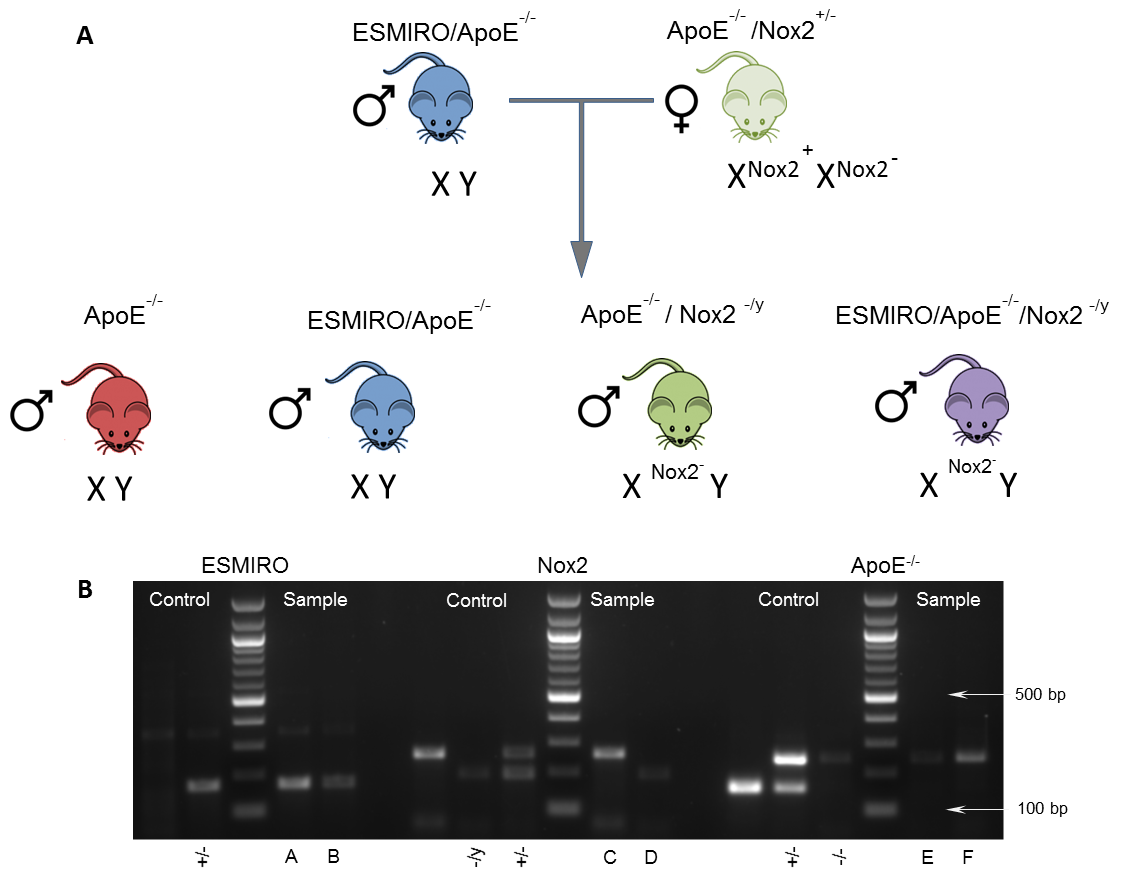
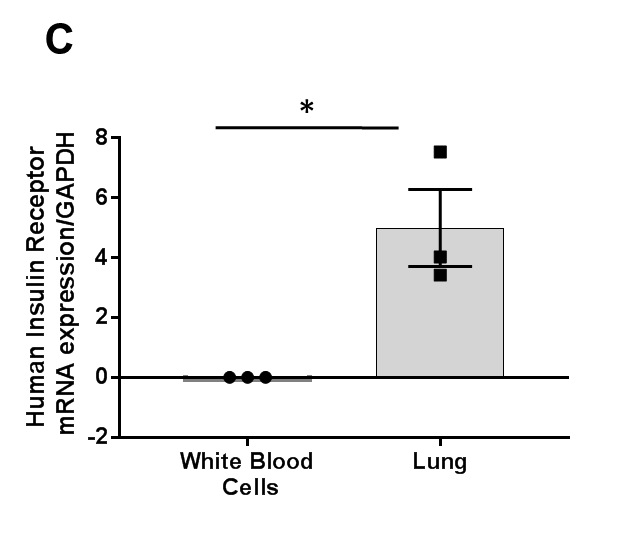
Divergent effects of genetic and pharmacological inhibition of Nox2 NADPH oxidase on insulin resistance related vascular damage  
  
