Environment International 158 (2022) 106997

Contents lists available at ScienceDirect

Environment International

journal homepage: www.elsevier.com/locate/envint

Toxicological transcriptome of human airway constructs after exposure to indoor air particulate matter: In search of relevant pathways of moisture damage-associated health effects

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ARTICLE INFO

Handling Editor: Prof. Yong-Guan Zhu

Keywords:
Indoor air
Health effects
Moisture damage
Gene expression
Particulate matter
In vitro

ABSTRACT

Background: Multiple health effects are associated with moisture damage in buildings. Studies explaining these associations and cell-level mechanisms behind the observed health effects are urgently called for.
Objectives: We focused on characterizing gene expression in human airway epithelium after exposure to indoor air particulate matter (PM) sampled from houses with and without moisture damage, alongside determination of general toxicological markers.
Methods: We performed detailed technical building inspections in 25 residential houses and categorized them based on the detection of moisture damages and the probability of occupant exposure. PM sampling was complemented by microbiological and volatile organic compound assessment. We exposed human airway constructs to three dilutions (1:16, 1:8, 1:4) of collected PM from moisture-damaged (index) and non-moisture-damaged (reference) houses and imaged selected constructs with electron microscopy. We analyzed general toxicological markers and the RNA of exposed constructs was sequenced targeting genes associated with toxicological pathways. We did groupwise comparisons between index and reference houses and pairwise comparisons in matched index/reference houses.
Results: In groupwise comparison, gene Cytochrome P450 Family 1 Subfamily A Member 1 (CYP1A1) was statistically significantly over-expressed in index houses at all dilutions of collected PM and Nuclear Factor Kappa B Subunit 1 (NFKB1) at dilution 1:4 of collected PM. In pairwise index/reference house comparison, several genes related to multiple toxicological pathways were activated, largest expression differences seen for CYP1A1. However, none of the genes was consistently expressed in all the matched pairs, and general toxicological markers did not differentiate index and reference houses.
Discussion: The exposure to PM from index houses activated toxicology -related genes in airway constructs. Differential expression was not consistent among all the index/reference pairs, possibly due to compositional differences of bioactive particles. Our study highlights CYP1A1 and NFKB1 as potential targets in moisture damage -associated cellular responses.

1. Introduction

Multiple adverse health effects, such as upper and lower respiratory tract symptoms, asthma exacerbation and development, are evidently associated with damp indoor environment and mold (Bornehag et al. 2001; Mendell et al. 2011; WHO 2009). High prevalence of moisture-damage in building stocks across many countries (Eurostat, 2020; Mudarri & Fisk, 2007) and people spending most of their time indoors

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https://doi.org/10.1016/j.envint.2021.106997
Received 18 July 2021; Received in revised form 16 November 2021; Accepted 20 November 2021
Available online 26 November 2021
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(Klepeis et al. 2001; Perez-Padilla et al. 2010) make this a relevant public health issue. Despite of decades of dedicated research, the substantial burden for public health and the enormous economic impact (Mudarri & Fisk, 2007), neither the mechanisms underlying the associations between indoor dampness and adverse health effects nor causally involved exposing agents have conclusively been revealed. Toxicological studies have supported the assumption of microbial exposure contributing to the observed health effects (Mendell et al. 2011; WHO 2009), but there are overall little and contradictory indications from the epidemiological literature that would strongly suggest a causal involvement of microbial exposure in moisture damage -related health effects. The existing knowledge gaps call for studies aiming to explain the cellular mechanisms behind the observed health effects as well as clarification of how indoor microbiota of damp indoor environments differs from a normal indoor microbial ecology and in how far they contribute to ill health.

Human exposure and related health effects can be investigated using epidemiological (e.g. Jaakkola et al. 2002; Thorn, Brisman & Toren, 2001; Nafstad et al. 1998), *in vivo* (e.g. Pieckova et al. 2006; Rand et al. 2006; Rosenblum Lichtenstein et al. 2006), and *in vitro* (e.g. Huttunen et al. 2003; Huttunen et al. 2004; Kamp et al 2005) studies. When studying mechanisms behind the observed health effects on the cellular level, *in vitro* studies such as models for human airway cells and tissues are most suitable. We have recently established the use of a human airway model in studying the effects of exposure to indoor air PM (Nordberg et al. 2020). In this model, primary normal human bronchial epithelial (NHBE) cells are cultivated in air–liquid interface (ALI) to induce differentiation to 3D pulmonary epithelium constructs (Prytherch, 2010; Rayner et al. 2019). Changes in expression of genes of interest upon exposure to indoor PM can be observed (Nordberg et al. 2020).

The aim of this study was to investigate whether the toxicological transcriptome of human airway constructs differ between exposure to indoor air PM from houses with moisture damage, as confirmed via detailed technical house inspection, and indoor PM from houses without such damages. We sequenced RNA from indoor air PM exposed airway constructs and assessed expression of 386 genes associated with toxicological responses. Transcriptome data was complemented with a spectrum of toxicological and microscopic assessments of the airway constructs, questionnaire for the occupants, and microbial and VOC determinations from air of the studied houses. We focus here on exploring associations between moisture damage categorization, gene expression and toxicological outcomes and explore correlations of respective findings with microbial levels and VOCs in the houses.

2. Methods

2.1. Assessment of moisture damages and house categorization

Houses likely to be affected by moisture damage were recruited to this study via municipal health protection officials and other collaborators of the Finnish Institute of Health and Welfare. Self-reported moisture observations as well as complaints about perceived indoor air quality and health symptoms linked to the house were recorded via occupant questionnaire and were part of initial inclusion criteria for potential index houses. Reference houses were recruited via handouts to house owners living in areas close-by index houses, as well as via email inquiries to personnel from University of Eastern Finland and Finnish Institute for Health and Welfare in Kuopio region. Altogether, 30 single family and row-houses (no apartments) located in Northern Savonia Area in Eastern Finland were recruited to the study, consisting of index houses with reported moisture damage and indoor air quality complaints, and reference houses without reported moisture problems and no complaints relating to indoor air quality.

To assess the actual damage status, all index and reference houses were inspected by trained civil engineers (Renovatek Oy, Tampere, Finland) using standardized inspection protocols and questionnaires. The information on building characteristics such as construction materials and heating, ventilation, and air conditioning (HVAC) systems as well as renovation activities were recorded during the inspections, with input from building occupants where needed. House assessments included various inspection methods (Table 1) with focus on investigating building structures and assessment of moisture risks within the building constructions.

The occupant exposure to moisture damage was assessed by applying categorization developed for workplaces by Finnish Institute of Occupational Health (Tähtinen et al. 2018), modified for use in residential environments. The categorization included the severity and extent of moisture damage and indoor air connections/flow between damaged areas and living spaces and is an expression of the probability and extent of exposure to moisture damage related indoor air impurities. Based on the detection of moisture damages and probability of human exposure, houses were grouped into four categories (Table 2).

From the original number of 30 houses that were recruited and inspected, we removed houses where renovations had already been initiated, houses that were neither considered good index or reference houses (i.e. unclear exposure status), and houses that did not have matching index or reference pairs. Houses were grouped into the four moisture damage exposure categories as follows: exposure to moisture damage unlikely (n = 7), possible (n = 5), likely (n = 10) and highly likely (n = 3). In this groupwise comparison, all houses where the probability of exposure to moisture damages was likely or highly likely (n = 13) were grouped together as index houses and compared to reference groups where the exposure to moisture damage was unlikely (n = 7) or possible (n = 5). In a second comparison approach using the same houses, nine index houses (probability of exposure to moisture damage likely or highly likely) were paired with nine reference houses (probability of exposure unlikely or possible). These index/reference pairs had similar construction type, house age, floor area, number of

Table 1
Overview of inspection methods used in the technical building assessments.

Inspection methods*	· Review of house blueprints where available · Inspection of structures with focus on moisture stress and moisture risks · General visual observation, documentation, and photographs · Moisture mapping of the house constructions with anticipated high moisture risk using dielectric surface moisture indicator · Structure moisture measurements inside wall assemblies using humidity and temperature probes · Examining the house constructions including opening of structures where necessary, based on the inspection and health inspection reports (opening of structures had to be carefully considered and was often not permitted in the reference houses) · Study of the air leakage routes (from structure to indoor air) by visual observation, smoke pen and thermal imaging · Determination of the pressure difference over the house envelope of the spaces to be examined (instantaneous measurement with ventilation at standard setting and maximum setting) · Sampling of house materials for microbial analysis to confirm or exclude microbial growth, when considered necessary
Assessed structures	· Soil surface/inclination near the house, soil material, underdrainage, etc. · Foundations, foundation walls, ground floor · Cellar (where applicable) · External walls, facades · Windows · Indoor spaces, -surfaces · Wet room spaces, -surfaces · Attic floor · Roof · Ventilation routes (no specific HVAC-examination)

* Following national guidance from Pitkäranta 2016 and The Ministry of Social Affairs and Health, 2003.

