1 **Exposure to the heated tobacco product IQOS generates apoptosis-mediated pulmonary**
2 **emphysema in murine lungs**

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18 **Running head:** Apoptosis-related emphysema in IQOS-exposed murine lungs
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21

22 **ABSTRACT**

23 Pulmonary emphysema is predominantly caused by chronic exposure to cigarette
24 smoke (CS). Novel tobacco substitutes, such as heated tobacco products (HTPs), have emerged
25 as healthier alternatives to cigarettes. IQOS, the most popular HTP in Japan, is advertised as
26 harmless compared to conventional cigarettes. Although some studies have reported its toxicity,
27 few *in vivo* studies have been conducted. Here, twelve-week-old C57BL6/J male mice were
28 divided into three groups and exposed to air (as control), IQOS aerosol, or CS for six months.
29 After exposure, the weight gain was significantly suppressed in the IQOS and CS groups
30 compared with the control (-4.93 g; IQOS *versus* air and -5.504 g; CS *versus* air). The serum
31 cotinine level was significantly higher in the IQOS group than in the control group. The
32 neutrophils and lymphocyte counts increased in the bronchoalveolar lavage fluid of the IQOS
33 and CS groups compared with those in the control group. Chronic IQOS exposure induced
34 pulmonary emphysema similar to that observed in the CS group. Furthermore, expression
35 levels of the genes involved in the apoptosis-related pathways were significantly upregulated
36 in the lungs of the IQOS-exposed mice. Cytochrome c, cleaved caspase-3, and cleaved poly
37 (ADP-ribose) polymerase-1 were overexpressed in the IQOS group compared with the control.
38 Single-stranded DNA and TdT-mediated dUTP nick-end labeling-positive alveolar septal cell
39 count significantly increased in the IQOS group compared with the control. In conclusion,
40 chronic exposure to IQOS aerosol induces pulmonary emphysema predominantly via
41 apoptosis-related pathways. This suggests that HTPs are not completely safe tobacco products.

42

43 **Keywords:** heated tobacco product, emphysema, apoptosis

44

45 **INTRODUCTION**

46 Chronic obstructive pulmonary disease (COPD) is mainly caused by chronic cigarette
47 smoking, and its mortality is ranked third in the world (1). Pulmonary emphysema is the major
48 pathological feature of COPD (2), and tobacco is known to contain numerous toxic compounds
49 responsible for emphysema. As nicotine increases dependence on smoking, establishing
50 alternative sources of nicotine consumption with reduced exposure to toxic elements is
51 considered a possible strategy for reducing the health hazards of smokers. To this end, novel
52 tobacco alternatives, such as electric cigarettes (e-cigarettes) and heated tobacco products
53 (HTPs), have been launched. Although e-cigarettes are claimed to be less harmful than
54 combustion cigarette smoke (CS), they have been implicated as a causative agent in lipoid
55 pneumonia (3, 4), reduced immunity against infections (5), lung adenocarcinoma (6), and
56 emphysema (7).

57 HTPs are newer electronic devices that generate aerosols by heat-processing tobacco
58 instead of combusting it. IQOS, a popular HTP, was launched in Japan and Italy in 2014, and
59 is presently available in more than 50 countries (8). IQOS limits tobacco pyrolysis and
60 combustion by operating at temperatures much lower than that associated with CS, thereby
61 resulting in the production of fewer toxic compounds (9). It has been reported that many users
62 regard IQOS to be a safer product than CS, increasing its social acceptability (10). In fact, while
63 the prevalence of conventional cigarette use is decreasing, the use of HTPs is increasing in
64 Japan (11). Several independent research groups have examined the major components in
65 IQOS and reported the presence of toxicants, such as tar, carbon monoxide, and aldehydes, in
66 much lesser amounts than that in conventional cigarettes (12-14). However, the safety of IQOS
67 in the context of human consumption remains to be established, and some are concerned about
68 its harmful effect (15). The most common reasons reported for HTP use were perceived
69 reduction in harm to self and others compared to cigarettes (11); by contrast, 37% of

70 participants complained of symptoms caused by HTPs aerosol produced by others (16). In
71 addition, the nicotine concentration in IQOS aerosol ranges from 57 % to 83 % of CS (17),
72 indicating that IQOS may lead to chronic nicotine addiction. *In vitro* reports have shown that
73 e-cigarettes are less toxic than HTPs (18), even though e-cigarettes are reported to cause
74 emphysema in mice (7). As results of few *in vivo* studies on the effects of long-term exposure
75 to IQOS are currently available, we investigated how the consumption of IQOS could affect an
76 organism.

77 To investigate the effect of IQOS *in vivo*, we exposed mice to IQOS aerosol for a six-
78 month period because a six-month exposure to conventional CS is known to cause emphysema
79 in the lungs of wild-type mice (19). Furthermore, we performed microarray and pathway
80 analyses to assess the mechanisms underlying emphysema in the exposed mice.

