SUPPLEMENTAL MATERIALS

Circulating immune cell signature analysis in HFpEF across species

Short title: Immune Cell Signatures in HFpEF Across Species

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Major Resources Table

Animals (in vivo studies)

Species	Vendor or Source	Background Strain	Sex	Persistent ID / URL
Mus musculus	Janvier Labs (Le Genest-Saint-Isle, France)	C57BL/6N	Male	https://janvier-labs.com/

Antibodies

Target antigen	Vendor or Source	Catalog #	Working concentration	Lot # (preferred but not required)
Phospho-NF-κB p65 (Ser536) (93H1) Rabbit mAb	Cell signaling	#3033S	1:1,000, Stock-concentration: 57 µg/ml	#19 (Best: 09/2027)
URL: https://www.cellsignal.com/products/primary-antibodies/phospho-nf-kb-p65-ser536-93h1-rabbit-mab/3033?srsltid=AfmBOoqFq0pcgJgtIbQuJ0fvDAZFZAY4lvSJtUjD4CPvP41TG07VBliJ				
Anti-IL-1 beta Antibody	antibodies	#A87561	1:1,000, Stock-concentration: 1 mg/ml	#41896
URL: https://www.antibodies.com/catalog/primary-antibodies/il-1-beta-antibody-a87561				
GAPDH Antikörper (0411)	Santa Cruz	#sc-47724	1:10,000; Stock-concentration: 200 µg/ml	#G1522
URL: https://www.scbt.com/de/p/gapdh-antibody-0411?srsltid=AfmBOorkUQuABvukTdbxkTBSAecaFIORKLU5yFZLzYpwSYvopIpVTM1P				
Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488	ThermoFisher	#A-21206	1:200, stock concentration: 2 mg/ml	#2668665
URL: https://www.thermofisher.com/antibody/product/Donkey-anti-Rabbit-IgG-H-L-Highly-Cross-Adsorbed-Secondary-Antibody-Polyclonal/A-21206				
Hoechst 33342 Stain	Biomol	#ICT-639	1:200, stock concentration: 200 µg/ml	#22D15
URL: https://www.biomol.com/de/produkte/chemikalien/farbstoffe-und-labeling/hoechst-33342-stain-1-ml-ict-639				
Acti-stain 555 phalloidin	Cytoskeleton	#PHDH1	1:140, stock concentration 14 µM	No information on vial
URL: https://www.cytoskeleton.com/phdh1				

Cultured Cells

Name	Vendor or Source	Sex (F, M, or unknown)	Persistent ID / URL

PBMCs	isolated from buffy coats of healthy blood donors (ethical vote 272-12-13082012, University Leipzig)	unknown	https://www.uniklinikum-leipzig.de/einrichtungen/blutbank
THP1	DSMZ, #ACC 16	M (established from the peripheral blood of a 1-year-old boy with acute monocytic leukemia (AML) at relapse in 1978)	https://www.dsmz.de/collection/catalogue/details/culture/ACC-16

Data & Code Availability

Description	Source / Repository	Persistent ID / URL
The mouse single-cell RNA-seq (scRNA-seq) and mouse and human bulk RNA sequencing data sets shown in this publication can be accessed at the National Center for Biotechnology Information Gene Expression Omnibus with accession number GSE298197.		

Study Design

Male C57BL/6N mice were fed a high-fat diet for 15 weeks, and diastolic dysfunction was induced by treatment with L-NAME. A random subset of these mice was additionally treated with nitro-oleic acid (NO_2-OA) for the last 4 weeks.

PBMCs were isolated from these animals for single cell and bulk RNA sequencing to analyze immune system dynamics. No samples were excluded from the analysis. All procedures were approved by the local animal care committee (LANUV, Germany).

Groups	Sex	Age	Number (prior to experiment)	Number (after termination)	Littermates (Yes/No)	Other description
Group 1 (Control)	Male	4-week + 15 weeks treatment	5	5	Yes	C57BL/6N; fed standard chow diet for 15 weeks
Group 2 (HFpEF)	Male	4-week + 15 weeks treatment	5	5	Yes	C57BL/6N; high-fat diet + L-NAME for 15 weeks
Group 3 (HFpEF + NO_2-OA)	Male	4-week + 15 weeks treatment	6	6	Yes	C57BL/6N; HFD + L-NAME for 15 weeks, NO_2-OA during last 4 weeks

Sample Size:

The sample size for the experimental groups was determined based on expected differences in target parameters and their variation, following standard practices for determining statistical power. Deviations in sample sizes between groups occurred due to animal loss from technical difficulties or disease burden.