Table 2
Categories for probability of occupant exposure to indoor air impurities related to moisture damages in residential house and their main criteria (adopted and modified from Tähtinen et al. 2018).

Categories	Main criteria for probability of exposure to moisture damage -associated indoor air impurities
Unlikely	No moisture or mold damage detected in the structures. No or little airflow through the building envelope. No risk structures identified. No abnormal smell observed in the living spaces.
Possible	One of the following criteria is true: • single isolated moisture damage observed • the structures include identified risk structures • a few or single air leaks through the building envelope • there are frequent routes between main floor living spaces and the riskier spaces such as e.g. cellar • the availability of fresh air through ducts or vents to the main living spaces is considered limited
Likely	One of the following criteria is true: • widespread moisture damage over a single structure/space OR moisture damage in multiple structures/spaces • the structures include identified risk structures AND single isolated moisture damage is observed • air leaks through the building envelope are observed AND single isolated moisture damage is observed • abnormal odors, such as mold odor, are observed in the indoor air AND there are frequent routes between main floor living spaces and the damaged spaces
Very likely	More than one of the following criteria is true: • widespread moisture damage over a single structure/space OR moisture damage in multiple structures/spaces • the structures include identified risk structures and air leaks through the building envelope are obvious • abnormal odors, such as mold odor, are observed in the indoor air AND there are frequent routes between main floor living spaces and the damaged spaces

occupants and pets, and geographical location to enable pairwise comparison of the results.

2.2. Sampling and sample preparation

2.2.1. Sampling indoor air particulate matter for toxicological studies

Indoor air particulate matter (PM) was sampled during winter seasons (largely December-March, with snow-cover on the ground) in 2016–2018 using NIOSH BC251 bio aerosol cyclone sampler (National Institute for Occupational Safety and Health, NIOSH, Morgantown, WV, USA) for 7 days intermittently 12 h per day (total sampling time ~ 84 h during daytime, flow rate ~ 10 L/min). Samples were collected in the living rooms and stored in −20 °C prior to the exposure experiments. Sampled air volume was calculated based on exact sampling duration and the average of start and end flow rate.

2.2.2. Preparation of indoor air particulate matter samples

Particulate matter sampled with the NIOSH sampler 1st stage (50% cut-off particle size 2.1 μm) and 2nd stage (collected particle size fraction 0.41–2.1 μm) tubes were combined with 2 mL of synthetic lung lining fluid (LLF) (Nordberg et al. 2020). Samples were mixed by using a vortex thoroughly for 30 s, sonicated for 15 min, and mixed again thoroughly by using a vortex for 30 s. Series of dilutions were prepared by mixing sample suspensions with LLF. First, the indoor air PM was suspended in 2 mL of LLF (dilution 1:2 of collected PM) and further diluted to three dilutions (1:16, 1:8, and 1:4) of collected PM for the exposure experiments. The justification for used dilutions is discussed more in detail in our previous study (Nordberg et al. 2020).

2.3. Culturing human airway constructs

Human airway constructs were differentiated from normal human bronchial epithelial (NHBE) cells (cat. CC-2540, lot. 0000580582, Lonza©, Walkersville, MD, USA) in four different sets during the spring 2018. The cells were isolated from a non-smoking Hispanic 57-year-old male. The NHBE cells were defrosted and seeded > 3500 cells/cm^2 into two T75 (75 cm^2) cell culture flasks containing LifeFactors® BronchiaLife™ tracheal epithelial cell culture medium (cat. LS-1047, lot. 06604) including Gentamicin-Amphotericin B (cat. LS-1104, lot. 06525), TM1 (cat. LS-1055, lot. 06343), HLL Supplement (cat. LS-1001, lot. 06459, L-Glutamine (cat. LS-1031, lot. 06531) and Extract-P (cat. LS-1037, lot. 06497) (Lifeline® Cell Technology, Troisdorf, Germany). The cells were incubated + 37 °C, 5% CO_2 for four days.

Transparent ThinCert™ cell culture inserts (pore size 0.4 μm) for 24-well plates (cat. 662 641, lot. 17 24 01 16, Greiner Bio-One®, Kremsmünster, Austria) were treated with 30 μg/mL rat tail collagen type I (cat. 354236, lot. 6116001, Corning, Massachusetts, USA) (i.e. 3 μg collagen per cell culture insert) for 45 min prior cell seeding. Cells were detached with 0.05% trypsin/EDTA solution (cat. CC-5012, lot. 605021, Clonetics® Lonza) and neutralized using trypsin neutralizing solution, TNS (cat. CC-5002, lot. 593714, Clonetics® Lonza). Cells were seeded ~ 140 000 cells/cm^2 to cell culture inserts and cultured for three days. Culture medium was changed daily.

Cell cultures were transferred to air–liquid interface (ALI) in HBTEC Air-Liquid Interface Differentiation Medium (cat. LM-0050, lot. 06246) including antimicrobial supplement Gentamicin 30 mg/mL, Amphotericin B 15 μg/mL (cat. LS-1104, lot. 06098) (Lifeline® Cell Technology) and cultured in ALI for 22 days before the exposure. The differentiation medium was changed every other day. The differentiation of the airway constructs was monitored by measuring transepithelial electrical resistance (TEER) using EndOhm chamber and EVOM2™ resistance meter (World Precision Instruments, Florida, USA) and by evaluating the cultures with light microscope.

2.4. Exposing human airway constructs to indoor air particulate matter

Before the exposure, the constructs with inadequate epithelial resistance (≤336 Ohm × cm^2) were discarded. The airway constructs were exposed to three dilutions (1:16, 1:8, 1:4) of collected indoor PM in air liquid interface (+37 °C, 5% CO_2, exposure time 24 h). The indoor PM samples were pre-warmed up to + 37 °C and mixed thoroughly for 30 s before applying 50 μL of each sample to the constructs. The exposure experiments were conducted in four batches during four months. Paired index and reference houses were always included in the same exposure set. Negative controls included controls that were exposed to only air–liquid interface and controls exposed to LLF.

2.5. General toxicological markers and microscopy of exposed human airway constructs

After exposure, transepithelial electrical resistance (TEER) of the airway constructs was measured. In addition, chemokine interleukin-8 (IL-8) was analyzed from the cell culture medium on the basal side of the constructs using Human IL-8 DuoSet cytokine kit (cat. DY208 and lot. P142084, R&D Systems, Minneapolis, MN, USA) and Victor^3 ™ multi label plate reader (model 1420–051, PerkinElmer, Waltham, MA, USA) at 570 nm. Furthermore, the protein concentration from the apical wash was analyzed using Bradford Reagent (cat. B6916, lot. SLBS2204V, Sigma-Aldrich), 2 mg/mL Protein Standard (cat. P0834, lot. not known, Sigma-Aldrich), and Victor^3 plate reader at 570 nm.

For microscopic imaging, 24 human airway constructs were fixed overnight (2.5 % glutaraldehyde, 0.1 mol/L phosphate buffer, pH 7.4). Sample preparations, transmission (TEM) and scanning electron microscopy (SEM) imaging was done in imaging unit SIB Labs (University of Eastern Finland, Kuopio, Finland). The procedure for TEM is described in our earlier study (Nordberg et al. 2020). For SEM imaging, exposed airway constructs were fixed (2.5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.4)) for two hours. After fixing, constructs were washed for ten minutes twice with buffer. Samples were gradually dehydrated in ethanol for five minutes in each concentration and dried

in hexamethyldisilazane (HMDS) twice for five minutes. Samples were attached to the stubs and gold-coated (~50 nm) for 90 s. SEM imaging was done using a field emission (Schottky type) Zeiss Σigma HD|VP scanning electron microscope (Zeiss, Cambridge, UK).

2.6. Gene expression profiling by next-generation sequencing (NGS), data processing and analysis

RNA was extracted from 432 human airway constructs using Qiagen RNeasy Plus Mini Kit (cat. 74136, lot. 160012202, Qiagen, Hilden, Germany) including DNase treatment. RNA samples were stored −70 °C and sent to Sequencing Unit of Finnish Institute for Molecular Medicine (FIMM, Helsinki, Finland) for further RNA quality control and sequencing.

RNA quality assessment, library preparation for sequencing, sequencing of RNA amplicons, and data analysis were done according to manufactureŕs instructions as described in our earlier study (Nordberg et al. 2020). Shortly, the quality and integrity of RNA samples was verified by Caliber GX RNA LabChip (PerkinElmer) and Qubit RNA BR system (Thermo Fischer Scientific, MA, USA). QIAseq Targeted RNA panel Human Molecular Toxicology Transcriptome (Qiagen) of 386 genes was used for first strand synthesis, molecular barcoding, gene-specific amplification, sample indexing, and library preparation for targeted RNA sequencing according to manufactureŕs instructions. Sequencing was done using Illumina HiSeq2000 platform by Sequencing Unit core facility at Finnish Institute for Molecular Medicine (FIMM) Technology Centre, HiLIFE, University of Helsinki supported by Biocenter Finland for RNA sequencing. All the samples were pooled in one lane of HiSeq flow cell with capacity of 238 million 100 bp reads.

