SUPPLEMENTAL MATERIAL

Endoplasmic reticulum stress mediates vascular smooth muscle cell calcification via increased release of Grp78-loaded extracellular vesicles

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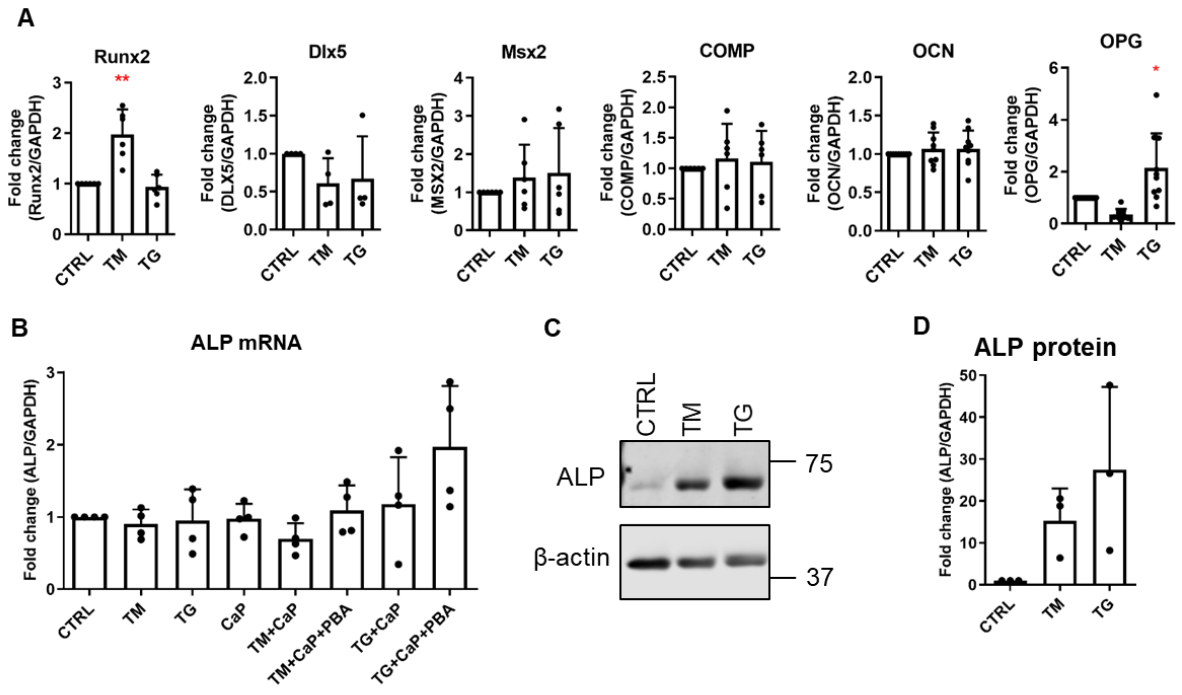
Supplemental Tables and Figures

Supplemental Table I. Information of vessel donors, whose samples were used for qPCR analysis.