Inclusion Criteria

All collected PBMC samples were included in the analysis.

Exclusion Criteria

No PBMC samples were excluded from the analysis.

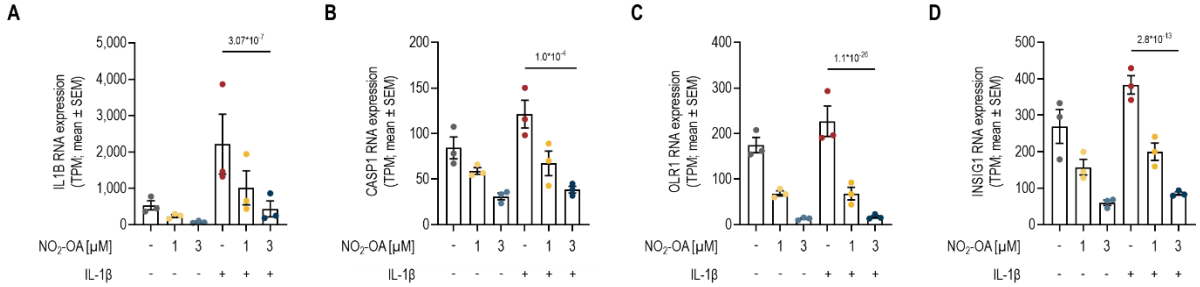
Randomization

Allocation of mice into experimental groups was random, except for allocation of mice after 11 weeks of HFD + L-NAME into treatment groups. Here, mice were allocated so that mean body weight was equal in all groups.

Blinding

All investigators were blinded to group allocation, except for the identification of chow or HFD + L-NAME while performing echocardiography, hemodynamic measurements and exercise test. Here, differences in body weight were obvious. However, the treatment of HFD + L-NAME with vehicle or NO_2-OA was concealed during these experiments as well.

Supplemental Figures

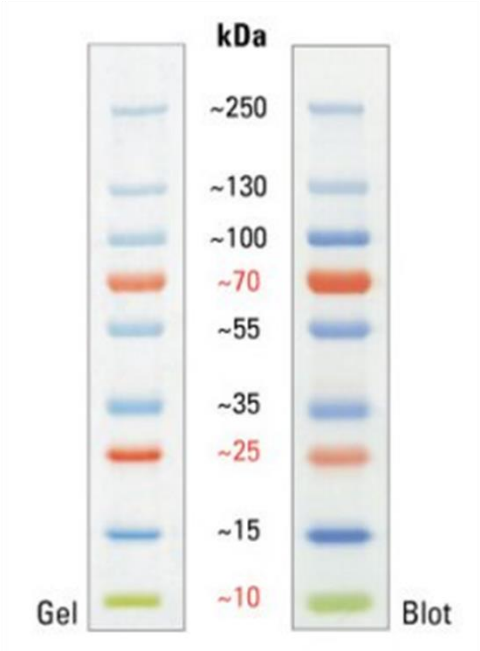


Supplemental Figure S1. NO_2-OA treatment reduces the inflammatory response of human immune cells

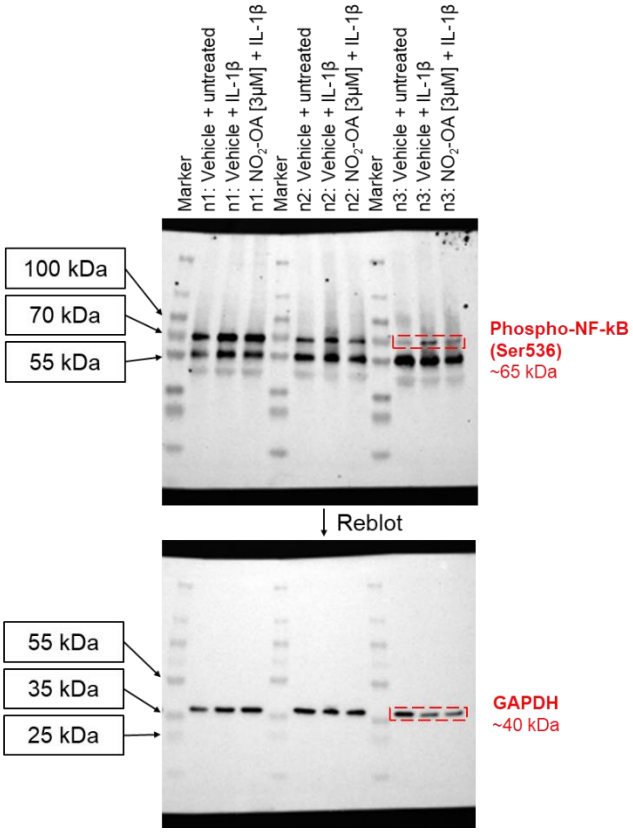
A-**D**, PBMCs were pre-treated with NO_2-OA ([1 µM] or [3 µM]) for 2.5h, then treated with IL-1β (10 ng/mL) for 4.5h. Bulk RNA seq. was performed (n=3, Cell culture). Using *edgeR* differentially expressed genes (exact negative binomial test, *P*-Values<0.05) were identified. Bar graphs show RNA expression of [A] IL1B, [B] CASP1, [C] OLR1, and [D] INSIG1 (all: TPM; n=3, Cell culture; only *P*-Value of exact negative binomial test between Vehicle + IL-1β vs. NO_2-OA [3 µM] + IL-1β shown).

