SUPPLEMENTAL INFORMATION FOR:

E-cigarette aerosol exacerbates cardiovascular oxidative stress in mice with an inactive aldehyde dehydrogenase 2 enzyme

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SUPPLEMENTAL METHODS:

ALDH2*2 knock-in mice

We initially measured ALDH2 expression for both male and female rodents. Based on these findings and since men tend to use tobacco products more than women (18% of men smoke e-cigarettes compared to 12% of women [1]), we used male mice for the *in vivo* e-cigarette exposure and *in vitro* cell based studies. In addition, using only male mice reduces the variability and increases the power of this study to comply with the 3Rs for animal use.

To study the effects of e-cigarettes on the ALDH2*2 variant, we used an ALDH2*2 knock-in mouse on a C57/BL6 background generated using homologous recombination [2]. For this study, male and female 8-10 week old mice were initially used to characterize ALDH2 expression for heart, lung and liver. Further studies were then performed using 8-10 week old male homozygous ALDH2*2 mice and age-matched male wild type ALDH2 mice. Mouse exposure to e-cigarette aerosol or room air were carried out by ZX and XY, and data analysis occurred blinded by XY and FX.

All animals were maintained in a constant 12-h dark/12-h light cycle in an AAALAC-accredited Veterinary Service Center at Stanford University. Food and water were available ad libitum. All animal procedures were performed in accordance with National Institutes of Health guidelines for the humane care of laboratory animals and approved by the Stanford University Administrative Panel on Laboratory Animal Care (APLAC). In accordance with approved guidelines, all mice were euthanized by intraperitoneal injection of 100 mg/kg inactin hydrate (Sigma), and euthanasia were confirmed with failure of responding to toe pinch.

ALDH2 expression and ALDH activity assay in organ homogenates

Organs including heart, lung and liver from unexposed wild type ALDH2 mice and ALDH2*2 mice were harvested, homogenized and centrifuged at 10000 rpm for 10 minutes at 4°C (in mannitol-sucrose buffer: 210 mM mannitol, 70 mM sucrose, 5 mM MOPS, and 1 mM EDTA, pH 7.4). Protein concentration was determined by Pierce BCA protein assay kit (Thermo Scientific). The ALDH2 protein expression for heart, lung, and liver homogenates were analyzed by western blot as described previously [3]. Samples were resolved on 10% SDS-polyacrylamide gels, transferred onto PVDF membrane and incubated with ALDH2 primary antibody (Abcam 1:1000) overnight. This was followed by incubation with anti-goat secondary antibody (Invitrogen 1:2000), and were incubated for chemiluminescence at room temperature for 5 minutes and imaged by using an Azure cSeries gel imaging system (Azure Biosystems, Dublin, CA).

ALDH enzymatic activity was measured as described [4]. Briefly, 200 μg of mitochondrial fraction was incubated with 2.5 mM cofactor NAD+ and 10 mM acetaldehyde, and the increase in NADH production was measured over time by spectrophotometer (DU800, Beckman Coulter, Indianapolis, IN) at 340 nm wavelength. The ALDH2 enzymatic activity was presented as μmol NADH /min/mg protein.

Primary mouse cardiac myocyte studies

Adult male cardiac myocytes were isolated from wild type ALDH2 and ALDH2*2 mice as previously described [5]. Briefly, hearts were rapidly excised and washed with calcium free Krebs-Henseleit perfusion buffer. After cannulating the aorta, blood was flushed and the hearts were

subjected to retrograde perfusion at 37°C with perfusion buffer. After 4 minutes of perfusion, collagenase type II (255 U/mg, Worthington Biochemical) was introduced and the heart was perfused for an additional 15 minutes until the heart became soft. The ventricles were minced and digested in Krebs-Henseleit buffer containing 1% BSA. Calcium then was reintroduced slowly to cardiac myocytes at a final concentration of 1.25mM. Cells were counted, plated on laminin (Sigma) coated-plates for calcium imaging or plated in 48-well plate at an equal cell density (2^10^4 cells) for cell viability studies. Cells were maintained in media 199 (Invitrogen) containing 1% BSA and placed in a 37° incubator for use the next day.

