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Supplemental Information

Pollen-derived RNAs

Are Found in the Human Circulation

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TRANSPARENT METHODS

Study Population

The Framingham Heart Study (FHS) is a long-term, ongoing population study (Kannel et al., 1979, Wilson et al., 1998). The FHS Offspring Study is a community-based, prospective study of general disease as well as cardiovascular disease (CVD) and its risk factors. Previous reports have detailed the sample composition and study design (Wilson et al., 1998). The study began in 1948 with 5,209 adult subjects and is now on its third generation of participants. Cohorts undergo an examination at the FHS once every ~4-8 years and have been densely phenotyped over multiple prior examinations with a wide variety of noninvasive tests. Participants in the Offspring Study have been examined every 4-8 years since the 1970s, for 8 prior exams. At exam 8, FHS participants had a mean age of 66 years, 55% were women (Table S1). Studies outlined by the FHS protocol were approved by and carried out in accordance with Boston University Medical Center and by UMass Medical School Institutional Review Board Committees. We postulated that the expression of specific genes is influenced by environmental factors and is related to subclinical phenotypes.

Cohort Blood Sample Processing and RNA Isolation

Blood samples were collected at the Framingham Heart Study (Framingham, MA, USA) on Offspring exam 8 (March 2005 - Jan. 2008) as previously described (Freedman et al., 2016). Blood collection tubes were centrifuged and plasma was separated from the cells and frozen at -80°C within 90 minutes of draw. RNA was isolated from citrated plasma samples using a miRCURY RNA Isolation Kit (Freedman et al., 2016).

FHS RNA Sequencing and Real-Time PCR Analyses

The exact protocols have been previously published in full (Freedman et al., 2016). Briefly, the Ion Total RNAseq Kit v2 (Cat. No: 4479789, Life Technologies, USA) was used for creating libraries for sequencing. RNA Sequencing was performed on the Ion Proton System with Ion PI Chip Kit v2 BC and Ion Proton System (Life Technologies, USA) using the Ion PI Sequencing 200 Kit v2 (Life Technologies, USA). Sequencing reads were at maximum 200 nucleotides.

Sequencing Data Analysis Using the Genboree Sequencing Pipeline

Small RNA-seq reads were processed and quantified using the exceRpt tool (Rozowsky et al., 2019) available on the Genboree Workbench [http://www.genboree.org/] (Freedman et al., 2016). ExceRpt (Rozowsky et al., 2019) incorporates several modifications to existing analysis methods used to assess cytosolic micro-RNAs (miRNAs) that specifically address experimental issues pertinent to exRNA profiling, such as variable contamination of ribosomal RNAs, the presence of endogenous non-miRNA small-RNAs, and the presence of exogenous small-RNA molecules derived from a variety of plant, bacteria, and viral species. Briefly, the software processes each sample independently through a cascade of read-alignment steps designed to remove likely contaminants and endogenous sequences before aligning to exogenous miRNAs as we have previously shown (Freedman et al., 2016). Exogenous alignment of the miRNAs and the genome was done via Genboree exceRpt small-RNA seq pipeline (v3.3.0) (Rozowsky et al., 2019).

Reverse Transcription for Pollen miRNAs (FHS and oxidized miRNAs)

Specific pollen miRNAs were chosen directly from the list generated by RNAseq. The most abundant and most commonly expressed pine pollen miRNAs were selected for measurement by RT-qPCR. RNA samples (2,782) were reverse transcribed and subjected to real time PCR (Freedman et al., 2016). For pine miRNAs, miScript Plant RT Kit (Cat. No: 218762, Qiagen, Frederick, MD, USA) was used due to 3' modifications. Real-Time PCR analysis was performed on the BioMark Real Time PCR System.

