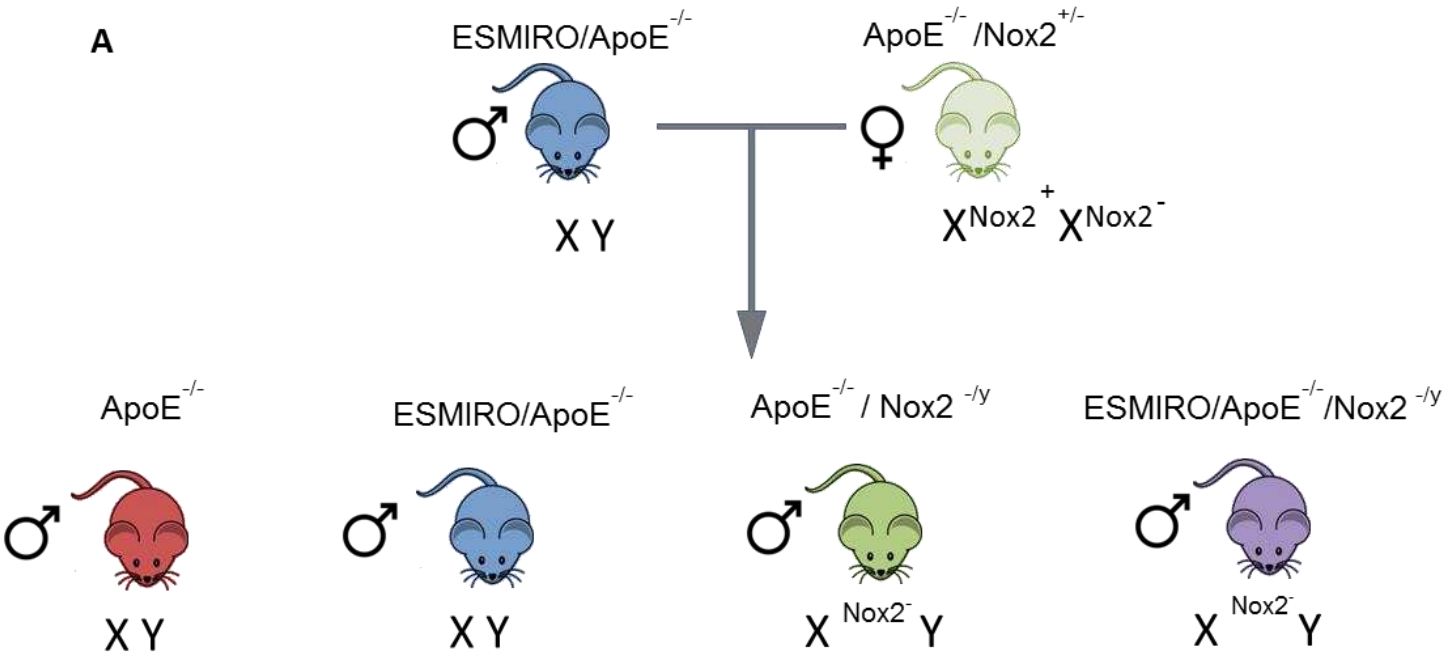
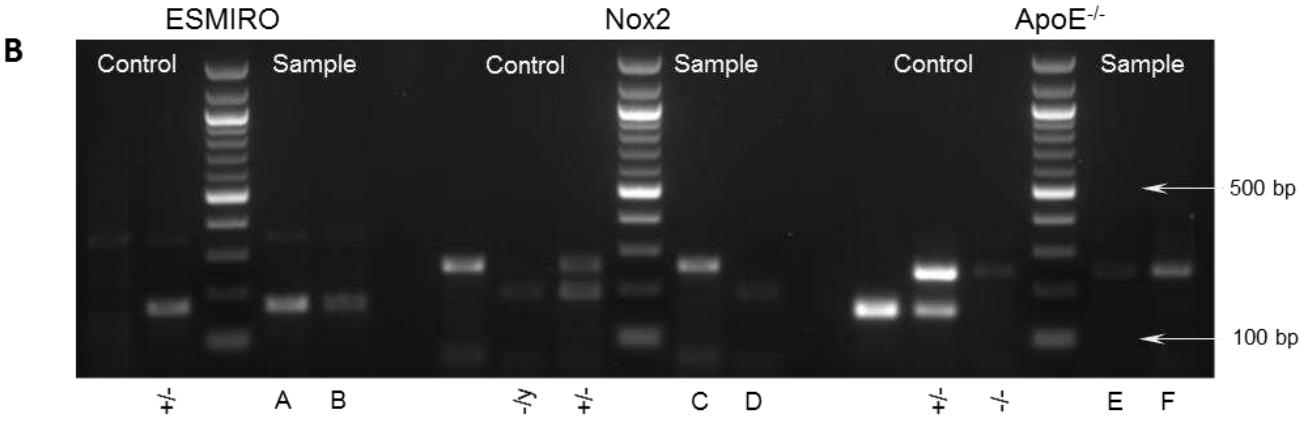
Divergent effects of genetic and pharmacological inhibition of Nox2 NADPH oxidase on insulin resistance related vascular damage