81

82 **MATERIALS AND METHODS**

83 Detailed methods and additional information are provided in the supplemental material
84 available online.

85

86 **Mice**

87 Twelve-week-old male C57BL/6J mice purchased from Oriental Yeast Co., Ltd.
88 (Tokyo, Japan) were used for all the experiments. The mice were fed a commercial chow (CRF-
89 1; Oriental Yeast) and water *ad libitum*. All the mice were maintained in a limited-access
90 humidity (55 % ± 10 %)- and temperature (24 ± 2 °C)-controlled barrier facility under a 12/12
91 h light/dark cycle. The protocols for animal experiments were approved by the Animal Care
92 and Use Committee of the Juntendo University Faculty of Medicine.

93

94 **Chronic exposure of mice to IQOS aerosol and CS**

95 The mice were divided into three groups; fresh air-exposed (termed air hereafter;
96 control), IQOS aerosol-exposed (termed IQOS hereafter), and CS-exposed (termed CS
97 hereafter) groups. A cigarette smoke exposure system for small animals (Model SIS-CS;
98 Shibata Scientific Technology; Tokyo, Japan) was used for this purpose, as previously
99 described (20, 21). Marlboro IQOS HeatStick Regular (Philip Morris; Tokyo, Japan) and Peace
100 non-filter cigarettes (Japan Tobacco; Tokyo, Japan) were used for tobacco exposure in the
101 IQOS and CS groups, respectively. The system for connecting the IQOS cartridge and SIS-CS
102 is shown in Figure S1. The experimental settings were as follows: 15 mL stroke volume, six
103 puffs per min, and 3.5 % IQOS aerosol or CS diluted with compressed air. The mice were
104 exposed for a duration of 30 min per day for five days per week over six months. During the
105 exposure period, the body weight of these mice was measured monthly.
106
107 **Measurement of serum cotinine levels**
108 The mice were anesthetized by an intraperitoneal injection of pentobarbital sodium
109 (105 mg/kg of body weight) and xylazine (18 mg/kg of body weight). Blood from the inferior
110 vena cava was collected into FG-SRMS tubes (Fuchigami; Kyoto, Japan) immediately after
111 exposure. The serum was isolated following centrifugation at 3,500 rpm for 5 min at 24 ℃.
112 Serum cotinine level was measured using the Cotinine (Mouse/Rat) ELISA Kit (Abnova;
113 Taipei, Taiwan).
114
115 **Bronchoalveolar lavage fluid and morphological evaluation of the lungs**
116 Mouse lungs were subsequently processed as previously described (22). The
117 bronchoalveolar lavage fluid (BALF) from each mouse lung was pooled, and the total number
118 of cells was counted by staining with Turk’s solution (Muto Pure Chemicals Co. Ltd; Tokyo,
119 Japan). BALF samples were centrifuged at 350 rpm for 10 min in a Shandon CytoSpin 4

120 cytocentrifuge (Thermo Fisher Scientific; Waltham, USA). The harvested cells were mounted
121 on glass slides and stained with Diff Quick (Sysmex; Hyogo, Japan). A total of 1,000 cells
122 were counted for estimation of cell populations.

123 Histological sections of 4 μm thickness were prepared and stained with hematoxylin
124 and eosin for morphometric evaluation. Airspace size was evaluated by determining the mean
125 linear intercept (MLI) value (23). Ten randomly selected fields in each section at ×20
126 magnification were used to calculate MLI. The destructive index (DI) was used to estimate the
127 extent of alveolar wall destruction (24). Twenty randomly selected fields in each section at ×10
128 magnification were utilized to measure DI. A DI >10% was considered significant destruction
129 of lung parenchyma (25). MLI and DI were measured using Adobe Photoshop v22.3.

130

131 **Pulmonary function test**

132 Following six months of exposure, pulmonary function tests were performed using the
133 flexiVent system (SCIREQ Scientific Respiratory Equipment Inc.; Montreal, Canada). The
134 mice were connected to the flexiVent system after tracheotomy and insertion of an 18G cannula.
135 Inspiratory capacity (IC), static compliance (Cst), static elastance (Est), and lung airway
136 resistance (Rn) were calculated using the flexiWare Version 7.2 (SCIREQ) (26).

