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Supplemental Information

miR-223-3p as a potential biomarker and player for adipose tissue dysfunction preceding type 2 diabetes onset

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SUPPLEMENTAL INFORMATION

SUPPLEMENTAL FIGURES

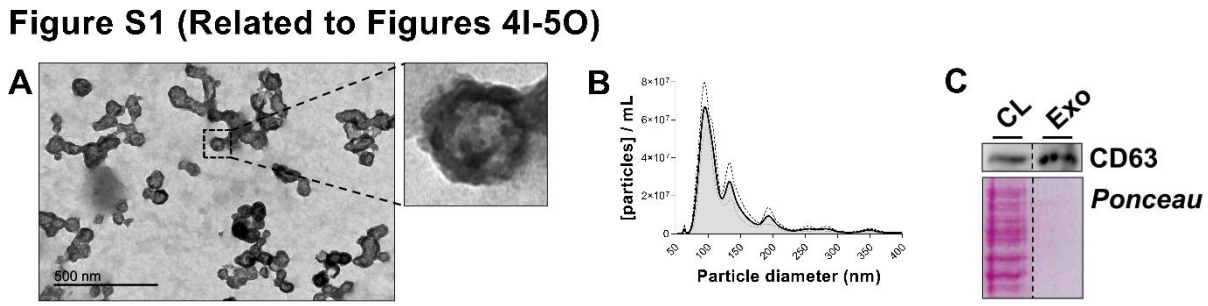

Figure S1 (Related to Figures 4I-5O). Exosome Isolation from 3T3-L1 Cells Culture Supernatant. Representative image and magnification of transmission electron microscopy (TEM) micrographs of exosome-like particles released by 3T3-L1 cells. Scale bar=500nm. (B) Representative size/concentration distribution of nanoparticles by NanoSight particle-tracking analysis (NTA). Mean is represented as a continue line and ±SEM is represented as dotted lines. (C) Representative blot of CD63 in 3T3-L1 cells total lysate (CL) and exosome purification fraction (Exo).

Figure S2 (Related to Figures 5A-O)

