Supplementary data

MATERIALS AND METHODS

Probes identification

Supplementary Table 1 describes the identification of TaqMan probes that were used to evaluate the gene expression of circulating microRNAs.

DNA extraction and sequencing of 16S rRNA

The bacterial DNA was isolated from the fecal samples using the QIAamp fast DNA stool mini kit (Qiagen, United States), following the manufacturer's instructions. The hypervariable V4 region from the rRNA gene was amplified by PCR using the following primer pair: 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). To pool different samples in the same reaction, we used the primer-fusion method and each sample had a distinct barcode attached on the corresponding PCR product. The purified products were subjected to emulsion PCR using Ion PGM™ Hi-Q™ view OT2 kit (Thermo Fisher Scientific, United States). After, the resulting enriched beads were sequenced in a next-generation sequencing (NGS) machine (Ion Torrent PGM, Life Technologies) using Ion PGM™ Hi-Q™ view sequencing kit (Thermo Fisher Scientific, United States).

Bioinformatics analyses

16S rRNA reads processing for downstream analyses: The sequence data exported from the Ion Torrent PGM™ System was processed using a custom pipeline in Mothur v.1.41.1[1]. Initially, sequences were depleted of barcodes and primers (where no mismatch was allowed) and then a quality filter was applied to eliminate low quality reads. Quality control was conducted by trimming the low-quality reads, those with incorrect length, those containing an ambiguous base, or containing homopolymers longer than 8 bp. All potentially chimeric sequences were identified and removed using VSEARCH[2].

After these initial quality filtering and trimming steps, the remaining sequences were clustered into operational taxonomic units (OTUs) based on a

99% identity level and were classified against the SILVA v132 reference database at 97% similarity[3]. Sequences that could not be classified (i.e., "unknown" sequences), as well as sequences identified as eukaryotes, mitochondria, and chloroplasts were removed prior to further analysis. To reduce spurious OTUs caused by PCR or sequencing errors, an additional filtering step was performed by removing OTUs with less than 10 reads. The resulting OTU table was normalized using the cumulative sum scaling (CSS) method. For alpha diversity analysis, the OTU table was rarefied to the smallest library size. Subsequent analyses of the sequence dataset were performed in R v. 3.6.1 (using vegan, phyloseq and ggplot2 packages) or QIIME v. 1.9.1[4].

Microbial ,community and statistical analysis

Alpha-diversity was assessed using species richness indices (ACE and Chao1) and species diversity indices (Shannon and Simpson). For overall comparison of significant differences among bacterial communities (*i.e.*, beta diversity), principal coordinates analysis (PCoA) was performed. A matrix using phylogenetic (weighted Unifrac) and non-phylogenetic (Bray–Curtis dissimilarity) metrics was calculated for each pair of samples. The distances were turned into points in space with the number of dimensions one less than the number of samples. To achieve statistical confidence for the sample grouping observed by PCoA, the ANOSIM multivariate test was performed on the distance matrix. To compare additional differences among the microbial communities, clustering methods based on Bray–Curtis dissimilarity were performed. The results of hierarchical clustering were visualized using heatmaps and dendrograms.

To detect potential taxa biomarkers, the linear discriminant effect size (LEfSe) method was performed[5]. The algorithm performs a nonparametric factorial Kruskal-Wallis sumrank test and LDA to determine statistically significant different features among taxa and estimates the effect size of the difference. Differences were considered significant for a logarithmic LDA score

threshold of ± 1.0 and a *P* value < 0.05 after adjusting for multiple hypotheses testing with the Benjamini–Hochberg method.

Metagenome prediction

Predictive functional gene profiling was based on 16S rRNA gene sequencing data using Piphillin[6] with updated KEGG database (May 2017) and a confidence cutoff value of 97. Piphillin uses direct nearest-neighbor matching between 16S rRNA amplicons and genomes to predict the represented genomes. This tool is not obliged to any unique data pre-processing protocol supporting KEGG and BioCyc as a reference database. The resulting table is then filtered to include only microbial metabolic pathways. Beta diversity of KEGG Orthology (KO) and Pathways (ko) abundances was calculated using the Bray−Curtis metric. Comparison of functional profiles of each population was performed using PERMANOVA. Differentially abundant features were determined using linear discriminant analysis (LDA) effect size (LefSe). Benjamini–Hochberg adjusted *p*-value was calculated to control the false discovery rate (FDR) in multiple testing. The KEGG groups were considered significantly enriched by satisfying an FDR corrected *p*-value of 0.05.