  
SUPPLEMENTAL MATERIAL

Maqbool *et al.* 2020

**SUPPLEMENTAL FIGURE S1: Breeding and Genotyping Strategy**

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**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 WTno bandand 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 |  |
| Annealing | 68 | 40 sec | 35 |
| 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 |  |
| Annealing | 60 | 1 min | 32 |
| Extension | 72 | 1 min |  |
| Final Extension | 72 | 15 min | 1 |

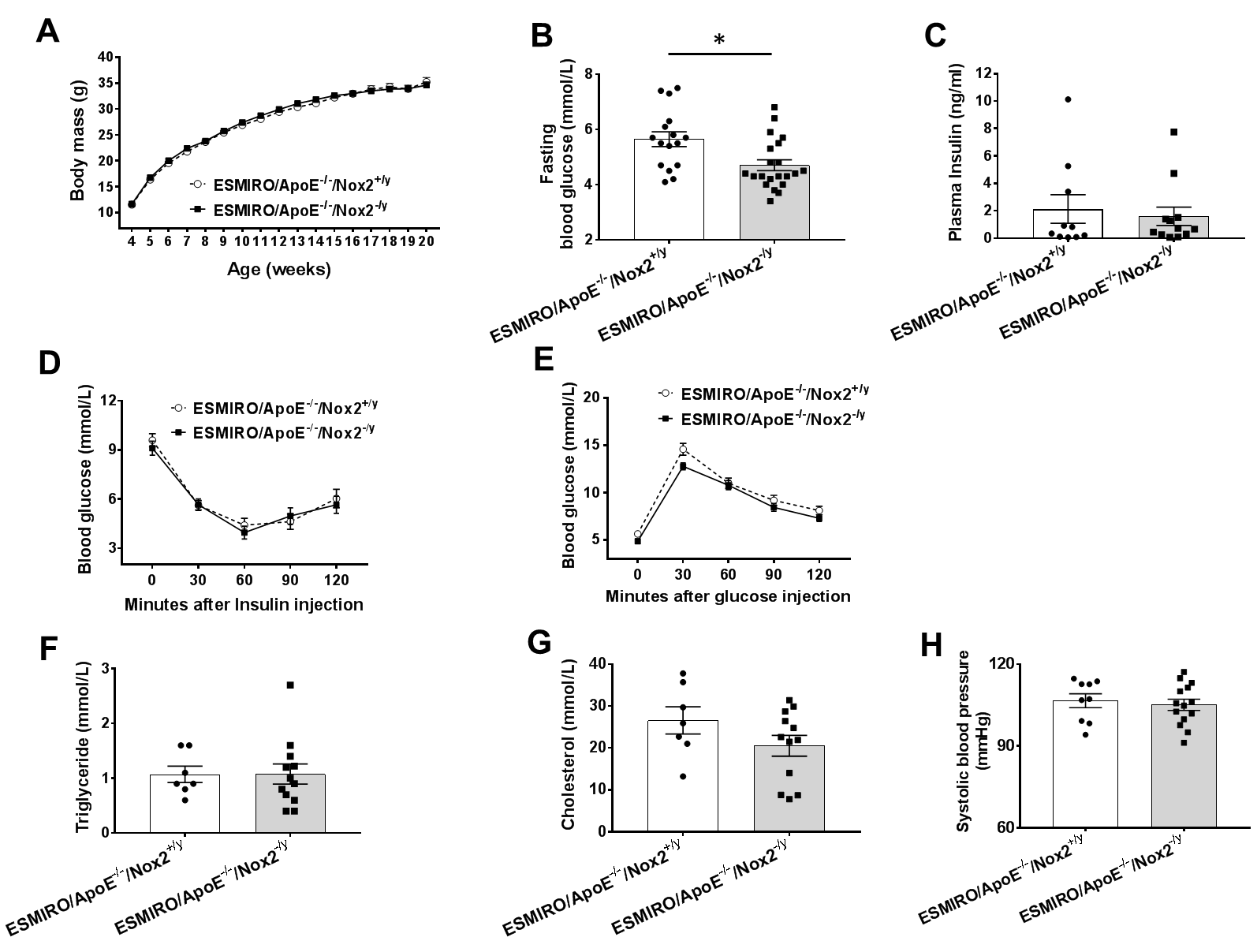
**SUPPLEMENTAL TABLE S3: Nox PCR Protocol**