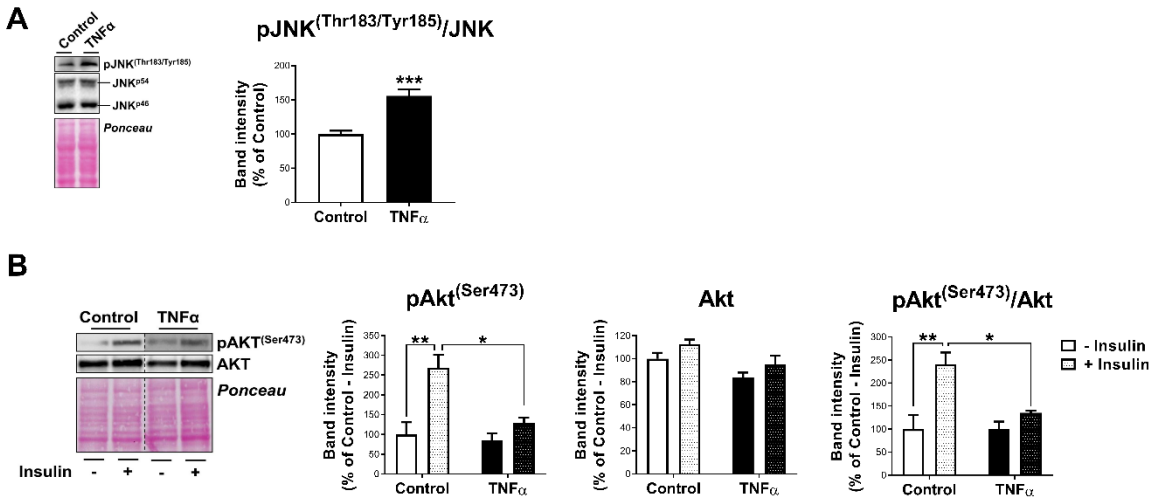

Figure S2 (Related to Figures 5A-O). Validation of TNFα-induced Inflammation and Insulin Resistance in 3T3-L1 cells. (A) Representative blot and quantification of pJNK$^{(Thr183/Tyr185)}$/JNK in 3T3-L1 cells at day 6 of differentiation (D6) upon 24h of treatment with 5nM TNFα or vehicle (control). ***P<0.001 vs. control. (B) Representative blot and quantification of pAkt$^{(Ser473)}$, Akt, and pAkt$^{(Ser473)}$/Akt ratio in control or TNFα-treated 3T3-L1 cells (D6) stimulated or not with insulin (100nM, 5min). *P<0.05, and **P<0.01 *vs.* indicated. n=6. Values are given as mean ± S.E.M.

Figure S3 (Related to Figures 5P-R)

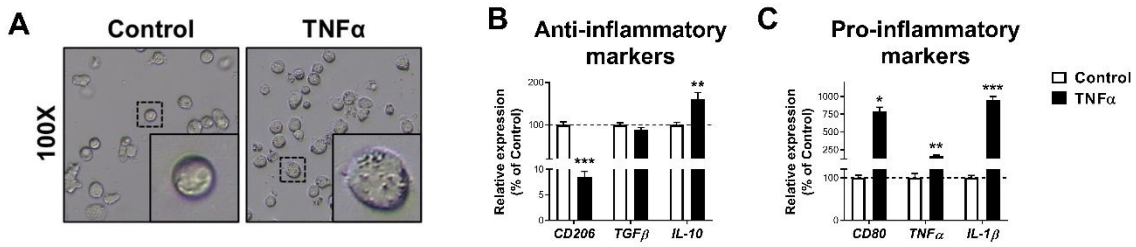

Figure S3 (Related to Figures 5P-R). THP1 Cells Exposed to TNFα. (A) Representative images from light microscopy of THP1 cells upon 24h of treatment with 5nM TNFα or vehicle (control). mRNA expression of anti-inflammatory markers (*CD206*, *TGFβ*, and *IL-10*) (B), and pro-inflammatory markers (*CD80*, *TNFα*, and *IL-1β*) (C) in THP1 cells treated with TNFα or vehicle (control). n=6. Values are given as mean ± S.E.M. *P<0.05, **P<0.01 and ***P<0.001 *vs.* control.

Figure S4 (Related to Figures 6-8)

