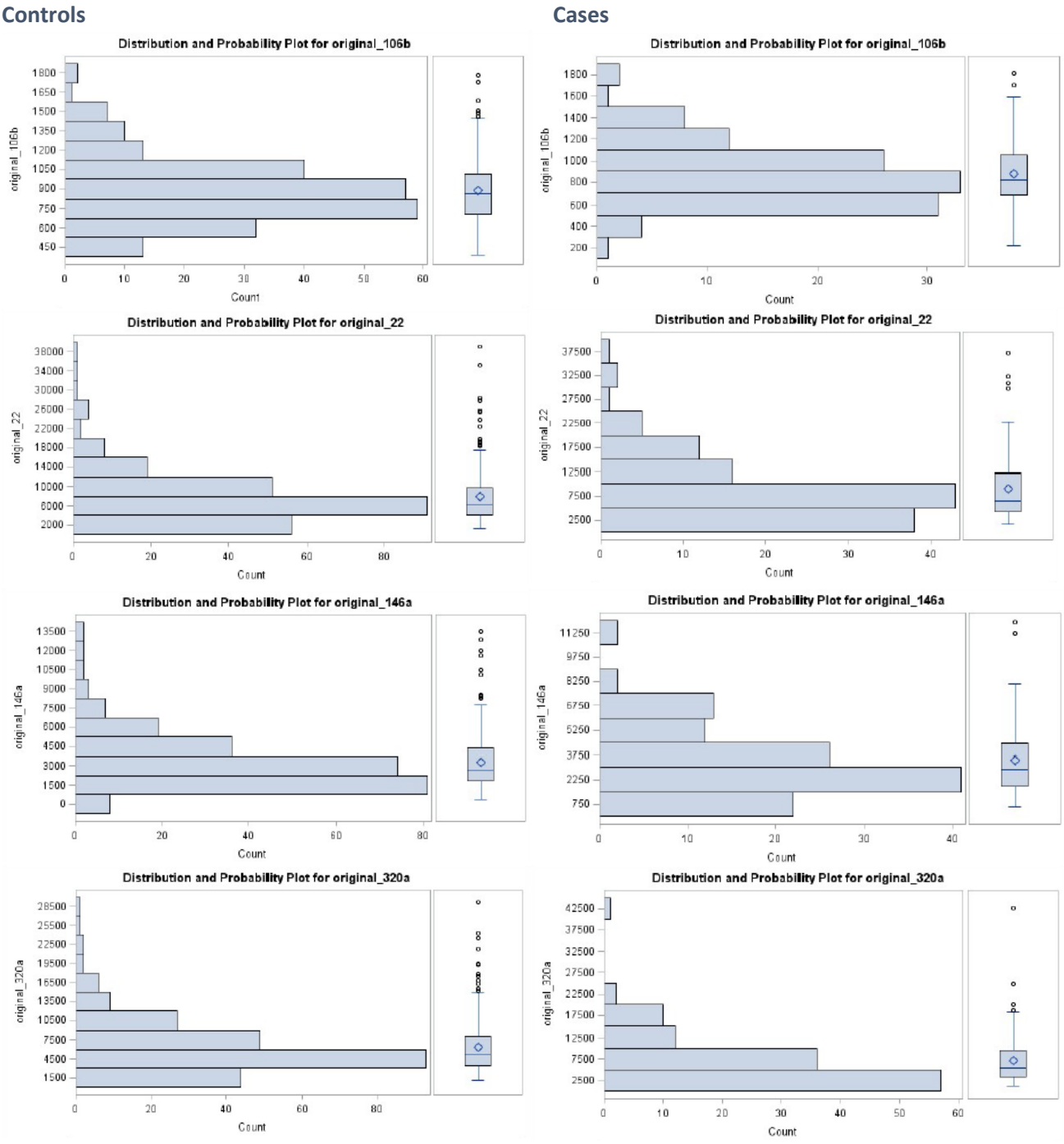






Supplemental Figure 3:

Distribution and probability plots for all miRNAs stratified by case/control status