Confirmation of plant miRNA origin utilizing $NaIO_4$ oxidation

Plasma RNA was isolated from healthy donors as described below in 14 µL elution volume. RNA was split evenly and one of the parts was subjected to oxidation with a final concentration of 10 mM sodium periodate ($NaIO_4$) for 40 min, in the dark, on ice. After oxidation the volume of both parts (oxidized and non-oxidized) was adjusted to 200 µL with H_2O. 1 mL of Qiazol was added and RNA was re-isolated using the plasma protocol of the Qiagen RNeasy Micro Kit according to the manufacturer's protocol. RNA was then subjected to RT-qPCR utilizing miScript chemistry. Plant miRNAs have a 3' modification (2'-O-methylation), instead of a hydroxyl group as in mammalian miRNAs, which makes them resistant to oxidation with sodium periodate. Since miScript technology utilizes polyadenylation of mature miRNAs in the first step of reverse transcription and the poly(A) polymerase requires a hydroxyl group at the 3' end, human miRNA cannot be amplified and detected when oxidation has occurred. TaqMan RT-qPCR is not appropriate for this verification since it does not involve polyadenylation in the first step of the reaction and does not account for the lack of oxidation at 3'-end of plant miRNAs.

Platelet pollen-miRNA transfer from lung Endothelial and Bronchial-epithelial Cells using Flow adhesion model

Primary lung endothelial cells (HMVEC-L, Lonza, cat. no. CC-2527, isolated from F age 52 y) or bronchial epithelial cells (normal, NHBE, Lonza cat. no. CC-2540, isolated from M age 42 y, or asthmatic, D-HBE-As, Lonza cat. no. 194911, isolated from F age 54 y) were plated on glass slides at 60,000 cells/slide overnight and then transfected with 5 nM of 2 synthetically designed pollen miRNAs (pta-miR948 and pde-miR1314, Integrated DNA Technologies, USA) or with miScript Inhibitor Negative Control (Qiagen, cat. no. 1027271) for 24h. Washed human platelets isolated from healthy human donors ($2x10^8$ platelets/slide) were run over the endothelial cells for 10 min at 3 mL/min (Clancy et al., 2017). Platelets were collected and assessed for pollen miRNA presence by RT-qPCR.(Clancy et al., 2017)

Co-culture of pollen-miRNA-transfected Primary Bronchial Epithelial Cells and HMVEC-L

Normal and asthma bronchial epithelial cells were cultured to Passage 4 and plated at $1.2 x10^5$ cells per well in a 12-well plate in 1.1 mL of BronchiaLife Complete Medium and were grown for two days prior to transfection. On the day prior to transfection, Human Microvascular Endothelial Cells – Lung (HMVEC-L, Lonza, cat. no. CC-2527) grown in VascuLife VEGF-Mv Endothelial Cell Culture Medium supplemented with VascuLife VEGF-Mv LifeFactors Kit (Lifeline Cell Technology, cat. no. LL-0005) and phenol red (Lifeline) to passage 6, were plated at $0.6 x 10^5$ cells per well in a 24-well plate in 0.550 mL of VascuLife Complete Medium. On the day prior to transfection, ThinCert Cell Culture Inserts, 0.4 µm membrane (Greiner Bio-One, Austria, cat. no. 662641) were coated with 200 µL of 50 µg/mL collagen (Chrono-Log Corp, PA, USA, cat. no. 385) diluted from stock with 60% ethanol made with sterile water. The coated inserts were dried in a closed biosafety cabinet under UV light overnight with the cabinet sash closed and fan off.

On the day of transfection, the HMVEC-L cells were washed with 1 mL pre-warmed PBS and 700 µL of media consisting of BronchiaLife Complete Media and VascuLife VEGF-Mv Complete Media at 1:1 proportion. Normal and asthma bronchial epithelial cells were transfected with 5 nM pde-miR1314 miRNA or miScript Inhibitor Negative Control, or in some cases cells were left untreated as described above for 6 hours. After transfection, epithelial cells were washed three times with pre-warmed Hepes-Buffered Saline Solution (Lonza, cat. no. CC-5022). Each well was trypsinized with 0.500 mL Trypsin-EDTA (Lonza, cat. no. CC-5012) until cells detached. The trypsin was neutralized with 1 mL of BronchiaLife Complete Media containing 2% FBS. Cells were transferred to a 15 mL tube and resuspended with a pipette. The wells were rinsed with 0.500 mL Hepes-BSS. The cells were centrifuged at 220 x g for 5 minutes. The cells were washed a second time with Hepes-BSS and