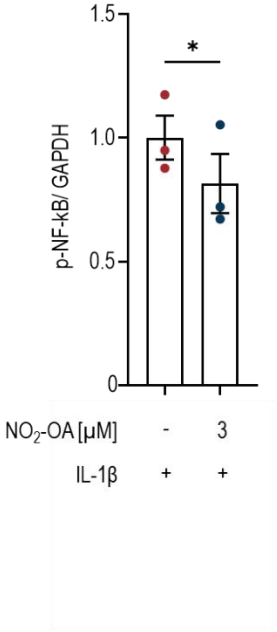
Unedited Blots

Used marker for Immunoblots: PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa (Thermo Scientific, #26620)

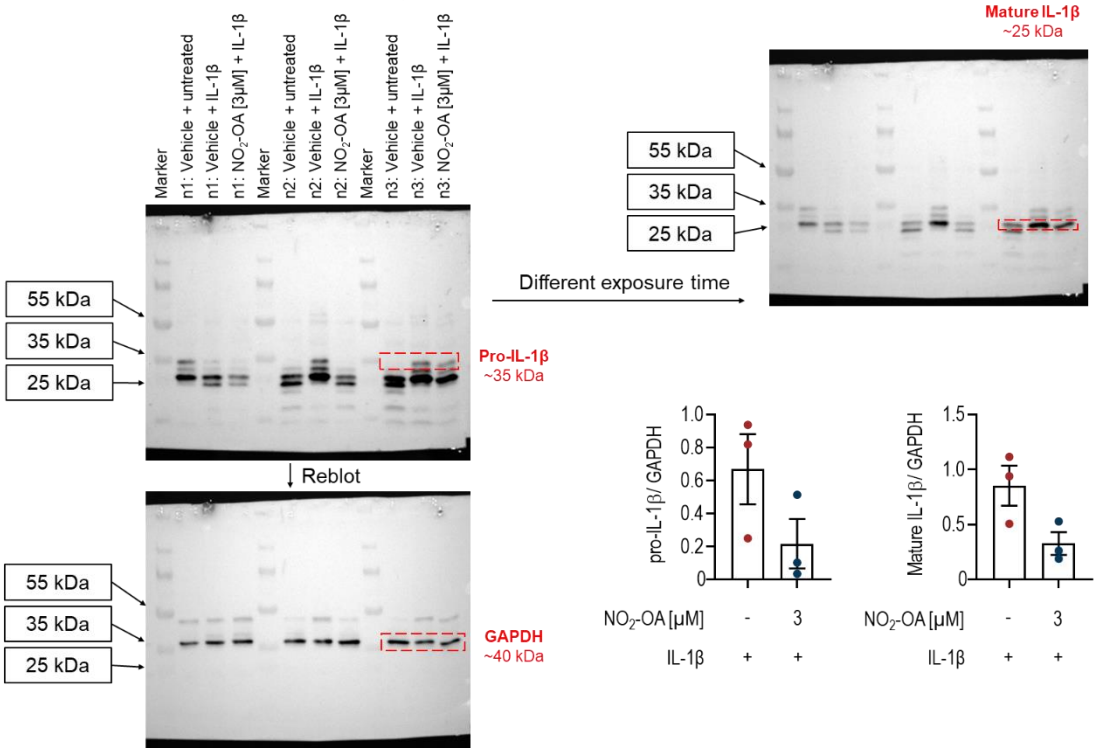


Full unedited immunoblots for Figure 3I: upper panel





Full unedited immunoblots for Figure 3I: lower panel



Statistical analysis: "Circulating immune cell signature analysis in HFpEF across species"

