Supplementary Materials

Region-specific human cardiac extracellular vesicles differentially regulate mitochondrial function

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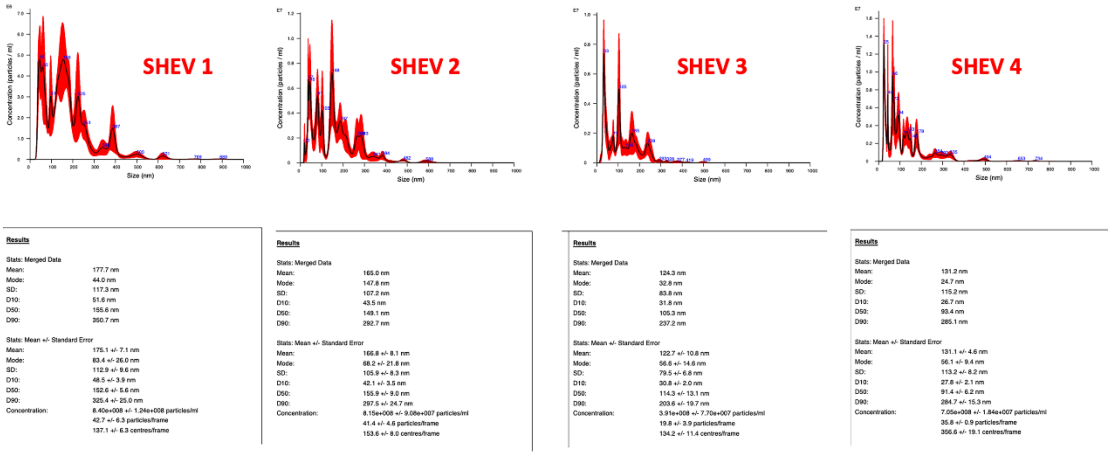
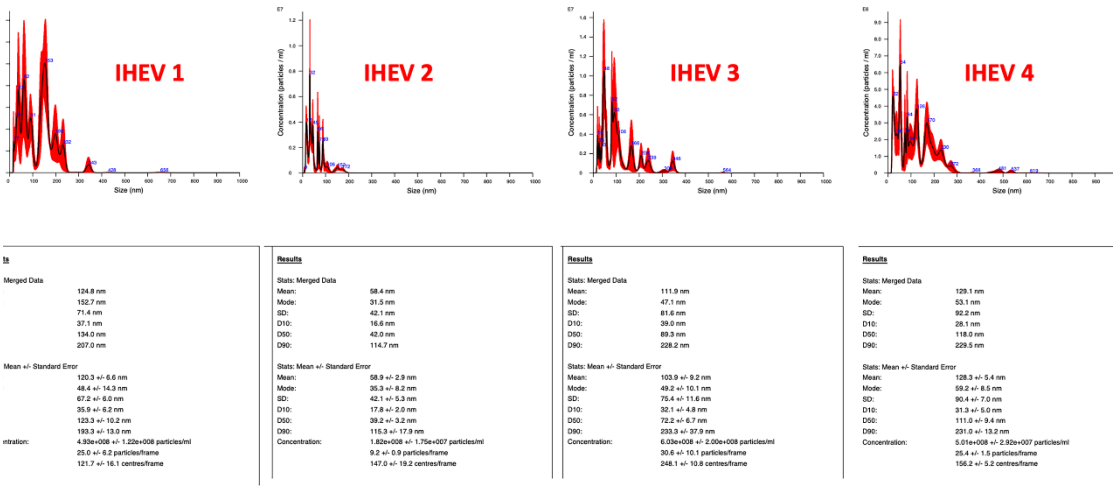
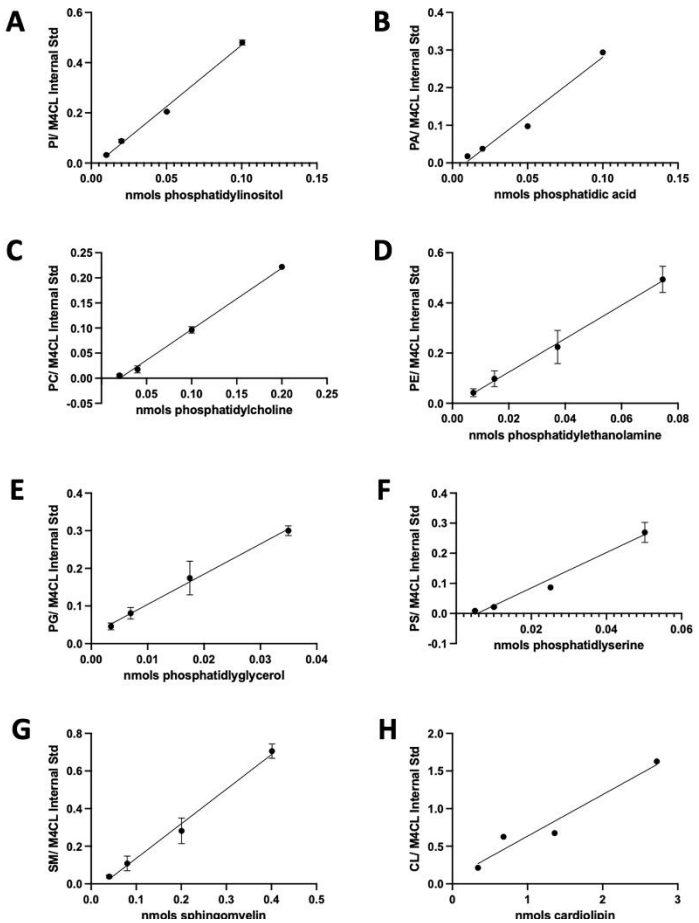