Calcium influx

The next day of primary cardiac myocytes isolation, to determine the impact of acetaldehyde on calcium influx, adult cardiac myocytes were incubated for 40 min at 37°C in DMEM serum free medium (Invitrogen). Cover slips containing cardiac myocytes were mounted on a Zeiss Axiovert inverted fluorescent microscope. Cardiac myocytes were superfused continuously with DMEM serum free media by a peristaltic pump at a flow rate of 2 mL/min. The basal intracellular calcium activity was recorded for 5 minutes. Cells were then superfused with a pulse of acetaldehyde (0.1, and 1 μM) in DMEM serum free media for 30 seconds to mimic an exposure occurring when inhaling an e-cigarette. This was followed by KCl (60mM) as a positive control. Images and ratio metric real-time calcium tracing data were acquired using an alternating excitation wavelength (340 and 380 nm) and emission wavelength (510 nm). Background fluorescence was corrected by Easy Ratio Pro software. The ratio of the 2 intensities acquired by Easy Ratio were used to measure changes in intracellular calcium levels, as previously described [6].

Cell viability assay

Wild type ALDH2 and ALDH2*2 knock-in primary cardiac myocytes were incubated with acetaldehyde (0.1 and 1 μM) for 4 hours and cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The yellow tetrazolium dye MTT was reduced to purple formazan in living cells. The absorbance of formazan was quantified at 560 nm using a Synergy 2 plate reader (BioTek, Winooski, Vermont, United States).

Measurement of Reactive Oxygen Species (ROS)

The level of ROS was measured by Amplex Red and Dichlorofluorescein diacetate (DCF-DA)[7]. DCF-DA was deacetylated by a viable cell to a non-fluorescent compound, which was further oxidized by reactive oxygen species (ROS) into a highly fluorescent compound DCF. Amplex red reacts in a 1 to 1 stoichiometry with H2O2 to produce fluorescent resorufin in the presence of horseradish peroxidase. Briefly, the wild type ALDH2 and ALDH2*2 knock-in primary cardiac myocytes were incubated with 10 μM H2-DCF-DA in medium at 37 °C for 30 min or mixture of 50 μM Amplex Red reagent and 0.1 U/mL horseradish peroxidase, and then the medium was removed and washed with PBS. Next cells were incubated with medium, medium, medium with 0.1 or 1 μM acetaldehyde or 20 μM 4-HNE as positive control. DCF fluorescence and Amplex Red react with H_2O_2 produced red-fluorescence was measured for 2 hours at ex/em = 485/535 nm and ex/em = 530/590 nm, respectively.

Immunohistochemistry and confocal microscopy

Primary cardiomyocytes from both wild type ALDH2 mice and ALDH2*2 mice were seeded on laminin precoated coverslip glass for immunofluorescence. Cardiomyocytes were preincubated

with M199 medium with vehicle or acetaldehyde 1 μM for 30 minutes. Cardiomyocytes were fixed with 4% paraformaldehyde for 10 minutes at room temperature. Next, cardiomyocytes were incubated with primary antibodies (anti-dystrophin 1:200, Abcam; anti-4HNE 1:200, Alpha Diagnostic) overnight at 4°C, then incubated with fluorophore-conjugated secondary antibodies the next day (Alexa Fluor 488 or Alexa Fluor 594, Invitrogen) at 1:1000 dilution. Cardiomyocytes were mounted to glass slides using anti-fade mountant with DAPI (Thermofisher Scientific) and imaged using a Zeiss LM900 confocal microscope.