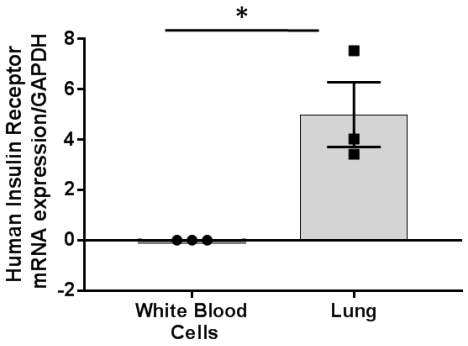
SUPPLEMENTAL MATERIAL

Maqbool *et al.* 2020

SUPPLEMENTAL FIGURE S1: Breeding and Genotyping Strategy







A: Observed male progeny from male ESMIRO/ApoE$^{-/-}$ after mating with female ApoE$^{-/-}$/Nox2$^{+/-}$

B: Genotype analysis of PCR products from progeny compare to established control animals: A-ESMIRO (+/-); B-ESMIRO (+/-); C-Nox2$^{+/y}$; D-Nox2$^{-/y}$; E&F-ApoE$^{-/-}$

C: Tie 2 promoter activity is restricted to (endothelial cells in) the lung with no leakage to white blood cells in ESMIRO/ApoE$^{-/-}$/Nox2$^{-/y}$ mice (n=3). For primer sequences see Refs 9. * denotes P<0.05,

SUPPLEMENTAL FIGURE S2: Genotyping Protocol

For ApoE:

0.5µl 10µM Common: 5'-GCCTAGCCGAGGGAGAGCCG-3'

0.5µl 10µM Wild type Reverse: 5'-TGTGACTTGGGAGCTCTGCAGC-3'

0.5µl 10µM Mutant Reverse: 5'-GCCGCCCCGACTGCATCT-3'

10µl x2 Bio mix red PCR MasterMix (Bioline BIO25006), 12.5µl water and 1µl extracted DNA. PCR cycle conditions are provided in Table S1. Cycles were run using a Verti 96 well thermo cycler (Applied Biosystems). PCR products were then run on a 1.5% agarose gel for 1 hr at 100 V, with a 100 bp ladder (New England Biolabs N0467S). Expected products sizes; were ApoE $^{+/+}$ 155 bp, ApoE $^{-/-}$ 245 bp, and ApoE $^{-/+}$ 155 & 245 bp.