Supplementary Figure 1. Nanosight average curves from SHEV preparations from four donors. Numbers correspond to the donors in Tables 1 and 2. SHEV: Subsarcolemmal heart extracellular vesicle.



Supplementary Figure 2. Nanosight average curves from IHEV preparations from four donors. Numbers correspond to the donors in Tables 1 and 2. IHEV: Interfibrillar heart extracellular vesicle.



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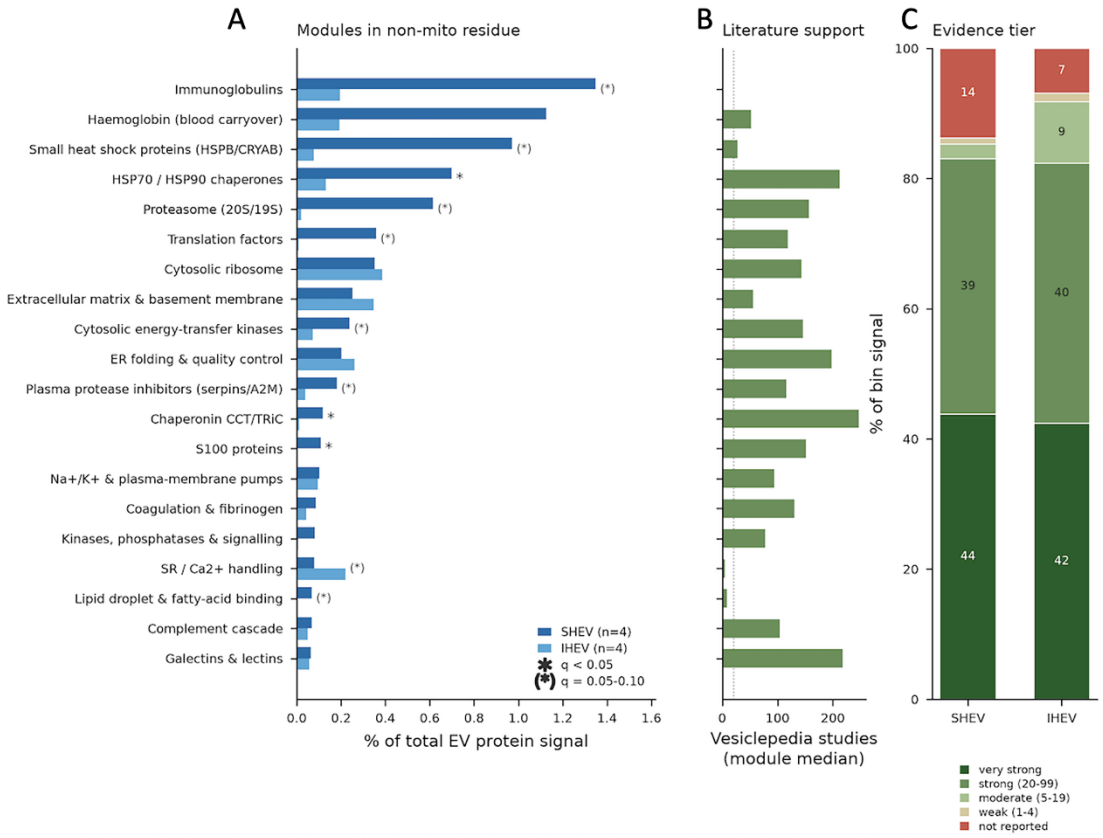
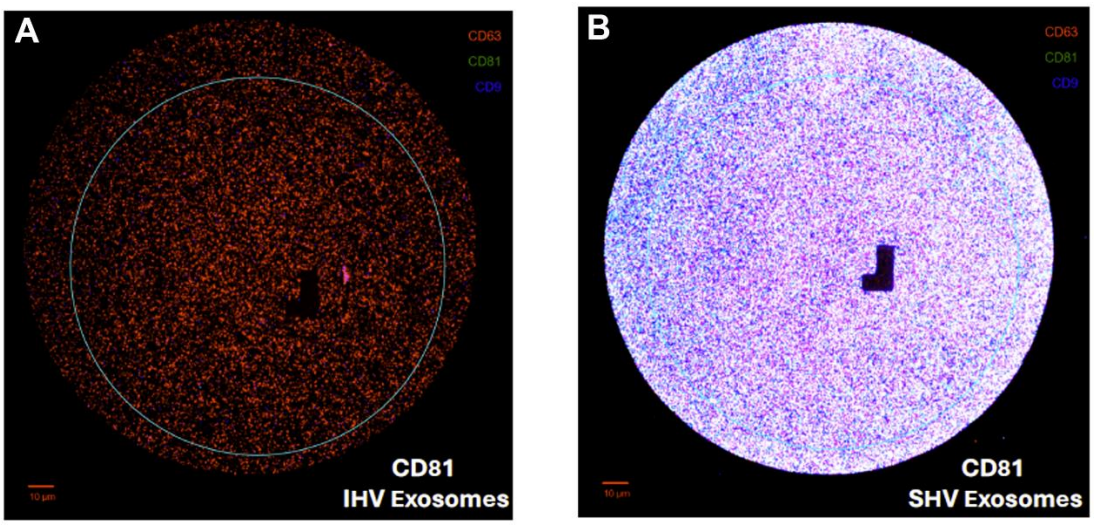
	slope	intercept
PG	4.22	0.28
PE	6.67	-0.01
PI	4.90	-0.02
PS	5.87	-0.03
PA	2.89	0.00
PC	1.23	-0.03
SM	1.84	-0.05
CL	0.55	0.08