Telemeter implantation for EKG recording

To monitor heart rate with e-cigarette exposures in conscious freely moving rodents, male mice were implanted with wireless telemetry systems to record the electrocardiogram (KAHA Sciences, Grafton, New Zealand). Mice were placed in the supine position on a heating pad to maintain the body temperature. Telemeters were implanted posteriorly under isoflurane anesthesia with EKG leads tunneled and placed in a lead II configuration. The surgical incision was closed with non-absorbable 4-0 veterinary sutures and observed for any infection or necrosis. After surgery, mice were given one week to recover. Animal were monitored continuously until able to ambulate freely, and post-operative analgesic was given (Buprenorphine, subcutaneous injection, 0.1 mg/kg) if severe pain was observed. Additionally, 4 days prior to e-cigarette exposure, telemeter functionality was tested by administering labetolol (10 mg/kg, intraperitoneal injection) to rodents while measuring heart rate from the remote telemeter captured by placing rodents on a wireless digital receiver tBase (Model: MT110, KAHA Sciences) with data continuously steamed and recorded by LabChart (AD Instruments, Sydney, Australia). After confirmation of the telemeter

functionality, heart rate was measured while rodents were exposed to room air or e-cigarette aerosol.

We implanted 36 male rodents (17 wild type and 19 ALDH2*2) with telemeters to continuously monitor heart rate in conscious and freely moving rodents. After telemeter instrumentation, 4 mice (1 wild type and 3 ALDH2*2 mice) were excluded prior to entering a protocol due to wound dehiscence at the site of the implanted telemeter.

E-cigarette aerosol exposure and tissue collection

After telemeter implantation and testing, rodents were divided into 4 groups: room air exposed wild type ALDH2 (WT) mice, room air exposed homozygous ALDH2*2 mice, e-cigarette exposed wild type mice, and e-cigarette exposed ALDH2*2 mice. The mice were exposed to e-cigarette aerosol within a 2L exposure chamber, where the aerosol to air ratio was 1:6. To monitor gas levels within the chamber, a multi-gas monitor (BW Honeywell, Charlotte, NC) continuously measured oxygen and carbon monoxide concentrations and was set to alarm if the environmental oxygen levels dropped by 1%.

The morning before exposure, the baseline heart rate for each of the four groups was recorded for 5 minutes. Exposures were then performed by pairing a wild type ALDH2 mouse with an ALDH2*2 mouse. Aerosol from a JUUL e-cigarette or room air was drawn and delivered to the exposure chamber by a peristaltic pump with a 20mL total puff volume per exposure. Mice were exposed to e-cigarette aerosol or room air for 4 sessions a day for 10 days. Next, for each exposure session, 7 puffs/min were drawn for the first two minutes (a total of 14 puffs), and the animal

whole body exposure to JUUL aerosol continued for an additional 5 minutes (exposure phase) followed by 23 minutes of a smoke-free interval for each session (recovery phase). Heart rate was recorded daily. The puff volume and exposure duration were based on prior studies [8-11].

After completing 10 days of exposure, rodents were anesthetized with 100 mg/kg Inactin (100mg/ml intraperitoneal, Sigma-Aldrich) and euthanasia were confirmed with failure of responding to toe pinch. The hearts were removed and homogenized in mannitol sucrose buffer (210 mM mannitol, 70 mM sucrose, 1 mM EDTA, 5 mM MOPS) and centrifuged at 10000 rpm for 10 minutes at 4°C to remove the cell debris. The supernatants were transferred to 1.5 ml Eppendorf tube and placed at -80° freezer for further molecular studies.

Protein carbonylation

Carbonyl groups introduced into protein side chains (reflecting aldehyde-induced oxidative modification of proteins) were measured by an OxyBlot protein oxidation detection kit (Millipore) by equally splitting the heart homogenate into two samples. One sample was derivatized by adding DNPH (2,4-dinitrophenylhydrazine) solution. The carbonyl groups in the protein side chains were derivatized to 2,4-dinitrophenylhydrazone (DNP-hydrazone) by reaction with DNPH. As a negative control, the other sample was mixed with derivatization-control solution. Specifically, 15-20 µg of protein was added into 10 µL of the positive/negative derivatization reaction solution, and the mixtures were incubated at room temperature for 15 minutes. Next, the DNP-derivatized protein were separated and detected by western blot.