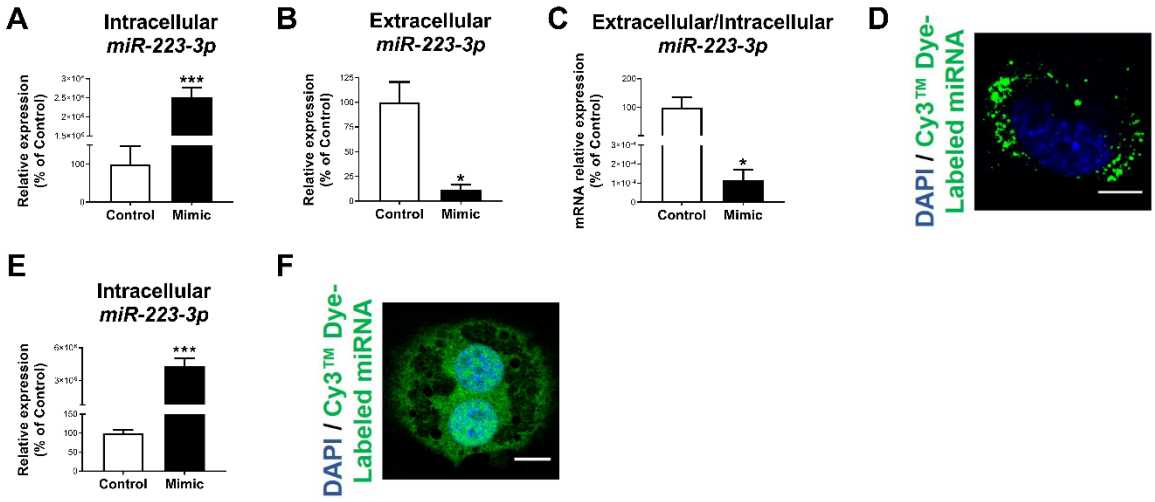

Figure S4 (Related to Figures 6-8). Validation of *miR-223-3p* Overexpression in Adipose Cells. Intracellular (A-F), extracellular (B) levels and extracellular/intracellular ratio (C) of *miR-223-3p*, and representative confocal micrographs (D-F) of 3T3-L1 cells transfected with negative miRNA-Control (control) or *miR-223-3p* (mimic) at day 3 (A-D) and day 6 (E-F) of differentiation. For confocal imaging cells were co-transfected with Cy3^{TM} labelled miRNA (green) and nuclei were stained with DAPI (blue). Scale bar=10µm. N=6. Values are means ± S.E.M. *P<0.05 and ***P<0.001 *vs.* control.

SUPPLEMENTAL TABLES

Table S1 (Related to Figure 1A). Correlations between Baseline Circulating Levels of miRNAs and ATIRI in the CORDIOPREV-DIAB Study Patients.

	ATIRI	
	r	***p***
hsa-miR-150	0.002	0.974
hsa-miR-103	-0.040	0.417
hsa-miR-28-3p	-0.035	0.470
hsa-miR-126	-0.032	0.511
hsa-miR-9	0.013	0.789
hsa-miR-30a-5p	-0.046	0.345
hsa-miR-375	0.024	0.620
hsa-miR-29a	-0.059	0.229

Correlations were determined by Pearson's correlation coefficient test. **r**, correlation coefficient.

Table S2 (Related to Figure 1A). Stepwise Akaike Information Criteria (AIC) Multivariable Logistic Regression.

	Model 1	**Model 2**	**Model 3**
AIC	297.15	295.19	293.94
hsa-miR-223-3p	X	X	X
Gender	X	X	X
Glucose	X	X	X
Triglycerides	X	X	X
BMI	X	X	X
Age	X	X	
CRP	X		

Stepwise Akaike information criterion (AIC) multivariate regression analysis including ATIRI as dependent variable and the plasma levels of the nine studied miRNAs (*miR-15*, *miR-123*, *miR-28-3p*, *miR-126*, *miR-9*, *miR-30a-5p*, *miR-223-3p*, *miR-375*, and *miR-29a*), and anthropometrical and clinical parameters as independent variables was performed in the R packet MASS using the stepAIC function. Three models were generated of which the best one with the lowest AIC value included gender, *hsa-miR-223-3p*, gender, glucose, triglycerides, and BMI. **CRP**, C-Reactive Protein; **BMI**, Body Mass Index.

Table S3 (Related to Figure 2). Selection of Predicted *miR-223-3p* Targets Genes Resulted from the *In-silico* Analysis, and Canonical Pathways and Upstream Regulators of the *miR-223-3p* Target Genes Set according to Ingenuity Pathway Analysis (IPA). Excel file.

Table S4 (Related to Figure 3). Biochemical and Anthropometric Characteristics of the Study Groups according to the Incidence of T2D during the Median Follow-up of 60 Months.