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

**SUPPLEMENTAL TABLE S4: Taqman Probes**

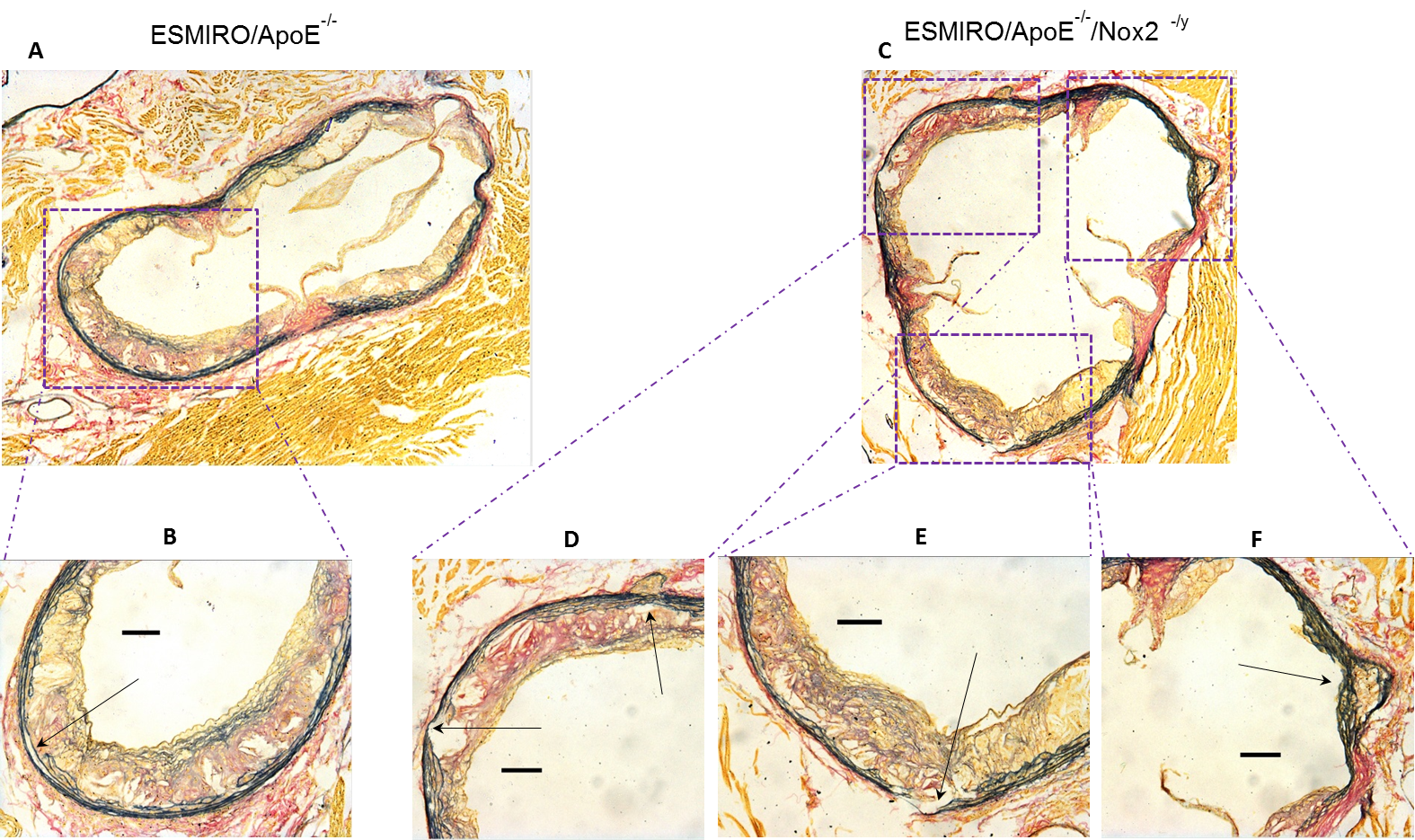
|  |  |
| --- | --- |
|  |  |
| **Target** | **Cat. No. (ThermoScientific**) |
|  |  |
| NOX2 | Mm01287743\_m1 |
| NOX4 | Mm00479246\_m1 |
| Catalase | Mm00437992\_m1 |
| SOD | Mm01313000\_m1 |
| IL-1β | Mm00434228\_m1 |
| TNFα | Mm00443258\_m1 |
| CCL2 | Mm00441242\_m1 |
| CCR2 | Mm99999051\_gH |
| HPRT | Mm03024075\_m1 |