Figure	Panel	Statistical test	Statistics with Software	Why was this test chosen?	Further information	Outlier detected	Number of Tests Adjusted	Adjustment Method	P-Values
1	A	x	x	x	x	x	x	x	x
1	B	x	x	x	x	x	x	x	x
1	C	x	x	x	x	x	x	x	x
1	D	x	x	x	x	x	x	x	x
1	E	Fisher's Exact test with subsequent Benjamini and Hochberg correction	R v4.3	GO analyses were performed using the TopGO R package, which uses Fisher's exact test as the default for calculating significance.	Differentially expressed genes (DEGs) were identified using the FindMarkers function, which by default uses the Wilcoxon rank sum test with Bonferroni-corrected *P* values. GO analysis was then performed on the DEGs.	x	x	Benjamini and Hochberg correction	Significantly upregulated DEGs (Wilcoxon rank-sum test, Bonferroni-corrected P-Values<0.05) were subjected to separate GO analyses.
1	F	Wilcoxon rank sum test with Bonferroni-corrected P values	R v4.3	Number of found DEGs is depicted, FindMarkers function uses by default Wilcoxon rank sum test with Bonferroni-corrected P values.	x	x	Number of genes	Bonferroni correction	Number of significantly upregulated DEGs (Wilcoxon rank-sum test, Bonferroni-corrected P-Values<0.05) in each disease and overlaps shown.
1	G	x	x	x	x	x	x	x	x
1	H	x	x	x	x	x	x	x	x
1	I	x	x	x	x	x	x	x	x
1	J	Fisher's Exact test with subsequent Benjamini and Hochberg correction	R v4.3	GO analyses were performed using the TopGO R package, which uses Fisher's exact test as the default for calculating significance.	Differentially expressed genes (DEGs) were identified using the FindMarkers function, which by default uses the Wilcoxon rank sum test with Bonferroni-corrected *P* values. GO analysis was then performed on the DEGs.	x	x	Benjamini and Hochberg correction	Significantly upregulated DEGs (Wilcoxon rank-sum test, Bonferroni-corrected P-Values<0.05) were subjected to separate GO analyses.
1	K	x	x	x	x	x	x	x	x

Figure	Panel	Statistical test	Statistics with Software	Why was this test chosen?	Further information	Outlier detected	Number of Tests Adjusted	Adjustment Method	P-Values
2	A	x	x	x	x	x	x	x	x
2	B	Ordinary one-way ANOVA with Tukey's multiple comparisons test, with a single pooled variance	GraphPad Prism 8.4.3	Shapiro-Wilk test: all groups passed normality; Control/ HFpEF/ HFpEF + NO2-OA: different individuals → no matching or pairing; Tukey was the recommended multiple comparisons test	Multiple comparisons: compare the mean of every other column	None	3 pairwise comparisons	Tukey's method for multiple comparisons	ANOVA: → Multiple comparisons: no comparison, ANOVA >0.05 *0,1044* → xx
2	C	Ordinary one-way ANOVA with Tukey's multiple comparisons test, with a single pooled variance	GraphPad Prism 8.4.3	Shapiro-Wilk test: all groups passed normality; Control/ HFpEF/ HFpEF + NO2-OA: different individuals → no matching or pairing; Tukey was the recommended multiple comparisons test	Multiple comparisons: compare the mean of every other column	None	3 pairwise comparisons	Tukey's method for multiple comparisons	ANOVA: → Multiple comparisons: Control vs HFpEF / HFpEF vs HFpEF + NO2-OA 0,0029 → **0,0026** / **0,0343**
2	D	Ordinary one-way ANOVA with Tukey's multiple comparisons test, with a single pooled variance	GraphPad Prism 8.4.3	Shapiro-Wilk test: all groups passed normality; Control/ HFpEF/ HFpEF + NO2-OA: different individuals → no matching or pairing; Tukey was the recommended multiple comparisons test	Multiple comparisons: compare the mean of every other column	None	3 pairwise comparisons	Tukey's method for multiple comparisons	ANOVA: → Multiple comparisons: Control vs HFpEF / HFpEF vs HFpEF + NO2-OA 1,7E-05 → **3,0E-05** / **2,6E-04**
2	E	x	x	x	x	x	x	x	x
2	F	x	x	x	x	x	x	x	x
2	G	x	x	x	x	x	x	x	x
2	H	x	x	x	x	x	x	x	x
2	I	x	x	x	x	x	x	x	x
2	J	Wilcoxon rank sum test with Bonferroni-corrected P values	R v4.3	Percentage of species-specific and shared orthologue DEGs per cell type is depicted, FindMarkers function uses by default Wilcoxon rank sum test with Bonferroni-corrected P values.	x	x	Number of genes	Bonferroni correction	Percentage of significantly up- and downreagulated DEGs (Wilcoxon rank-sum test, Bonferroni-corrected P-Values<0.05) in each cell type and species shown.
2	K	x	x	x	x	x	x	x	x
2	L	x	x	x	x	x	x	x	x
2	M	x	x	x	x	x	x	x	x
2	N	x	x	x	x	x	x	x	x
2	O	x	x	x	x	x	x	x	x