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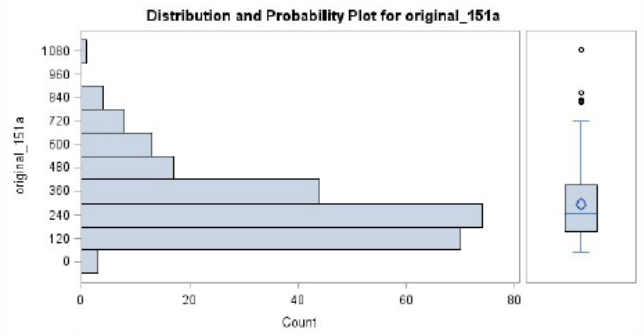

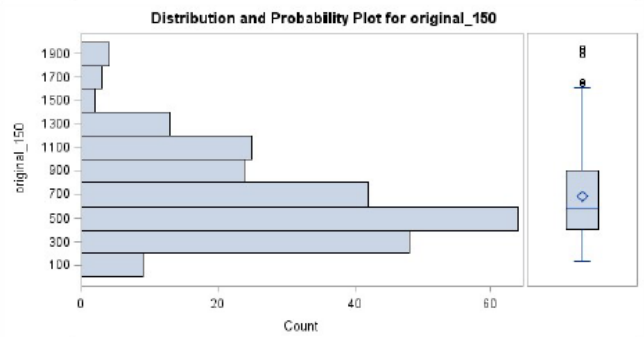

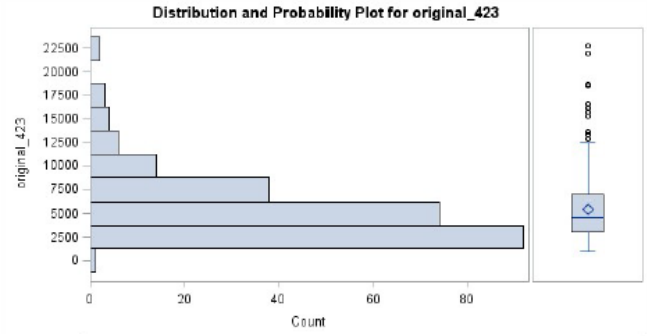

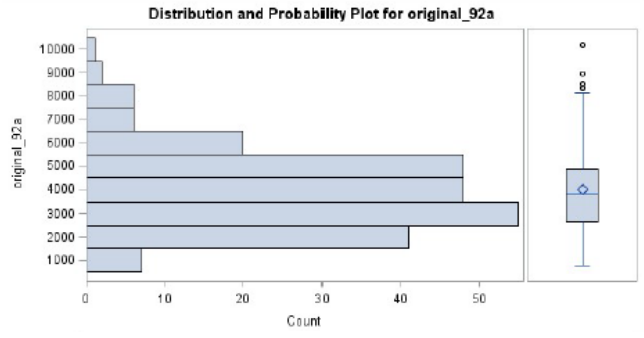

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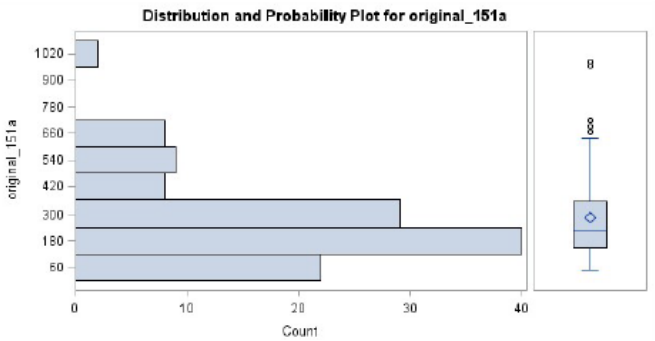