Correlations between the analyzed markers

Complementary data on correlations between cardiomyocyte morphometry variables with inflammatory and histopathological markers of liver injury, atherogenic indices, microRNAs, biochemical parameters and anthropometric parameters of the animals were evaluated.

RESULTS

Correlations Between the Cardiomyocyte Morphometry and Liver Injury Markers and Cardiovascular Risk

With all variables in hands, we then did a correlation analysis among cardiomyocyte morphometry (*i.e.* the percentage of normal cardiomyocytes, the percentage of atrophic cardiomyocytes and the average area of these cells) and

this information is described in the Supplementary Table 2. This correlation analysis was performed between all the anthropometric, inflammatory, fibrogenesis, atherogenic ratios and microRNAS parameters described in this article, as well as with the previously published results[7]. Several translationally relevant results were found. Firstly, we found a negative correlation between the average area and the percentage of normal cardiomyocytes with NAFLD score. Complementary to this, histopathological NAFLD score correlated positively with the percentage of atrophic cardiomyocytes. Considering endothelial markers, monocyte chemoattractant protein (MCP)-1 and tissue inhibitor of metalloproteinase (TIMP)-1 correlated negatively with the percentage of normal cardiomyocytes and positively with the percentage of atrophic cardiomyocytes. Furthermore, the average area of cardiomyocytes correlated negatively with cardiovascular disease (CVD) risk factors and metabolism of lipids (Castelli's Risk Index-I, Castelli's Risk Index-II and atherogenic coefficient). miR-33a and miR-126 correlated negatively and positively with the percentage of normal cardiomyocytes, respectively. Several markers of systemic inflammation correlated negatively with the percentage of normal cardiomyocytes, such as NOD-like receptor protein (NLRP)-3, caspase (Casp)-1, interleukin (IL)-18, IL-1β and myeloid differentiation primary response (Myd)-88, while the percentage of atrophic cardiomyocytes correlated positively with Nlrp-3, toll-like receptor (TLR)-9, Tlr-4, IL-18, IL-1β and Myd-88. Several anthropometric data associated with obesity correlated negatively with the percentage of normal cardiomyocytes and averaged area of cardiomyocytes, while correlating positively with the percentage of atrophic cardiomyocytes. Finally, IL-10 hepatic levels correlated positively with the average area of cardiomyocytes and negatively with the percentage of cardiomyocytes. Corroborating this, an increased ratio of pro/anti-inflammatory cytokines in the liver was positively associated with the percentage of atrophic cardiomyocytes and negatively correlated with the average area of these cells.

Supplementary Table 1 Probes identification

Assay name	Assay ID
hsa-miR-33a-5p	002135
hsa-miR-126-3p	002228
mmu-miR-499-5p	001352
hsa-miR-186-5p	002285
hsa-miR-146a-5p	000468
cel-miR-39-3p	000200

Supplementary Table 2 Correlation among variables of cardiomyocyte morphometry and severity of liver disease progression and cardiovascular risk markers.

Variables[1]	Statistical test	% Normal cardiomyocytes	Average area of cardiomyocytes	% Atrophic cardiomyocytes
NAFLD score	Pearson Correlation	-0.519[3]	-0.630[2]	0.721[2]
	Sig. (2-tailed)	0.019	0.003	0.0027
Quantification of collagen (picrosirius)	Pearson Correlation	-0.205	-0.312	0.238
	Sig. (2-tailed)	0.385	0.181	0.312
IL-1β	Pearson Correlation	-.437[3]	-.393	.382
	Sig. (2-tailed)	0.061	0.096	0.107
MCP-1	Pearson Correlation	-.490[3]	-.390	.498[3]
	Sig. (2-tailed)	0.028	0.090	0.025
TIMP-1	Pearson Correlation	-.694[2]	-.405	.607[2]
	Sig. (2-tailed)	0.001	0.076	0.005
PAI-1	Pearson Correlation	-.317	.389	-.289
	Sig. (2-tailed)	0.173	.090	0.216
CRI-I	Pearson Correlation	-.234	-.459[3]	.386
	Sig. (2-tailed)	0.336	0.048	0.103
CRI-II	Pearson Correlation	-.399	-.492[3]	.551[3]
	Sig. (2-tailed)	0.091	0.032	0.014
AC	Pearson Correlation	-.236	-.457[3]	.389