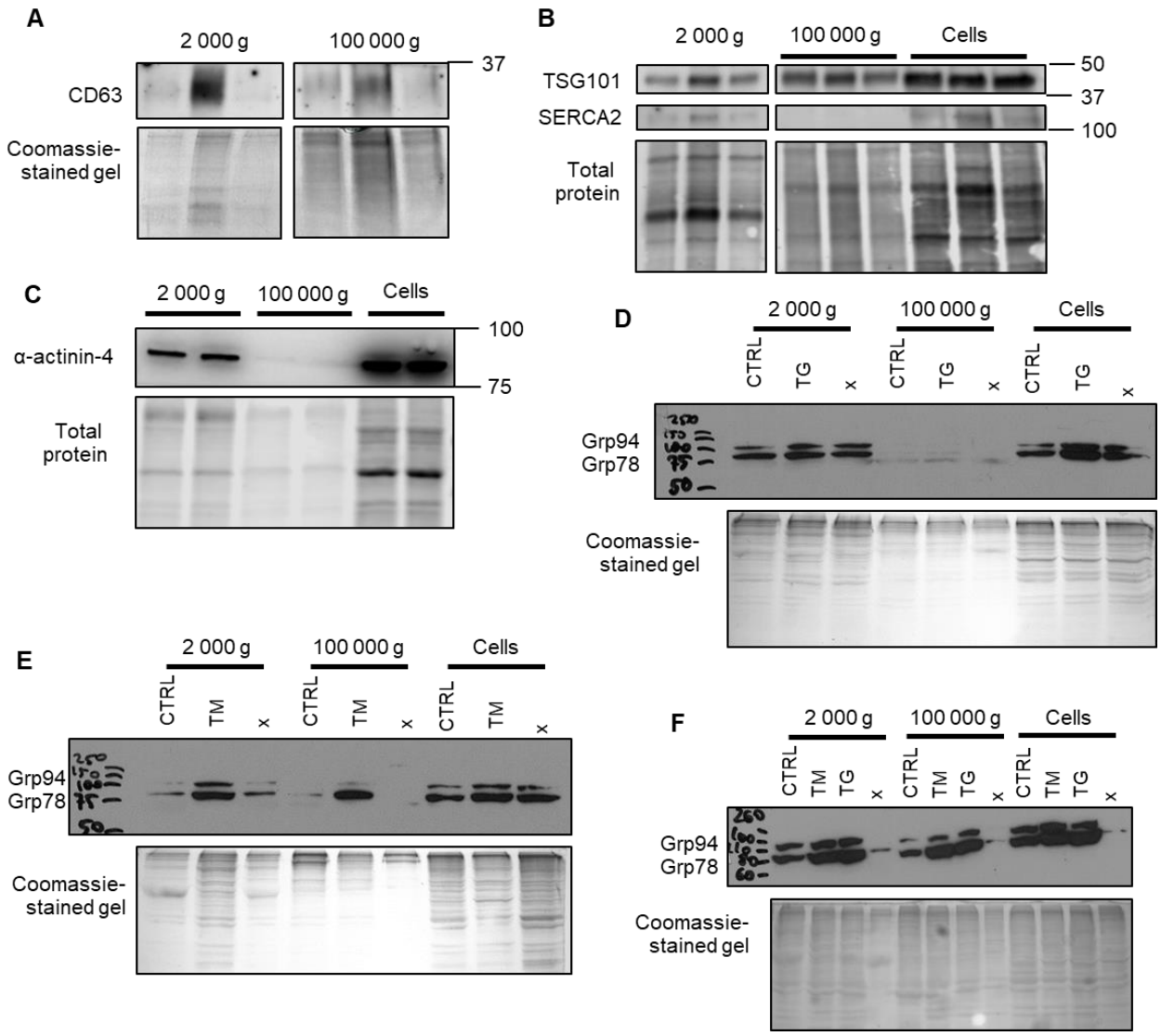
Group	Mean Age	Age SD	Females	Males	n
Healthy	38	9	8	2	10
Fatty Streak	43	12	8	2	10
Calcified	51	7	6	5	11

Supplemental Table II. Information of vessel donors, whose samples were used for histology stains and immunohistochemistry. First column indicates age in years, sex (M – male, F - female) and vessel identification code (A - aorta, C - carotid). Gradually increasing score of calcium deposit indicates: 0 = no calcium and 4 more than 75 % of mineral deposit in that particular vessel.