centrifuged at 220 x g for 5 minutes. Cells were re-suspended in media consisting of BronchiaLife Complete Media and VascuLife VEGF-Mv Complete Media at 1:1 proportion, counted on a hemacytometer, and plated onto the collagenized inserts (which had been washed with 300 µL of mixed media just prior) at 0.275 x 10^5 cells per insert in 300 µL of mixed media. The inserts were transferred to the wells containing the HMVEC-L cells and then co-cultured at 37°C for 24 hours. After 24 hours the inserts were removed. 500 µL of media was removed from the HMVEC-L cell wells and 700 µL of QIAzol Lysis Reagent (Qiagen, cat. no. 79306) was added. The HMVEC-L cells were washed with pre-warmed PBS. 700 µL of QIAzol was added to the wells and after 10 minutes the cells were collected and frozen at -80°C.

RT-qPCR for human genes targeted by pollen miRNA

Genes for measurement were selected based on target score calculated as a function of complementarity between the seed sequence and targeted sequence in the 5'-UTR region of human mRNA using mirdb.org. Transfected endothelial or bronchial epithelial cells were collected at the appropriate time points, 700 µL of Qiazol reagent was added and after 10 minutes, the samples were frozen at -80°C until processing. RNA was isolated from these samples using the miRNeasy Mini Kit (Qiagen, cat. no. 217004) with on-column DNA digestion using the RNase-free DNase kit (Qiagen, cat. no. 79254) and eluted in 30 µL. RNA was normalized to total RNA concentration and cDNA was synthesized in a 10 µL reaction using the Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Life Technologies, cat. no. 4368813). cDNA was preamplified in a 5 µL reaction using the TaqMan PreAmp Master Mix (Life Technologies, cat. no. 4391128). Preamplification was performed for 10 human genes targeted by pollen miRNA as well as ACTB. Preamplification product was diluted with 40 µL of DNA Suspension Buffer (Teknova, cat. no. T0223) prior to qPCR. qPCR was performed in a 10 µL reaction using TaqMan Gene Expression Master Mix (Life Technologies, cat. no 4369016), on a QuantStudio 3 Real-Time PCR system (Thermo Fisher Scientific, MA, USA). The following genes were screened for using TaqMan assays (Life Technologies, cat. no. 4331182): PAPD5 (Hs00900790_m1), MMP24 (Hs00198580_m1), PRR11 (Hs00383634_m1), FRS2 (Hs00183614_m1), MAFG (Hs00361648_g1), CSDE1 (Hs00918650_m1), CREB3L3 (Hs00962115_m1), PIK3AP1 (Hs00381030_m1), ARF4 (Hs01070798_g1), HIVEP3 (Hs00962949_m1), ACTB (Hs99999903_m1).

***In vivo* Pollen Inhalation Model and TaqMan RT-qPCR**

Plant pollens were delivered to mice via the intranasal route and pollen miRNA presence was assessed in platelets and plasma after 24 hours. To ensure horizontal transfer, PBS dissolved pine pollen was intranasally delivered to C57BL/6J mice for 3 consecutive days. Pollen from *Pinus taeda* and *Pinus*

strobus (Greer, cat# RM132 and RM130) was resuspended at 50 µg/µL in sterile saline and 30 µL of the suspension were delivered intranasally. At the times indicated, after the final delivery, blood was collected by heart puncture and plasma and platelets were immediately isolated. RNA was isolated from 100 µL of plasma or from washed murine platelets using miRNeasy Micro Kit (Qiagen cat no. 217084) according to the manufacturer's protocols (Freedman et al., 2016). RT-qPCR for pine pollen miRNA analysis used TaqMan gene expression assays and the TaqMan® MicroRNA Reverse Transcription Kit (ThermoFisher Scientific, MA, USA, cat. no. 4366597).

