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Urinary VOCs as biomarkers of early stage lung tumour development in mice

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28 **Abstract :**

29 **Background:** Lung cancer is the primary cause of cancer-induced death. In addition to
30 prevention and improved treatment, it has increasingly been established that early detection is
31 critical to successful remission. **Objective:** The aim of this study was to identify volatile
32 organic compounds (VOCs) in urine that could help diagnose mouse lung cancer at an early
33 stage of its development. **Methods:** We analysed the VOC composition of urine in a
34 genetically engineered lung adenocarcinoma mouse model with oncogenic EGFR
35 doxycycline-inducible lung-specific expression. We compared the urinary VOCs of 10
36 cancerous mice and 10 healthy mice (controls) before and after doxycycline induction, every
37 two weeks for 12 weeks, until full-blown carcinomas appeared. We used SPME fibres and gas
38 chromatography – mass spectrometry to detect variations in cancer-related urinary VOCs over
39 time. **Results:** This study allowed us to identify eight diagnostic biomarkers that help
40 discriminate early stages of cancer tumour development (i.e., before MRI imaging techniques
41 could identify it). **Conclusion:** The analysis of mice urinary VOCs have shown that cancer
42 can induce changes in odour profiles at an early stage of cancer development, opening a
43 promising avenue for early diagnosis of lung cancer in other models.

44

45 **Keywords:** Lung cancer, early detection, biomarkers, urine, volatile organic compounds,
46 EGFR oncogenic mutation, *Mus musculus*.

47

48 **1. Introduction**

49 Cancer is one of the deadliest diseases in the world, with more than nine million casualties in 2022 [1].
50 Of the many cancers affecting humans, 18% are lung cancers. In 90% of these cases, deaths are due to
51 metastasis rather than primary tumours [2,3]. Lung cancers, like many other cancers, are difficult to
52 detect at early stages. In addition to improvements in prevention and treatment, there is growing
53 evidence that early detection is often critical to successful remission: patients whose cancer was
54 diagnosed and treated at an early stage were shown to have better long-term survival than patients
55 whose tumours were not detected before the appearance of symptoms (e.g., cervical, colorectal, and
56 breast cancers [4,5]). The current diagnostic techniques are radiography, magnetic resonance imaging,
57 and biopsy [6,7]. These methods are effective, but they have the disadvantages of being either
58 invasive, expensive, time-consuming, or necessitate facilities only present in large hospitals [8–10]. In
59 addition to these diagnostic techniques, the search for specific biomarkers has increased with the
60 progress of high-throughput techniques [11]. Diagnostic biomarkers include proteins, tumour antigens,
61 and gene transcripts found in urine or blood [5,12]. However, none of the biomarkers identified so far
62 has sufficient sensitivity, specificity, or reproducibility to diagnose lung cancer early enough to
63 prevent metastasis. The identification and validation of non-invasive biomarkers that could effectively
64 help diagnose lung cancer at an early stage remain critical to address the main cause of human death
65 by cancer in the world [13].

66 Over the past 20 years, increased attention has been devoted to exploring the potential use of volatile
67 organic compounds (VOCs) as non-invasive biomarkers of early stage cancers. Two-thirds of these
68 studies involved lung cancer [14]. Although many studies have indicated significant differences in the
69 VOC profiles of healthy versus cancerous patients in humans [15,16] or mice [17,18], it is still
70 unknown how early in cancer development variations in VOCs composition may take place.

71 The aim of this study was to identify candidate VOCs that may be associated with early cancer
72 development. We used a genetically engineered mouse model (GEMM) harbouring two different
73 transgenes, the lung-specific CCSP-rtTA and a human EGFR with an activating mutation (EGFR^{L858R})

74 that after eight to 12 weeks of doxycycline treatment induce detectable EGFR-driven lung
75 adenocarcinoma [19]. We compared VOC profiles of cancerous mice (CC) and their negative control
76 littermates (NC). Since doxycycline is an antibiotic and may induce changes in gut microbiota,
77 influencing mouse urinary VOCs [20], we compared VOCs in the urine of CC and NC mice before
78 and after doxycycline treatment. We also compared VOC profiles of CC and NC mice at different
79 stages of tumour development, seeking to identify specific VOCs that could help diagnose cancer
80 development at earlier stages than the current diagnostic tools for lung cancer [19].

81 **2. Materials and methods**

82 2.1. Ethical clearance

83 Our study involved a transgenic mouse model for which all precautions for animal welfare were taken.
84 The project received ethical clearance by the Ethical Committee for Animal Experimentation (French
85 Ministry of Higher Education, Research and Innovation) number 1645-22123.