The data was analyzed in GeneGlobe Data Analysis Center. In brief, after uploading the RNAseq data in fastq format the data was trimmed according to manufactureŕs instructions and aligned to GRCh38 reference genome using STAR. After processing the alignments, unique molecular identifiers (UMIs) were counted for 386 genes including 6 genomic DNA controls and 10 reference genes. For further data analysis, raw UMI counts were downloaded and gene annotations were combined with sample annotations in R (version 3.6.1) (R Core Team, 2017) using SummarizedExperiment (version 1.16.1). For uses other than differential gene expression (DEG) analysis, read counts were normalized using R function DESeq2::varianceStabilizingTransformation in "blind" mode (v1.26.1) (Love et al. 2014). To identify technical bias, quality control and exploration were performed (multidimensional scaling, principal component analysis, and unsupervised hierarchical clustering) in R/Bioconductor (R Core Team, 2017; Huber et al. 2015); individual outliers were not detected, but bias assignable to sample preparation batch was identified and adjusted for in statistical analysis (see below).

Statistically differentially expressed genes (DEGs) were identified using R package DESeq2 (v1.26.1), using Wald as test type, FDR for *p*-value adjustment and the R function DESeq2::lfcShrink (type-="normal") for shrinking fold changes of low expressed RNAs. In the first scenario the nine house pairs were tested separately per each of the three dilutions of collected indoor PM (3–4 replicates per house). In the second scenario, the houses with original definition "unlikely" were set as "no moisture" group (n = 7 houses) and "likely" and "highly likely" combined to "moisture" group (n = 13 houses) and tested between the groups by adjusting for the sample preparation batch identified during quality control. Pairwise test results were visualized as a Circos plot using R package circlize (v0.4.11) (Gu et al. 2014).

2.7. Microbial determinations from indoor air

Air samples for DNA based microbial determinations were collected in parallel with collection of indoor PM for toxicological analyses (section 2.2.1) in the houses' living rooms. Sample collection took place during daytime hours (typically 12 h between 8 a.m. to 8p.m.) over seven consecutive days using the Button Inhalable Aerosol sampler (SKC, Eighty Four, PA, USA; cat. no. 225–360; Kalatoor et al. 1995) operated at a flow rate of 4 L/min. 7 days integrated samples (á 12 h, total approx. 84 h) were collected onto polycarbonate membrane filters (Millipore, Billerica, MA, USA; pore size 0.45 μm). Filters were stored at −20 °C until DNA extraction. Temperature (T), relative humidity (RH), and CO_2 were monitored in the study houses over this same one-week period with a ClimaBox 3 device (Hanwell Solutions Ltd, UK; model RL5406) in 5-minute logging interval.

For DNA extraction, the filters were placed into sterile 2-mL Eppendorf tubes with sterile glass beads and cell lysis was initiated with a bead-milling step for mechanical cell disruption (Haugland et al. 2002) in lysis buffer of Chemagic DNA Plant kit (cat. no. CMG-194, lot N-0112098; PerkinElmer Chemagen Technologie GmbH, Germany), using MiniBeadbeater-16 for 1 min (cat. no. 607EUR; Biospec Products Inc., USA). Manufacturer's instructions were followed in subsequent DNA extraction and clean up using the Chemagic DNA Plant kit on a KingFisher mL DNA extraction robot (701–989; Thermo Scientific, Finland). As an internal standard, 0.64 μg of deoxyribonucleic acid sodium salt from salmon testes (cat. no. D1626, lot. SLBF9870V; Sigma Aldrich Co., USA) (Haugland et al. 2005) was added to the samples prior to extraction. Internal standard was used to correct for the presence of inhibitors and to assess the performance of the DNA extraction. DNA was stored at −20 °C until subsequent analysis. Negative (reagents) and positive controls as well as blank polycarbonate filters were included in the DNA extraction along with air samples. Quantitative PCR (qPCR) was based on previously published qPCR assays for quantitation of following target microbial groups: Gram-positive and Gram-negative bacteria (Kärkkäinen et al. 2010); *Streptomyces spp.* group (Rintala and Nevalainen, 2006); group of *Penicillium* spp., *Aspergillus* spp., and *Paecilomyces variotii,* and total fungal DNA (Haugland et al. 2004 and 2002) and internal standard salmon testis DNA (Haugland et al. 2005). QPCR reactions were performed as written in the original publications with minor modifications detailed in Hyytiäinen et al. (2018). Reactions were performed in 0.2 mL 96-well plates (cat. no. 401334; Agilent Technologies Inc., CA, USA) on Stratagene Mx3005P qPCR System (Agilent Technologies Inc., CA, USA). Positive, negative, and no-template controls were included in the qPCR runs. Relative quantification method was used to calculate numbers of cell equivalents (CE) in the samples (Haugland et al. 2004). Results were blank corrected and normalized for sampled air volume, to be finally expressed as CE/m^3.

2.8. Volatile organic compounds

Volatile Organic Compounds (VOCs) were sampled by means of Radiello passive samplers (Radiello^R, RAD120) based active charcoal adsorbent (RAD130), to determine time-weighted average air concentrations. VOC sampling was performed in the living room of the house during a period of seven days. VOC sampling cartridges were stored in the respective glass containers post sampling at + 4 °C in the dark until analysis at the collaborator VITO in Belgium. Field and laboratory blanks were included in the sample processing and analysis. Prior to the analysis, the passive samplers were desorbed using carbon disulfide (CS_2). The resulting extracts were analyzed by means of gas chromatography coupled mass spectrometry (GC–MS), using 2-fluorotoluene as internal standard. MS detection was based on simultaneous full scan and selected ion monitoring (SIM). Based on the total peak surface of the total ion chromatogram from the full scan (TIC), and using the response of the internal standard, the total load of volatile organic compounds (TVOC) was calculated.

2.9. Symptom questionnaires

The occupants of the houses filled questionnaires related to their health status, symptoms, and perceived indoor air quality in their house, using previously developed questionnaires (Järvi et al., 2018; Reijula et al., 2004). Questionnaires addressed adults, school-aged children, and

smaller children separately. The questionnaire included a section on respiratory and general symptoms and their frequency of occurrence during the past 12 months (daily, weekly, or rarely/never) The symptoms were divided into four groups (Fig. 1) and symptom scores were calculated for upper and lower respiratory symptoms and general symptoms, using dichotomized variables as described in detail in Järvi et al. (2018).

2.10. Statistical analyses

Statistical analyses and figures were done using Graph Pad Prism 8.4.3 (GraphPad Software). The normality of each data set was tested with Anderson-Darling (Stephens, 1974), D'Agostino & Pearson (D'Agostino et al. 1990), Shapiro-Wilk (Srivastava & Hui, 1987) and Kolmogorov-Smirnov (Lilliefors, 1967) tests. For comparing TEER, protein and IL-8 secretion of human airway constructs exposed to indoor PM, two-way ANOVA and Bonferronís multiple comparisoń s test, $p < 0.05$, were used. For analyzing correlation between toxicological markers, gene expression and microbial factors, Spearman correlation test, $p < 0.05$, was used. For assessing correlation between the microbial factors, toxicological markers (i.e. TEER, protein, IL-8), and gene expression within nine house pairs, the value of the reference house was subtracted from the value of the index house for all these determinants.

House characteristics, symptom and microbial data were analyzed in SAS 9.3 (SAS Institute, Cary NC, USA). Differences in levels of microbial and other exposure measurement variables between groups (likely and very likely, possible, unlikely) were tested using Wilcoxon Scores (Rank Sums) and Kruskal Wallis statistical test; pairwise comparisons were performed using Wilcoxon signed-rank test, all using significance level of $p < 0.05$. Building data as well as occupant reports of perceived indoor air quality and symptoms were analyzed using Chi-square or Fisher's exact test. Symptom scores were created as previously described in Järvi et al. 2018 in detail, including sums and frequencies of individual reported symptoms as follows: upper respiratory symptom score (0–14; based on daily/weekly/rarely or never report of stuffy nose, sore throat, rhinitis, hoarseness, night cough, dry cough, phlegm); lower respiratory symptom score (0–6; based on report of wheezing cough, wheezing, shortness of breath); and general symptom score (0–20; based on reports of muscle pain, back pain, pain in joints, urinary problems, eye symptoms, stomach ache, headache, tiredness, sleeping difficulties, concentration difficulties).