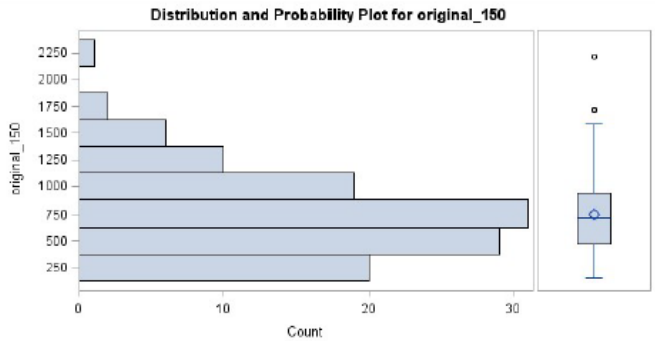

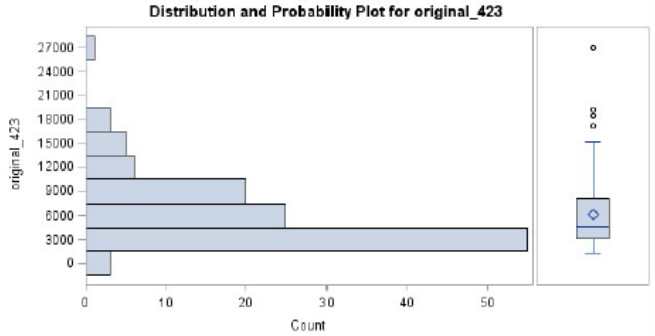

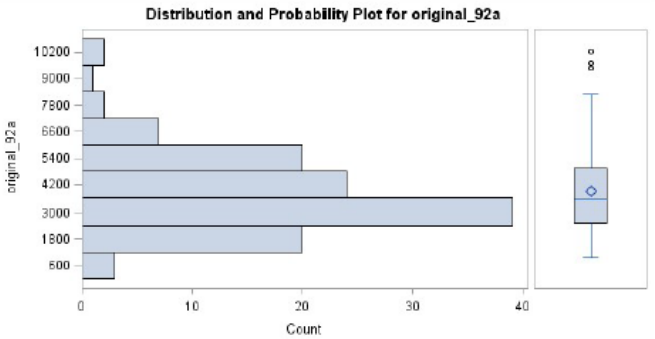

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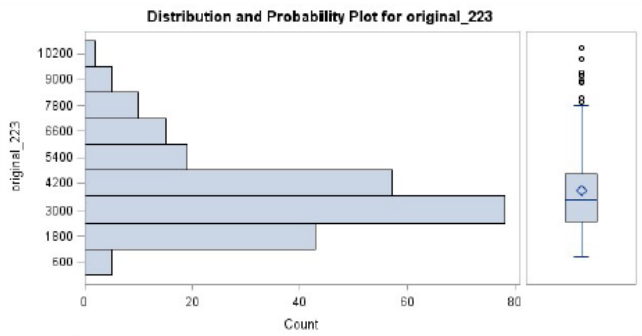