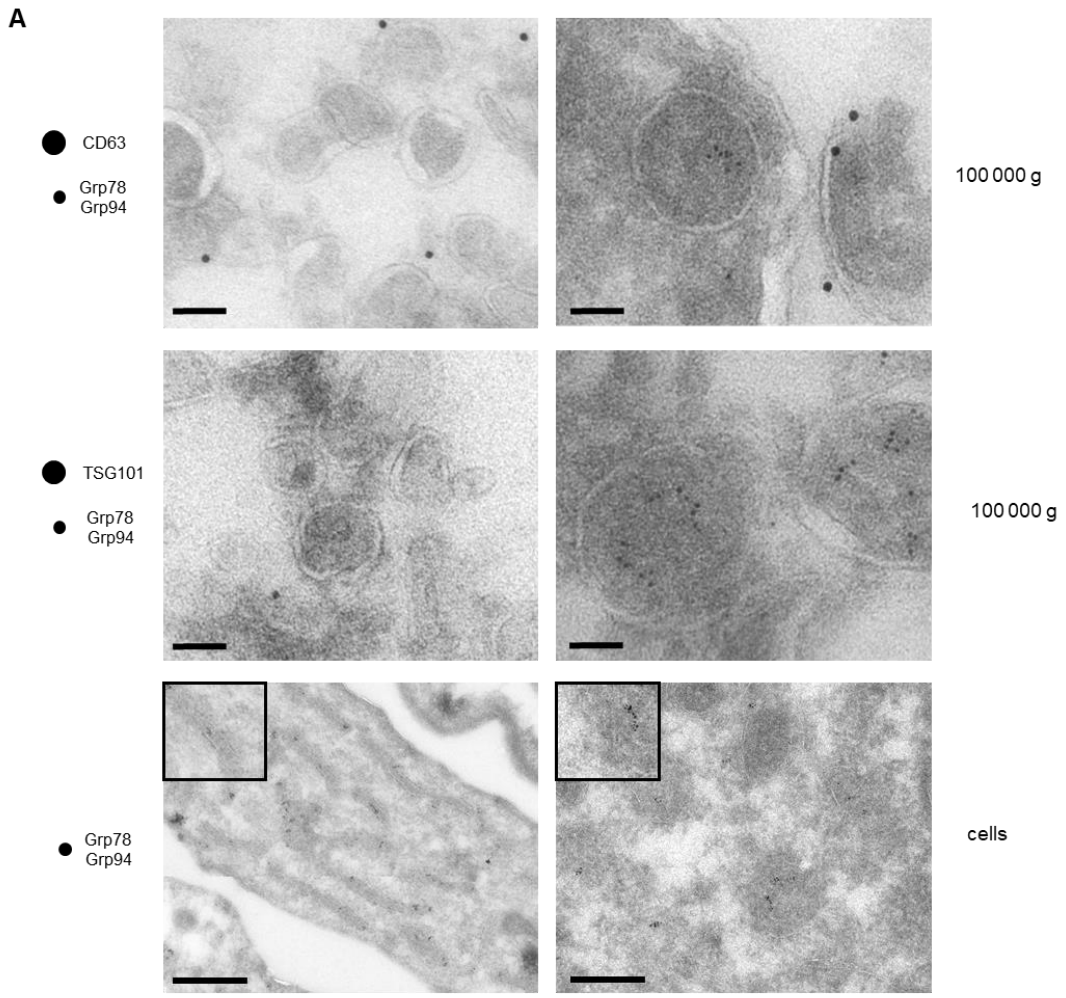
Age/sex, ID code	Atherosclerosis stage	Calcium deposit score
18M,9 A	I	0
22M, 2A	I	0
22M, 18A	II	1
44F, 5A	IV	1
53F, 4A	IV	1
52M, 3A	IV	2
54M, 26A	V	3
55F, 10A	IV	2
56M, 3A	V	4
59F, 9A	IV	3
60F, 11A	V	4
64F, 10A	V	2
71F, 2A	V	3
77F, 4A	IV	1
59M, 25C	VII	4
62M, 35C	VII	1
65M, 22C	VII	1
66F, 26C	VII	0
68M, 64C	VII	2
69F, 5C	VIII	2
74F, 17C	VIII	4
74F, 62C	VIII	3

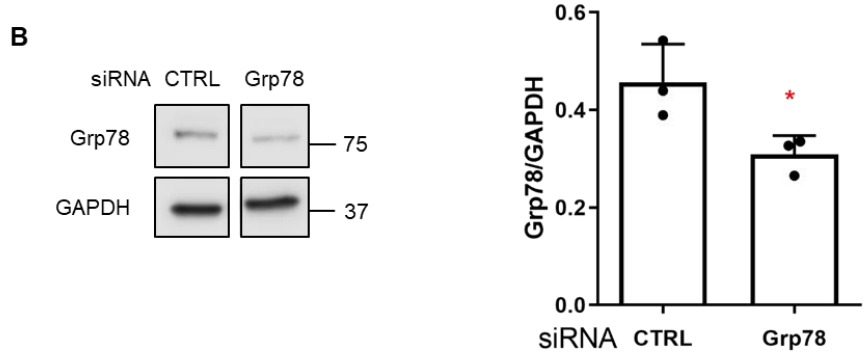


Supplemental Figure I. ER stress regulates osteogenic differentiation. A. VSMCs were treated with 0.4 µg/ml TM or 0.2 µg/ml TG for 24 hours. Osteogenic gene expression in response to ER stress was analysed by real-time PCR. Statistical significance was tested using one sample t-tests. **B.** VSMCs were treated with 2.7 mM Ca^{2+}, 2.5 mM PO_4^{3-}, 0.04 µg/ml tunicamycin (TM) or 0.01 µg/ml thapsigargin (TG), 0.5 mM PBA for 8 days in M199 with 5% FBS. ALP mRNA levels in a calcifying VSMCs (real-time PCR). Statistical significance was tested using the Kruskall-Wallis test. **C.** Western blotting and **D.** quantification of ALP protein levels. Statistical significance was tested using one sample t-tests. Graphs show mean and individual data points. Dots denote individual data points, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.