Cell Count in blood or BAL

Blood (500 µl) from mice was collected by heart puncture in 200 µl of CPD after CO_2-asphyxiation. Blood cell count was determined using an AcT8 hematology analyzer (Beckman Coulter, USA). Broncho-alveolar lavage (BAL) was collected in sterile 1 mL PBS supplemented with CPD buffer (9:1 ratio). Immediately after euthanasia, murine lungs were gently flushed and BAL was fixed and stained as described below. 50 µL of BAL was run for blood cell differential on a Hemavet HV950 (Drew Scientific Group, FL, USA). Of note, the numbers measured using this blood hematology analyzer (Figure 3a and Figure S2) represent only cells that are singular; any aggregates that are formed between white blood cells (Figure 3c) cannot be accounted for by this method or by flow cytometry.

Confocal Microscopy and Antibodies

The volume of isolated BAL, or human platelets treated with pollen, was adjusted to 1 mL with PBS and fixed with 333 µL of 16% PFA for 10 min at constant rotation of 1000 rpm using magnetic stir bars (Bio/Data Corporation, cat. no. 105990) in glass tubes (Bio/Data Corporation, cat. no. 101521). Cells were spun down for 7 min at 1200 x g and washed once with ice-cold PBS. Cells were then resuspended in 100 µL of HEPES-modified Tyrode's buffer supplemented with 2% FBS and blocked for 1h at room temperature, then antibodies were added for an additional hour. At the end of incubation, samples were washed with 1 mL of 1X PBS and mounted on slides. The following antibodies were used throughout this study: anti-human: CD41-FITC or CD41-APC (clone HIP8, eBioscience, CA, USA, cat. no. 11-0419 and cat. no. 17-0419); anti-mouse: CD41-FITC (clone MWReg30, eBioscience, cat. no. 11-0411), Ly6G-APC (clone RB6-8C5, eBioscience, cat. no. 17-5931), F4/80-APC (Biolegend, cat. no. 123116).

Washed Human Platelets and Murine Platelet/Plasma Isolation

Human platelets were isolated from venous citrated blood of healthy human donors by a two-step centrifugation method (Koupenova et al., 2014). Platelets were re-suspended in 37°C HEPES-

Tyrode Buffer (140 nM NaCl, 6.1 KCl, 2.4 mM $MgSO_4.7H_2O$, 1.7 mM Na_2HPO_4, 5.8 mM Na-HEPES, pH 7.4, supplemented with 0.35% BSA and 0.1% Dextrose) and used in the transfer experiments.
Murine plasma/platelets were isolated from citrated blood collected by heart puncture. To control for variations in volume from each mouse, 500 µL of blood was collected in 200 µL of CPD buffer throughout the entire study. Blood counts were measured immediately after draw, using a Coulter AcT 8 hematology analyzer. Blood was immediately spun down at 500 x g for 10 min at room temperature. The top plasma layer was removed and centrifuged for an additional 10 min at 2000 x g at room temperature and 200 µL of plasma was removed and immediately frozen on dry ice. Isolation of platelets for RNA: the remaining blood fraction was gently transferred to Corning Falcon Round Bottom Polystyrene Tubes (Corning Incorporated - Life Sciences, Oneonta, NY, USA, cat. no. 352058) containing 4 mL of CPD Buffer (16 mM Citric acid, 102 mM Sodium citrate, 18.5 mM NaH_2PO_4, 142 mM Dextrose, pH 7.4) and centrifuged at room temperature for 5 min at 300 x g with no brake. The top layer (~1 cm left above the RBC layer) was transferred to a new polystyrene tube and centrifuged at room temperature for 7 min at 3500 x g with no brake. Any remaining red blood cells in the platelet pellet were manually removed with a pipette; platelets were immediately lysed in 700 µL of QIAzol Lysis Reagent.
Isolation of platelets for platelet aggregation: The bottom cell layer was re-suspended in 4 mL of platelet wash buffer (10 mM sodium citrate, 150 mM NaCl, 1 mM EDTA, 1% (w/v) Dextrose, pH 7.4) supplemented with 100 ng/mL PGE_2 (Koupenova et al., 2014) in 5 mL Corning Falcon Round Bottom Polystyrene Tubes. The rest of the procedure was carried out similarly as for RNA, with the exception that the platelet pellet was re-suspended in 300-500 µL of HEPES-Tyrode Buffer.