Next page: Figure 3

Figure	Panel	Statistical test	Statistics with Software	Why was this test chosen?	Further information	Outlier detected	Number of Tests Adjusted	Adjustment Method	P-Values			
3	A	x	x	x	x	x	x	x	x			
3	B	x	x	x	x	x	x	x	x			
3	C	x	x	x	x	x	x	x	x			
3	D	Exact negative binomial test	R v4.3	edgeR uses by default exact negative binomial test	x	x	x	x	Using edgeR differentially expressed genes (exact negative binomial test, P-Values<0.05) were identified. Heat map shows selection of genes significantly up-regulated after IL-1β treatment and down-regulated with NO2-OA [3 µM] treatment prior IL-1β treatment.			
3	E	RM one-way ANOVA with Tukey's multiple comparisons test, with a single pooled variance	GraphPad Prism 8.4.3	Shapiro-Wilk test: all groups passed normality; Cells from a different donor were used for all 6 conditions of each sample replicate → paired values; Dunn was the recommended multiple comparisons test	Multiple comparisons: compare the mean of every other column	None	3 pairwise comparisons	Tukey's method for multiple comparisons	ANOVA:	Multiple comparisons: Vehicle + IL-1β vs. NO2-OA [3 µM] + IL-1β	Multiple comparisons: Vehicle + Untreated vs. NO2-OA [3 µM] + IL-1β	
									1,4E-03	**0,0010**	**0,0372**	
3	F	RM one-way ANOVA with Tukey's multiple comparisons test, with a single pooled variance	GraphPad Prism 8.4.3	Shapiro-Wilk test: all groups passed normality; Cells from a different donor were used for all 6 conditions of each sample replicate → paired values; Dunn was the recommended multiple comparisons test	Multiple comparisons: compare the mean of every other column	None	3 pairwise comparisons	Tukey's method for multiple comparisons	ANOVA:	Multiple comparisons: Vehicle + Untreated s. Vehicle + IL-1β	Multiple comparisons: Vehicle + IL-1β vs. NO2-OA [3 µM] + IL-1β	
									1,9E-05	**3,6E-05**	**1,5E-04**	
3	G	RM one-way ANOVA with Tukey's multiple comparisons test, with a single pooled variance	GraphPad Prism 8.4.3	Shapiro-Wilk test: all groups passed normality; Cells from a different donor were used for all 6 conditions of each sample replicate → paired values; Dunn was the recommended multiple comparisons test	Multiple comparisons: compare the mean of every other column	None	3 pairwise comparisons	Tukey's method for multiple comparisons	ANOVA:	Multiple comparisons: Vehicle + IL-1β vs. NO2-OA [3 µM] + IL-1β	Multiple comparisons: Vehicle + Untreated vs. NO2-OA [3 µM] + IL-1β	
									6,2E-05	**1,1E-04**	**4,6E-04**	
3	H	RM one-way ANOVA with Tukey's multiple comparisons test, with a single pooled variance	GraphPad Prism 8.4.3	Shapiro-Wilk test: all groups passed normality; Cells from a different donor were used for all 6 conditions of each sample replicate → paired values; Dunn was the recommended multiple comparisons test	Multiple comparisons: compare the mean of every other column	None	3 pairwise comparisons	Tukey's method for multiple comparisons	ANOVA:	Multiple comparisons: Vehicle + IL-1β vs. NO2-OA [3 µM] + IL-1β	Multiple comparisons: Vehicle + Untreated vs. NO2-OA [3 µM] + IL-1β	
									9,1E-04	**6,5E-04**	**0,0365**	
3	I	x	x	x	x	x	x	x	x			
3	J	x	x	x	x	x	x	x	x			
3	K	Exact negative binomial test	R v4.3	edgeR uses by default exact negative binomial test	x	x	x	x	Using edgeR differentially expressed genes (exact negative binomial test, P-Values<0.05) were identified which were down-regulated when pre-treated with NO2-OA [3 µM] before IL-1β treatment, compared to IL-1β treatment following prior vehicle treatment.			Venn diagram shows number of genes significantly downregulated by NO2-OA in the cell culture experiment and the HFpEF-animal model (Cohort 4) as well as orthologue overlaps.
									Using *edgeR* differentially expressed genes (exact negative binomial test, *P*-Values<0.05) were identified which were down-regulated in HFpEF + NO_2-OA mice compared to HFpEF mice.			
3	L	x	x	x	x	x	x	x	x			
3	M	x	x	x	x	x	x	x	x			
3	N	x	x	x	x	x	x	x	x			