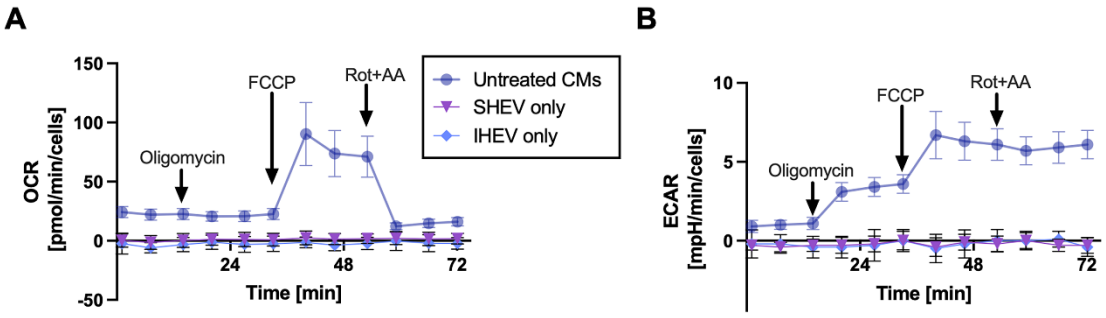
Supplementary Figure 3. Standard curves, slopes, and intercepts for each of the eight phospholipids against tetramyristoylcardiolipin. (A) PI; (B) PA; (C) PC; (D) PE; (E) PG; (F) PS; (G) SM; (H) CL; (I) slopes and intercepts of the curves in (A–H). PI: Phosphatidylinositol; PA: phosphatidic acid; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PG: phosphatidylglycerol; PS: phosphatidylserine; SM: sphingomyelin; CL: cardiolipin.