Western blot detection for 4HNE protein adducts, protein carbonylation and NF-κB

The formation of 4-hydroxynonenal-induced protein adducts, carbonylated protein, and nuclear factor kappa B (NF-κB/p65) in heart homogenates were analyzed by western blot. The protein content was determined by Pierce BCA protein assay kit (Thermo Scientific). Samples were resolved on 10% SDS-polyacrolamide gels at 200 volts for 2 hours at room temperature and transferred to PVDF membranes at 100 volts for 90 minutes at 4°C. The membranes were then incubated with primary antibody (anti-4HNE from Millipore 1:1000; anti-DNP 1:500; anti-phospho-NFκB from Invitrogen 1:1000; anti-NFκB from Invitrogen 1:1000) at 4°C overnight, followed by incubation with secondary antibody (anti-rabbit from Invitrogen 1:2000) at room temperature for 2 hours. Membranes were incubated for chemiluminescence at room temperature for 5 minutes and imaged by using an Azure cSeries gel imaging system (Azure Biosystems, Dublin, CA).

Free MDA production

Free malondialdehyde (MDA) was measured by reacting with thiobarbituric acid (TBAR) to generate an MDA-TBAR adduct according to the manufacturer's instructions (Abcam, Cambridge, MA). Briefly, the heart homogenates were lysed in manufacturer provided lysis buffer with butylated hydroxytoluene (BHT) and centrifuged at 13,000g for 10 minutes to collect the supernatants. For each well containing MDA standard and samples, 3x volumes of TBA reagent was added to generate the MDA-TBA adduct. The absorbance of MDA-TBA was measured at 532nm using a Synergy 2 plate reader (BioTek, Winooski, Vermont, United States).

SUPPLEMENTAL TABLES AND FIGURES:

Supplemental Table 1.

Brand Name	Listed Ingredients	Flavor	Nicotine (mg/mL)
Blu Plus+ Rechargeable	Vegetable Glycerin, Propylene Glycol, Nicotine, Natural and Artificial Flavors, Water	Classic Tobacco	24
Halo G6 Rechargeable	Propylene Glycol, Glycerin, Flavorings, Nicotine	Prime 15	24
Juul	Glycerol, Propylene Glycol, Flavor, Nicotine, Benzoic acid	Classic Tobacco	50

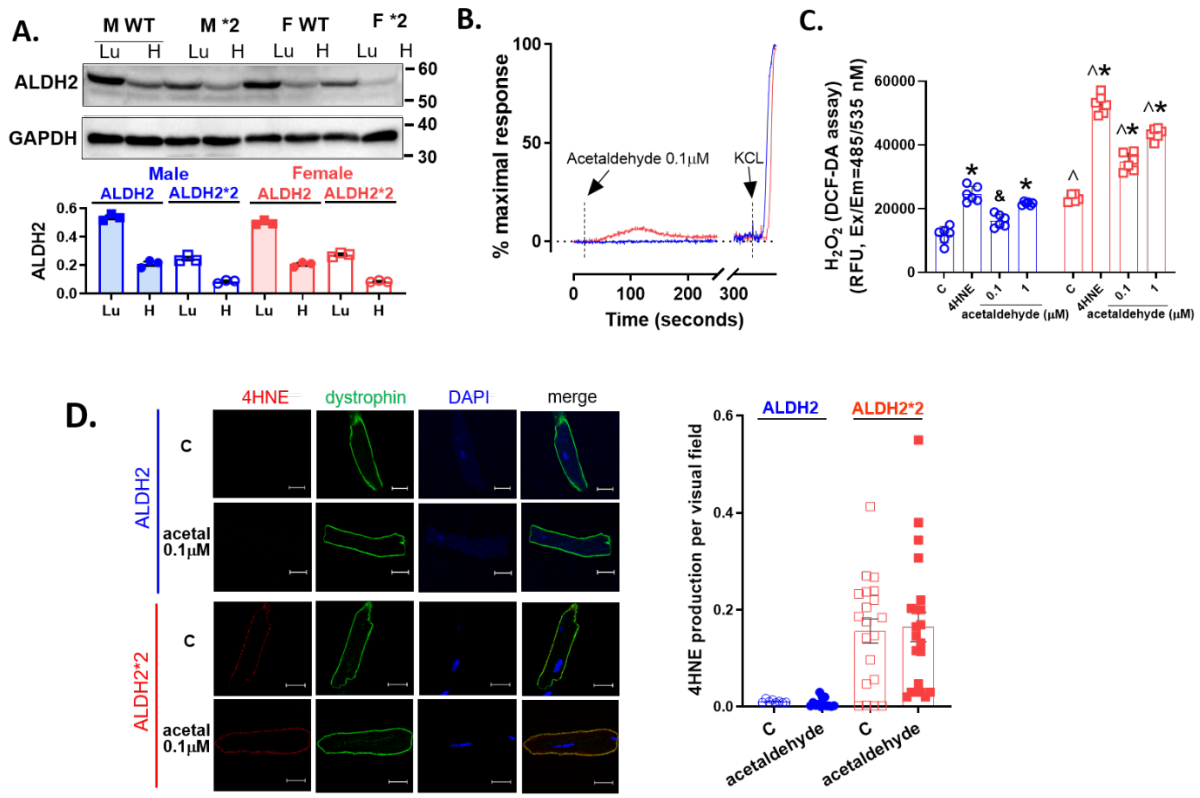
Supplemental Table 1. E-cigarette chemical composition. Three e-cigarette brands were quantified for aerosolized nicotine and aldehyde content including Blu, Halo and Juul. Brand name, chemical composition on the packaging insert, and nicotine concentration are listed.