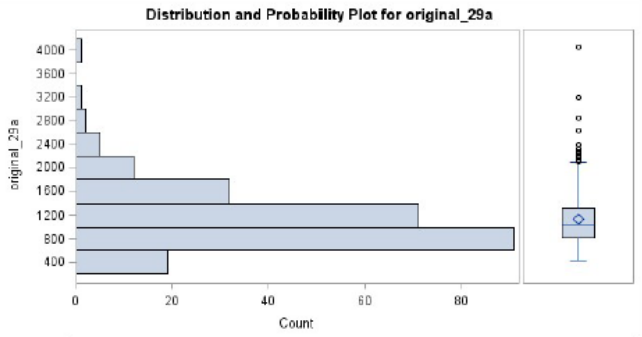

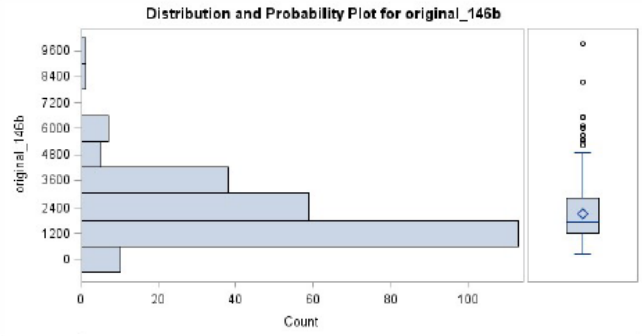

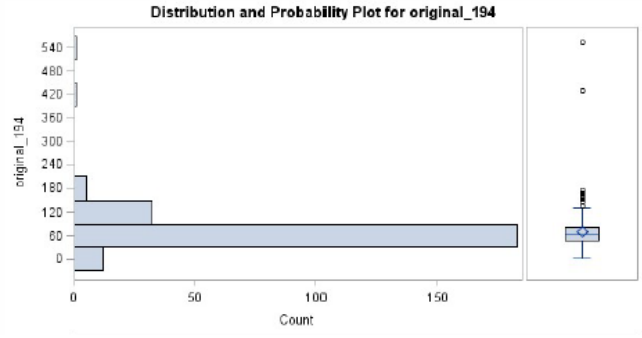

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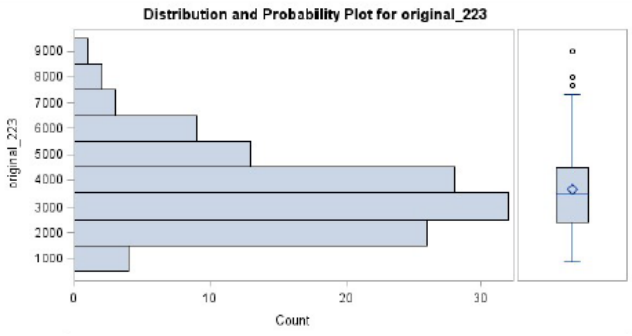

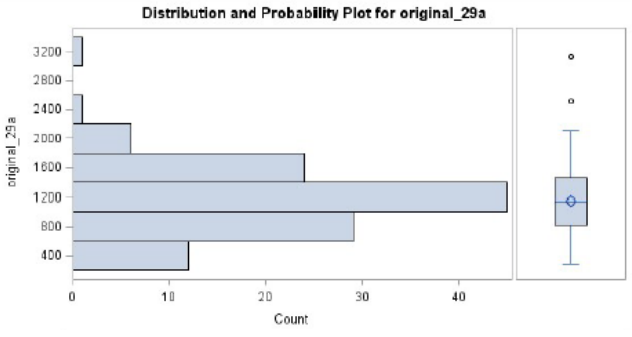

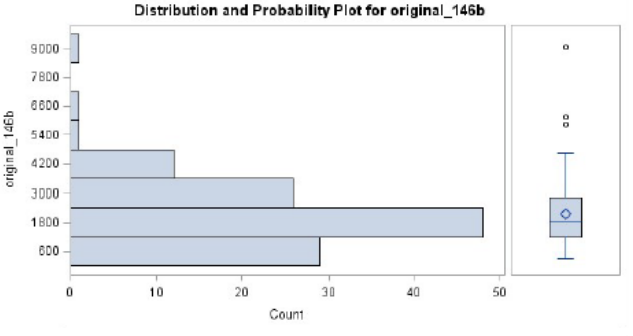

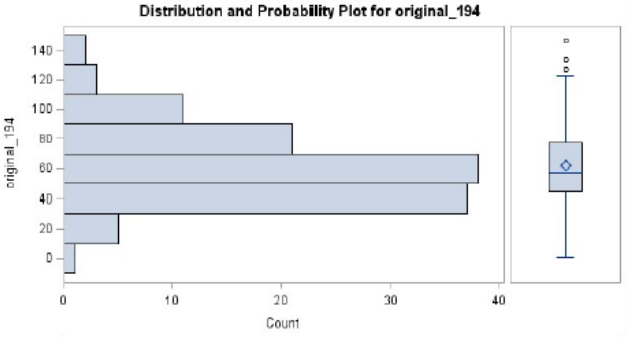

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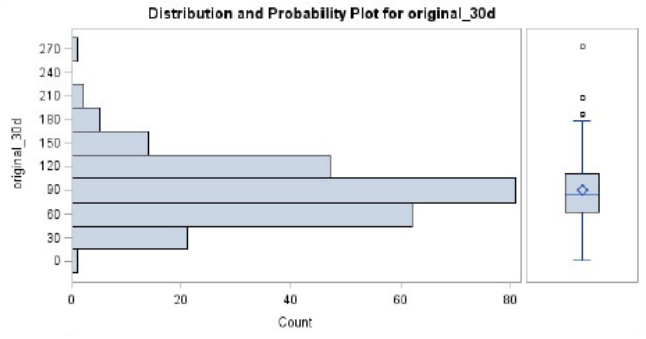