Human Platelet and Pollen Interaction

Human platelets were isolated as described above. $2x10^5$ platelets/µL, in 200 µl of HEPES-Tyrode Buffer supplemented with Ca^{2+}/Mg^{2+}, (Koupenova et al., 2014) were incubated with 10 ng/µL of *Pinus strobus* pollen dissolved in PBS. Samples were incubated for the times indicated in the figure legends, at 37°C in a PAP-8E aggregometer (Bio/Data Corporation), at constant rotation of 1000 rpm. At the end of each time point, the volume was adjusted and platelets were fixed and stained as described in the “Confocal Microscopy and Antibodies” section.

Mice

All plasma and platelet isolation, and in vivo models were carried out in male C57BL/6J mice (The Jackson Laboratory, cat.no. 000664), age 12-14 weeks. All procedures were approved by the University of Massachusetts Institutional Animal Care and Use Committee and conducted accordingly.

Murine Platelet Function

Washed murine platelets at $2x10^5$ platelets/µl in 200 µl of HEPES-Tyrode Buffer (supplemented with 1 mM $CaCl_2$, 2 mM $MgCl_2$, and 0.3 µM fibrinogen) were tested for aggregation potential in the presence of murine thrombin for 10 minutes at 37°C (Koupenova et al., 2014) using a PAP-8 aggregometer.

Murine Pulmonary Function

Whole body plethysmography (PenH): Pulmonary resistance quantifying breathing in awake spontaneously breathing mice was measured non-invasively. Unanesthetized, unrestrained mice were placed in a clear Plexiglas chamber, and both ambient and chamber pressure and temperature were used to calculate mouse ventilation.[8,14,15] Baseline measurements were taken under conditions of normoxia (21% oxygen; nitrogen balance) followed by a 10-minute exposure to hypercapnia (7% carbon dioxide; 21% oxygen; nitrogen balance). This method assesses the ability of mice to respond to a respiratory challenge. During baseline measurements, PenH was used to assess airway responsiveness. Penh is an empirical and dimensionless parameter and increased PenH signifies increased bronchoconstriction (Verheijden et al., 2014). In order to confirm this increased airway responsiveness, we directly measured airway resistance and pulmonary mechanics. *Pulmonary Mechanics:* Pulmonary mechanics at baseline and in response to incremental doses of methacholine were performed using forced oscillometry (FlexiVent system, SCIREQ, Montreal, Canada) in tracheotomized and anesthetized mice. Mice were anesthetized and placed on a computer controlled piston-ventilator FlexiVent system (SCIREQ, Montreal, Canada) (Keeler et al., 2017). Measurements were obtained by analyzing pressure and volume signals acquired in reaction to predefined, small amplitude, oscillatory airflow waveforms (perturbations) applied to the subject's airways. After an initial mechanical scan protocol, animals were subjected to incremental doses of nebulized methacholine. Respiratory system resistance (Rrs) was obtained by assessing the mouse's response to a single frequency forced oscillation maneuver (McGovern et al., 2013). Resistance of central airways (Rn) and small airway and tissue resistance (G) was measured (McGovern et al., 2013). To ensure that the animal was alive during these *in vivo* pulmonary mechanical scans, the heart beat was visualized between each dose during the deep inflation and at the end of the study.

H&E staining of Murine Lungs

After the Murine Pulmonary Function Experiment, lungs were inflated with 1 mL of 4% formaldehyde and fixed overnight. After 24h, lungs were transferred to 1X PBS for 24h, paraffin embedded, sectioned, and stained by the UMass DERC Morphology core. Images were taken with an Amscope IN300T-FL microscope and ToupViewX software (ToupTek, China).

Statistical Analyses of the Pollen miRNAs from the FHS

As previously described (Freedman et al., 2016), we studied plasma samples from the FHS offspring 8 cohort. There were 2,782 subjects for analysis. All statistical analyses related to the FHS were performed using STATA 13.0. Descriptive statistics are displayed as mean ± SD for continuous variables and count (percentage) for categorical variables. The RT-qPCR Cq values were not normalized because a precise and homogeneous plasma volume was used for each included sample. Ordinary least squares linear regression models were used to test for association with median age and sex and the Cq value for each pine pollen miRNA was assessed. In the mechanistic *in vitro* or *in vivo* studies, all values were expressed as the average ± SD of at least 3 different experiments. Fold changes in gene expression (RT-qPCR) were calculated based on beta-actin normalization and standard calculation methods. Statistical analyses were performed by either student-t test or ANOVA single factor test followed by Bonferroni test post analysis or unpaired t-test using Prism software (GraphPad Prism v7; La Jolla, CA, USA) using α=0.05.