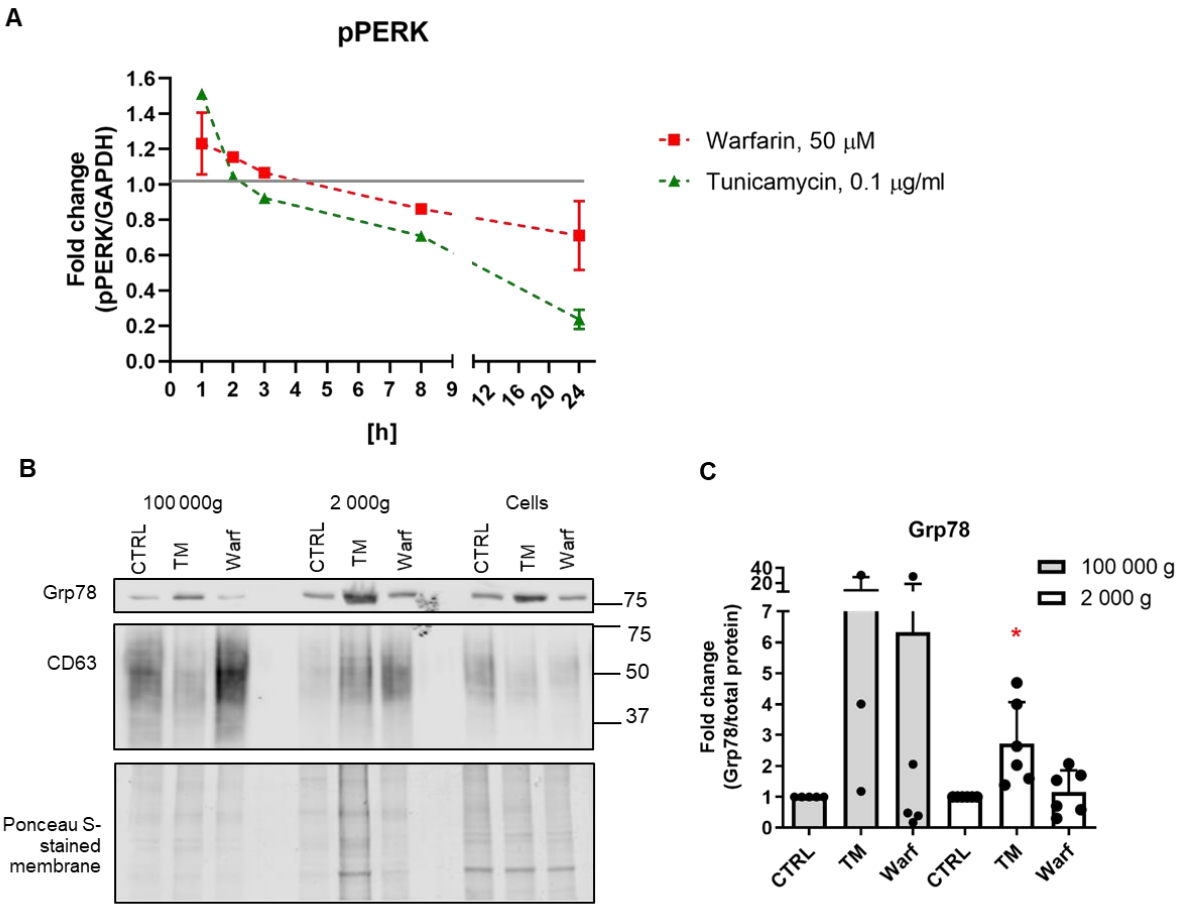
Supplemental Figure II. EVs isolated from VSMCs by ultracentrifugation express exosome markers. Western blots showing enriched expression of exosome markers **A.** CD63 and **B.** TSG101 in 100 000 g pellets compared to 2000 g pellets (apoptotic bodies) and cell lysates and a lack of **B.** SERCA-2 (an ER resident protein) and **C.** α-actinin-4 (a microvesicle marker) in 100 000 g fractions, suggesting that fractionation of exosomes and apoptotic bodies was successful. VSMCs were incubated in M199 with 0.5% FBS for 24 h; EVs were isolated from culture media by ultracentrifugation. All samples were run on the same gel/membrane, images were cropped for clarity of presentation. Loading controls were performed by staining the gels with Coomassie or membranes with REVERT after transfer. **D-F.** Uncropped western blots corresponding to data in Figure 4G and 4H. VSMCs were treated with 0.2 μg/ml TM or TG in phenol red-free DMEM with 0.1% BSA (FBS free) for 24h. X – condition unrelated to this study.