For ESMIRO:

0.5µl 10µM ESMIRO Forward: 5'-TGGCAGCTTTCCCCAACACT -3'

0.5µl 10µM ESMIRO Reverse: 5'-CCGTTCCTCAGGGGTGTCC -3'

10µl x2 Bio mix red PCR MasterMix (Bioline BIO25006), 13µl water and 1µl extracted DNA. PCR cycle conditions are provided in Table S2. Cycles were run using a Verti 96 well thermo cycler (Applied Biosystems). PCR products were then run on a 1.5% agarose gel for 1 hr at 100 V, with a 100 bp ladder (New England Biolabs N0467S). Expected products sizes; were WT no band and ESMIRO 172 bp.

For Nox2:

0.5µl 10µM Common: 5'-AAGAGAAACTCCTCTGCTGTG AA -3''

0.5µl 10µM Wild type: 5'-CGCACTGGAACCCCTGAGAAAGG -3''

1µl 10µM Mutant: 5'-GTTCTA ATTCCATCAGAAGCTTAT CG -3'

10µl x2 Bio mix red PCR MasterMix (Bioline BIO25006), 12µl water and 1µl extracted DNA. PCR cycle conditions are provided in Table S3. Cycles were run using a Verti 96 well thermo cycler (Applied Biosystems). PCR products were then run on a 1.5% agarose gel for 1 hr at 100 V, with a 100 bp ladder (New England Biolabs N0467S). Expected products sizes; were Nox2 $^{+/+}$ 240 bp, Nox2 $^{-/-}$ 195 bp, and Nox2 $^{-/+}$ 195 & 240 bp.

SUPPLEMENTAL TABLE S1: APOE PCR Protocol

PCR	Temperature (°C)	Time	Cycles
Initial denaturation	94	3 min	1
Denaturation	94	20 sec	35
Annealing	68	40 sec	
Extension	72	2 min	

SUPPLEMENTAL TABLE S2: ESMIRO PCR Protocol

PCR	Temperature (°C)	Time	Cycles
Initial denaturation	94	4 min	1
Denaturation	94	1 min	32
Annealing	60	1 min	
Extension	72	1 min	
Final Extension	72	15 min	1