Data and Software Availability

All RNA sequencing data in this manuscript has been previously deposited in dbGaP, accession number phs000007.v27.p10; the RNA-seq data can be accessed under Jane Freedman at http://genboree.org/exRNA-atlas/exRNA-Grids.rhtml?grid=analysisTable.

SUPPLEMENTAL EXCEL FILE

Raw Cq values from RT-qPCR that generated the plots in Figure 1c

Cq after miScript RT-qPCR after oxidation of plasma-derived RNA with $NaIO_4$ confirming that the pollen miRNA reads are of plant origin. Plant miRNAs have a methyl group at the 3' end that cannot be oxidized and the miRNA can be transcribed and detected by miScript chemistry.

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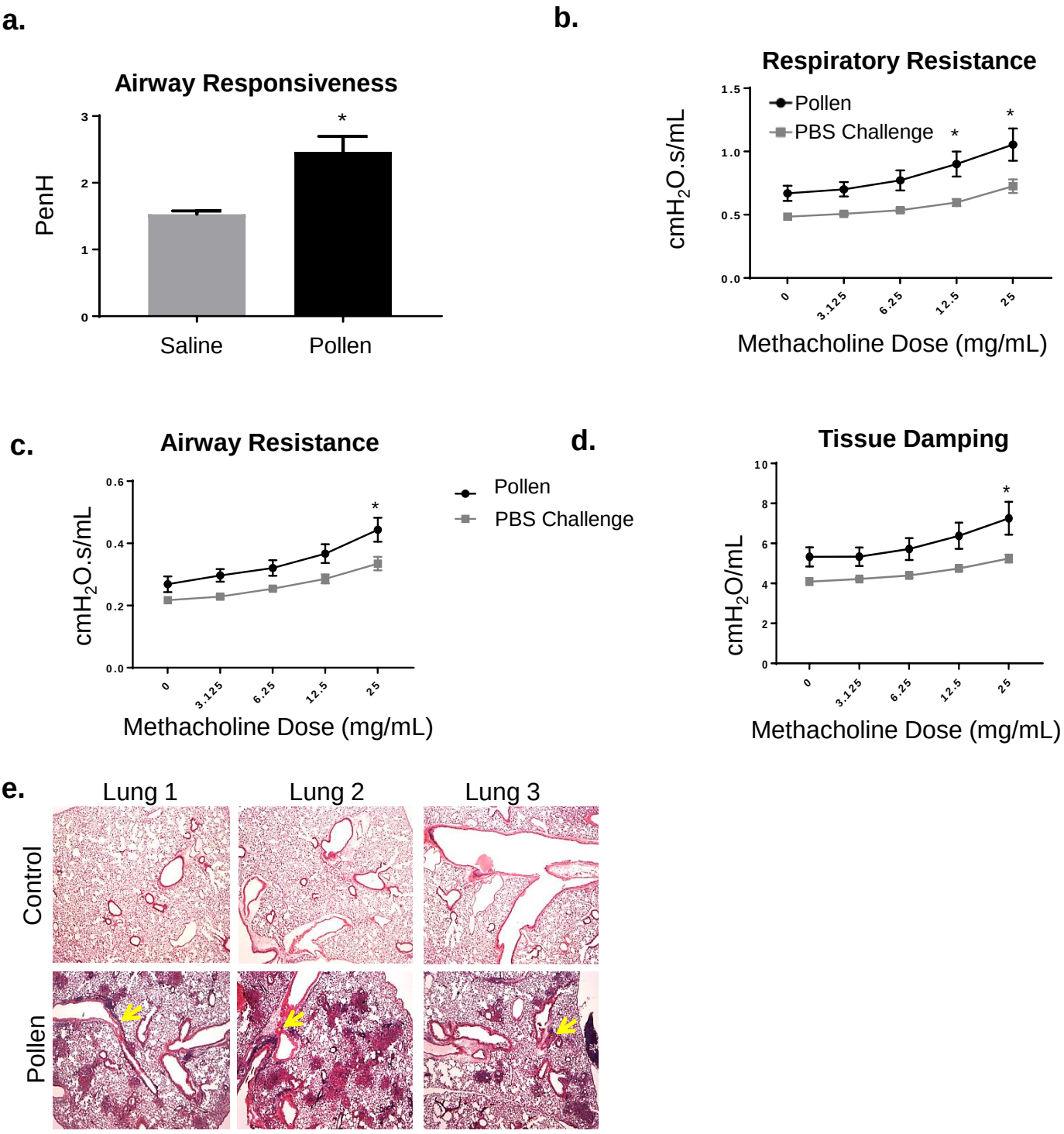