3. Results

3.1. Study houses and occupants

Basic descriptive statistics of the study houses, including key building characteristics, are presented in Table A.1, separated for three moisture damage exposure groups "unlikely", "possible", "likely or highly likely". Buildings were detached or semi-detached houses with one exception (row house), were of comparable age and size between groups, and most often built with wooden frame and slab on ground foundation. Predominant ventilation type was natural ventilation, and heating was mostly achieved with electricity and/or wood/pellets. Buildings of the "unlikely" category had somewhat more often a basement and a fireplace; smoking indoors was not reported for any of the study houses. Similarly, basic descriptive of the study population with focus on symptom reports and perceived indoor air quality are presented in Table A.2. Upper, lower, and general symptoms were significantly more often reported by occupants likely or very likely exposed to moisture damage, as to be expected per the design and inclusion criteria of this study. Occupants of damaged houses reported also moldy odor and noise annoyance more frequently (borderline non-significant). Descriptives of the microbial and VOC levels determined from air samples collected in the houses are presented in Table A.3. We did not observe any statistically significant differences in microbial and VOC measurements between exposure categories. Correlations between gene expression and microbial components/VOC levels are presented more in detail in Table A.4 (Excel -file).

3.2. Microscopic evaluation of the airway constructs

Based on transmission and scanning electron microscopy images carried out prior to the exposure experiments, the airway constructs produced for the toxicological and gene expression experiments were uniformly formed, but not fully differentiated to pseudostratified structure. Nevertheless, we did observe multiple structures characteristic of differentiated human airway epithelium, such as 500 nm long microvilli (Figure B.1.A), tight junctions, adherens junctions, and desmosomes (Figure B.1.B). Scanning electron microscopy images showed respiratory epithelium with tightly joined different types of cells (Figure B.2.A). One house was selected to these microscopic analyses as an example based on its' categorization in "highly likely" group. After the exposure to indoor PM collected from this house, we observed multiple unidentified particles on top of the culture (Figure B.2.B-D).

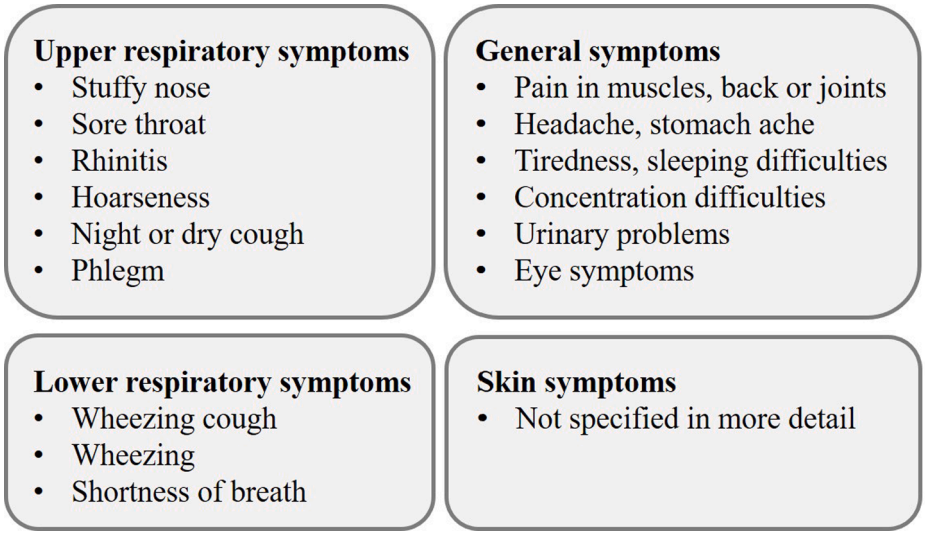

Fig. 1. The occupants of the studied houses filled in questionnaires regarding their health. The symptoms were categorized to four groups: upper and lower respiratory symptoms, general, and skin symptoms.

3.3. Effect of indoor PM exposure on toxicological markers

We analyzed multiple toxicological markers including protein secretion from the apical wash, tissue transepithelial resistance (TEER), and interleukin-8 (IL-8) secretion from the cell culture medium after exposure to indoor PM from houses in different exposure categories.

3.3.1. Pairwise index/reference house comparison

Neither resistance of the tissue, nor concentration of IL-8 in the culture media or protein levels in the apical wash were consistently different between index and reference houses within the nine index/reference house pairs. Only a few statistically significant differences were demonstrated; increase in IL-8 level at 1:4 dilution of collected indoor PM in Pair 5 (Padj 0.0328) and at dilution 1:8 of collected indoor PM (Padj < 0.0001) and at dilution 1:4 of collected indoor PM (Padj < 0.0001) in Pair 7 (Fig. 2).

In addition, decrease in IL-8 level at dilution 1:4 of collected indoor PM was demonstrated in Pairs 2 (Padj $= 0.0007$) and 6 (Padj $= 0.0071$) (Fig. 2) and decrease in TEER levels was demonstrated at dilution 1:16 of collected indoor PM in Pairs 3 (Padj $= 0.0110$) and 9 (Padj $= 0.0267$) (Fig. 3).

However, we did observe a non-significant trend for dilution of collected indoor PM and TEER (i.e. TEER of cell layer decreasing when exposed to less diluted i.e. higher dose of indoor PM) (Fig. 3), IL-8 secretion (i.e. IL-8 concentration increasing in the cell culture medium when exposed to less diluted i.e. higher dose of indoor PM) (Fig. 2) and possibly an U-shaped trend for protein secretion (i.e. protein concentration in apical wash being higher when exposed to dilutions 1:16 and 1:4 of collected indoor PM than when exposed to dilution 1:8 of collected indoor PM) (Fig. 4).

3.3.2. Groupwise house comparison

Protein levels in the apical wash were not significantly different between the groups of houses categorized based on the probability of exposure to moisture-damage (Fig. 5A).

There were some statistically significant differences in cell layer resistance and chemokine IL-8 concentration in cell culture medium between the three house categories which are presented in Fig. 5B-C (two-way ANOVA, Bonferronis multiple comparison's test, $p < 0.05$, CI = 95 %). In each of the categories, less diluted indoor air PM-samples (i.e. higher indoor PM dose) tended to induce higher production of IL-8 (Fig. 5C), whereas with cell layer resistance values the response to less diluted (i.e. higher dose) indoor PM-samples was more likely to be negative (Fig. 5B). Including the houses with possible exposure in the reference group did not bring forth any significant differences.

3.4. Effect of indoor PM exposure on gene expression

3.4.1. Pairwise index/reference house comparison

When comparing the gene expression induced by indoor air PM from nine index/reference house pairs in a set of 386 genes (QIAseq Targeted RNA Human Toxicology Transcriptome Panel), we identified statistically significant difference in expression of 165 out of 386 genes that were assessed (Fig. 6) (padj < 0.01).

The highest number of genes was differentially expressed in gene groups linking to mitochondrial energy metabolism, endoplasmic reticulum stress & unfolded protein response, apoptosis, fatty acid metabolism and phospholipidosis -pathways (Fig. 6). However, the

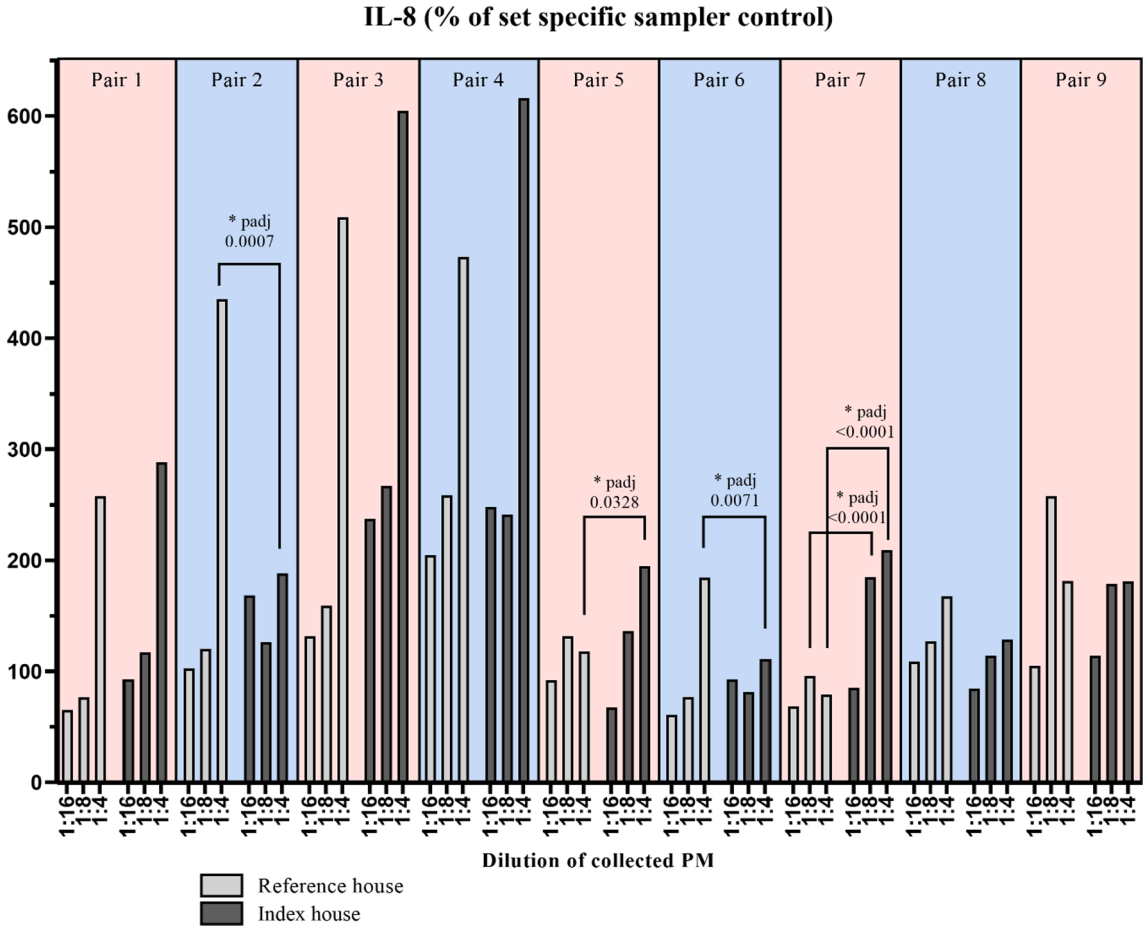

Fig. 2. Chemokine Interleukin 8 (IL-8) values in cell culture medium of human airway constructs exposed to three dilutions (1:16, 1:8 and 1:4) of indoor air particulate matter (PM) sampled from nine index (moisture-damaged)-reference (non-moisture-damaged) house pairs.