**SUPPLEMENTAL FIGURE S3: Metabolic phenotype of ESMIRO/ApoE-/-/Nox2-/y and ESMIRO/ApoE-/-/Nox2+/y mice**



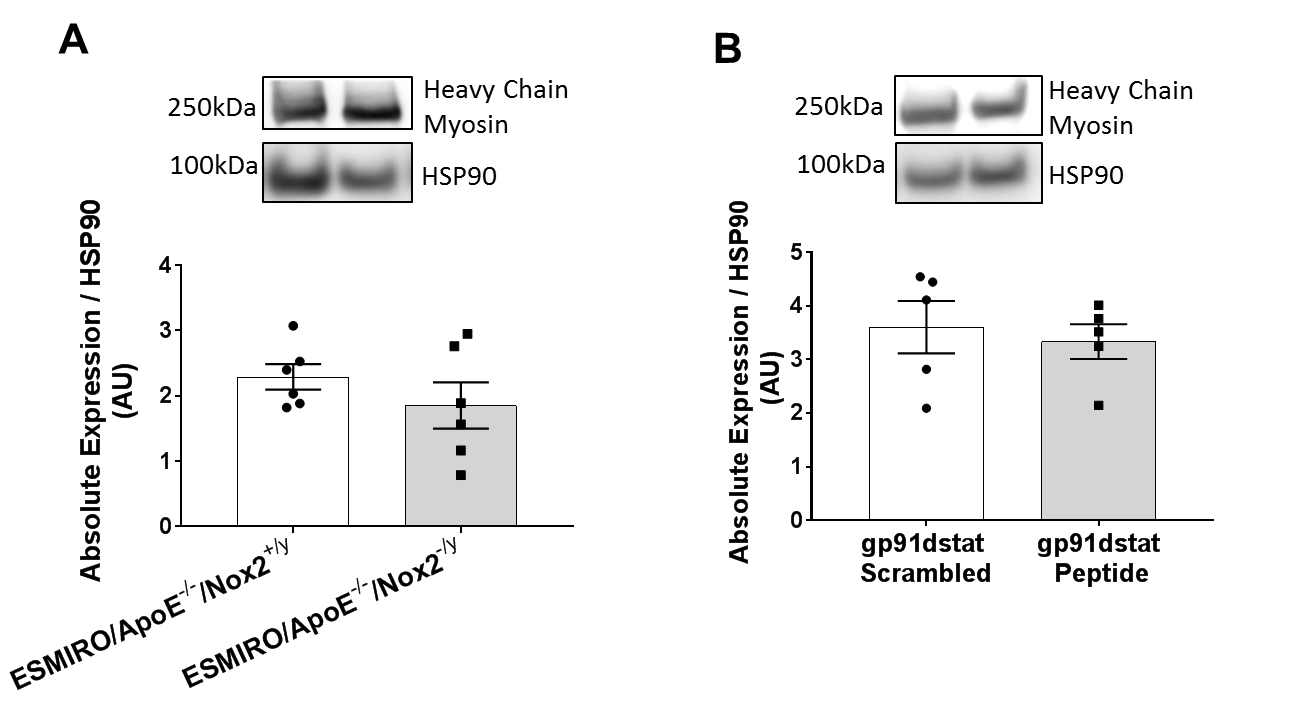
**A**: No difference in growth when comparing ESMIRO/ApoE-/-/Nox2-/y (n=90) and ESMIRO/ApoE-/-/Nox2+/y mice (n=59), arrow denotes commencement of western diet. **B**: ESMIRO/ApoE-/-/Nox2-/y mice had lower fasting blood glucose (n=22) than ESMIRO/ApoE-/-/Nox2+/y (n=16). **C**: No difference in random 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.