Figure S1 (Related to Figure 1). Effect of pine pollen on pulmonary mechanics. Pollen was delivered intranasally in mice over 3 consecutive days. **a.** Platelets were isolated 24h post the 3rd dose and tested for pollen miRNA presence by RT-qPCR. Effect of pollen on pulmonary mechanics: **a.** Airway responsiveness measured non-invasively in unanesthetized mice by unrestrained whole body plethysmography, as a function of enhanced pause (PenH). PenH is an empirical parameter assessing increases in bronchoconstriction. b-d. Pulmonary mechanics assessed in the same mice as in a. (after PenH) by the ventilated lung resistance method in anesthetized animals by increased exposure to methacholine. **b.** Total respiratory system resistance; **c.** Central airway resistance; **d.** Tissue damping, a measurement of the function of the smallest airways in tissue resistance. **e.** H&E Histology of the lungs of 3 of 4 different mice after the highest dose of methacholine exposure. Pictures of the lobes of each mouse are compared to the same lobe position as in control. Blue staining indicates a dramatic increase in leukocyte presence in the lungs of pollen mice. Yellow arrows point to constricted airway structures. a-d. Each test involved n=8 mice/group (male). Data is represented as the average ± SEM. Significance is established using unpaired t-test in b. and 2-way repeated-measures ANOVA followed by Bonferoni post hoc analysis in c-e. Significance in all cased is considered at $p<0.05$.