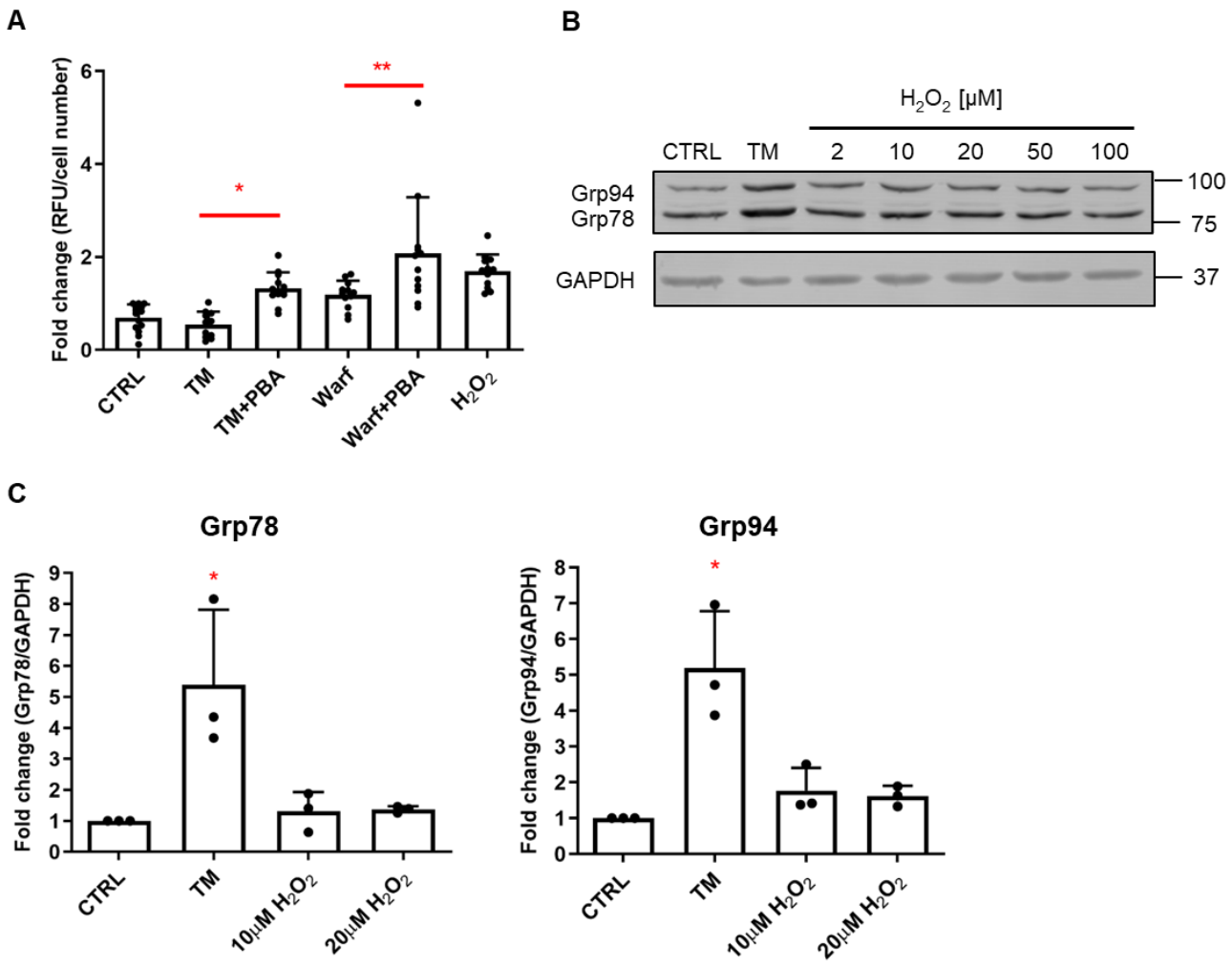


Supplemental Figure III. Grp78 in VSMCs and EVs. A. Electron microscopy showing Grp78/Grp94 in VSMCs and EVs isolated by from VSMCs using ultracentrifugation. Immunogold staining and electron microscopy was carried out on sections of fixed EV pellets (Grp78/Grp94 - 6 nm gold, CD63 and TSG101 - both 10 nm gold), Cells were stained for Grp78/Grp94 only. Scale bars are 100 nm for EV images, 400 nm for cells. **B** Grp78 siRNA knock-down results in a 30% decrease in Grp78 protein expression in VSMCs after 24 h. Representative western blot and. quantification. Statistical