137

138 **Microarray analysis**

139 Total RNA samples extracted from the right lung using miRNeasy mini kits (Qiagen,
140 Hilden, Germany) were submitted for microarray analysis (n = 3 for each group). Details about
141 the microarray method are available in the online supplement. Genes that were differentially
142 expressed in the IQOS or CS groups compared with that in the air group were identified. Data
143 from the microarray analysis were plugged into the Ingenuity Pathway Analysis platform

144 version 46901286 (QIAGEN; Hilden, Germany), which yielded functional analysis and
145 canonical pathway analysis results.
146
147 **Western blot analysis**
148 Proteins were extracted from the whole lung as described previously (20). Protein
149 concentrations were measured using a Pierce BCA Protein Assay Kit (Thermo Fisher
150 Scientific). Proteins were resolved by SDS-PAGE and transferred on to PVDF membranes.
151 The membranes were blocked with 5 % skim milk in Tris-buffered saline with Tween 20 and
152 incubated overnight at 4 °C with cleaved caspase-3 (Asp175) antibody (#9661; Cell Signaling
153 Technology; Danvers, MA, USA; 1:1000 dilution), caspase-3 antibody (#9662; Cell Signaling
154 Technology; 1:1000 dilution), anti-cleaved PARP1 antibody [E51] (ab32064; Abcam;
155 Cambridge, UK; 1:1000 dilution), anti-PARP1 antibody [EPR18416] (ab191217; Abcam;
156 1:1000 dilution), anti-caspase-9 antibody [EPR18107] (ab202068; Abcam; 1:2000 dilution),
157 cytochrome c (136F3) (#4280; Cell Signaling Technology; 1:1000 dilution), and β-Actin
158 (13E5) monoclonal antibody (#4970, Cell Signaling Technology; 1:1000 dilution). Next,
159 membranes were incubated with the appropriate horseradish peroxidase-conjugated secondary
160 antibodies, followed by detection on a Clarity Western enhanced chemiluminescence substrate
161 (Bio-Rad Laboratories; Hercules, CA, USA). Western blot images were captured using an
162 ImageQuant LAS 4000 mini biomolecular imager (GE Healthcare; Pittsburgh, PA, USA) and
163 quantified using the Multi-Gauge image V3.0 software (Fujifilm Life Science; Tokyo, Japan).
164 Quantified data were normalized using β-actin as a loading control.
165
166 **Immunohistochemical analysis**
167 Paraffin-embedded lung sections were deparaffinized as previously described (21).
168 Tissue sections were first blocked with 5 % goat serum albumin in phosphate-buffered saline,

169 and then incubated at 4 °C overnight with anti-single-stranded DNA (ssDNA) antibody
170 (Immuno-Biological Laboratories Co.; Tokyo, Japan; 1:1000 dilution). Next, the tissue
171 sections were incubated with biotin-labeled anti-rabbit IgG antibody (Agilent Technologies;
172 1:300 dilution) and avidin (Vector Laboratories; Burlingame, CA, USA; 1:50 dilution) for 30
173 min between 25 °C – 28 °C. The signal was detected using hydrogen peroxide and 3,3′-
174 diaminobenzidine tetrahydrochloride. Tissue sections were counterstained with hematoxylin
175 and dehydrated in xylene. The ratio of ssDNA-positive nuclei to the total number of nuclei
176 detected in a specific field was determined in ten different areas of the lung per mouse at a
177 magnification of ×20.
178
179 **TdT-mediated dUTP nick-end labeling (TUNEL) assay**
180 The paraffin-embedded lung sections were subjected to a TUNEL assay using an *in situ*
181 Apoptosis Detection Kit (Takara Bio, Shiga, Japan), as per the manufacturer's instructions.
182 The ratio of TUNEL-positive nuclei to the total number of nuclei detected in a specific field
183 was determined in ten different areas of the lung per mouse at a magnification of ×20.
184
185 **cDNA synthesis and real-time quantitative polymerase chain reaction (qPCR)**
186 cDNA was synthesized from total RNA using ReverTra Ace qPCR RT Master Mix
187 with gDNA Remover (Toyobo Co. Ltd., Osaka, Japan). Real-time qPCR was performed using
188 Thunderbird SYBR qPCR Mix (Toyobo) and StepOnePlus real-time PCR system (Applied
189 Biosystems, Foster City, CA, USA). *Cyclooxygenase (COX)-2*, *interleukin (IL)-6*, *nfn2l2*,
190 *hmox-1*, *Ccl2*, and *Apaf1* were measured by RT-qPCR. *Glyceraldehyde-3-phosphate*
191 *dehydrogenase* (*GAPDH*) was measured as an internal control. The primers used for the assay
192 are shown in the supplemental material.
193

194 **Statistical analysis**

195 The data were expressed as mean ± standard error of the mean and were analyzed using
196 GraphPad Prism Version 7.03 for Windows (GraphPad Software; San Diego, CA, USA).
197 Analysis of variance was performed using the Kruskal-Wallis test followed by the Dunn's
198 multiple comparisons test, and *p*-values were calculated for IQOS and CS with air as a control.
199 Differences were considered significant when $P < 0.05$.

