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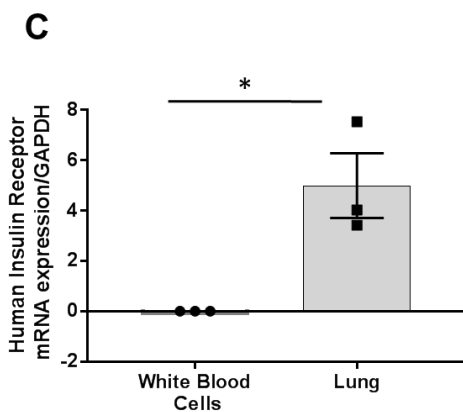
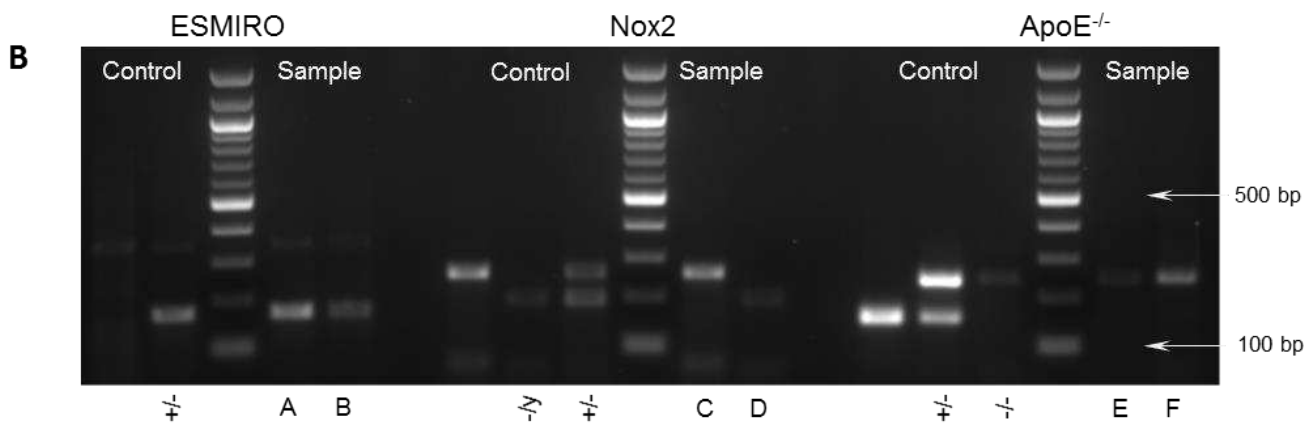
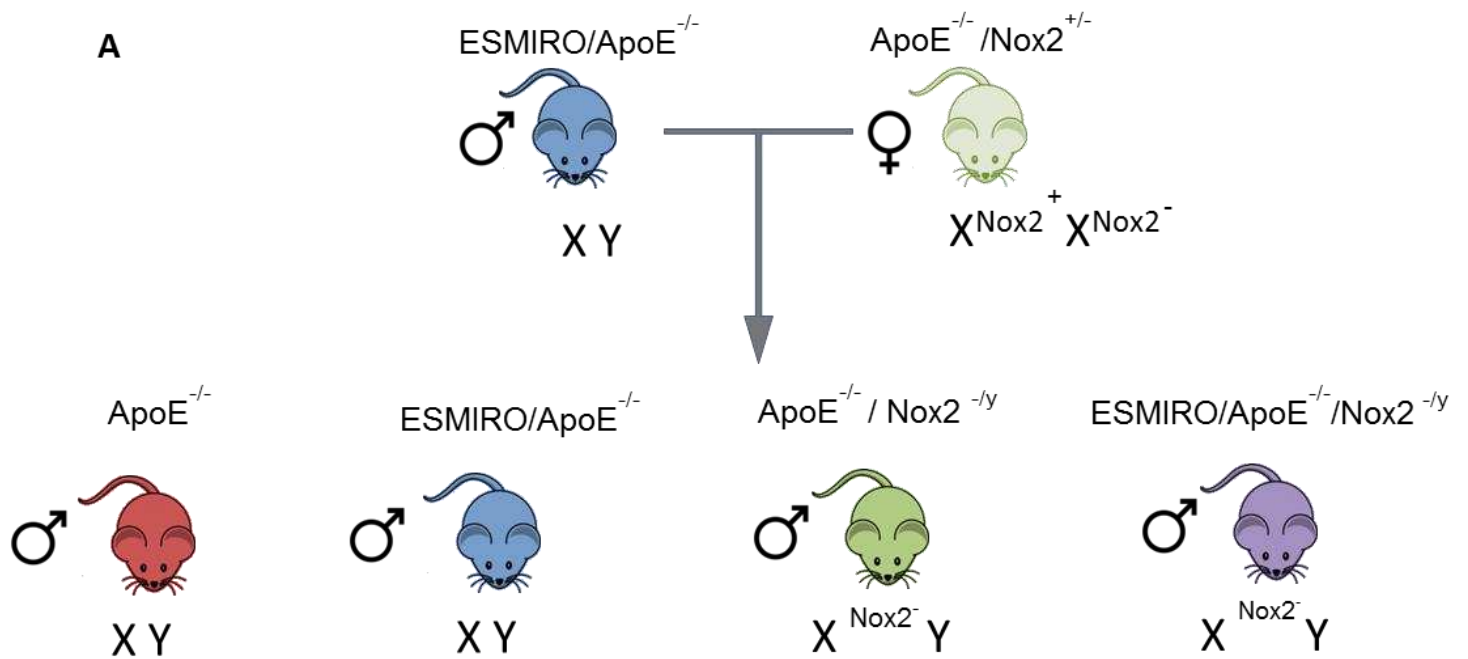
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Divergent effects of genetic and
pharmacological inhibition of Nox2 NADPH
oxidase on insulin resistance related
vascular damage