	Non-T2D	**Incident-T2D**	***P Value***
n	32	32	-
Age (years)	60.88 ± 1.30	62.09 ± 1.29	0.508
WC (cm)	104.06 ± 1.84	103.44 ± 1.57	0.799
BMI (Kg/m^2)	29.94 ± 0.55	29.88 ± 0.63	0.941
TG (mg/dL)	102.38 ± 8.87	117.71 ± 10.14	0.259
Chol (mg/dL)	159.81 ± 5.72	156.52 ± 5.16	0.671
c-HDL (mg/dL)	44.42 ± 1.22	42.48 ± 1.23	0.269
hs-CRP (mg/L)	2.35 ± 0.53	2.66 ± 0.59	0.695
Glucose (mg/dL)	91.97 ± 1.87	95.19 ± 2.08	0.253
HbA1c (%)	5.78 ± 0.07	5.97 ± 0.08	0.069
Insulin (mU/L)	7.07 ± 0.78	8.80 ± 0.85	0.140
HOMA-IR	2.36 ± 0.21	2.23 ± 0.19	0.640

Values are expressed as mean ± SEM. **Non-T2D**, Subjects who did not develop T2D; **Incident-T2D**, subjects who developed T2D; **WC,** Waist circumference; **BMI**, body mass index; **TG**, triglycerides; **c-HDL**, high-density lipoprotein; **hs-CRP**, high-sensitivity C-reactive protein; **HbA1c**, glycosylated hemoglobin; **HOMA-IR**, homeostasis model assessment-insulin resistance.

Table S5 (Related to Materials and Methods). Sequences and Transcript Sizes of Primers used in Quantitative Real-time PCR (qRT-PCR).

	Primer sequences (5' → 3')		
Gene (NCBI reference)	**Forward**	**Reverse**	**Size (bp)**
Acly (NM_001199296.1)	CACCTCCAAGAAGCCAAATC	CCAATGAAGCCCATACTCCTT	87
Actb (NM_007393.5)	GCCTTCCTTCTTGGGTATGG	AGCACTGTGTTGGCATAGAGG	108
Ago2 (NM_153178.4)	ATGCCTTCAAACCTCCACCT	TGCTCCACAATTTCCCTGTT	169
Agpat3 (NM_053014.3)	CCTCATCCTGACGTTCTTGG	CGCATCAGGTTATGGGTGTT	69
Atgl (NM_001163689.1)	ATGGTCCTCCGAGAGATGTG	AGGGTTGGGTTGGTTCAGTAG	68
Bscl2 (NM_001136064.3)	CGTGATCGGGTACTGATGTATG	CACTGAGCGTGAAGAAGTGG	57
Cd36 (NM_001159558.1)	GGCAAAGAACAGCAGCAAA	CAACAGACAGTGAAGGCTCAAA	73
Cd63 (NM_001042580.1)	CAAGGAATCCACTATCCATACCC	TTCCCAAGACCTCCACAAAA	119
Cebpa (NM_001287523.1)	GTGGACAAGAACAGCAACGA	TCACTGGTCAACTCCAGCAC	128
Cidea (NM_007702.2)	ATGGGATTGCAGACTAAGAAGG	TAACCAGGCCAGTTGTGATG	47
Cidec (NM_178373.4)	TCCCAGAAGCCAACTAAGAAGA	CAGGTCATAGGAAAGCGAGTATG	54
Dgat2 (NM_026384.3)	CTACTCCAAGCCCATCACCA	CAGTTCACCTCCAGCACCTC	50
Fabp1 (NM_017399.5)	ATCCGTCTGGTCAAGGTCAA	GGGCAATCTTCTTGTTGGTG	69
Fabp4 (NM_024406.3)	AAGAAGTGGGAGTGGGCTTT	CTGTCGTCTGCGGTGATTT	84
Fabp5 (NM_010634.3)	AGGATCTCGAAGGGAAGTGG	CTCGGTTTTGACCGTGATGT	44
Fasn (NM_007988.3)	ATACAATGGCACCCTGAACC	TTACAGAGGAGAAGGCCACAA	159
Gapdh (NM_001289726.1)	GTGGCAAAGTGGAGATTGTTG	CTCCTGGAAGATGGTGATGG	164
Glut4 (AB008453.1)	AAGAGTCTAAAGCGCCTGACC	TTGGACGCTCTCTCTCCAAC	94
Hnrnpa2b1 (NM_016806.3)	GCGATGGAGAGAGAAAAGGAA	GATCCCGCATAACCACACA	133
Hprt (NM_013556.2)	TGGATACAGGCCAGACTTTGTT	TTGCGCTCATCTTAGGCTTT	153
Hsl (NM_010719.5)	TCTAAATCCCACGAGCCCTAC	AAGGCATATCCGCTCTCCA	69
Insr (NM_010568.3)	GTTCAAGACCAGACCCGAAG	TCCAGACCATAGACACGGAAG	155
Lpl (NM_008509.2)	AGCCAAGAGAAGCAGCAAGA	CCATCCTCAGTCCCAGAAAA	72
Mgl (NM_001166251.1)	TCCACAGAATGTTCCCTACCA	GCTCATCATAACGGCCACA	80
Pck1 (NM_011044.3)	CTTTGGAAGCGGATATGGTG	TGCCTTCGGGGTTAGTTATG	59
Plin1 (NM_175640.2)	TGACGACCAGACAGACACAGA	TCACTGCGGAGATGGTGTT	51