200

201 **RESULTS**

202 **Chronic exposure to IQOS suppresses weight gain in mice**

203 We measured the body weight of mice monthly and calculated weight gain. Long-term
204 exposure to CS had previously been reported to induce systemic inflammation and muscular
205 atrophy in the host, leading to the suppression of weight gain (27). In this study, mice in the air
206 group gained weight steadily. However, weight gain was significantly suppressed in the IQOS
207 group compared with the air group after 3 months of exposure (Table S1). Although weight
208 gain suppression began earlier in the CS group, the amount of weight gain in the IQOS group
209 was almost identical to that of the CS group after a six-month exposure period ($P < 0.001$ for
210 both *versus* air; Figure 1). The mean difference in weight gain after six months of exposure
211 was −4.93 g (95 % CI: −3.44 to −6.42 g; IQOS *versus* air) and −5.504 g (95 % CI: −3.936 to
212 −7.072 g; CS *versus* air) (Table S1).

213

214 **Increased levels of serum cotinine in the IQOS and CS groups**

215 Serum cotinine, the predominant metabolite of nicotine, levels were measured due to
216 the short half-life of nicotine. While cotinine in the air group was nearly absent, it was
217 detectable in the IQOS (330 ± 54 ng/mL) and CS (254 ± 32 ng/mL) groups (Figure 2).

218

219 **Increased neutrophil and lymphocyte count in BALF of IQOS group**

220 An increase in cell numbers in BALF after exposure to CS has been reported because
221 of the accumulation of macrophages, neutrophils, and lymphocytes, reflecting lung
222 inflammation (28). Herein, the total cell count in BALF was higher in the CS group than in the
223 air group, but not in the IQOS group. Exposure of mice to IQOS led to an increase in the
224 percentage of neutrophils and lymphocytes, similar to the effect of CS exposure (Figure 3).

225

226 **Chronic IQOS exposure induces pulmonary emphysema**

227 Representative histological hematoxylin–eosin-stained images of the lungs from each
228 group are shown in Figures 4A and S2. These results indicated that chronic exposure to IQOS
229 aerosol caused pulmonary emphysema in mice, similar to conventional CS. To quantify the
230 degree of emphysema, we evaluated airspace enlargement and alveolar wall destruction.
231 Compared with the air group, the IQOS group showed significantly greater MLI (P = 0.0096)
232 (Figure 4B) and DI (P = 0.0027) (Figure 4C). The CS group also showed higher MLI (P =
233 0.0061) and DI (P = 0.029) scores than those in the air group.

234

235 **Lung mechanics following chronic exposure to IQOS and CS**

236 IC and lung compliance measured using the flexiVent are known to increase in a COPD
237 mouse model (29). Our experiments showed increased IC and Cst and decreased Est in the CS
238 group; however, these changes were not significant in the IQOS group (IC; P = 0.36, Cst and
239 Est; P = 0.65). No changes in the Rn were observed in the IQOS or CS groups compared to
240 that in the air group (Figure 5).

241

242 **Differences in gene expression profiles in IQOS and CS groups**

243 To assess the mechanisms underlying emphysema development in the IQOS group, we
244 performed microarray analysis using whole lung tissue. The microarray analysis data were
245 deposited in the Gene Expression Omnibus database (ID: GSE161869). As shown in Figure
246 6A, 1,181 probes were upregulated due to IQOS exposure compared with the air group, while
247 1,463 probes were upregulated on CS exposure. Only 116 probes were upregulated in both the
248 IQOS and CS groups. In contrast, 725 and 1,595 probes were down-regulated due to IQOS and
249 CS exposure, respectively, compared with the air group. 196 probes were down-regulated in
250 both groups. Based on the probes up- or down-regulated by IQOS exposure, we constructed a
251 heatmap depicting definitive differences in lung gene expression profiles between the CS and
252 IQOS groups (Figure 6B) and the top 50 up- and down-regulated genes, respectively (Figure
253 S3). The gene lists in Figure 6A and 6B are available along with the online supplementary data.
254 To elucidate the biological significance of the altered gene expression profiles in the
255 IQOS and CS groups, we performed functional analysis and pathway analysis using the
256 Ingenuity Pathway Analysis® software. The results of the functional analysis, obtained from
257 probes that were up- and down-regulated compared with the control, are presented in Tables
258 S2 and S3, respectively. Functions related to cell survival and structural maintenance such as
259 'Cellular Function and Maintenance,' 'Cell Death and Survival,' 'Cellular Growth and
260 Proliferation,' and 'Organismal Injury and Abnormalities' were identified in the IQOS group.
261 In contrast, inflammation and immune-related functions such as 'Inflammatory Response,'
262 'Inflammatory Disease,' 'Immune Cell Trafficking,' and 'Immunological Disease' were more
263 highly ranked in the CS group, as previously reported (30). The results of the canonical
264 pathway analysis are presented in Tables S4 and S5. Pathway analysis revealed apoptosis-
265 related pathways such as 'Nur77 Signaling in T Lymphocytes,' 'Calcium-induced T
266 Lymphocyte Apoptosis,' and 'Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells'
267 among the top 10 pathways associated with the genes upregulated in the IQOS group. In

268 contrast, pathways related to inflammation such as 'Neuroinflammation Signaling Pathway'
269 and 'IL-10 Signaling' and immunity such as 'Communication between Innate and Adaptive
270 Immune Cells' were ranked higher in the CS group.