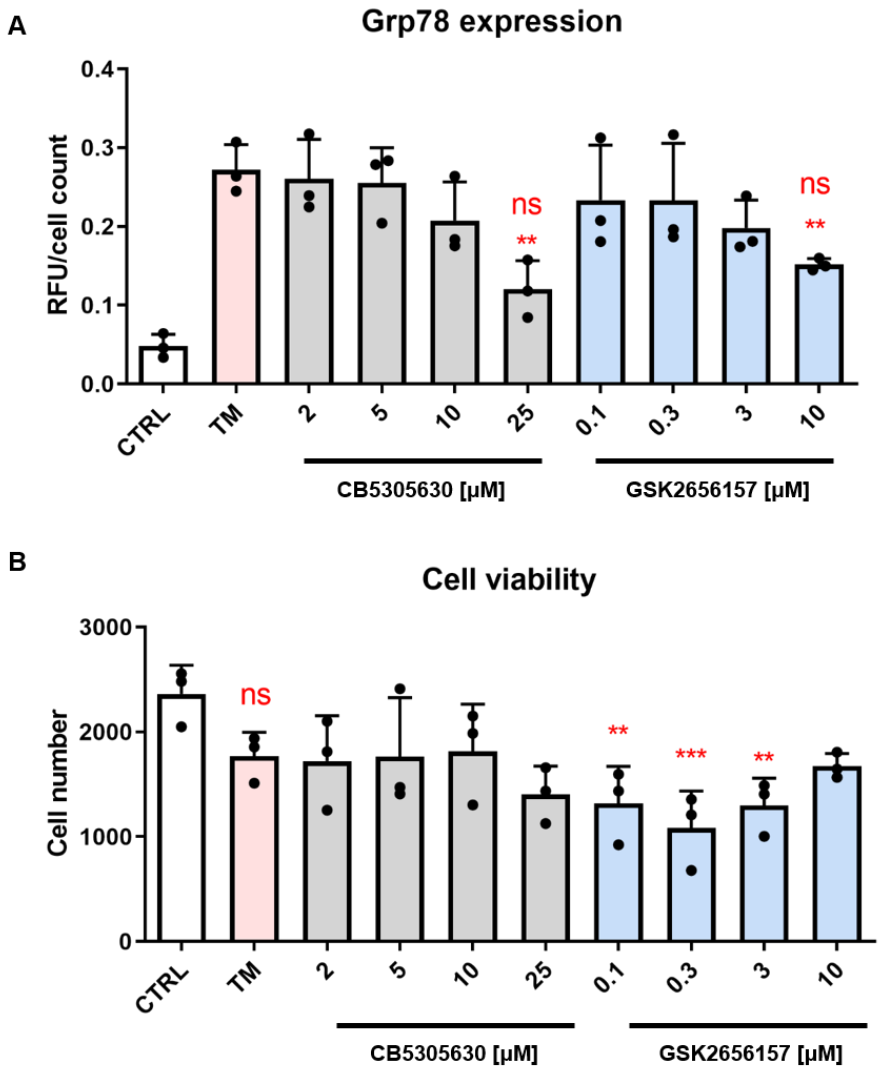
significance was tested using t-test. Dots denote individual data points, * p < 0.05, ** p < 0.01, *** p < 0.001.