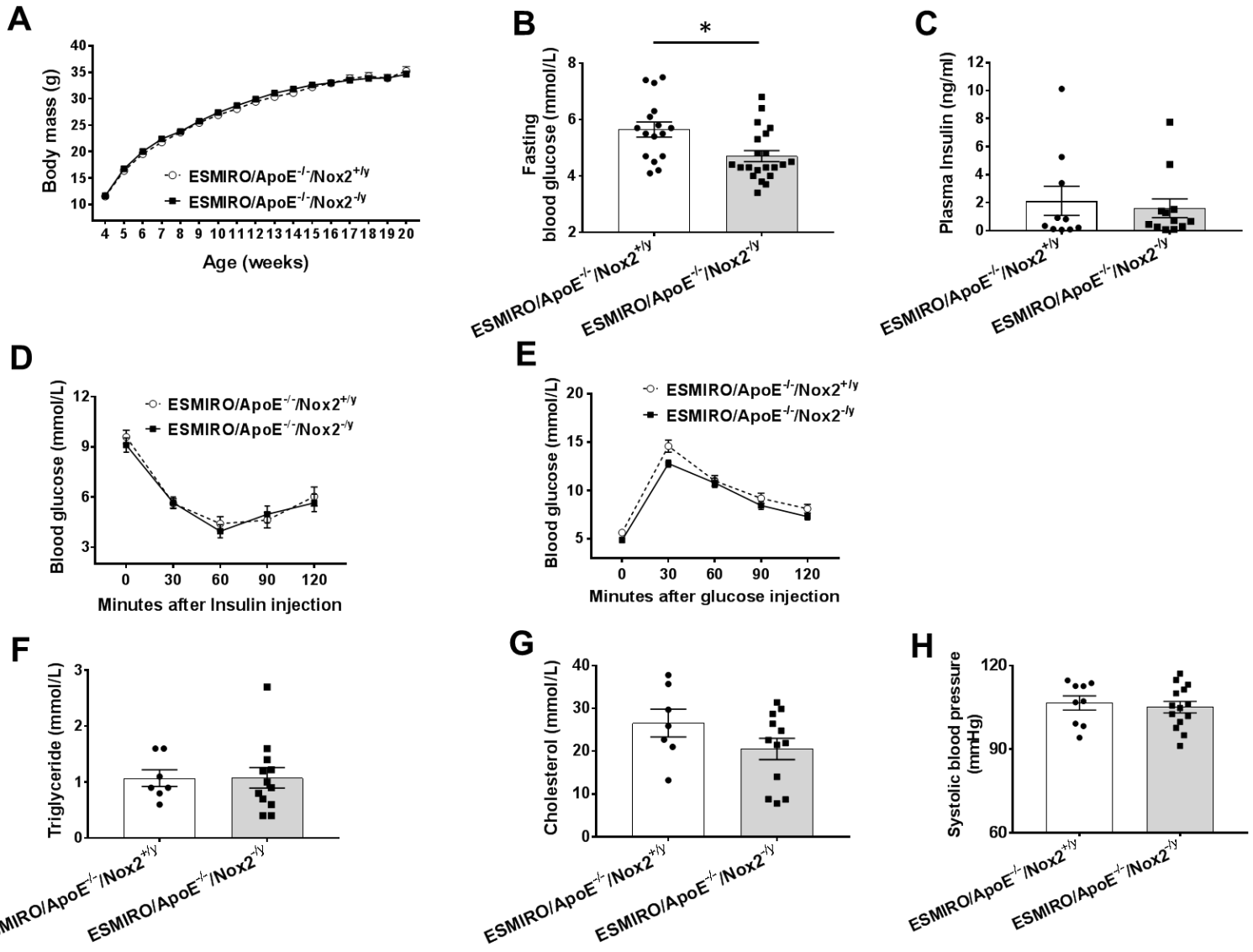
SUPPLEMENTAL TABLE S3: Nox PCR Protocol

PCR	Temperature (°C)	Time	Cycles
Initial denaturation	94	5 min	1
Denaturation	94	30 sec	35
Annealing	55	30 sec	
Extension	72	30 sec	
Final Extension	72	7 min	1