271

272 **Upregulation of apoptosis-related proteins in the IQOS group**

273 Based on the pathway analysis results, we hypothesized that apoptosis was the
274 predominant mechanism underlying emphysema in the IQOS-exposed lung. To verify this
275 hypothesis, we performed western blot analysis and found significantly higher expression
276 levels of cytochrome c ($P = 0.047$), cleaved caspase-9 ($P = 0.0048$), cleaved caspase-3 ($P =$
277 0.032), and cleaved PARP1 ($P = 0.010$) in the IQOS group compared with those in the air
278 group (Figure 7A–C, E, G). In addition, the ratios of cleaved analogues (caspase-3/9 and
279 PARP1) to the corresponding intact proteins (total caspase-3/9 and PARP1) also increased (P
280 = 0.016, 0.0064, and 0.027, respectively) (Figure 7D, F, H). Cytochrome c ($P > 0.99$), cleaved
281 PARP1 ($P = 0.065$), and ratio of cleaved PARP1 to total PARP1 ($P = 0.057$) in the CS group
282 were not significantly increased. Full blot images are shown in Figures S4 and S5.

283

284 **Chronic IQOS exposure causes alveolar cell apoptosis**

285 To examine apoptosis at the tissue level, we performed immunohistochemical analysis.
286 We observed a greater number of ssDNA-positive alveolar septal cells in the IQOS group than
287 in the air group ($P = 0.0071$) (Figure 8A, B). We also performed a TUNEL assay and found a
288 significantly greater number of TUNEL-positive alveolar septal cells in the IQOS group
289 compared with the air group ($P < 0.001$) (Figure 8C, D). However, these changes were not
290 significant in the CS group ($P = 0.084$ and $P = 0.078$, respectively).

291

292 **Several mechanisms other than apoptosis are enhanced in the CS group**

293 To assess the other mechanisms that cause emphysema, we performed RT-qPCR
294 using mRNA extracted from whole lungs. As shown in Figure 9, gene expression levels of
295 inflammation markers *COX-2* ($P = 0.032$) and *IL-6* ($P = 0.011$), oxidative stress markers
296 *nfe2l2* ($P = 0.023$) and *hmox-1* ($P = 0.017$), and immune cell trafficking marker *CcL2* ($P =$
297 0.013) were significantly upregulated in the CS but not in the IQOS group. In contrast, the
298 expression of apoptosis marker, *Apaf1*, was upregulated only in the IQOS group ($P = 0.028$)
299 (Figure 9).
300
301 **DISCUSSION**
302 Emphysema is the predominant anatomical feature of COPD and is usually triggered
303 by cigarette smoking. Tobacco industries have marketed HTPs as safer alternatives to CS and
304 insisted that their consumption leads to reduced health concerns in smokers. However, *in vitro*
305 studies carried out at independent research institutes have reported negative effects of HTPs,
306 including IQOS, such as impaired cell viability and metabolic activity, and inflammation (15,
307 18, 31). The effects of HTPs on animals, particularly its long-term exposure, remains unknown.
308 Therefore, we exposed C57BL/6J mice for a long time to evaluate IQOS toxicity. To the best
309 of our knowledge, this is the first study to demonstrate emphysema induction in murine lungs
310 because of chronic exposure to IQOS aerosol, predominantly mediated by apoptosis.
311 Emphysema is a form of alveolar destruction induced by several mechanisms, including
312 inflammation, oxidative stress, protease-antiprotease imbalance, and senescence. We focused
313 on apoptosis based on insights obtained from functional and pathway analyses of the
314 microarray results. As suggested by the microarray data, emphysema may be induced by a
315 different gene expression pattern and not simply due to a differential degree of exposure
316 compared to CS. Bhat *et al*. reported that acute exposure to IQOS increased albumin levels,
317 lung epithelial cell damage, several pro-inflammatory cytokines/chemokines, and

318 inflammatory T cells in mice BALF (32). Moased *et al.* reviewed the application from the
319 manufacturer and concluded that there was evidence of severe pulmonary inflammation and
320 immune toxicities in rats BALF exposed to IQOS aerosols, albeit better than a conventional
321 cigarette (33). These *in vivo* studies will complement our finding that apoptotic pathways
322 involving T cells were enhanced in the IQOS exposed lung. Additionally, the significant
323 suppression of weight gain in IQOS and CS may be associated with systemic effects such as
324 inflammation and changes in muscle structure (27). Indeed, IQOS aerosol exposure is known
325 to impair vascular endothelial cell function in rats (34). Hence, further research on the systemic
326 effects of IQOS is essential.