86 2.2. Mice

87 Urine samples were obtained from CCSP/EGFRTL transgenic mice, that were generated by crossing
88 the Tet-ON-^{EGFRT790M/L858R} transgenic mouse strain (hereinafter EGFRTL) with the CCSP-rtTA strain
89 (hereafter CCSP) carrying rtTA, an inverse tetracycline-responsive element under the control of the
90 lung-specific promoter CCSP. In CCSP/EGFRTL mice, the EGFR gene contains both L858R
91 mutations (causing EGFR-constitutive activation in the absence of ligands such as EGF) and the
92 T790M gate-keeper mutation (conferring resistance to the first generation of EGFR inhibitors, i.e.,
93 gefitinib/erlotinib). Crosses were kept in heterozygosity, so littermates could have any of the four
94 possible genotypes: WT/WT, WT/EGFRTLtg, CCSPtg/WT, and CCSPtg/EGFRTLtg. In our
95 experimental setting, we used WT/WT mice—lacking both the CCSP and EGFRTL transgenes—and
96 so unable to develop tumours upon doxycycline induction (hereafter non-cancerous [NC] mice), and
97 CCSPtg/EGFRTLtg mice carrying both transgenes so lung tumours develop upon doxycycline
98 induction (hereafter cancerous [CC] mice). This system allows the expression of EGFRTL specifically
99 in the lungs, but only upon doxycycline exposure, thus leading to the development of peripheral

100 adenocarcinomas with bronchioloalveolar features in alveoli as well as papillary adenocarcinomas
101 [19,21]. Mice were 13 weeks old when doxycycline was added to their food for lung-specific
102 oncogene induction.

103 Twenty transgenic male mice were involved in our study, 10 CC and 10 NC. They were bred at the
104 IRCM (Montpellier Cancer Research Institute) and maintained at the breeding facilities of the IRD
105 (Institute of research and development) of Montpellier at six weeks of age. Mice were kept in groups
106 of two to four in transparent cages (26.8 cm × 21.5 cm × 14.1 cm height) before starting the
107 experiment. The cages contained sawdust, a cellulose square, hay, and a cardboard tunnel. Six weeks
108 later, mice were kept isolated to avoid any influence of social interactions on their VOC profiles [23,
109 24]. Their mass was checked once a week over the 12 weeks of doxycycline treatment to monitor their
110 health (i.e., euthanasia if loss of 10% of mass). Lung adenocarcinoma was induced by ingestion of
111 food pellets containing doxycycline (1 mg/kg), available ad libitum, starting at 13 weeks of age for 12
112 weeks (Figure 1).

113 At the age of 25 weeks, all mice (NC and CC) were euthanized at the IRCM, where their lungs were
114 examined. The presence or not of lung adenocarcinoma was confirmed by analysis of formalin-fixed
115 paraffin-embedded lung sections that had been stained with hematoxylin-eosin (Figure 2). After
116 necropsy, two mice of the CC genotype showed no apparent lesions; although their VOCs were
117 analysed, they were not included in our data set (Table S1). We therefore present below our analysis of
118 the VOC profiles of urine samples obtained from 10 NC and eight CC mice.

119 2.3. Urine sampling

120 Seven urine samples from each of the 18 male mice were obtained, one every two weeks. The first
121 sample was collected before the addition of doxycycline (T0) and the last one 12 weeks later (T12)
122 (Figure 1). The urine-collection protocol was as follows: each mouse was placed individually in an
123 empty cage previously washed with alcohol and left to dry. Mice were left to urinate in the cage for a
124 maximum of one hour. The urine was collected with a micropipette, transferred to an Eppendorf tube,
125 and kept frozen at -20°C. Each mouse was sampled both in the morning (between 9 and 10 am) and in

126 the afternoon (between 2 and 4 pm) to capture at least part of the circadian variation in secretion for 3
127 days. The samples were pooled into one tube per individual before being frozen. In addition, distilled
128 water was deposited on a cleaned cage and collected following the same protocol as for the urine to
129 serve as a technical control for chemical analyses.