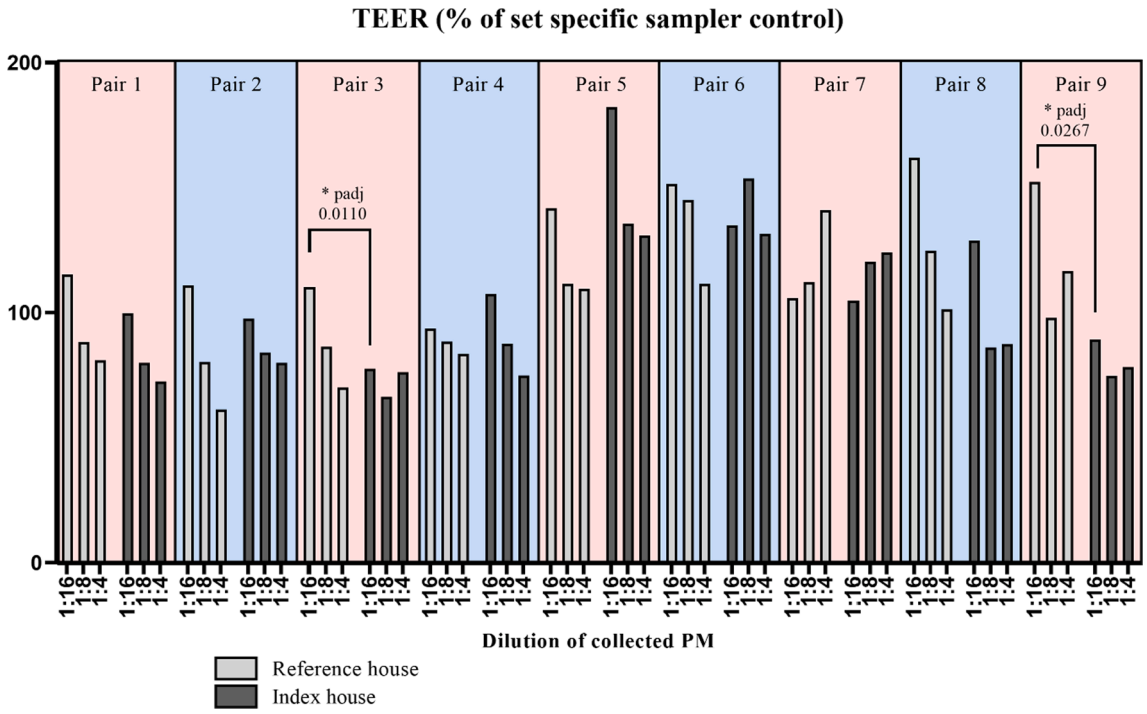

Fig. 3. Tissue electric resistance (TEER) values of human airway constructs exposed to three dilutions (1:16, 1:8 and 1:4) of indoor air particulate matter (PM) sampled from nine index (moisture-damaged)-reference (non-moisture-damaged) house pairs.

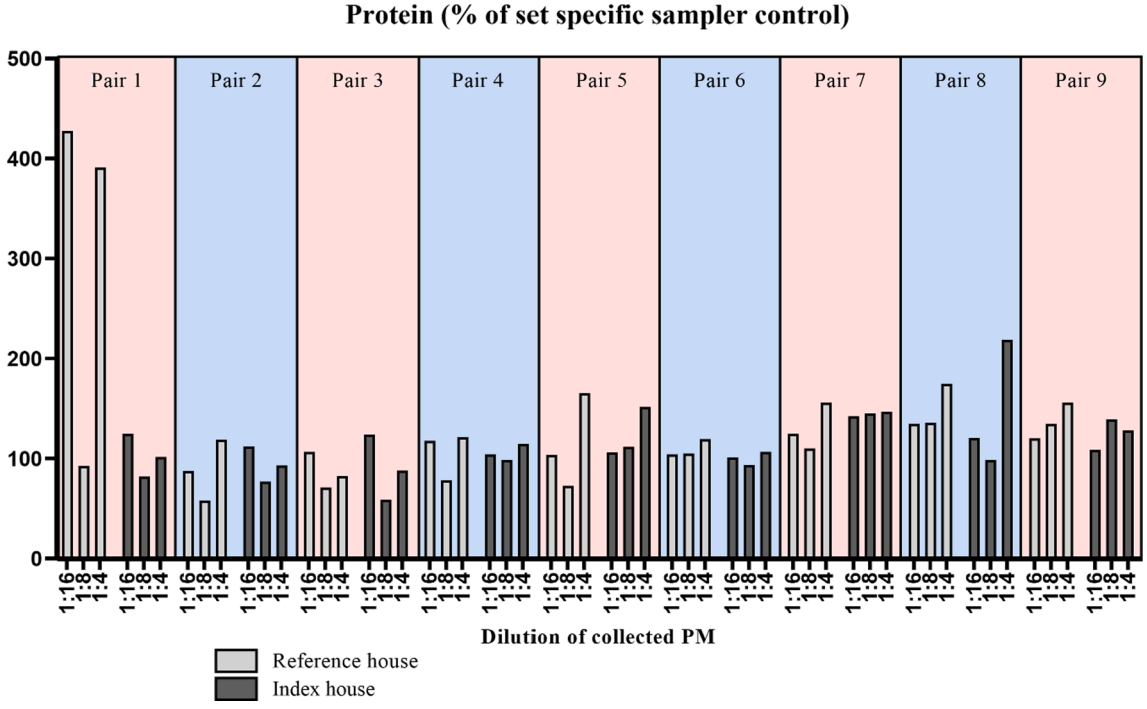

Fig. 4. Protein concentration in apical wash of human airway constructs exposed to three dilutions (1:16, 1:8 and 1:4) of indoor air particulate matter (PM) sampled from nine index (moisture-damaged)-reference (non-moisture-damaged) house pairs.

expression was not systematically differential across all house pairs for any of the 165 genes. Most over-expressed (i.e. log2FC > 1.0, padj < 0.01) and under-expressed (i.e. log2FC < -1.0, padj < 0.01) genes are presented in Table 3.

3.4.2. *Groupwise house comparison*

In addition to pairwise comparisons, index houses (exposure to moisture damage likely or very likely; n = 13) were compared to reference houses (moisture damage exposure unlikely; n = 7) in groupwise comparisons. In this comparison, Cytochrome P450 1A1 (CYP1A1) was statistically significantly over-expressed in index houses at all dilutions of collected PM (i.e. dilution 1:16 of collected PM: log2FC = 0.007, padj = 0.03; dilution 1:8 of collected PM: log2FC = 0.077, padj = 0.018; dilution 1:4 of collected PM: log2FC = 0.270, padj