327 Previous studies support the crucial role of apoptosis in the development of lung
328 emphysema. While apoptosis can directly cause emphysema (35), it also interacts with
329 oxidative stress and protease/antiprotease imbalance to promote tissue destruction (36).
330 Kasahara *et al.* found that apoptosis of septal endothelial cells and decreased expression of lung
331 vascular endothelial growth factor (VEGF) and its receptor 2 (VEGFR2) occurred in
332 emphysema (37) and that VEGF receptor inhibitor induced apoptosis of alveolar septal cells
333 and caspase inhibitor suppressed this change (38). Koike *et al.* showed that anti-endothelial
334 monocyte-activating protein 2 (EMAPII) inhibited CS-induced apoptosis and had a therapeutic
335 effect on emphysema (39). Thus, apoptosis contributes to emphysema formation, but we
336 predict that lung emphysema by IQOS is induced through a complex and intertwined
337 mechanism that is not yet perfectly understood.

338 Several reports about the major aerosol components of IQOS have been published by
339 independent groups (12-14). Conventional cigarettes contain over 7,000 substances, many of
340 which are toxic and carcinogenic. Many of these components have not been investigated in
341 IQOS. However, 56 novel ingredients, in addition to those already reported by the tobacco
342 companies, are present in a greater percentage in IQOS compared to CS. Propylene glycol and

343 glycerol, the main ingredients of IQOS, act as solvents for nicotine and flavoring components.
344 Although these products are used as food additives and in medical supplies and are considered
345 safe for oral consumption, their aerosols reduce cell viability and enhance oxidative stress in
346 primary human bronchial epithelial cells (40). Furthermore, safety concerns associated with
347 their inhalation have not been addressed satisfactorily. Several ingredients present in IQOS
348 cannot be ignored, although their percentages are lesser compared to CS. Acrolein induces
349 tobacco-related diseases and induces reactive oxygen species production and DNA damage,
350 resulting in apoptosis in human lung epithelial cells (41-43). Similarly, formaldehyde—
351 recognized by the International Agency for Research on Cancer as a Group 1 carcinogen—is
352 known to induce oxidative stress in lung epithelial cells and causes apoptosis via p38 MAPK
353 activation (44). Smoking one pack of IQOS a day leads to 65% – 70% less exposure to
354 formaldehyde and 85% less exposure to reactive oxygen species compared to CS, although the
355 exposure is twice as high as compared to ambient air (45). When e-cigarette liquid is
356 aerosolized by thermal decomposition, flavoring compounds act as additional sources of toxic
357 aldehydes (46). Thus, flavored IQOS could lead to similar toxicity. In addition, the unique
358 structure of IQOS cannot be ignored, as the heat of a heated tobacco product device can be
359 transferred to the tobacco stick filter, resulting in the generation of harmful compounds such
360 as formaldehyde, acrolein, and acetone from the heated filter (47). These components of IQOS,
361 even in small amounts, can increase the risk of carcinogenesis, asthma, and pneumonia, as well
362 as emphysema, if exposed for long periods (48).

363 Nicotine is a well-known addictive chemical that leads to the continuous use of tobacco
364 products and results in long-term exposure to harmful compounds. We estimated the amount
365 of nicotine ingested by measuring the concentration of serum cotinine, a metabolite of nicotine
366 with a long half-life. Serum cotinine concentration in the IQOS group was higher than in the
367 CS group. This suggested that IQOS exposure may induce nicotine addiction and chronic

368 smoking habits, resulting in negative effects upon long-term exposure. Previous reports have
369 shown that the amount of nicotine in IQOS aerosol is approximately 57 %–83 % of CS (17, 45,
370 49). However, a prior study where rats were exposed to IQOS aerosol and CS under similar
371 conditions showed significantly higher blood nicotine and cotinine levels in the IQOS group
372 and may be due to the particle size of the IQOS aerosol (34). Moreover, a previous study on e-
373 cigarettes showed that nicotine induces COPD features such as emphysema, mucin production,
374 and enhanced cytokine and protease levels in murine lungs (7). Thus, nicotine not only induces
375 tobacco addiction but also exhibits harmful effects on the respiratory system, even if other
376 toxicities associated with IQOS are much lower than that in CS.