130

131 2.4. Analysis and identification of VOCs in mouse urine

132 VOCs emanating from mouse urine were analysed using solid phase micro extraction (SPME, 65 μ m
133 diameter PDMS-DVB fibre composed of polydimethylsiloxane-divinylbenzene; Sigma-Aldrich,
134 Bellefonte PA, USA). To control for ambient temperature, analyses took place in an oven at 22°C. An
135 SPME fibre was introduced into a 4 ml vial with a silicone/PTFE septum, into which we pipetted 40 μ l
136 of urine. After 3 min of equilibration time, the fibre was exposed to the vial headspace for one hour
137 (Figure 3). The position of the fibre in the vial was controlled so that the distance from the urine
138 sample (~1 cm) was similar for all samples. After extraction, the same fibre was introduced into a gas
139 chromatograph coupled to a mass spectrometer injector (GC-MS, quadrupole mass spectrometer
140 Shimadzu QP2010-SE, Kyoto, Japan). The GC-MS was equipped with an Optima 5-MS fused silica
141 capillary column (30 m \times 0.25 mm \times 0.25 μ m film thickness; Macherey-Nagel, Düren, Germany) with
142 helium as the carrier gas (1 ml/min). All spectra were analysed with resident software (GCMS
143 Solution, Shimadzu, Kyoto, Japan). During the injection of the SPME fibre in the GC for desorption
144 the injector was at 250°C. The oven temperature was maintained at 40°C for 2 min, after which the
145 temperature increased by 5°C every minute until it reached 175°C and by 12°C per minute until it
146 reached 220°C.

147 As described elsewhere [22], VOCs were tentatively identified using peak retention time (RT) and
148 mass spectra for each compound visible on the chromatogram. Retention indexes (RI) were calculated
149 with reference to n-alkanes injected in the same GC-MS (Alkanes standard solution, 04070; Sigma-
150 Aldrich, Darmstadt, Germany). Final identification of compounds was based on comparisons with
151 mass spectrum databases (NIST 2007, Wiley Registry Ninth), with databases of compound RIs (e.g.,

152 Adams, 2007, Pubchem, <https://pubchem.ncbi.nlm.nih.gov/>), and standards when available. After
153 tentative identification of all compounds and exclusion of those present in the technical controls, we
154 calculated the surface of each compound's peak area based on the total ion current chromatogram
155 (TICC). The surface area of compounds that were only present as traces could not be calculated, so
156 they were given an arbitrary value corresponding to 10% of the value obtained for the smallest peak
157 present in our dataset. Because SPME extraction reflects the relative proportions of components in
158 each sample, our calculations needed to consider this constraint for each compound and each
159 individual to accurately compare VOC profiles of different individuals. For this, we divided a
160 compound's peak surface area by the sum of the peak areas of all compounds present in the individual
161 profile. Hereafter, we refer to these values as relative quantities expressed in percentage.

162 2.5. Statistics

163 All statistical analyses were performed with RStudio software version 3.4.4 [23]. We used the
164 following packages: ade4 [24], vegan [25], mixOmics [26], ggplot2 [27], RVAideMemoire [28],
165 Hotelling [29], and nlme [30]. Normality and heteroscedasticity of residuals were checked visually
166 after plotting the model residuals, and data were transformed when necessary (see below).

167 To compare variations in the relative proportions of VOCs emitted by NC and CC mice across
168 experimental conditions (T0, T2, T4, T6, T8, T10, T12), a multivariate redundancy analysis (RDA)
169 followed by permutation F tests were realised [31]. Relative proportions were CLR-transformed prior
170 to RDAs. Because our data included zeroes, a small constant an order of magnitude smaller than the
171 smallest non-zero value in our data was added to all values prior to transformations (e.g., 0.01 if the
172 smallest non-zero value was 0.1). Data were then auto scaled to give equal weight to all compounds in
173 the analyses. Factors included in the RDA depended on the hypothesis tested. We first questioned the
174 impact of the doxycycline treatment, i.e., experimental condition (seven modalities, T0 to T12), on
175 urine VOC composition. To address this question, we separately analysed CC and NC mice. These
176 models included the experimental condition as an explanatory variable with conditional variables
177 being fibres (two fibres used) and mouse identity. To assess VOC differences between NC and CC
178 mice across experimental conditions, we designed a model including mouse identity nested within