Pparg (NM_001127330.2)	GCCTCCCTGATGAATAAAGATG	AGGCTCCATAAAGTCACCAAAG	108
Scd1 (NM_009127.4)	CAAAGAGAAGGGCGGAAAA	AGCACCAGAGTGTATCGCAAG	89
Srebf1 (NM_011480.4)	AGGTCACCGTTTCTTTGTGG	AATACAGTTCAACGCTCGCTCT	151
Ybx1 (NM_011732.2)	GTCATCGCAACGAAGGTTTT	TCAAACTCCACAGTCTCTCCATC	176

bp, base pairs

Table S6 (Related to Materials and Methods). Antibodies used in Immunoblotting Analyses.

Antibody	Commersial Source	Reference	Host Specie	Dilution
Anti-AKT	Cell Signaling Technology	9272	Rabbit	1/1,000
Anti-pAKT$^{(Ser473)}$	Cell Signaling Technology	4060	Rabbit	1/750
Anti-ARF6	Santa Cruz Biotechnology	sc-7971	Mouse	1/1,000
Anti-AS160	Merck Millipore	07-741	Rabbit	1/1,000
Anti-pAS160$^{(Ser666)}$	Merck Millipore	09-489	Rabbit	1/750
Anti-CD63	Sigma-Aldrich	SAB4301607	Rabbit	1/1,000
Anti-CHOP	Cell Signaling Technology	2895	Mouse	1/1,000
Anti-GLUT4	Abcam	ab35826	Mouse	1/1,000
Anti-GRP78/BiP	Santa Cruz Biotechnology	sc-376768	Mouse	1/1,000
Anti-GSS	Abcam	Ab133592	Rabbit	1/1,000
Anti-IRS1	Santa Cruz Biotechnology	sc-7200	Rabbit	1/1,000
Anti-pIRS1$^{(Ser307)}$	Santa Cruz Biotechnology	sc-33956	Rabbit	1/750
Anti-pIRS1$^{(Tyr612)}$	Merck Millipore	09-432	Rabbit	1/750
Anti-JNK	R&D Systems	AF387	Rabbit	1/1,000
Anti-pJNK$^{(Thr185/Tyr185)}$	R&D Systems	AF1205	Rabbit	1/1,000
Anti-PGC1α	Abcam	ab54481	Rabbit	1/1,000
Anti-SOD1	Sigma-Aldrich	HPA001401	Rabbit	1/1,000
Anti-UCP1	Abcam	ab23841	Rabbit	1/750
Anti-Mouse IgG peroxidase	Sigma-Aldrich	A-9044	Rabbit	1/2,500
Anti-Rabbit IgG peroxidase	Sigma-Aldrich	A-8275	Goat	1/2,500