SUPPLEMENTAL TABLE S4: Taqman Probes

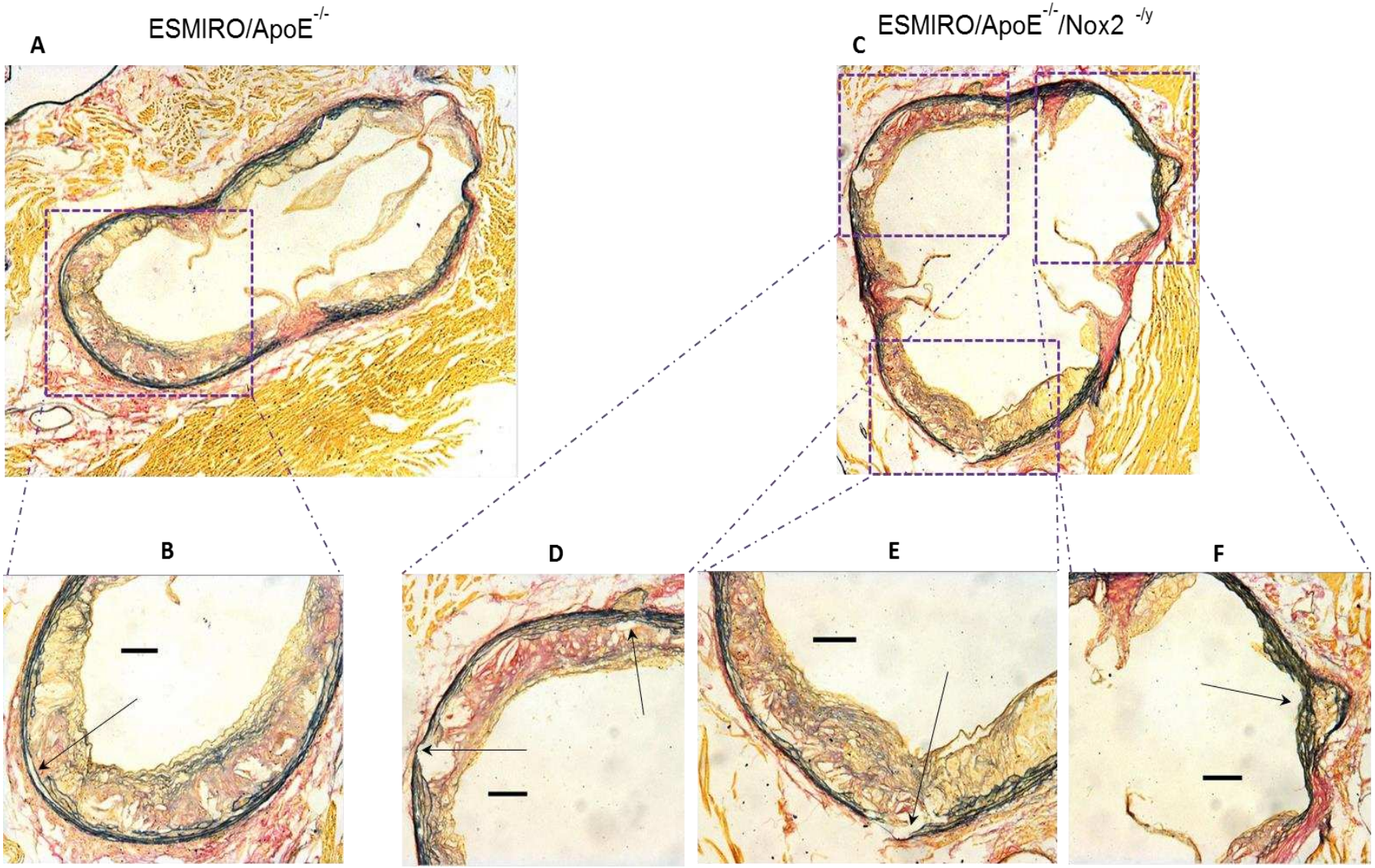
Target	Cat. No. (ThermoScientific)
NOX2	Mm01287743_m1
NOX4	Mm00479246_m1
Catalase	Mm00437992_m1
SOD	Mm01313000_m1
IL-1β	Mm00434228_m1
TNFα	Mm00443258_m1
CCL2	Mm00441242_m1
CCR2	Mm99999051_gH
HPRT	Mm03024075_m1

SUPPLEMENTAL FIGURE S3: Metabolic phenotype of ESMIRO/ApoE$^{-/-}$/Nox2$^{-/y}$ and ESMIRO/ApoE$^{-/-}$/Nox2$^{+/y}$ mice