Supplementary Figure 4. Protein modules in the "Other Non-mitochondrial" fraction of cardiac EV proteomes, with EV-literature support as analyzed by Claude Science. (A) Summed abundance of each functional module in the "Other Non-Mitochondrial" category as a percentage of total EV protein signal, mean of n=4 SHEV and n=4 IHEV preparations. Significance is a paired t-test of module sums across the four donor-matched SHEV/IHEV pairs, Benjamini-Hochberg corrected across all 35 modules tested: *$q < 0.05$; (*) $q = 0.05–0.10$; (B) Module median number of distinct published EV experiments reporting each member protein (Vesiclepedia 5.1, human protein records, joined on Entrez Gene ID); dotted line, 20 studies; (C) Composition of each EV type's fraction signal by per-protein evidence tier, as % of that EV type's bin total; tiers are cut on Vesiclepedia study count (very strong ⩾100; strong 20–99; moderate 5–19; weak 1–4; not reported). EV: Extracellular vesicle; SHEV: subsarcolemmal heart extracellular vesicle; IHEV: interfibrillar heart extracellular vesicle.

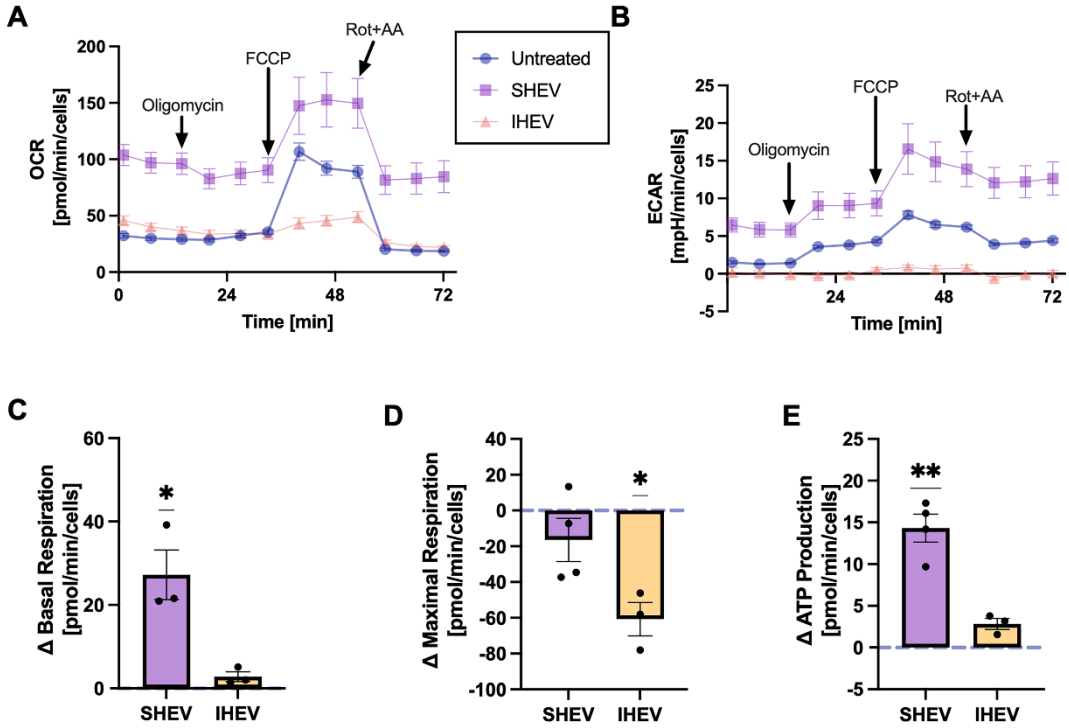


Supplementary Figure 5. CD81 exosomes are elevated in SHEV compared with IHEV as determined by Leprechaun analysis. (A) Example of CD81-captured exosomes from IHEV co-stained for the tetraspanins CD63, CD81, and CD9; (B) CD81-captured exosomes from SHEV stained in the same manner. EVs were from the same human heart. SHEV has more CD81 exosomes and more that co-express other tetraspanins. SHEV: Subsarcolemmal heart extracellular vesicle; IHEV: interfibrillar heart extracellular vesicle; EV: extracellular vesicle.