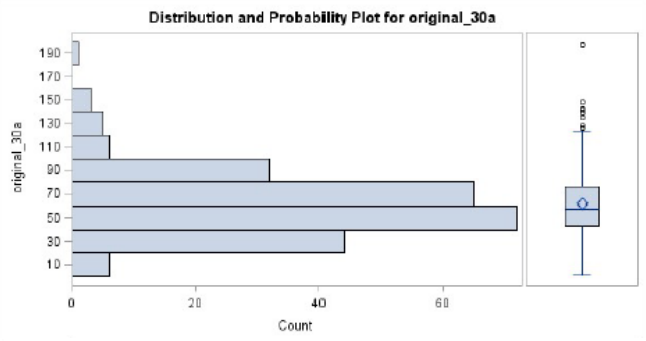

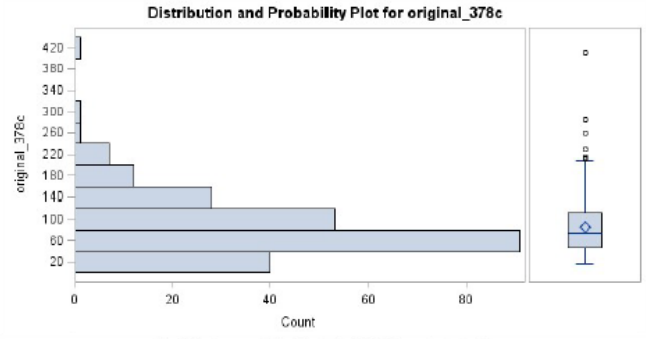

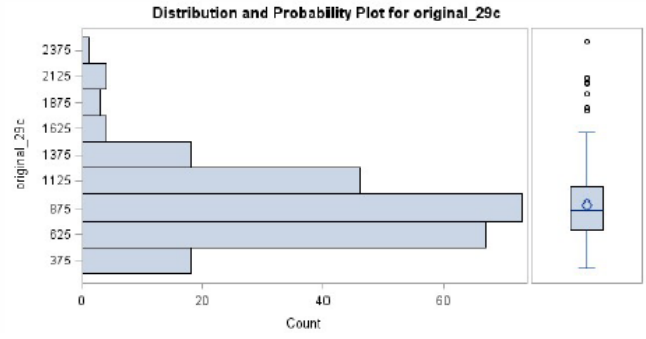

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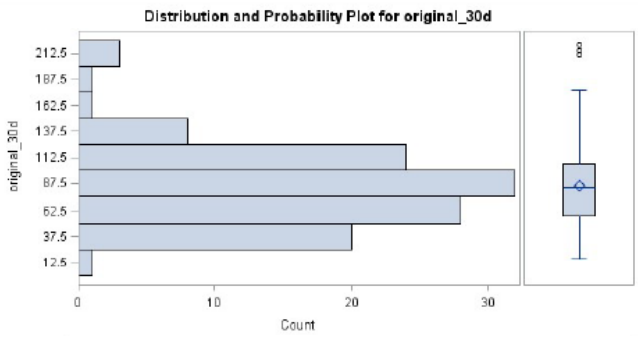