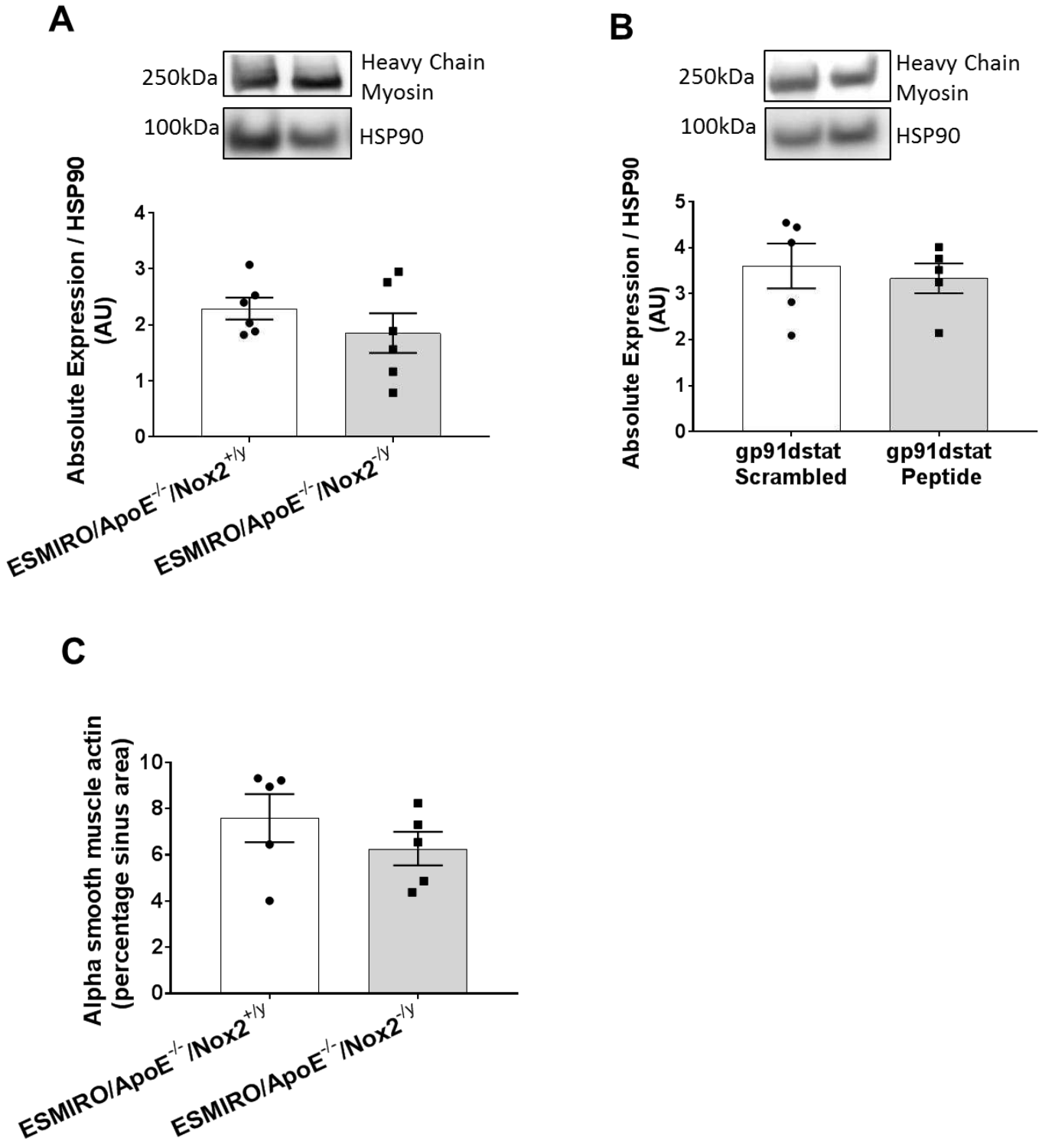
A: No difference in growth when comparing ESMIRO/ApoE$^{-/-}$/Nox2$^{-/y}$ (n=90) and ESMIRO/ApoE$^{-/-}$/Nox2$^{+/y}$ mice (n=59), arrow denotes commencement of western diet. **B**: ESMIRO/ApoE$^{-/-}$/Nox2$^{-/y}$ mice had lower fasting blood glucose (n=22) than ESMIRO/ApoE-/-/Nox2+/y (n=16). **C**: No difference in random insulin when comparing ESMIRO/ApoE$^{-/-}$/Nox2$^{-/y}$ (n=12) and ESMIRO/ApoE$^{-/-}$/Nox2$^{+/y}$ mice (n=10). **D**: No difference in intraperitoneal insulin tolerance tests when comparing ESMIRO/ApoE$^{-/-}$/Nox2$^{-/y}$ (n=23) and ESMIRO/ApoE$^{-/-}$/Nox2$^{+/y}$ mice (n=13). **E**: Glucose tolerance was significantly better at 30 min when comparing ESMIRO/ApoE$^{-/-}$/Nox2$^{-/y}$ (n=22) and ESMIRO/ApoE$^{-/-}$/Nox2$^{+/y}$ mice (n=16). **F**: No difference in fasting triglyceride when comparing ESMIRO/ApoE$^{-/-}$/Nox2$_{-/y}$ (n=12) and ESMIRO/ApoE$^{-/-}$/Nox2$^{+/y}$ mice (n=7). **G**: No difference in fasting cholesterol when comparing ESMIRO/ApoE$^{-/-}$/Nox2$^{-/y}$ (n=12) and ESMIRO/ApoE$^{-/-}$/Nox2$^{+/y}$ mice (n=7). **H**: No difference in systolic blood pressure when comparing ESMIRO/ApoE$^{-/-}$/Nox2$^{-/y}$ (n=14) and ESMIRO/ApoE$^{-/-}$/Nox2$^{+/y}$ mice (n=9). Data expressed as mean (±SEM), n=number of mice per genotype * denotes P<0.05.