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^{+/-}; D-Nox2^{-/-}; 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^{-/-} 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'-GCCGCCCGACTGCATCT-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'-CCGTTCTCAGGGGTGTCC -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'-AAGAGAACTCCTCTGCTGTG 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

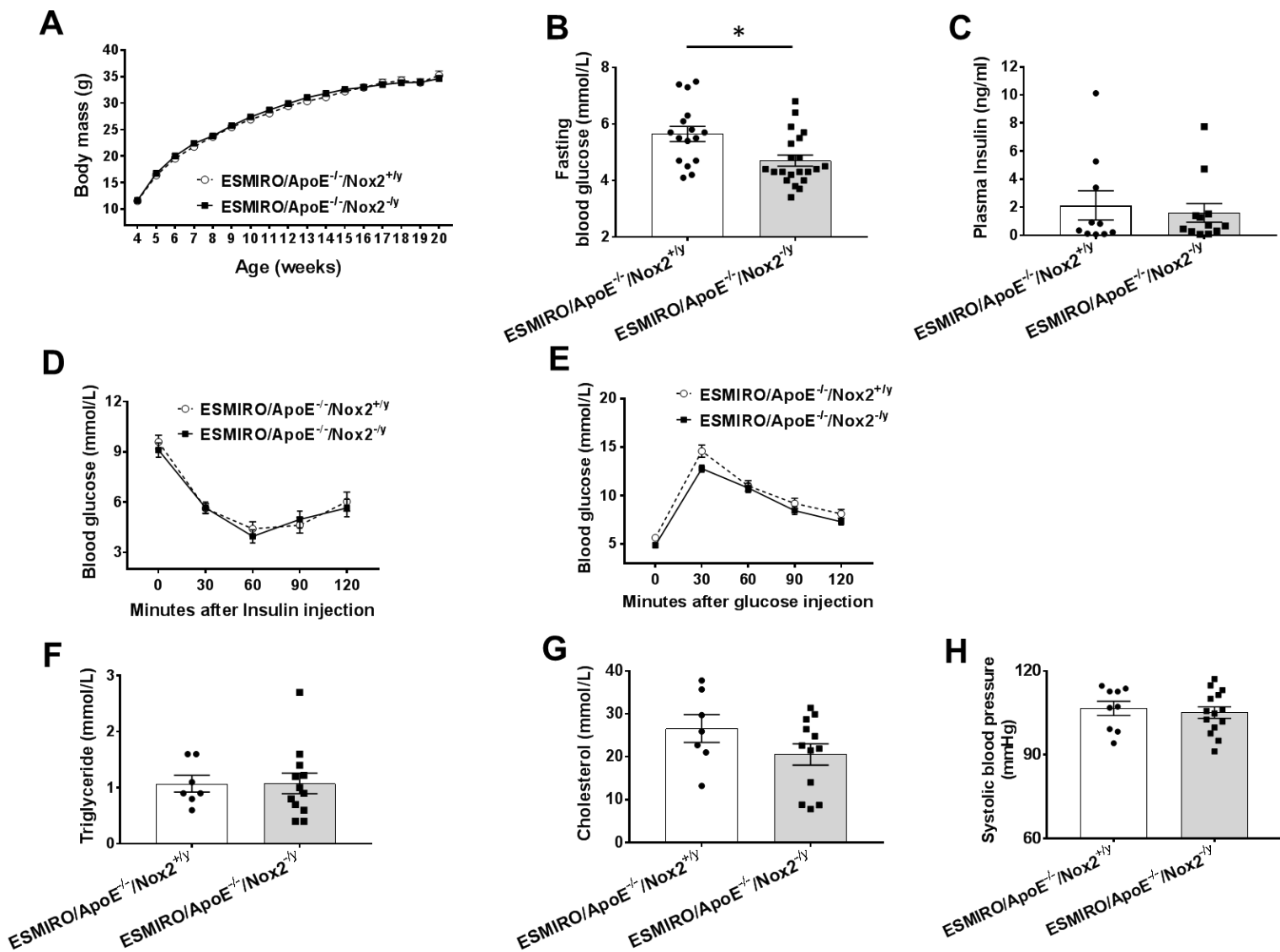
SUPPLEMENTAL TABLE S3: Nox PCR Protocol

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

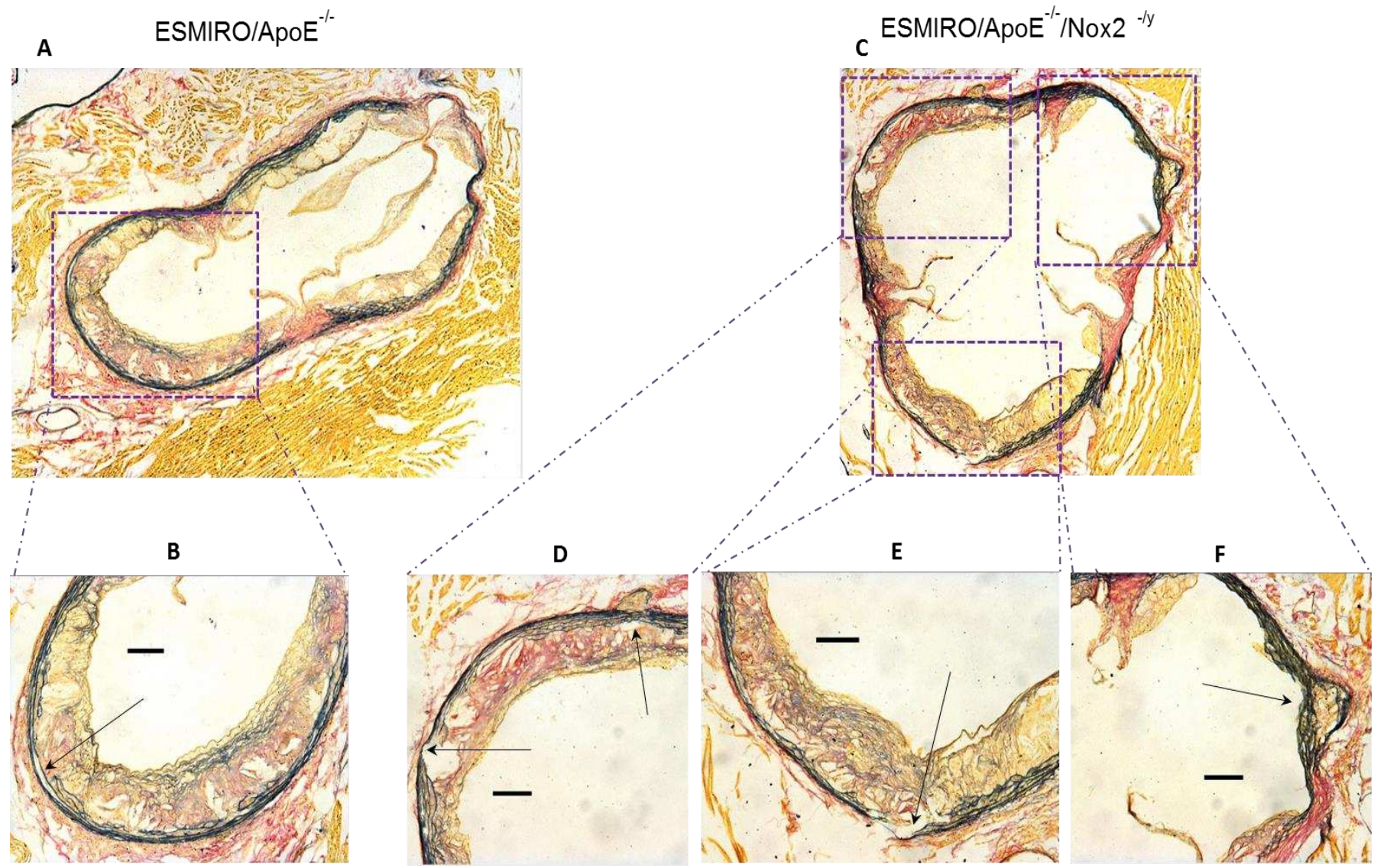
SUPPLEMENTAL TABLE S4: Taqman Probes

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

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

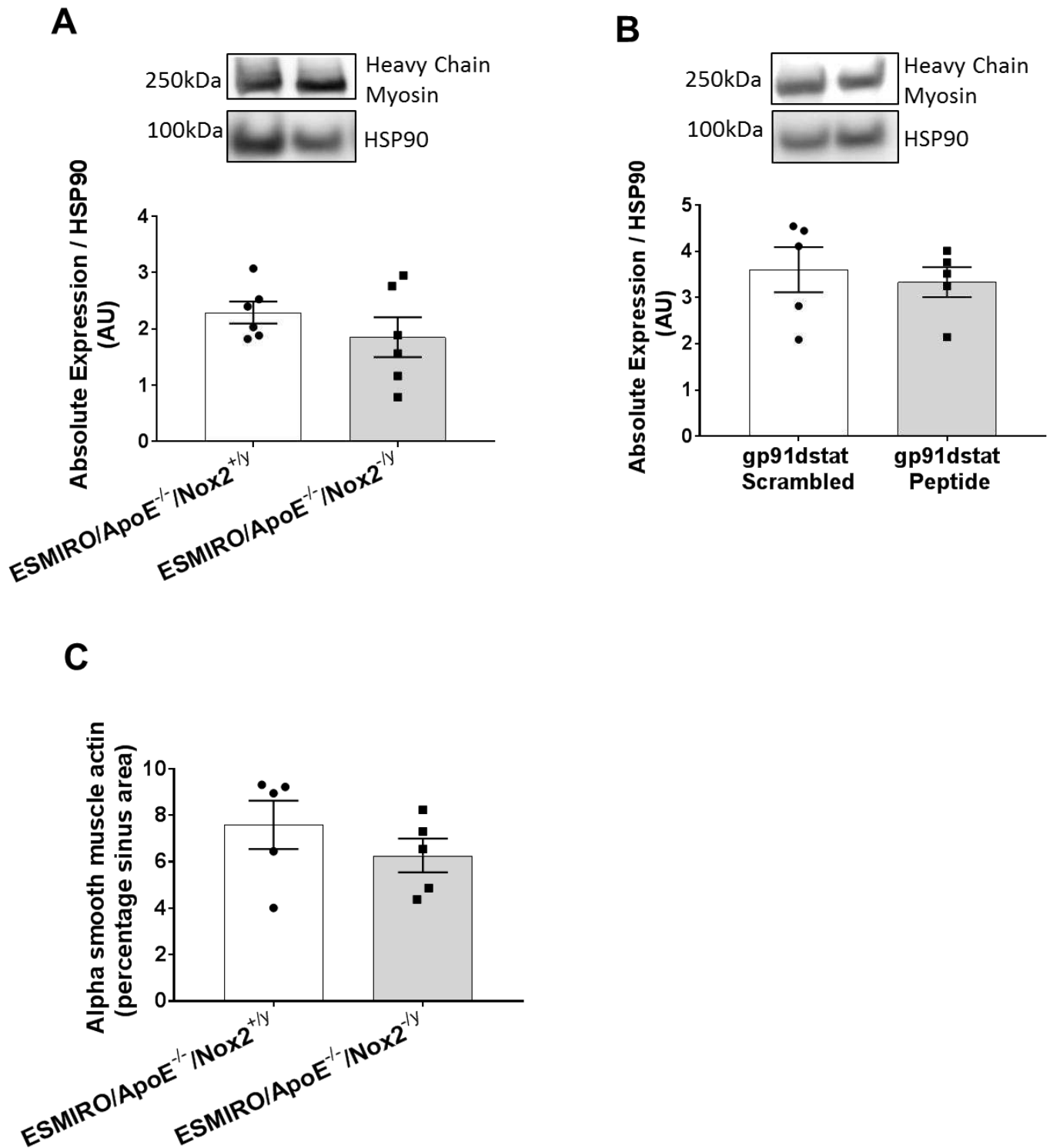


A: No difference in growth when comparing ESMIRO/ApoE^{-/-}/Nox2^{-/-} (n=90) and ESMIRO/ApoE^{-/-}/Nox2^{+/-} mice (n=59), arrow denotes commencement of western diet. **B:** ESMIRO/ApoE^{-/-}/Nox2^{-/-} mice had lower fasting blood glucose (n=22) than ESMIRO/ApoE^{-/-}/Nox2^{+/-} (n=16). **C:** No difference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