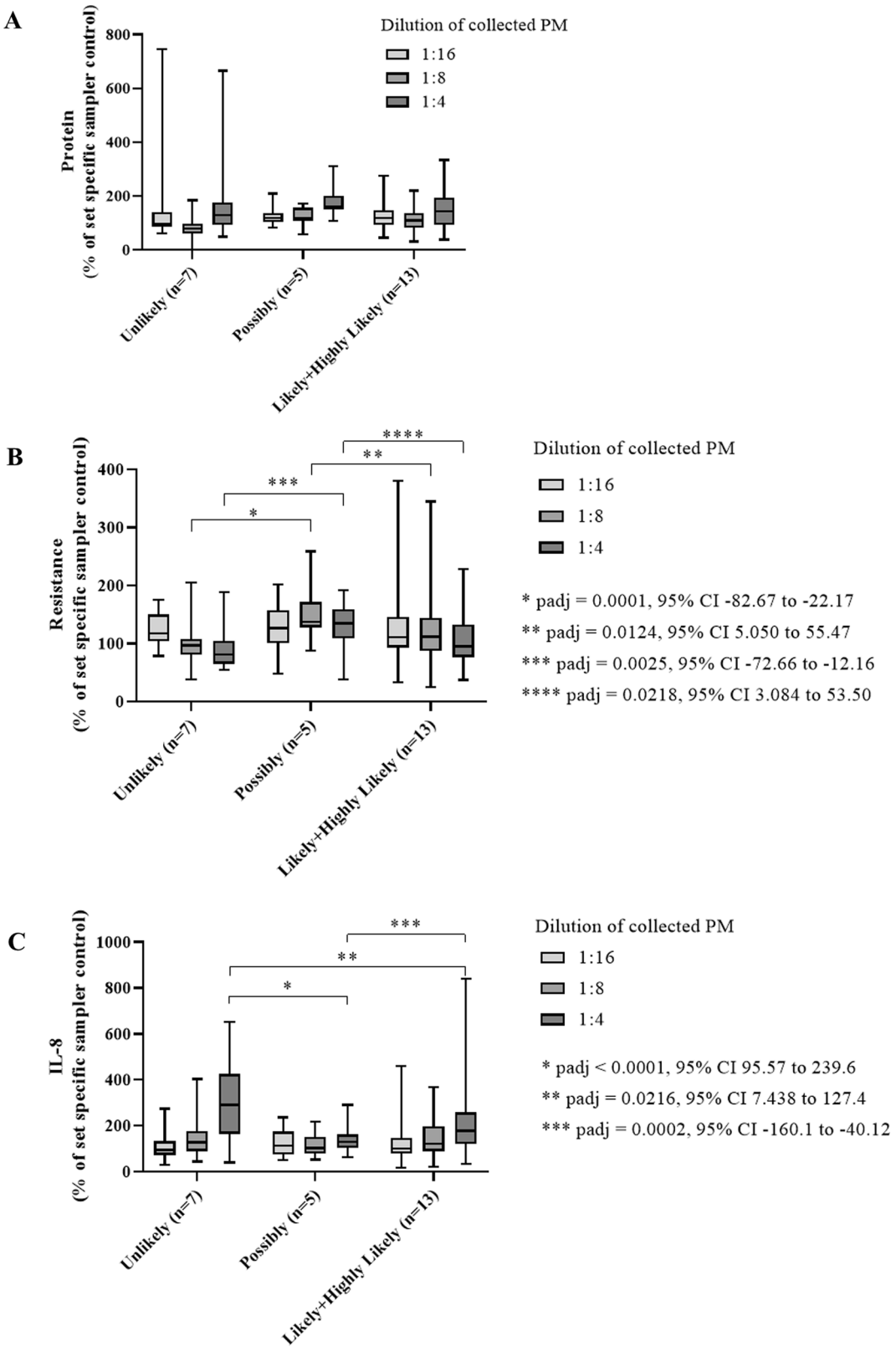

Fig. 5. A) Protein concentration in the apical wash, B) tissue transepithelial electrical resistance (TEER), and C) interleukin-8 (IL-8) concentration in cell culture medium (percentage compared to the set-specific sampler controls) in three house categorization groups (i.e. unlikely, possibly and likely + highly likely) after exposed to three dilutions (1:16, 1:8, 1:4) of collected indoor PM. Box plots represent 25th median and 75th percentile and whiskers represent min–max values. Each sample was prepared in quadruplets. Normality was tested by Shapiro-Wilk, $p < 0.05$ and statistical significance was determined by using two-way ANOVA and Bonferronís multiple comparison's test, $p < 0.05$, CI 95%.

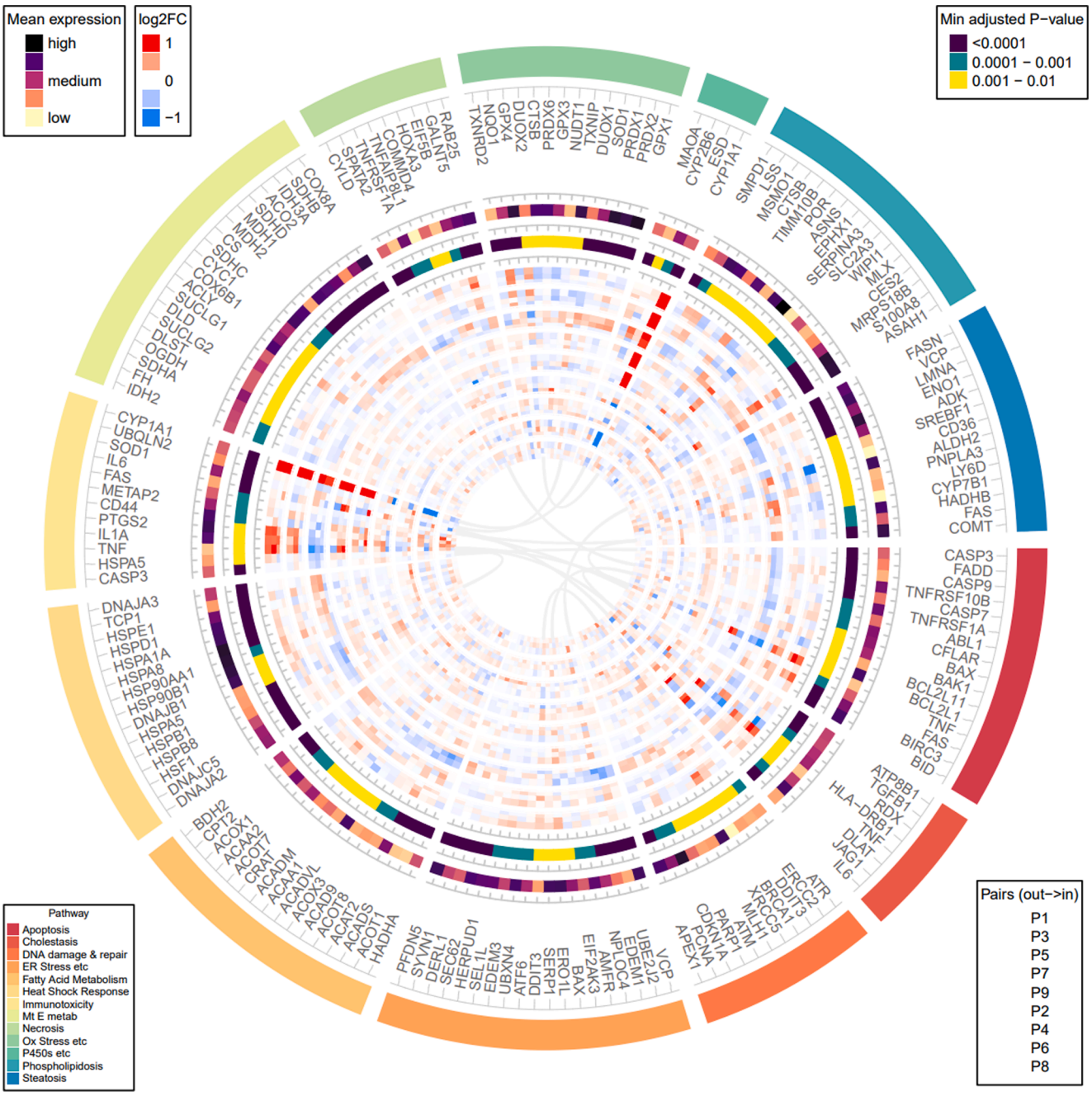

Fig. 6. Circos plot of differentially expressed genes of human airway constructs after exposure to three dilutions (1:16, 1:8, 1:4) of indoor air PM from nine matched index/reference house pairs. The gene was included in the plot if the pairwise test showed high statistical significance (padj < 0.01) for any dilution of collected indoor PM in any pair. The outmost color band indicates the toxicology-related pathways for the genes. Inward from the gene names, the first colored band indicates the mean expression level across all samples for the gene, categorized as five quantiles as indicated. Next, the most extreme adjusted p-value for each gene across all comparisons is indicated using three categories. The nine innermost band sets, colored in blue-white-red scale, indicate the log2FC for each pairwise comparison; within each band set, the three individual bands are for the three dilutions of collected indoor PM, ordered inward by more diluted (decreasing dose) indoor PM samples. The arcs at the center indicate genes that are duplicated in the plot due to their association in more than one pathway.

= 0.002) and Nuclear factor NF-kappa-B p105 subunit (NFKB1) at the dilution 1:4 of collected PM (log2FC = 0.124, padj = 0.029).

3.4.3. *Correlations between expression of selected genes and levels of microbes and TVOCs*

Seven genes that were highlighted as statistically significant in pairwise comparisons (Table 3), were correlated with different microbial groups and total volatile organic compound (TVOC) levels determined from air samples in these houses (Fig. 7).