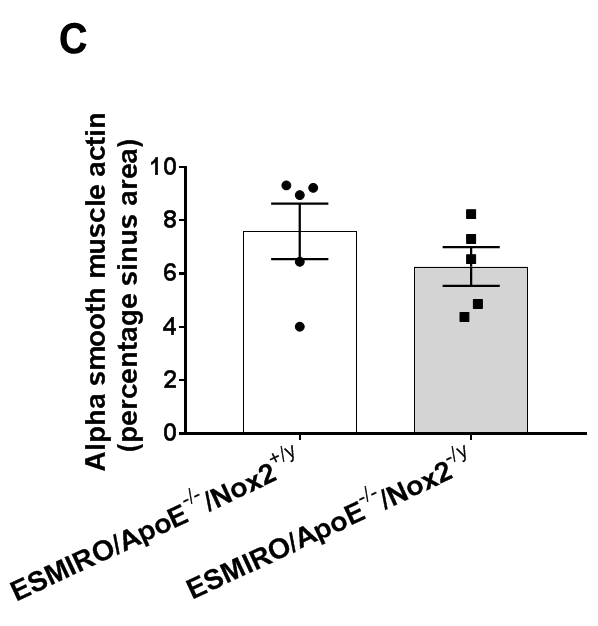
**SUPPLEMENTAL FIGURE S4: Elastin fibre fragmentation**



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

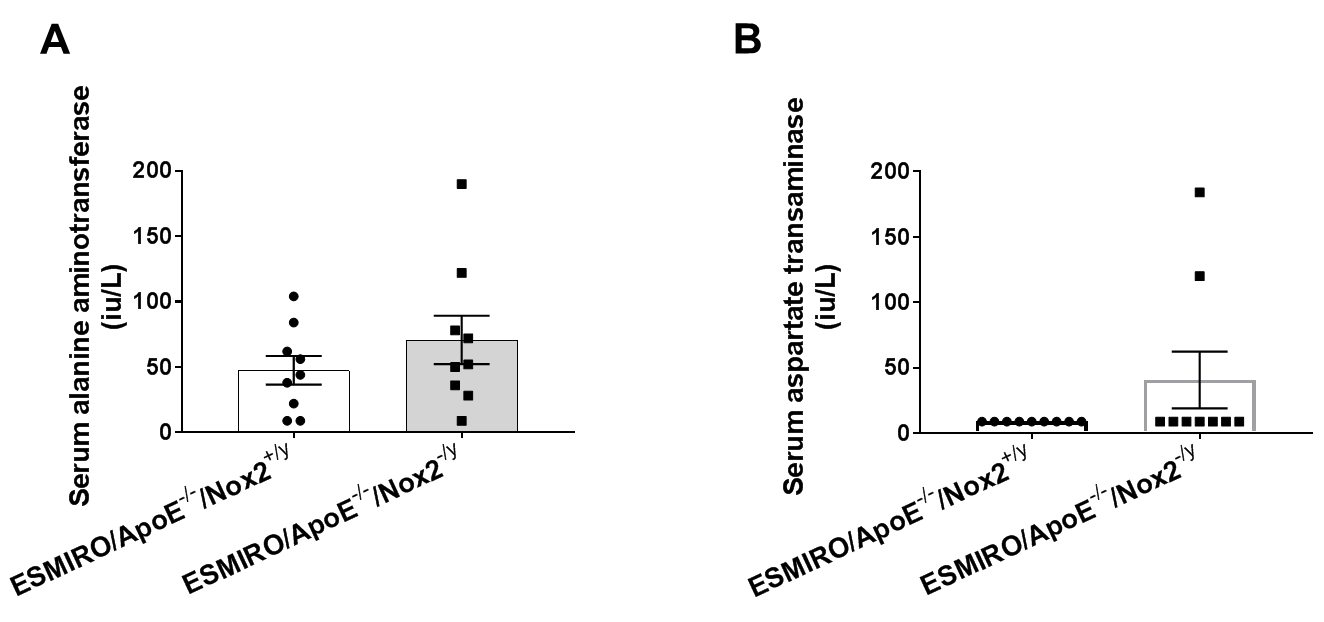
**SUPPLEMENTAL FIGURE S5: The effect of genetic ablation or pharma -cological inhibition of Nox2 on Heavy Chain Myosin expression and alpha smooth muscle actin in aortas of mice**