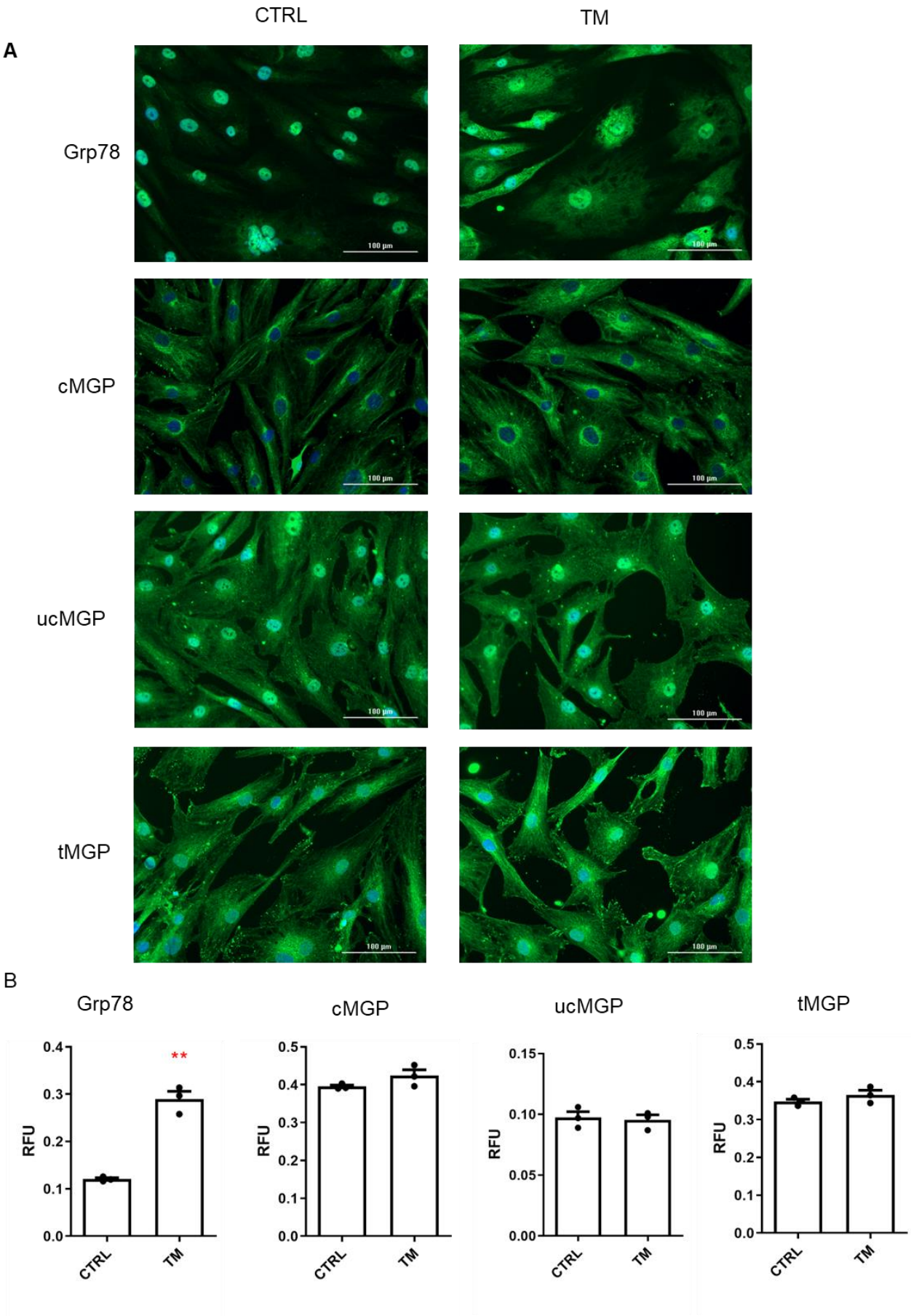
Supplemental Figure IV. Warfarin induces ER stress and enhances loading of Grp78 into EVs. A. Western blot quantification, plotted as a time course analysis of phosphorylated PERK levels. Human VSMCs were treated with 50 μM warfarin or 0.1 μg tunicamycin for 1, 2, 3, 8 or 24 hours in M199 with 0.5% FBS. In response to ER stress, PERK phosphorylation is transiently induced and then reduced compared to baseline levels. **B, C.** Western blot and quantification showing Grp78 expression in EVs released from cells treated with warfarin. VSMCs were treated with 50 μM warfarin or 0.2 μg/ml TM in M199 with 0.5% FBS; EVs were isolated from culture media by ultracentrifugation. Graph shows mean + SD and individual data points. Statistical significance was tested one sample t-tests and Wilcoxon test (Warfarin-treated 100000 g pellet). Dots denote individual data points, * p < 0.05, ** p < 0.01, *** p < 0.001.



Supplemental Figure V. ER stress is uncoupled from oxidative stress in VSMCs. A. VSMCs were loaded with DCFDA and then treated with 0.2 μg/ml tunicamycin (TM), 1 mM PBA, 50 μM warfarin and 50 mM H_2O_2 in KRPG buffer with Hoechst. Fluorescence was measured and normalised to cell count using the Cytation 3 imaging reader. Pooled data from 3 independent experiments. Statistical significance was tested using ANOVA. **B, C.** VSMCs were treated with H_2O_2 or 0.4 μg/ml TM in the presence of 0.5% FBS for 24 hours. Western blotting for Grp78 and Grp94 chaperones - dose response to H_2O_2. Quantification of Western blots repeated with just two concentrations of H_2O_2. Statistical significance was tested using the Kruskall-Wallis test. All graphs show mean and individual data points. Dots denote individual data points, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.