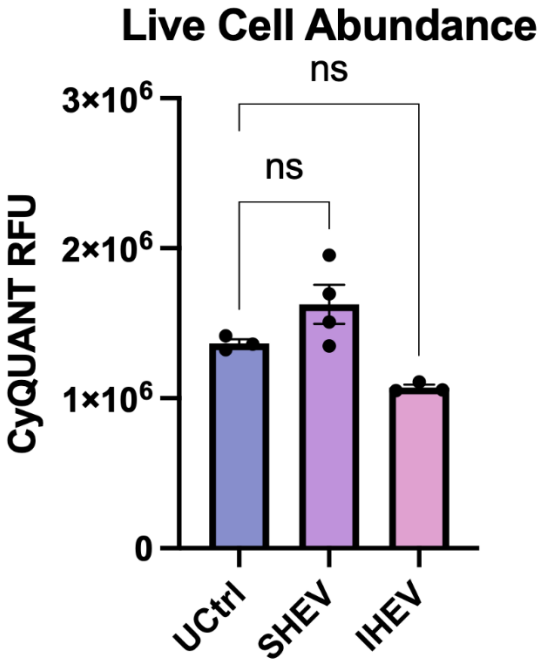
Supplementary Figure 6. SHEV and IHEV preparations exhibit no detectable intrinsic respiratory activity. (A) OCR and (B) ECAR were measured in Seahorse wells containing SHEV or IHEV preparations in the absence of NRVMs and compared with untreated cardiomyocytes (CMs). SHEV and IHEV preparations alone exhibited no detectable oxygen consumption or extracellular acidification under these assay conditions, indicating that the EV preparations did not contribute measurable intrinsic respiratory activity to the responses observed in EV-treated NRVMs. Data represent technical Seahorse wells; error bars indicate SEM. Arrows indicate substrate or inhibitor additions. SHEV: Subsarcolemmal heart extracellular vesicle; IHEV: interfibrillar heart extracellular vesicle; OCR: oxygen consumption rate; ECAR:

extracellular acidification rate; NRVM: neonatal rat ventricular myocyte; CM: cardiomyocyte; EV: extracellular vesicle; SEM: standard error of the mean.



Supplementary Figure 7. Independent replication of SHEV- and IHEV-associated effects on cardiomyocyte bioenergetics. An independent NRVM preparation was treated for 48 h with the same donor-derived SHEV and IHEV preparations used in the particle-normalized experiment shown in Figure 9. In this experiment, equal volumes (10 µL/well) of each EV preparation were administered. Retrospective calculation using NTA-derived particle concentrations indicated EV exposures ranging from approximately 1.82×10^8 to 8.40×10^8 particles/well. (A) OCR and (B) ECAR in untreated, SHEV-treated, and IHEV-treated NRVMs. Arrows indicate substrate or inhibitor additions. (C) Change in basal respiration, (D) maximal respiration, and (E) ATP production relative to the mean untreated response from the same NRVM preparation (Δ = EV-treated donor mean − untreated mean). Each point in C–E represents one independently derived human EV donor preparation and is the mean of technical Seahorse wells. The dashed horizontal line at Δ=0 represents the untreated NRVM reference. Donor-level Δ values were compared with zero using two-sided one-sample t-tests. *$p<0.05$; **$p<0.01$. Error bars indicate SEM. NRVM: Neonatal rat ventricular myocyte; SHEV: subsarcolemmal heart extracellular vesicle; IHEV: interfibrillar heart extracellular vesicle; EV: extracellular vesicle; NTA:

nanoparticle tracking analysis; OCR: oxygen consumption rate; ECAR: extracellular acidification rate; ATP: adenosine triphosphate; SEM: standard error of the mean.



Supplementary Figure 8. CyQUANT assessment of viable-cell abundance following EV treatment. Viability of SHEV- or IHEV-treated cardiomyocytes compared with untreated control cardiomyocytes (Uctrl). Analysis was performed using ordinary one-way ANOVA with multiple comparisons to untreated cells corrected using Tukey's test. Each data point for SHEV and IHEV treated cells is the average of five separate wells per condition. Data points for untreated cells represent individual Seahorse wells. Error bars show SEM. EV: Extracellular vesicle; SHEV: subsarcolemmal heart extracellular vesicle; IHEV: interfibrillar heart extracellular vesicle; Uctrl: untreated control; ANOVA: analysis of variance; SEM: standard error of the mean.