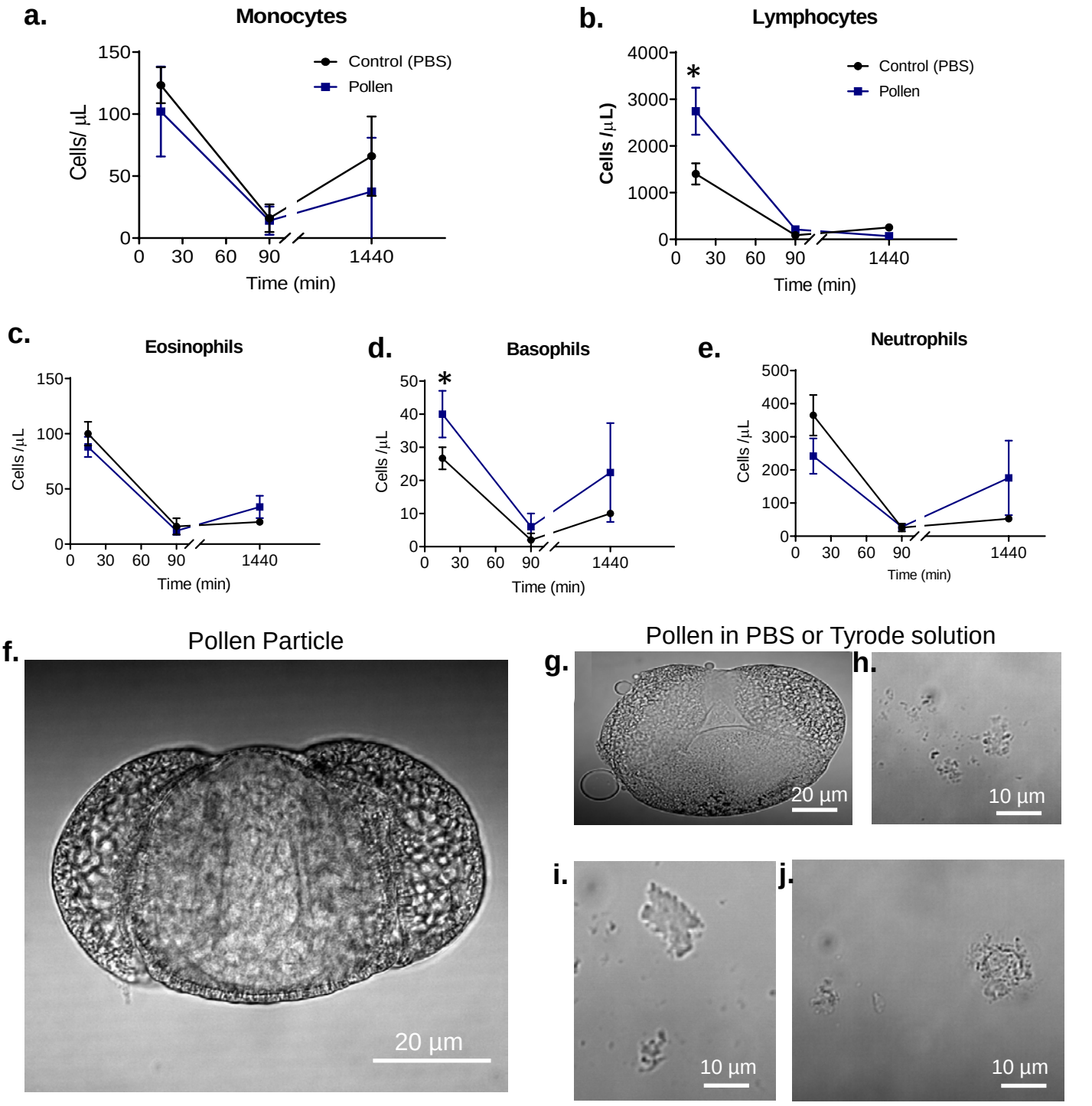

Figure S2 (Related to Figure 3). Blood cell numbers in BAL post pollen delivery and spontaneous formation of sub-pollen particles from pollen under hydrophilic conditions. Pollen was delivered intranasally in mice over 3 consecutive days. BAL was collected at the indicated time points and 50 µl of blood was tested for blood differential with a Hemavet HV950 blood counter. **a.-e.** Counts of different blood cells as indicated in each graph. Graphs represent mean ± SD. Significance was assessed by two-way ANOVA with $p<0.05$, followed by Bonferroni post test. Of note, this method only tests singular cells and does not account for cell aggregates as seen in Figure 3c. f-j: Sub-pollen particle formation from pine pollen particles under hydrophilic conditions assessed by differential interference contrast (DIC) microscopy of: **f.** Intact pine-pollen particle visible after immediate fixation of dissolved pollen in PBS. **g.** Pollen in BAL (PBS flush) 15 min after 3 days of sequential delivery of intranasal pollen in mice. **h-j:** Sub-pollen particles of various sizes formed in PBS or HEPES buffered Tyrode solution after 5 min of mixing. Scale bars are indicated on the images.

Table S1 (Related to Table 1): **FHS Sample Characteristics**

	Offspring 8 (n=2,776)	
Characteristics	**mean / n**	**SD / %**
Age (years)	66.3	9.0
Sex (female)	1499	54%
Systolic BP	128.5	17.2
Diastolic BP	73.5	10.1
BMI (mg/kg2)	28.3	5.4
Total Cholesterol to HDL Ratio	3.5	1.1
Triglycerides	118.4	69.7
Fasting Blood Glucose	106.7	23.7
Diabetes	378	14%
Blood Sugar Treatment	252	10%
Anti-hypertensive Treatment	1330	48%
Lipid Lowering Treatment	1179	43%
COPD	61	2%
Asthma	232	9%
Hay Fever	798	29%

BP-blood pressure; BMI- body mass index; HDL-high density lipoprotein; COPD- Chronic obstructive pulmonary disease