Next page: Figure 4

Figure	Panel	Statistical test	Statistics with Software	Why was this test chosen?	Further information	Outlier detected	Number of Tests Adjusted	Adjustment Method	P-Values				
4	A	Wilcoxon rank sum test with Bonferroni-corrected P values	R v4.3	Number of found DEGs is depicted, FindMarkers function uses by default Wilcoxon rank sum test with Bonferroni-corrected P values.	x	x	Number of genes	Bonferroni correction	Number of significantly upregulated DEGs (Wilcoxon rank-sum test, Bonferroni-corrected P-Values<0.05) in each disease separated by BMI and overlaps shown.				
4	B	x	x	x	x	x	x	x	x				
4	C	x	x	x	x	x	x	x	x				
4	D	x	x	x	x	x	x	x	x				
4	E - DYNC1I2	Kruskal-Wallis test with Dunn's multiple comparisons test	GraphPad Prism 8.4.3	Non-parametric test due to n<10; Control/ HFpEF ...: different individuals → no matching or pairing; Dunn was the recommended multiple comparisons test	Multiple comparisons: compare the mean of every other column	None	6 pairwise comparisons	Dunn's method for multiple comparisons	ANOVA:	Multiple comparisons			
										Control lean vs HFpEF obese	Control obese vs HFpEF obese		
									4,9E-03	**0,0291**	**0,0345**		
4	E - COPS9	Kruskal-Wallis test with Dunn's multiple comparisons test	GraphPad Prism 8.4.3	Non-parametric test due to n<10; Control/ HFpEF ...: different individuals → no matching or pairing; Tukey was the recommended multiple comparisons test	Multiple comparisons: compare the mean of every other column	None	6 pairwise comparisons	Dunn's method for multiple comparisons	ANOVA:	Multiple comparisons			
										Control lean vs HFpEF obese	Control obese vs HFpEF obese		
									2,0E-02	*0,1052*	*0,0668*		
4	E - H4C5	Kruskal-Wallis test with Dunn's multiple comparisons test	GraphPad Prism 8.4.3	Non-parametric test due to n<10; Control/ HFpEF ...: different individuals → no matching or pairing; Tukey was the recommended multiple comparisons test	Multiple comparisons: compare the mean of every other column	None	6 pairwise comparisons	Dunn's method for multiple comparisons	ANOVA:	Multiple comparisons			
										HFpEF lean vs HFpEF obese			
									6,0E-02	*0,0895*			
4	E - TMEM14A	Kruskal-Wallis test with Dunn's multiple comparisons test	GraphPad Prism 8.4.3	Non-parametric test due to n<10; Control/ HFpEF ...: different individuals → no matching or pairing; Tukey was the recommended multiple comparisons test	Multiple comparisons: compare the mean of every other column	None	6 pairwise comparisons	Dunn's method for multiple comparisons	ANOVA:	Multiple comparisons			
										no comparison, ANOVA >0.05			
									1,2E-01	*xx*			
4	E - CRYZ	Kruskal-Wallis test with Dunn's multiple comparisons test	GraphPad Prism 8.4.3	Non-parametric test due to n<10; Control/ HFpEF ...: different individuals → no matching or pairing; Tukey was the recommended multiple comparisons test	Multiple comparisons: compare the mean of every other column	None	6 pairwise comparisons	Dunn's method for multiple comparisons	ANOVA:	Multiple comparisons			
										Control lean vs HFpEF obese			
									3,2E-02	*0,0505*			
4	E - TOMM22	Kruskal-Wallis test with Dunn's multiple comparisons test	GraphPad Prism 8.4.3	Non-parametric test due to n<10; Control/ HFpEF ...: different individuals → no matching or pairing; Tukey was the recommended multiple comparisons test	Multiple comparisons: compare the mean of every other column	None	6 pairwise comparisons	Dunn's method for multiple comparisons	ANOVA:	Multiple comparisons			