Supplementary Table 1. Number of side chain carbons and double bonds in phospholipid species analyzed for this study

CARDIOLIPIN			PHOSPHATIDIC ACID			PHOSPHATIDYLGLYCEROL		
Mass/Charge	**Carbons**	**Double Bonds**	**Mass/Charge**	**Carbons**	**Double Bonds**	**Mass/Charge**	**Carbons**	**Double Bonds**
CL 1394	68	7	**PA 671**	34	2	**PG 745**	34	2
CL 1396	68	6	**PA 673**	34	1	**PG 747**	34	1
CL 1398	68	5	**PA 675**	34	0	**PG 773**	36	2
CL 1422	70	5	**PA 695**	36	4	**PG 775**	36	1
CL 1424	70	6	**PA 697**	36	3			
CL 1448	72	8	**PA 699**	36	2	**PHOSPHATIDYLINOSITOL**		
CL 1450	72	7	**PA 701**	36	1	**PI 857**	36	4
CL 1452	72	6				**PI 859**	36	3
CL 1454	72	5	**PHOSPHATIDYLCHOLINE**			**PI 861**	36	2
CL 1456	72	4	**PC 790**	32	1	**PI 885**	38	4
CL 1470	74	11	**PC 792**	32	0	**PI 887**	38	3
CL 1472	74	10	**PC 816**	34	2	**PI 889**	38	2
CL 1474	74	9	**PC 818**	34	1	**PI 891**	38	1
CL 1476	74	8	**PC 840**	36	4	**PI 909**	40	6
CL 1494	76	13	**PC 842**	36	3	**PI 911**	40	5
CL 1496	76	12	**PC 844**	36	2	**PI 913**	40	4

CL 1498	76	11	**PC 864**	38	6			
CL 1518	78	15	**PC 866**	38	5	**PHOSPHATIDYLSERINE**		
CL 1520	78	14				**PS 758**	34	2
CL 1522	78	13	**PHOSPHATIDYLETHANOLAMINE**			**PS 760**	34	1
CL 1524	78	12	**PE 714**	34	2	**PS 762**	34	0
CL 1544	80	16	**PE 716**	34	1	**PS 786**	36	2
CL 1546	80	15	**PE 738**	36	4	**PS 788**	36	1
CL 1548	80	14	**PE 740**	36	3	**PS 810**	38	4
			PE 742	36	2	**PS 834**	40	6
MONOLYSOCARDIOLIPIN			**PE 762**	38	5			
mlcl 1160	52	5	**PE 764**	38	4	**SPHINGOMYELIN**		
mlcl 1162	52	4	**PE 766**	38	3	**SM 789**	18	0
mlcl 1186	54	6	**PE 788**	40	5	**SM 817**	20	0
mlcl 1188	54	5	**PE 790**	40	6	**SM 843**	22	1
mlcl 1210	56	8				**SM 845**	22	0
mlcl 1212	56	7				**SM 869**	24	2
						SM 871	24	1

CL: Cardiolipin; PA: phosphatidic acid; PG: phosphatidylglycerol; PC: phosphatidylcholine; PI: phosphatidylinositol; PE: phosphatidylethanolamine; PS: phosphatidylserine; SM: sphingomyelin; mlcl: monolysocardiolipin.

Supplementary Table 2. Statistics for protein function bar graphs in Figure 5 using ordinary one-way ANOVA corrected for multiple comparisons using Tukey's test

Tukey's multiple comparisons test	Mitochondrial & glycolysis	Sarcomere cytoskeleton	Vesicle trafficking	ER, Golgi & secretory	Other non-mitochondrial
SSL Mito *vs.* IF Mito	**0.0032**	**0.0372**	**0.027**	0.9797	0.9512
SSL Mito *vs.* SHEV	**< 0.0001**	**< 0.0001**	**0.0016**	**0.027**	0.1614
SSL Mito *vs.* IHEV	**< 0.0001**	**< 0.0001**	**< 0.0001**	0.0777	**0.0039**
SSL Mito *vs.* Heart	**< 0.0001**	**< 0.0001**	**0.0001**	**< 0.0001**	0.057
IF Mito *vs.* SHEV	**< 0.0001**	**< 0.0001**	0.5676	**0.011**	0.05
IF Mito *vs.* IHEV	**< 0.0001**	**< 0.0001**	**0.0015**	**0.0309**	**0.0117**
IF Mito *vs.* Heart	**< 0.0001**	**< 0.0001**	**0.0092**	**< 0.0001**	**0.0206**
SHEV *vs.* IHEV	0.1663	**0.0043**	**0.0029**	0.8946	**< 0.0001**
SHEV *vs.* Heart	**0.0044**	**< 0.0001**	**0.0296**	**< 0.0001**	0.7384
IHEV *vs.* Heart	**0.0004**	**< 0.0001**	0.9527	**< 0.0001**	**< 0.0001**

SSL: Subsarcolemmal; IF: interfibrillar; Mito: mitochondria; SHEV: subsarcolemmal heart extracellular vesicle; IHEV: interfibrillar heart extracellular vesicle; ANOVA: analysis of variance.