377 Tobacco industries have publicized the importance of providing safer alternatives to
378 conventional tobacco products, popularly referred to as 'harm reduction.' However, the U.S.
379 Food and Drug Administration had clarified that IQOS consumption could reduce exposure to
380 harmful chemicals only if smokers switched to it completely, although they did not claim that
381 these products were safe. The Food and Drug Administration also argued that young
382 individuals or individuals who do not currently use tobacco products should not adopt IQOS
383 consumption (50). Although some consumers misunderstand HTPs to be harmless, there is no
384 evidence that switching to IQOS reduces tobacco-related diseases. Contrarily, our study
385 revealed that long-term inhalation of IQOS caused lung emphysema in mice. There have also
386 been case reports of eosinophilic pneumonia in humans after using electric inhalation devices,
387 including HTPs (51). Furthermore, some *in vivo* studies have shown that IQOS exposure can
388 cause vascular endothelial dysfunction (30) and hepatotoxicity (52). A human study showed
389 that the use of HTPs was associated with asthma, allergic rhinitis, and atopic dermatitis in
390 adolescents (53). From these facts, we should be concerned about pulmonary toxicity as well
391 as the systemic effect of HTP use. Hence, a comparative study between IQOS and conventional

392 CS on the health of subjects was not performed. Quitting the consumption of all tobacco-related
393 products is recommended for a healthy life.
394 This study had a few limitations; first, we focused on apoptosis despite the possibility
395 of several other mechanisms that cause lung emphysema in mice. Hence, further studies on the
396 other mechanisms of emphysema are required. Second, we did not assess which cells or tissue
397 are responsible for developing emphysema. In Figure 8, the apoptotic response seems to appear
398 mainly in the alveolar epithelium, but we could not work out the details. We are investigating
399 the possibility of using methods such as single-cell analysis to identify the responsible cells,
400 which is a future challenge. Third, other HTPs, flavors, and comparisons with e-cigarettes were
401 not investigated. Fourth, we could not examine the mass concentration of the total particle
402 matter of IQOS in the same way as CS (1141 ± 64 mg/m^3) because of the high water content
403 in the aerosol. Finally, the number of animal samples assessed in this study was restricted.
404 Typical COPD lung function features were not detected in the IQOS group, although the
405 characteristic morphological changes were observed. A previous study showed that C57BL/6J
406 male mice exposed to CS for six months did not differ significantly from the control group
407 except for tissue elastance (54). We suspect that a six-month exposure may be too short to alter
408 respiratory function for wild-type mice or non-aging model mice. Prolonged exposure may
409 potentially show significant changes in lung function as well.
410 In conclusion, this is the first study that demonstrated that long-term exposure to IQOS
411 aerosol induced emphysema in murine lungs via apoptosis. HTPs are, thus, not safer compared
412 to conventional cigarettes, and work in ways different from conventional cigarettes.
413

414 **Supplemental data:** https://doi.org/10.6084/m9.figshare.19343456

415

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423

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426

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429

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431 performed animal experiments. N.N.A., M.K., and Y.S. performed all other experiments and
432 analyzed data. F.T., Y.K., S.K., and K.T. supervised the experiments. N.N.A. and T.S.
433 drafted the manuscript. E.K., F.T., Y.K., S.K., and K.T. revised the manuscript. All authors
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622 **Figure Legends**

623 **Figure 1.** Chronic IQOS exposure suppressed weight gain in mice, similar to that in the
624 cigarette smoke (CS) group at the end of six months of exposure. Data presented are mean ±
625 Standard error of mean (SEM) of n = 5–7 mice per group. Statistical significance was
626 determined using Dunn's multiple comparisons test. The P-values were calculated for IQOS
627 and CS with air as a control. CS = cigarette smoke.

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629 **Figure 2.** Serum cotinine concentration immediately after exposure. Cotinine level in the IQOS
630 group was significantly higher than in the air group ($P < 0.001$). Data presented are mean ±
631 SEM of $n = 6$ mice per group. Statistical significance was determined using Dunn's multiple
632 comparison test. The P-values were calculated for IQOS and CS with air as a control. CS =
633 cigarette smoke.

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635 **Figure 3.** Cell numbers and percentages in bronchoalveolar lavage fluid (BALF) collected
636 from mice after exposure. (A) Images of BALF after cytocentrifuge with Diff Quick stain.
637 Scale bars: 200 μm. (B) Total cell number in the CS group ($P = 0.029$), but not in the IQOS
638 group ($P = 0.64$), increased in comparison to that in the air group. (C) Macrophage number
639 remained unchanged while (D) macrophage percentage decreased in both the IQOS (P =
640 0.0018) and CS groups ($P = 0.021$). Neutrophil (E) number and (F) percentage increased in
641 both the IQOS (P = 0.038 and 0.0091, respectively) and CS groups (P = 0.0043 and 0.010,
642 respectively). Lymphocyte (G) number and (H) percentage also increased in the IQOS (P =
643 0.0019 and < 0.001) and CS (P = 0.021 and 0.042, respectively) groups. Data presented are
644 mean ± SEM of n = 5–7 mice per group. Statistical significance was determined using Dunn's
645 multiple comparison test. The P-values were calculated for IQOS and CS with air as a control.
646 CS = cigarette smoke.