SUPPLEMENTAL FIGURE S4: Elastin fibre fragmentation



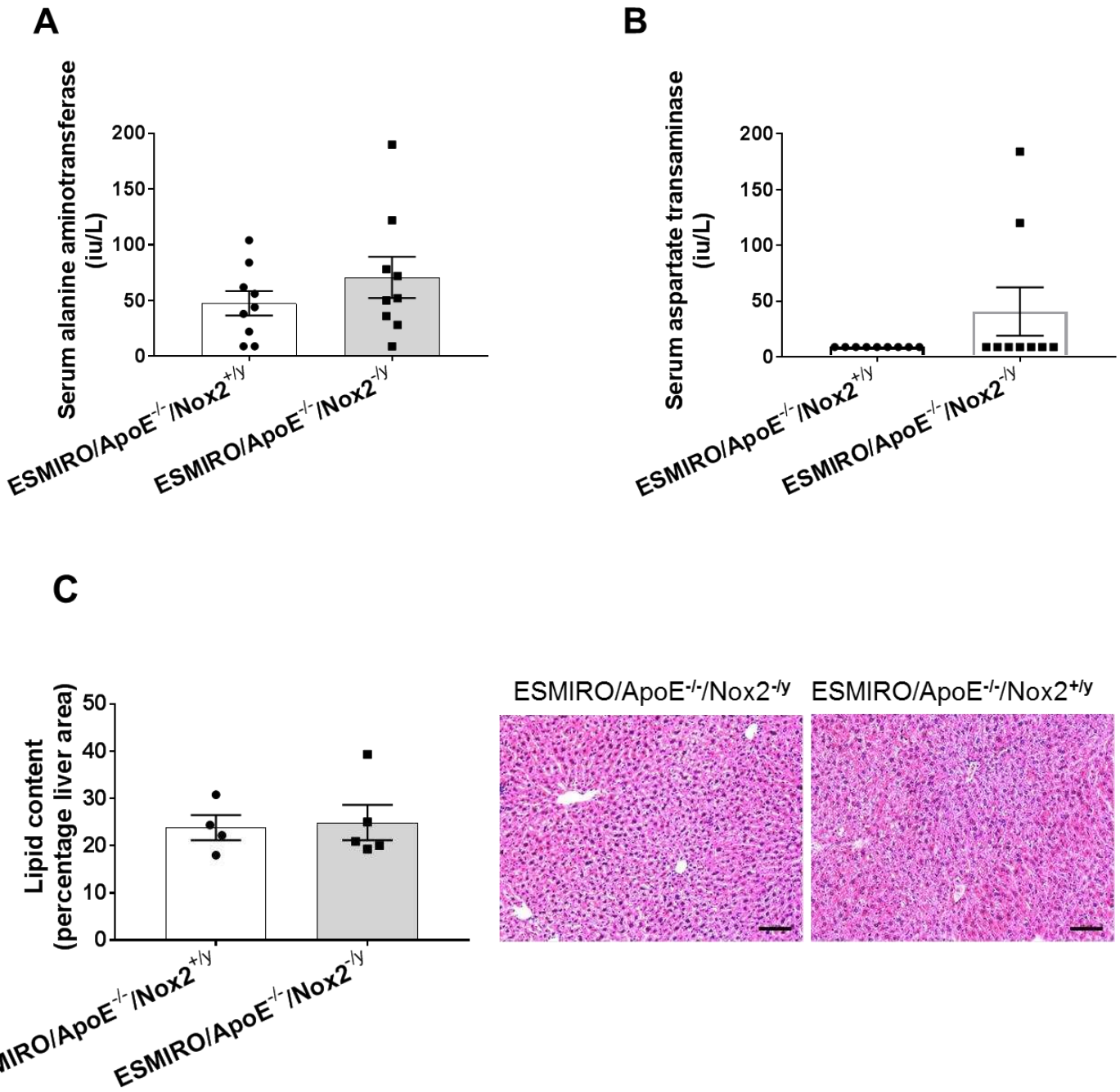
Photomicrographs of aortic sinus from ESMIRO/ApoE$^{-/-}$ (A) and ESMIRO/ApoE$^{-/-}$/Nox2 $^{-/y}$ (C) showing elastin fiber fragmentation after 12 weeks Western Diet. Multiple sites of fragmentation of elastic lamina are noted in ESMIRO/ApoE$^{-/-}$/Nox2 $^{-/y}$ (high powered views in D, E, F) compared to ESMIRO/ApoE$^{-/-}$ (image B). Scale bar – 100μm