Supplemental Table 2

Metabolite	Reagent Ion	Reaction Ratio	Mass (m/z)
Acetaldehyde	H_3O^+	3.70×10^{-9}	45, 63
	NO^+	6.00×10^{-9}	43, 61
Formaldehyde	H_3O^+	3.40×10^{-9}	31, 49
Acrolein	H_3O^+	4.20×10^{-9}	57, 75
	NO^+	1.40×10^{-9}	55, 86
Nicotine	H_3O^+	3.0×10^{-9}	163
	NO^+	2.5×10^{-9}	162
	O_2^+	1.8×10^{-9}	162

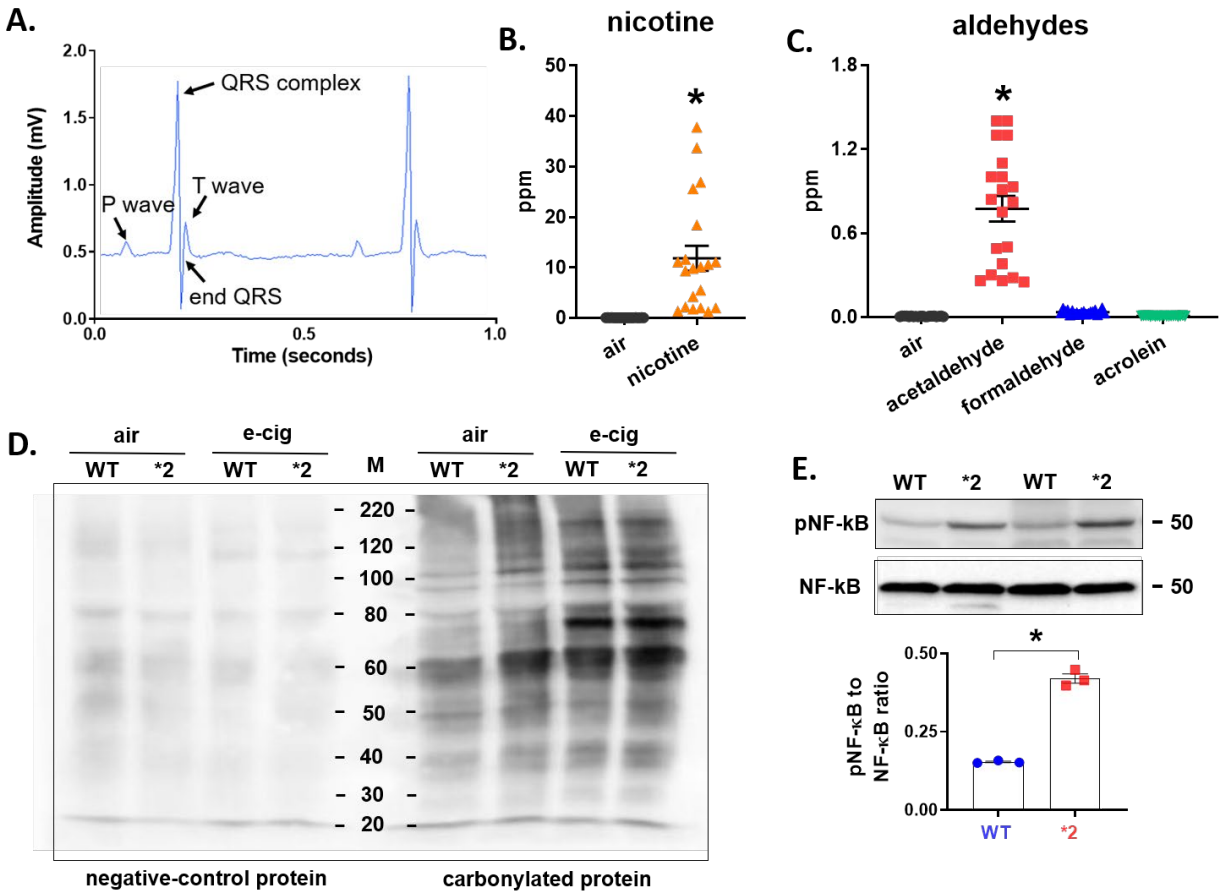
Supplemental Table 2. Reagent ions, reaction ratio and mass used for SIFT-MS to detect and identify nicotine and aldehydes within e-cigarette aerosol.

Supplemental Figure 1



Supplemental Figure 1. A. Western blot of male and female wild type ALDH2 and ALDH2*2 rodents. ALDH2 protein expression is gender independent for both wild type ALDH2 and ALDH2*2 rodents. n= 3 mice/gender. **B**. Calcium response in primary cardiac myocytes exposed to acetaldehyde. Calcium influx induced by 0.1 μM acetaldehyde (n = 21 cells for ALDH2 and n= 20 cells for ALDH2*2 from 4 biological replicates). **C**. H_2O_2 activity by 0.1 or 1μM acetaldehyde with 4-HNE (20μM) as a positive control assessed by DCF-FA (n=6/group). **D**. Immunostaining of primary cardiomyocytes for 4-hydroxynoneal (4-HNE) protein adducts in vehicle or 0.1 μM acetaldehyde treated cells (left panel, bar = 20 μm). Green: dystrophin, Red: 4-HNE protein adducts, Blue: DAPI. Right: Quantification of 4-HNE protein adducts.

Supplemental Figure 2



Supplemental Figure 2. **A.** EKG telemeter recording. Representative EKG waveform captured from mouse telemeter. Arrows show P wave, QRS complex and T wave respectively. **B**. E-cigarette aerosol average concentration of nicotine or **C.** aldehydes in the rodent exposure chamber. n=20/group, *p<0.001 comparison between e-cigarette aerosol and air, calculated one-way ANOVA with Bonferroni correction. **D**. Protein carbonylation blot. Uncut blot of the negative control (left half of panel) and DNP-derivatized protein carbonyls (right half of panel). **E**. Basal level of nuclear factor kappa B (NF-κB) signaling. Representative western blot of phospho- and total nuclear factor kappa B (NF-κB, 65 kDa) in heart homogenates from unexposed wild type ALDH2 and ALDH2*2 mice. Blue in bar graph represents wild type ALDH2 mice and red represents the ALDH2*2 mice. WT= wild type, *2 = ALDH2*2

Reference

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