CYP1A1 correlated positively with total fungi at dilutions 1:4 and 1:8 of collected PM (dilution 1:4 of collected PM: Spearman R = 0.44, Spearman p = 0.03 and dilution 1:8 of collected PM: Spearman R = 0.45, Spearman p = 0.03). TNF correlated positively with *Streptomyces* at dilution 1:8 of collected PM (Spearman R = 0.55, Spearman p = 0.006). IL1A correlated positively with *Streptomyces* at dilution 1:8 of collected PM (Spearman R = 0.53, Spearman p = 0.009). IL6 correlated negatively both with *Penicillium/Aspergillus* (Spearman R = -0.52, Spearman p = 0.010) and total fungi (Spearman R = -0.47, Spearman p = 0.023) at dilution 1:16 of collected PM. HLA-DRB1 correlated negatively with *Grampositive* bacteria at dilution 1:4 of collected PM (Spearman R =

Table 3
Most over- and under-expressed genes in pairwise comparisons of index and reference houses. Differentially expressed (adjusted p-value < 0.01) genes with fold change of >1 (over-expression, marked with orange) or less than −1 (under-expression, marked with blue) are presented along with the gene function category as described in Qiagen's webpages for QIAseq Targeted RNA Human Molecular Toxicology Transcriptome Panel (Qiagen, 2021). Fold change = log2 of the index/reference ratio; CYP1A1 = Cytochrome P450 1A1; IL-6 = Interleukin 6; TNF = Tumor necrosis factor; IL1A = Interleukin 1-alpha; CD36 = Platelet glycoprotein 4; HLA-DRB1 = HLA class II histocompatibility antigen; DRB1 beta chain; TNFAIP8L1 = Tumor necrosis factor alpha-induced protein 8-like protein 1.

Gene	Fold Change	Adjusted p-value	Gene function category
CYP1A1	4.0	2.65×10^{-43}	Cytochrome CYP450s & Phase I Drug Metabolism, Immunotoxicity
IL-6	1.6	$4{,}51 \times 10^{-6}$	Cholestasis, Immunotoxicity
TNF	1.1	0.008	Apoptosis, Cholestasis, Immunotoxicity
IL1A	1.0	0.006	Immunotoxicity
TNFAIP8L1	−1.0	0.001	Necrosis
HLA-DRB1	−1.0	0.005	Cholestasis
CD36	−1.1	0.005	Steatosis
CYP1A1	−2.5	1.99×10^{-14}	Cytochrome CYP450s & Phase I Drug Metabolism, Immunotoxicity

-0.43, Spearman p = 0.037). No statistically significant correlations were seen between gene expression data and TVOC -levels. Correlation data is presented in more detail in Table A.4 (Excel -file).

4. Discussion

In this study, we characterized the changes in the toxicological markers and transcriptome of human airway constructs to identify potential mechanistic pathways related to exposure to moisture damaged indoor environments. Our findings reveal the plethora of responses evoked by indoor air particulate matter and highlight the variability between the exposure environments regardless of the moisture damage status. By comparing houses with high probability of moisture-damage -related problems to reference houses, we were able to identify few genes such as CYP1A1 and NFKB1 as interesting candidates for future studies, as well as get further indication of the central role of immunotoxicity and xenobiotic metabolism in the responses of the respiratory epithelium challenged with particulate matter from moisture-damaged houses.

Moisture-damage -associated indoor air problems in buildings are widely assumed to have a connection with microbe concentrations, microbial components, and metabolites of microbes (Cabral, 2010; Lappalainen et al. 2013; Nevalainen et al. 2015). Microbes are always present in indoor environments, but there are several studies suggesting that moisture damages and indoor dampness may modify the ecology and levels of the bacterial and fungal indoor microbiota (Jayaprakash et al. 2017; Hegarty et al. 2019; Sylvain et al. 2019; Adams et al. 2020; Emerson et al. 2015). It is likely that synergistic effects of multiple different exposure agents indoors, including possibly particles and chemicals in addition to microbes, rather than a single component leads to the adverse health effects, and there could be multiple mechanisms involved (Korkalainen et al. 2017; Hirvonen et al. 2005; Penttinen et al. 2006).

Health effects of moisture-damaged indoor environment and particularly the contribution of microbial exposure to human health are notoriously difficult to study due to the lack of established causal agents or even good indicators of relevant exposure, beyond observational measures such as visible mold or mold odor. The association between adverse health effects and dampness in buildings has been demonstrated consistently, but the attempts to link moisture damage, altered indoor microbiota and health endpoints have produced unclear and even contradictory findings (Borràs-Santos et al. 2013; Jacobs et al. 2014). Revealing the cell-level mechanisms behind the effects of exposure to moisture-damaged indoor environment would be groundbreaking for offering more specific diagnostic tools and for developing more accurate assays for recognizing housing conditions relevant for occupants' health, which also motivated this current research.

When comparing the indoor air of houses with highest and lowest likelihood for exposure to moisture damage in our study, we observed no significant differences between the levels of microbial markers in different exposure groups. However, when comparing the responses of respiratory epithelium to particulate matter collected from these groups of houses, genes CYP1A1 and NFKB1 did stand out. Interestingly, both CYP1A1 and NFKB1 are related to immunotoxicity, and CYP1A1 is involved also in Cytochrome P450s & Phase I drug metabolism. Out of the studied genes, the observed changes in the expression of CYP1A1 were the most striking. Irrespective of the moisture-damage status, the exposure to indoor PM increased the expression of CYP1A1 dose-dependently in 15 out of 18 houses.

Our results strongly indicate variability within the moisture damage exposure in that the pairs of index and reference houses could not be unambiguously separated based on differential expression of one single gene or a specific set of genes. This is likely due to the variation in the levels of bioaerosols, volatile organic compounds or airborne PM in general both in moisture-damaged as well as non-damaged residential environments. However, comparing pairs of houses with different exposure status highlighted changes in multiple genes. These differentially expressed genes were related to Cytochrome CYP450s and phase I drug metabolism, immunotoxicity, cholestasis, apoptosis, phospholipidosis, oxidative stress & antioxidant response, steatosis, necrosis, endoplasmic reticulum stress & unfolded protein response and fatty acid metabolism according to the categorization of Qiagen (2021).

Other measured toxicological markers such as electrical resistance of the epithelium and concentration of IL-8 or proteins were not able to differentiate the groups nor pairs of houses categorized based on the probability of moisture damage. These findings support earlier studies suggesting that the moisture-damaged indoor environments cannot be identified based on any individual toxicological indicators (Mahiout et al. 2019).

To offer possible explanations for the observed key changes in gene expression we linked the data on gene expression with levels of microbial markers and volatile organic compounds in the studied houses. The microbial markers were measured from filters sampled with Button Inhalable Aerosol Sampler collected in parallel and during the same time as PM used in the exposure experiments. While the microbial and PM gene expression measurements were not done from one and the same sample, our approach ensured sufficient and ideally suited sample material for the two dedicated analyses. We found some correlations between specific exposure agents, most notably positive correlations between fungal levels and CYP1A1 as well as *Streptomyces* and inflammatory markers TNF and IL1A, along with negative correlations between fungal levels and IL-6. However, considering that there were <10 statistically significant findings among more than a hundred comparisons, and these were representing both negative and positive associations depending on the dilution of collected PM level and microbe in question, overall there was no clear indication of the response being dependent on microbial exposures. We acknowledge that studying the link between microbial exposures and gene expression would require a wider analysis of microbial communities present in the houses instead of a few selected species studied here. However, our main aim of detailed exploration of gene expression responses to moisture damage limited the number of houses included in the study, which in turn ruled out the possibility of statistically meaningful comparison of hundreds of exposure variables against a large set of candidate genes. While limited, our approach of using selected set of quantitative PCR assays enabled the use of air samples typically low in biomass, and consideration of absolute quantity of these biomarkers instead of relative abundance sequence-