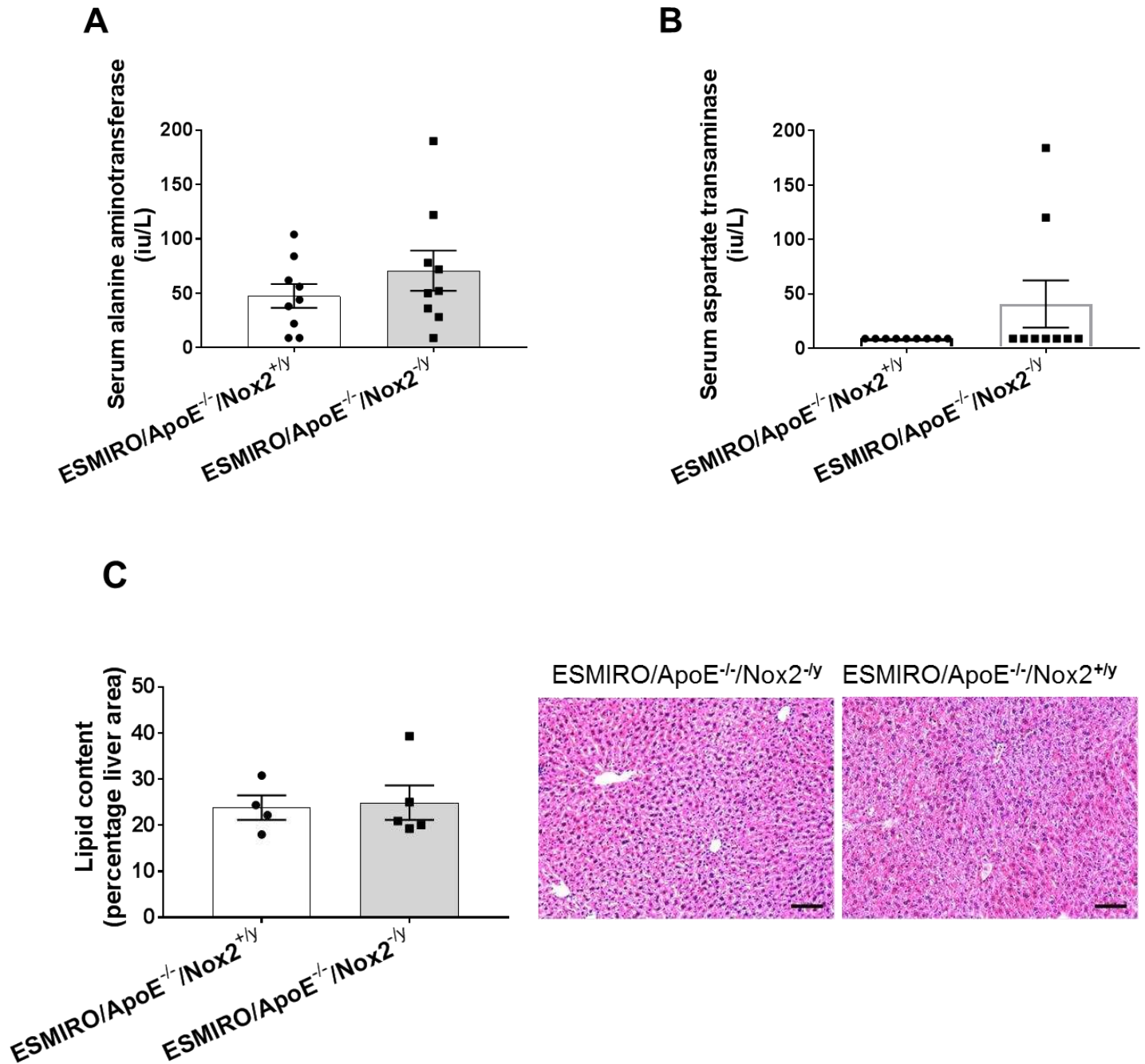
Photomicrographs of aortic sinus from ESMIRO/ApoE^{-/-} (A) and ESMIRO/ApoE^{-/-}/Nox2^{-/-y} (C) showing elastin fiber fragmentation after 12 weeks Western Diet. Multiple sites of fragmentation of elastic lamina are noted in ESMIRO/ApoE^{-/-}/Nox2^{-/-y} (high powered views in D, E, F) compared to ESMIRO/ApoE^{-/-}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

SUPPLEMENTAL FIGURE S5: The effect of genetic ablation or pharmacological 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^{-/-} (n=6) and ESMIRO/ApoE^{-/-}/Nox2^{+/-} 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^{-/-} (n=5) and ESMIRO/ApoE^{-/-}/Nox2^{+/-} mice (n=5).

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



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