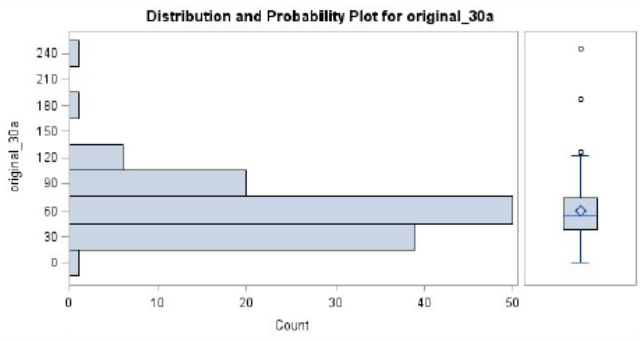

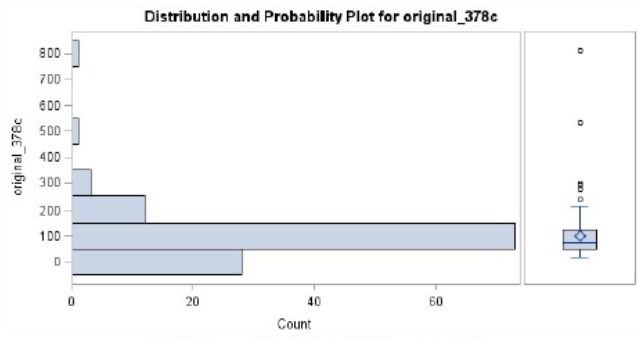

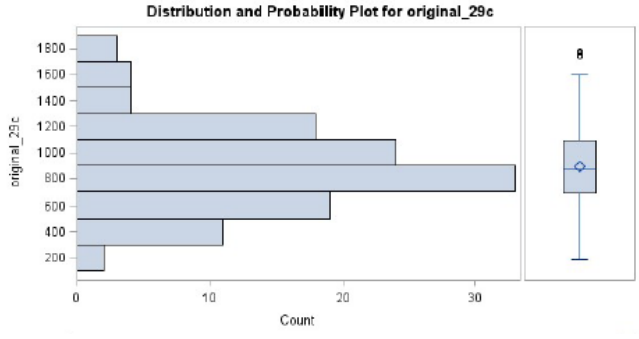

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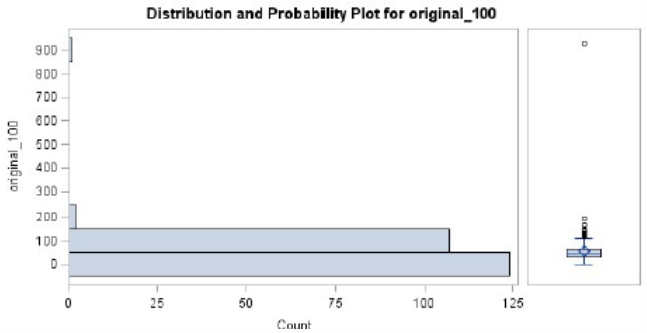