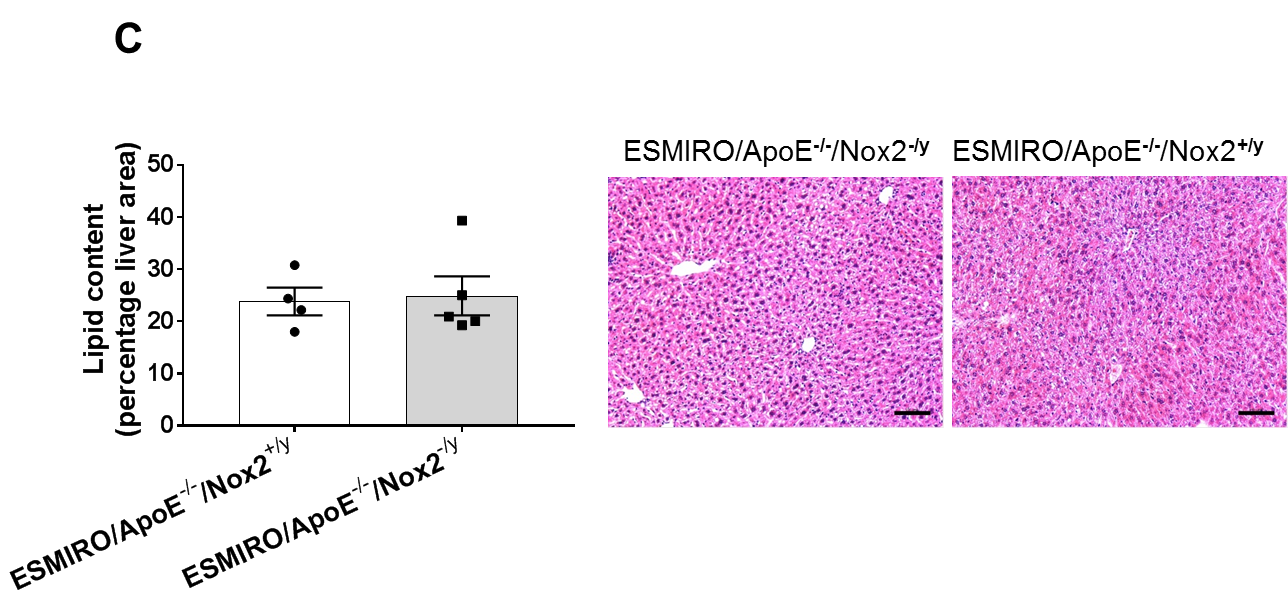


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**A**: No difference in heavy chain myosin expression in the aortas of ESMIRO/ApoE-/-/Nox2-/y (n=6) and ESMIRO/ApoE-/-/Nox2+/y mice (n=6). **B**: No difference in heavy chain myosin expression in the aortas of ESMIRO/ApoE-/- mice treated with the NOX2 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