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648 **Figure 4.** Chronic IQOS exposure induced lung emphysema similar to the effect of CS. (A) Histological sections stained with hematoxylin-eosin. Scale bars: main image, 200 µm; inset on the upper right, 50 µm. (B) Airspace enlargement quantified by mean linear intercept (MLI) were increased in both the IQOS (P = 0.0096) and CS (P = 0.0061) groups. (C) Alveolar wall destruction quantified by destructive index (DI) were increased in both the IQOS (P = 0.0027) and CS (P = 0.029) groups. Data presented are mean ± SEM of n = 4–7 mice per group. Statistical significance was determined using Dunn's multiple comparison test. The P-values were calculated for IQOS and CS with air as a control. CS = cigarette smoke.

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657 **Figure 5.** Lung mechanics following six months of exposure, as measured by the flexiVent system. (A) IC was increased in the CS group (P = 0.015), but not in the IQOS group (P = 0.36). (B) Cst was increased in the CS group (P = 0.049), but not in the IQOS group (P = 0.65). (C) Est was decreased in the CS group (P = 0.049), but not in the IQOS group (P = 0.65). (D) Rn was not changed both IQOS and CS group. Data presented are mean ± SEM of n = 5–7 mice per group. Statistical significance was determined using Dunn's multiple comparisons test. The P-values were calculated for IQOS and CS with air as a control. CS = cigarette smoke, IC = inspiratory capacity, Cst = static lung compliance, Est = static lung elastance.

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666 **Figure 6.** Gene expression profiles of mouse lung after exposure. (A) Venn diagram showing up- and down-regulated probe numbers compared to that in the air group. (B) Heatmap displaying up- and down-regulated genes in the IQOS group compared to the air group and expression of those genes in the CS group. CS = cigarette smoke. n = 3 for each group.

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671 **Figure 7.** Upregurlation of apoptosis-related proteins in homogenate lung tissue collected from
672 the IQOS group, as compared to the air group. (A) Cytochrome c, caspase-9, cleaved caspase-
673 9, caspase-3, cleaved caspase-3, PARP1, cleaved PARP1, and β-actin expression levels were
674 analyzed by western blotting. Quantified protein levels (normalized to β-actin) of (B)
675 cytochrome c, (C) cleaved caspase-9, (D) cleaved caspase-9/caspase-9, (E) cleaved caspase-3,
676 (F) cleaved caspase-3/caspase-3, (G) cleaved PARP1 (G), and (H) cleaved PARP1/PARP1 (H).
677 Data presented are mean ± SEM of n = 5–7 mice per group. Statistical significance was
678 determined using Dunn's multiple comparison test. CS = cigarette smoke.

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680 **Figure 8.** Single-stranded DNA (ssDNA) and TdT-mediated dUTP nick-end labeling
681 (TUNEL) expression were significantly higher in the lungs of mice exposed to IQOS aerosol
682 compared to ambient air. (A) Immunohistochemical images of ssDNA, with arrows showing
683 ssDNA-positive alveolar septal cells. (B) The ratio of ssDNA-positive cells to the total cell
684 number in the IQOS group was significantly increased ($P = 0.0071$), but not in the CS group P
685 $= 0.084$). (C) Representative image for TUNEL assay, with arrows showing TUNEL-positive
686 alveolar septal cells. (D) The ratio of TUNEL-positive cells to the total cell number in the IQOS
687 group was significantly increased ($P < 0.001$), but not in the CS group ($P = 0.078$). Data
688 presented are mean ± SEM of n = 5–7 mice per group. Statistical significance was determined
689 using Dunn's multiple comparison test. The P-values were calculated for IQOS and CS with
690 air as a control. Scale bars: 50 µm. CS = cigarette smoke.

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692 **Figure 9.** Gene expression of inflammation, oxidative stress, and immune cell trafficking in
693 the CS group and apoptosis in the IQOS group were upregulated by the RT-qPCR. (A) *COX-*
694 *2*, (B) *IL-6*, (C) *nfe2l2*, (D) *hmox-2*, (E) *Ccl2* were upregulated in the CS group (each P =

695 0.032, 0.011, 0.023, 0.017, and 0.013) but not in the IQOS group. (F) *Apaf1* was upregulated

696 in the IQOS group (P = 0.028) but not in the CS group.