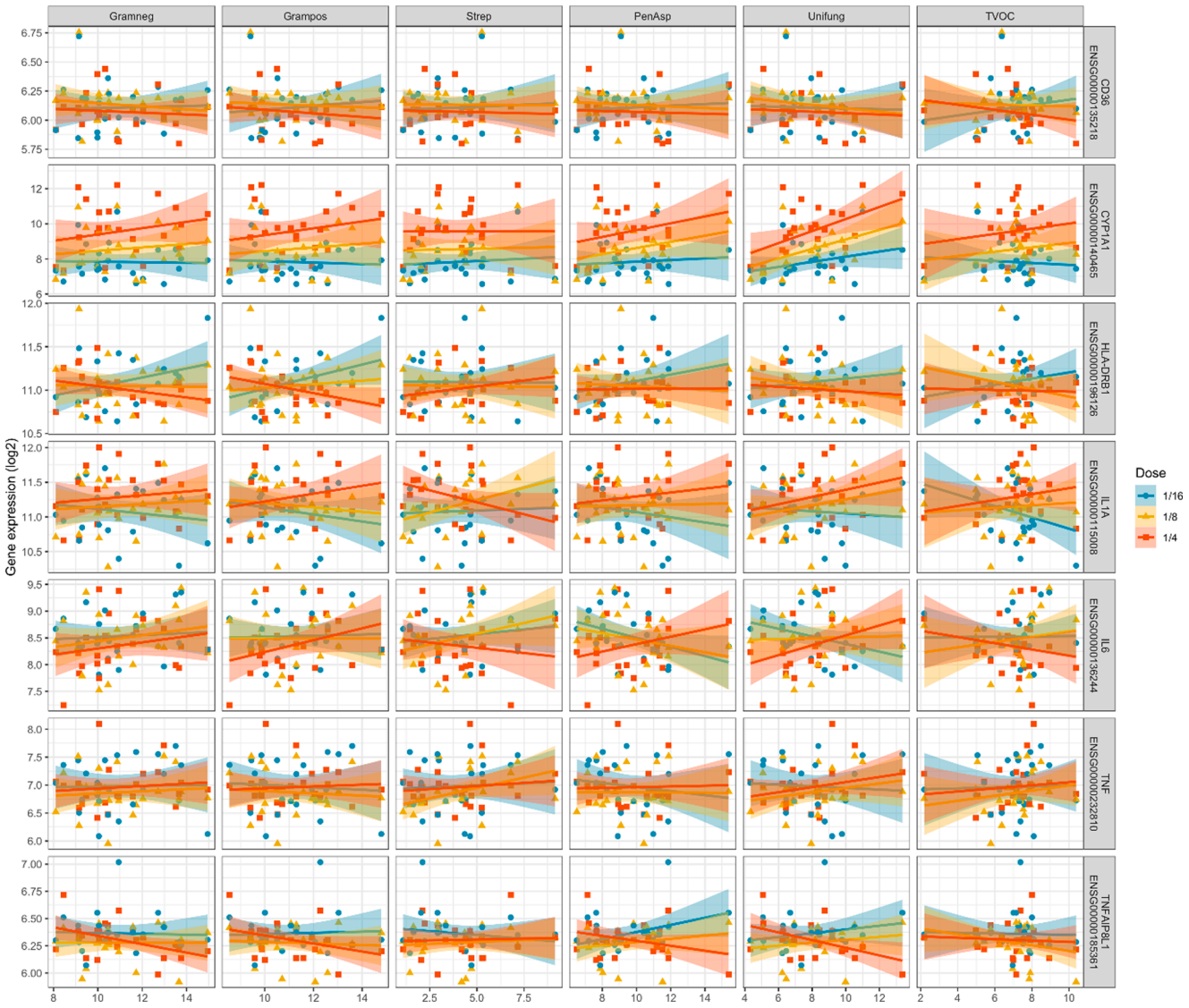

Fig. 7. Correlation of the expression of seven genes (i.e. CYP1A1 = Cytochrome P450 1A1; IL-6 = Interleukin 6; TNF = Tumor necrosis factor; IL1A = Interleukin 1-alpha; CD36 = Platelet glycoprotein 4; HLA-DRB1 = HLA class II histocompatibility antigen; DRB1 beta chain; TNFAIP8L1 = Tumor necrosis factor alpha-induced protein 8-like protein 1) and the levels of microbes and TVOCs. Abbreviations: Gramneg = Gram-negative bacteria, Grampos = Gram-positive bacteria, Strep = Streptomyces spp., PenAsp = Penicillium/Aspergillus/Paecilomyces variotii group, Unifung = total fungal DNA, TVOC = total volatile organic compounds.

based data provides. Our next steps will include a thorough characterization of exposure variables, including microbiota and secondary metabolites of these houses and exploration of their associations with the moisture damage status.

In addition to microbial exposures, also levels of VOCs have been suggested to be implicated with moisture-damage -related health effects, although the suggested effects generally occur at higher levels compared to what we see in indoor environments (Kwon et al. 2018; Baloch et al. 2020). High indoor air VOC levels do not always correlate with microbial concentrations indoors (Gallon et al. 2020; Schleibinger et al. 2008), due to the fact that there are also other significant indoor (and outdoor) sources for these compounds other than microbes. In our study, there were no statistically significant correlations between VOC-levels indoors and gene expression data, nor did the TVOC levels differentiate the moisture-damaged buildings from non-damaged ones.

In our experimental model we observed multiple characteristics familiar to human airway epithelium, and the constructs consistently presented attributes of functional respiratory epithelium such as high resistance values and presence of ciliated cells. However, we also acknowledge the limitations of our model, as the electron microscopy images indicated that the human airway constructs were not fully differentiated. Our approach using dilution series of PM in the exposure experiments instead of weighed mass has the benefit of avoiding labor intensive and imprecise weighing of the particles as well as excess handling (i.e. extraction) of the dust possibly changing the properties of the particles. The reasoning behind this exact method is further discussed in our earlier study (Nordberg et al. 2020).

We noted that the properties of the constructs were slightly different between the sets of experiments regardless of using primary cells from the same donor and the same culturing conditions. The bias from these differences was countered in group-wise testing by adjusting the statistical model for experiment sets; in pairwise testing adjustment was unnecessary because for all pairs both houses represent the same experimental set. In statistical analyses, considering the multitude of endpoints analyzed in the same dataset, the likelihood of false positive results increases. We did, however, adjust our analyses for multiple comparisons and strove to put more weight on the findings that were seen consistently in different dilution of collected PM and endpoints.

One of the strengths of our study design was the thorough categorization of the houses based on the probability of exposure to moisture

damage. We are confident that the categorization was successful, as the occupants living in houses where the probability to be exposed to moisture damages was likely or highly likely indeed reported significantly more symptoms compared to occupants living in houses where the probability was unlikely or possible.

We did not attempt to investigate correlations between moisture-damage -related gene expression and human symptoms, and this was for two reasons: one, our study design forces correlation between damage status and the symptoms because the initial inclusion criteria included indoor air complaints and symptom reports for potential index houses as well as the absence of such complaints for potential reference houses. Second, exploring associations between gene expression in our model and symptom reports in occupants of the respective study houses would require a large number of participants, whereas our study population was small and heavily limited due to the intensive inspection, sampling and analysis efforts of samples from each house. Our goal was to provide a short list of possibly relevant genes to target more specifically in follow-up studies, and by doing so we hope our study findings will indeed motivate future research to clarify associations between the markers associated with damage status and respiratory health in exposed building occupants.

5. Conclusions

In summary, our findings suggest that the exposure to indoor PM from houses in which the probability of human exposure to moisture damage is high activates especially genes related to immunotoxicity, phospholipidosis, oxidative stress & antioxidant response, steatosis, Cytochrome P450s & Phase I drug metabolism, cholestasis and fatty acid metabolism. The effect does not manifest in the same way in every moisture-damaged house, which is understandable knowing the variability in the composition of bioactive particles within indoor PM samples of different houses. In line with the previous research findings, the measured general toxicological markers did not reliably identify the houses with different levels of moisture-damage.

The follow-up of this study will include sequencing the full transcriptome of airway constructs exposed to indoor PM from four index vs. reference house pairs. Additionally, the airway construct model will be further optimized, and genes related to inflammation and immunity will be studied more thoroughly. Our novel study approach may serve as a promising tool for future research studying the health effects and toxicity related to indoor air particles.

6. Author statement

The FASTQ, raw count and vst-normalize counts matrix files of gene expression data have been deposited in Gene Expression Omnibus under accession number: GSE175878. The measured toxicological markers, microbial analyses, and health questionnaires are currently being analysed also for other, yet unpublished research articles and a doctoral thesis, but the raw data will be made available in the future from research data storage service IDA. In the meantime, individual data requests concerning the herein published data will be handled on a case-by-case basis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This study is a part of REMEDIAL consortium which is funded by Academy of Finland (grant n:o 296724 and 296587) and University of Eastern Finland (UEF). Kuopion Seudun Hengityssäätiö (Kuopio, Finland) funded this study with personal grant in 2017, 2019, and 2020.

We acknowledge William G. Lindsley and Bean T. Chen (National Institute for Occupational Safety and Health, Morgantown, West Virginia, USA) for providing us indoor particulate matter sampling device. We sincerely acknowledge engineer office Renovatek Oy (Tampere, Finland) for inspecting the houses. In addition, we thank the Sequencing Unit core facility at FIMM Technology Centre, HiLIFE, University of Helsinki supported by Biocenter Finland for RNA sequencing. Furthermore, we acknowledge SIB Labs (University of Eastern Finland, Kuopio, Finland) for processing and imaging our samples. From Finnish Institute for Health and Welfare, Environmental Health Unit, we wish to thank Kristiina Myller for heavy field work efforts, Maria Valkonen, Mervi Ojala and Heli Martikainen for sampling logistics and excellent laboratory processing and analysis of samples, as well as Asko Vepsäläinen for data management and statistical support. The computational analyses were partly performed on servers provided by UEF Bioinformatics Center, University of Eastern Finland, Finland.

Data sharing

The FASTQ, raw count and vst-normalize counts matrix files have been deposited in Gene Expression Omnibus under accession number: GSE175878.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2021.106997.

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