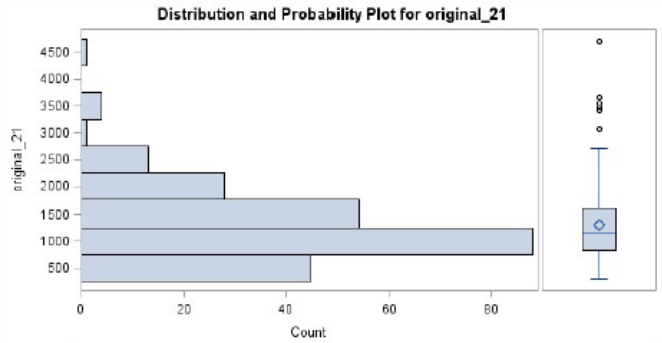

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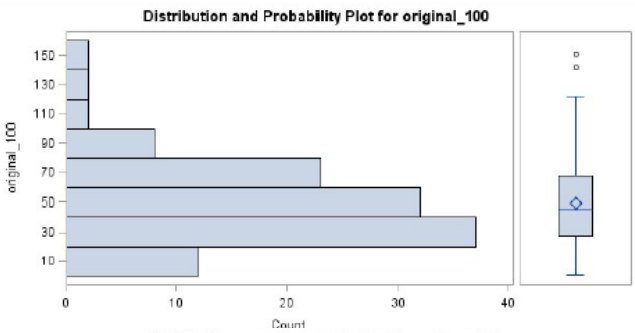
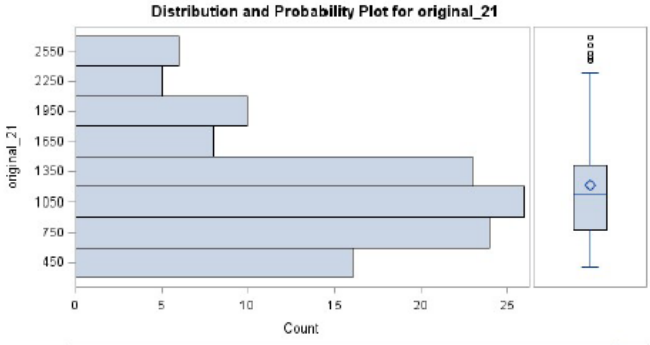
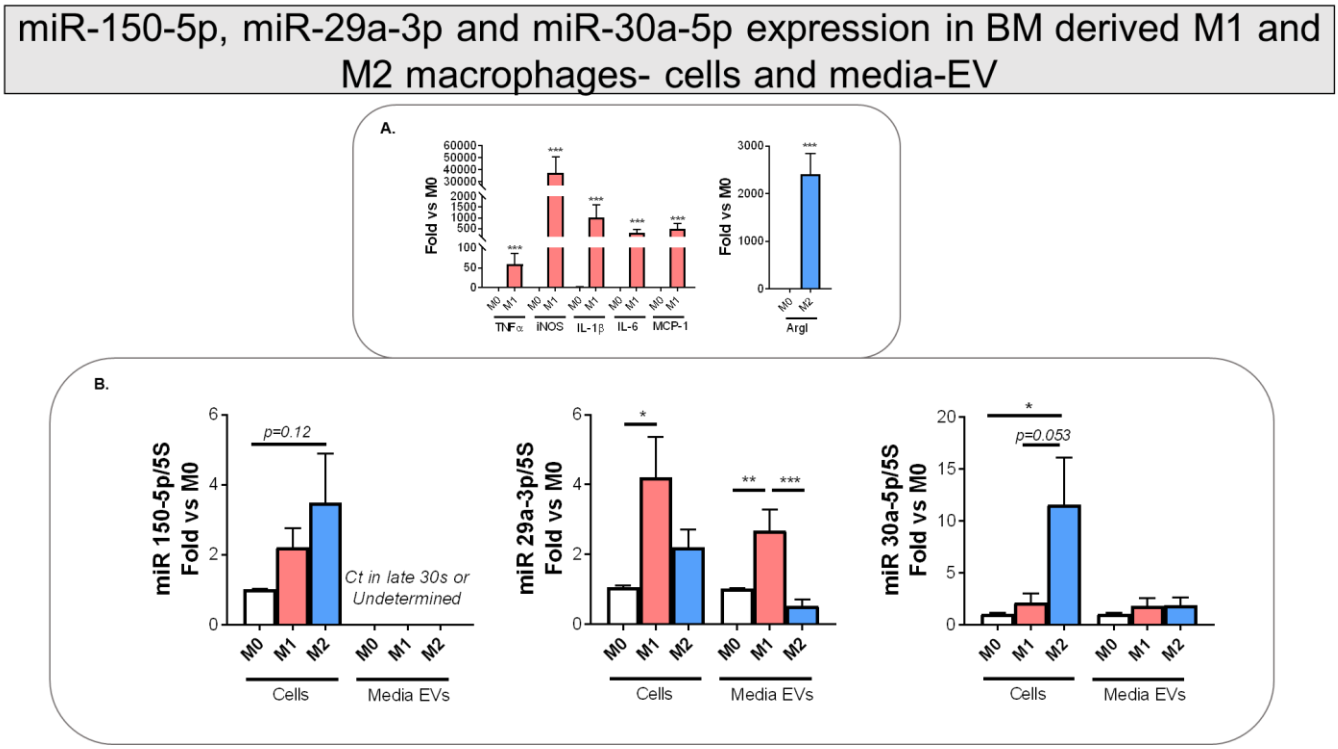




Supplemental Figure 4:

Expression levels of miR-150-5p, miR-29a-3p, and miR-30a-5p in bone marrow derived monocytes



BM = bone marrow; EV = extracellular vesicle

A. Signature mRNA marker expression was determined in BM derived M1 and M2 macrophages to ensure in vitro polarization. Tnfa, inos, il1b, il6 and ccl2 were quantified as M1 macrophage markers and arg1 as M2 macrophage marker. Data is represented as fold vs M0. n=8, Statistics Mann-Whitney non-parametric test.

B. Quantification of the expression of the miRNA of interest (miR-150-5p, miR-29a-3p and miR-30a-5p) in BM derived M1 and M2 macrophages, as well as their respective culture media EVs. Data is represented as fold vs M0 cells or EVs from M0's culture media, respectively. n=8, Statistics 1-way ANOVA Turkey's multiple comparisons test. *≤0.05, **≤0.01, ***≤0.001.

References

1) Rio DC, Ares M Jr, Hannon GJ, Nilsen TW. Purification of RNA using TRIzol (TRI reagent). Cold Spring Harb Protoc. 2010 Jun;2010(6):pdb.prot5439.