Supplemental Figure VI. PERK and IRE1 inhibitors decrease ER stress measured by Grp78 expression. A. IRE1 inhibitor CB53 (CB5305630) most efficiently blocks ER stress (Grp78 expression) at 25 µM and PERK inhibitor GSK (GSK2656157) at 10 µM. Cells were treated with 0.2 µg/ml TM and ER stress inhibitors for 24 hours in M199 with 0.5% FBS, fixed and stained with anti-Grp78 primary antibody, fluorescent-labelled secondary antibody and DAPI. Fluorescence was quantified using Cytation 3 reader and normalised to nuclei counts. *denotes significant difference compared to TM, “ns” denotes lack of significant difference compared to CTRL. Data from a single experiment performed in triplicate (3 wells with cells/condition). Statistical significance for each compound was assessed using ANOVA with Tukey’s test. **B.** Cell viability assessed by counting nuclei after a 24 hour treatment is not significantly affected by 10 µM GSK or 25 µM CB53. Cells were treated and analysed as described above. Statistical significance compared to CTRL was tested using ANOVA and Tukey’s *post hoc* test. All graphs show mean with individual data points. Dots denote individual data points, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.


A
CTRL
TM
Grp78
cMGP
ucMGP
tMGP
100 µm
B
Grp78
cMGP
ucMGP
tMGP
RFU
**
CTRL
TM

Supplemental Figure VII. ER stress does not change MGP carboxylation status. VSMCs were treated with 0.1 µg/ml tunicamycin for 24 hours in M199 with 0.5% FBS. Cells were fixed, immunostaining was performed with carboxylation-specific anti-MGP antibodies (ucMGP –uncarboxylated, cMGP – carboxylated, tMGP – total (unphosphorylated) and anti-Grp78. Cells were imaged and quantified in the Cytation 3 imaging reader. **A.** Representative images. Scale bars are 100 µm. **B.** Quantification of fluorescence, normalised to cell counts. Statistical significance was tested using t-tests. Graphs show representative data from 1 of 3 experiments; mean with individual data points. Dots denote individual data points, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Major Resources Tables

Animals (in vivo studies)

Species	Vendor or Source	Background Strain	Sex
Rats	Charles River Laboratories (UK)	Sprague-Dawley	M

Animal breeding

	Species	Vendor or Source	Background Strain	Other Information
Parent - Male	n/a	n/a	n/a	n/a
Parent - Female	n/a	n/a	n/a	n/a

Antibodies

Target antigen	Vendor or Source	Catalog #	Working concentration	Lot # (preferred but not required)
KDEL (Grp78 and Grp94)	Assay Designs	SPA-827	1:2500 (WB) 1:500 (IHC) 1:50 (EM)	
GAPDH	Santa Cruz	sc-25778	0.04 µg/ml (WB)	
CNN1	Abcam	ab108337	0.01 µg/ml (WB)	
CD63	BD Bioscience	556019	1 µg/ml (WB) 100 µg/ml (EM)	
SM22α	Abcam	ab14106	1 µg/ml (WB)	
p-MLC	Cell Signalling	36755	1:500 (WB)	
ALP	Abcam	ab108337	1:10000 (WB)	
Mouse IgG	Li-Cor	926-32210	0.1 µg/ml (WB)	
Rabbit IgG	Li-Cor	926-68071	0.1 µg/ml (WB)	
ATF4	Santa Cruz	sc-200	0.8 µg/ml (IHC)	
TSG101	Sigma	T5701	4 µg/ml (WB) 400 µg/ml (EM)	
SERCA2	Calbiochem	564702	1:500 (WB)	
α-actinin-4	Abcam	ab108198	1:500 (WB)	
Grp78	Santa Cruz	sc-13968	2 µg/ml (ICC)	
pPERK	Abcam	ab192591	0.7 µg/ml (WB)	
MGP total (unphosphorylated)	VitaK B.V.	3-15	1:75 (ICC)	Schurgers et al. ATVB 2005; 25:1629-1633
MGP carboxylated	VitaK B.V.	35-54	6.7 µg/ml (ICC)	Schurgers et al. ATVB 2005; 25:1629-1633
MGP uncarboxylated	VitaK B.V.	35-49	3.6 µg/ml (ICC)	Schurgers et al. ATVB 2005; 25:1629-1633
Rabbit IgG-FITC	Dako	F0205	3.2 µg/ml (ICC)	
Mouse IgG-FITC	Jackson	115-095-068	1:250 (ICC)	

Cultured Cells

Name	Vendor or Source	Sex (F, M, or unknown)
Human primary vascular smooth muscle cells	1. Deceased adult organ donors with approval of Cambridge Local Research Ethics Committee LREC 97/084, characterised and archived in the laboratory. All human materials were handled in compliance with the Human Tissue Act (2004, UK). 2. Patients undergoing open aortic surgery at Maastricht University Medical Centre, in agreement with the Dutch Code for Proper Secondary Use of Human Tissue (http://www.fmwv.nl). This study complies with the Declaration of Helsinki.	F, M