SUPPLEMENTAL FIGURE S5: The effect of genetic ablation or pharma -cological inhibition of Nox2 on Heavy Chain Myosin expression and alpha smooth muscle actin in aortas of mice



A: No difference in heavy chain myosin expression in the aortas of ESMIRO/ApoE$^{-/-}$/Nox2$^{-/y}$ (n=6) and ESMIRO/ApoE$^{-/-}$/Nox2$^{+/y}$ mice (n=6). **B**: No difference in heavy chain myosin expression in the aortas of ESMIRO/ApoE$^{-/-}$ mice treated with the NOX2 inhibitor, gp91dstat (n=5) compared to ESMIRO/ApoE$^{-/-}$ mice treated with scrambled peptide (n=5). C: No difference in alpha smooth muscle actin expression in the aortic sinus in ESMIRO/ApoE-/-/Nox2-/y (n=5) and ESMIRO/ApoE-/-/Nox2+/y mice (n=5).

SUPPLEMENTAL FIGURE S6: Liver function and lipid deposition in ESMIRO/ApoE$^{-/-}$/Nox2$^{-/y}$ and ESMIRO/ApoE$^{-/-}$/Nox2$^{+/y}$ mice



A: No difference in serum alanine aminotransferase (ALT) levels of ESMIRO/ApoE$^{-/-}$/Nox2$^{-/y}$ (n=9) and ESMIRO/ApoE$^{-/-}$/Nox2$^{+/y}$ mice (n=9). **B**: No difference in serum aspartate transaminase (AST) levels of ESMIRO/ApoE$^{-/-}$/Nox2$^{-/y}$ (n=9) and ESMIRO/ApoE$^{-/-}$/Nox2$^{+/y}$ mice (n=9). **C**: No difference in lipid deposition in the liver of ESMIRO/ApoE$^{-/-}$/Nox2$^{-/y}$ (n=4) and ESMIRO/ApoE$^{-/-}$/Nox2$^{+/y}$ mice (n=5). Scale bar: 100µm