	Sig. (2-tailed)	0.331	0.049	0.099
miR-33a	Pearson Correlation	-.704[2]	.038	.232
	Sig. (2-tailed)	0.001	0.881	0.354
miR-126	Pearson Correlation	.459[3]	.320	-.364
	Sig. (2-tailed)	0.042	0.169	0.114
Ppar-α	Pearson Correlation	.205	.616[2]	-.613[2]
	Sig. (2-tailed)	0.386	0.004	0.004
Nlrp-3	Pearson Correlation	-.554[3]	-.278	.549[3]
	Sig. (2-tailed)	0.011	0.236	0.012
Casp-1	Pearson Correlation	-.668[2]	.020	.273
	Sig. (2-tailed)	0.002	0.934	0.258
Tlr-9	Pearson Correlation	-.371	-.339	.558[3]
	Sig. (2-tailed)	0.129	0.169	0.016
Tlr-4	Pearson Correlation	-.253	-.310	.487[3]
	Sig. (2-tailed)	0.296	0.196	0.034
IL-18	Pearson Correlation	-.618[2]	-.203	.497[3]
	Sig. (2-tailed)	0.004	0.391	0.026
IL-1β	Pearson Correlation	-.642[2]	-.404	.703[2]
	Sig. (2-tailed)	0.002	0.078	0.001
Myd-88	Pearson Correlation	-.746[2]	-.258	.524[3]
	Sig. (2-tailed)	0.002	0.273	0.018

Abdominal circumference	Pearson Correlation	-.419	-.480[3]	.491[3]
	Sig. (2-tailed)	0.066	0.032	0.028
Abdominal fat tissue	Pearson Correlation	-.648[2]	-.346	.438
	Sig. (2-tailed)	0.003	0.147	0.061
∆ Weight	Pearson Correlation	-.641[2]	-.449[3]	.508[3]
	Sig. (2-tailed)	0.002	0.047	0.022
Liver weight/body weight ratio	Pearson Correlation	-.599[2]	-.592[2]	.712[2]
	Sig. (2-tailed)	0.005	0.006	0.000
Total cholesterol	Pearson Correlation	-.557[3]	-.154	.421
	Sig. (2-tailed)	0.013	0.528	0.073
HDLc	Pearson Correlation	.074	.554[3]	-.441
	Sig. (2-tailed)	0.757	0.011	0.051
IL-10 (pg/mg)	Pearson Correlation	.419	.648[2]	-.681[2]
	Sig. (2-tailed)	0.066	0.002	0.001
TNF-α (pg/mg)	Pearson Correlation	-.120	-.571[2]	.559[3]
	Sig. (2-tailed)	0.614	0.009	0.01
TNF-α/IL-10	Pearson Correlation	-.194	-.649[2]	.645[2]
	Sig. (2-tailed)	0.413	0.002	0.002
IL-1β/IL-10	Pearson Correlation	-.241	-.615[2]	.492[3]
	Sig. (2-tailed)	0.306	0.004	0.028
IL-6/IL-10	Pearson	-.490[3]	-.467[3]	.648[2]

	Correlation			
	Sig. (2-tailed)	0.028	0.038	0.002
Nile red (Liver Fat Deposition)	Pearson Correlation	-.525[3]	-.512[3]	.535[3]
	Sig. (2-tailed)	0.018	0.021	0.015

[1]Variables was represented for Spearman's correlation coefficient; [2]Correlation is significant at the 0.01 level; [3]Correlation is significant at the 0.05 level. AC: Atherogenic coefficient; Casp-1: Caspase-1, CRI: Castelli's Risk Index; IL: Interleukin; MCP: Monocyte chemoattractant protein; Myd-88: Myeloid differentiation primary response-88; NAFLD: Non-alcoholic fatty liver disease; NLRP: NOD-like receptor protein; PAI: Plasminogen activator inhibitor; PPAR: Peroxisome proliferator-activated receptor; TIMP: Tissue inhibitor of metalloproteinase; TLR: Toll-like receptor; TNF: Tumor necrosis factor.

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