										Control lean vs HFpEF obese	Control obese vs HFpEF lean	Control obese vs HFpEF obese	
									1,3E-03	*0,0561*	*0,0581*	**0,0021**	
4	F - DYNC1I2	Kruskal-Wallis test with Dunn's multiple comparisons test	GraphPad Prism 8.4.3	non-parametric: n<10; Control/ HFpEF ...: different individuals → no matching or pairing; Tukey was the recommended multiple comparisons test	Multiple comparisons: compare the mean of every other column	None	6 pairwise comparisons	Dunn's method for multiple comparisons	ANOVA:	Multiple comparisons			
										Control vs HFpEF	HFrEF ischemic vs HFpEF	HFrEF non-ischemic vs HFpEF	
									6,3E-05	**0,0062**	**0,0030**	**0,0030**	
4	F - COPS9	Kruskal-Wallis test with Dunn's multiple comparisons test	GraphPad Prism 8.4.3	non-parametric: n<10; Control/ HFpEF ...: different individuals → no matching or pairing; Tukey was the recommended multiple comparisons test	Multiple comparisons: compare the mean of every other column	None	6 pairwise comparisons	Dunn's method for multiple comparisons	ANOVA:	Multiple comparisons			
										Control vs HFrEF non-ischemic	HFrEF non-ischemic vs HFpEF		
									2,2E-03	*0,9278*	**0,0011**		
4	F - H4C5	Kruskal-Wallis test with Dunn's multiple comparisons test	GraphPad Prism 8.4.3	non-parametric: n<10; Control/ HFpEF ...: different individuals → no matching or pairing; Tukey was the recommended multiple comparisons test	Multiple comparisons: compare the mean of every other column	None	6 pairwise comparisons	Dunn's method for multiple comparisons	ANOVA:	Multiple comparisons			
										Control vs HFrEF ischemic	Control vs HFrEF non-ischemic	HFrEF ischemic vs HFpEF	HFrEF non-ischemic vs HFpEF
									1,8E-06	**0,0396**	**1,5E-04**	**0,0409**	**2,1E-05**
4	F - TMEM14A	Kruskal-Wallis test with Dunn's multiple comparisons test	GraphPad Prism 8.4.3	non-parametric: n<10; Control/ HFpEF ...: different individuals → no matching or pairing; Tukey was the recommended multiple comparisons test	Multiple comparisons: compare the mean of every other column	None	6 pairwise comparisons	Dunn's method for multiple comparisons	ANOVA:	Multiple comparisons			
										HFrEF ischemic vs HFpEF			
									0,0354	*0,1016*			
4	F - CRYZ	Kruskal-Wallis test with Dunn's multiple comparisons test	GraphPad Prism 8.4.3	non-parametric: n<10; Control/ HFpEF ...: different individuals → no matching or pairing; Tukey was the recommended multiple comparisons test	Multiple comparisons: compare the mean of every other column	None	6 pairwise comparisons	Dunn's method for multiple comparisons	ANOVA:	Multiple comparisons			
										no comparison, ANOVA >0.05			
									0,0747	xx			
4	F - TOMM22	Kruskal-Wallis test with Dunn's multiple comparisons test	GraphPad Prism 8.4.3	non-parametric: n<10; Control/ HFpEF ...: different individuals → no matching or pairing; Tukey was the recommended multiple comparisons test	Multiple comparisons: compare the mean of every other column	None	6 pairwise comparisons	Dunn's method for multiple comparisons	ANOVA:	Multiple comparisons			
										Control vs HFpEF	HFrEF ischemic vs HFpEF	HFrEF non-ischemic vs HFpEF	
									4,6E-05	**0,0084**	*0,0721*	**1,2E-04**	