179 health status, health status (NC, CC), experimental condition, and the interaction between the last two
180 factors as explanatory variables. Fibre identity was included as a conditional variable. VOCs showing
181 an absolute correlation coefficient >0.8 with the main RDA axes were considered as potential
182 diagnostic molecules and were analysed using univariate approaches. These consisted of linear mixed
183 models including experimental condition, status, and their interaction as fixed factors as well as mouse
184 identity and fibre as random factors. Data were log-transformed prior to these analyses to normalize
185 the residuals distribution. Post-hoc pairwise comparisons were performed with the Estimated Marginal
186 Means method (EMMeans). Significance level was set at $\alpha = 0.05$ and adjusted for multiple
187 comparisons with the sequential Bonferroni method [32]. To assess for correlation between
188 discriminate compounds the “corplot” package was used. To get insight into possible biochemical
189 links between molecules, we first used the KEGG database resource [33] in which information on
190 mice metabolic pathways could be found (*Mus musculus*;
191 *animals/chordata/mammalia/rodentia/murcidae/mus*). Second, we used a deep learning user-friendly
192 open-source toolkit (the BioNavi-NP developed by Zhen et al. [34]), a device that allows to predict the
193 biosynthetic pathways of a given molecule. We used the programme on line
194 (<http://biopathnavi.qmclab.com/job.html>, with the Default settings).

195 **3. Results**

196 Thirty-six compounds were detected in our biological samples and absent from the technical controls
197 (Table S2). Nineteen of these compounds were present in all samples while the other 27 were found in
198 both CC and NC samples, although not in every sample (Table S3). Twenty-six of these compounds
199 could be chemically identified, while the other 11 were unknown (Table S3). Among the molecules
200 identified in this study, 10 had already been reported in human cancer studies and 11 in mouse cancer
201 studies (details in Table S3).

202 The RDA analyses indicated that VOC profiles of both CC and NC were influenced by the
203 doxycycline treatment (Models 1 and 2, Table 1; all post-hoc tests comparing T0 with T2–T12,
204 $p < 0.05$). However, the influence of experimental conditions was more marked among the CC profiles

205 ($p = 0.005$) than among the NC ones ($p = 0.041$), and their variation across experimental conditions
206 did not follow the same pattern (Figure S1), suggesting that the antibiotic treatment alone may not
207 explain the overall variation observed in the VOC profiles of CC mice (Figure 4). When comparing
208 the profiles of CC and NC at the different experimental conditions, the RDA analysis showed
209 significant effects of health status, experimental conditions, and their interaction (model 3, $p < 0.005$;
210 Table 1, Figure 5). Post-hoc pairwise comparisons indicated that the VOC profiles of CC and NC mice
211 at T0 were not significantly different ($p = 0.897$). However, from the onset and till the end of the
212 doxycycline treatment (T2-T12), the VOC profiles of NC and CC mice were systematically and
213 significantly different ($p < 0.01$ for four of the comparisons and $p < 0.05$ for the two others).

214 Given these results, we sought to identify specific compounds that could explain the above-described
215 differences between CC and NC and hence would constitute potential biomarkers of cancer
216 development. With this aim, we retained 17 VOCs showing an absolute correlation coefficient higher
217 than 0.8 with the main RDA axes, and we further analysed their variations across health status and
218 experimental conditions with univariate analyses (Table 2). Among these VOCs, six compounds
219 showed no variation across treatments or between status (Table 2). Three other compounds, 3-
220 methylcyclopentanone, 3-ethylcyclopentanone, and butan-2-one, varied by treatment but not between
221 CC and NC, suggesting a direct effect of doxycycline but not a cancer effect. The relative proportions
222 of two other VOCs, pentan-2-one and 3-methylbutan-2-one, were affected both by the doxycycline
223 treatment per se and cancer development, displaying a highly heterogeneous pattern of variation
224 (Figure 6). While the variation of 3-methylbutan-2-one was impacted by both doxycycline and cancer
225 development, its pattern of variation suggests that it could be a good candidate to diagnose later stages
226 (T10, T12) of cancer development (Figure 6). Remarkably, the last five VOCs showed significantly
227 different relative proportions in CC and NC mice profiles starting at T2, a very early stage when only
228 cancer lesions are observed, and through to T12 (Figure 6). Three of these five compounds were
229 present in small relative proportions (1–10%) in the mice profiles (phenylacetone, 4-methylhept-6-en-
230 3-one, and hept-4-en-2-one), while two others, which are also male mouse pheromones, were present
231 in large relative proportions ($< 50\%$; 2-*sec*-butyl-4,5-dihydro-thiazole and 3,4-dehydro-*exo*-

232 brevicomine). Variation in the relative proportions of the two above mentioned pheromones was not
233 significantly correlated (Figure 7), while 3-methylbutan-2-one, pentan-2-one, hept-4-en-2-one and 3,4-
234 dehydro-exo-brevicomine variation showed a positive relation, although not necessarily similar
235 responses to experimental conditions (Figure 7). Other compounds were negatively correlated (i.e.
236 phenylacetone and the 4 other compounds; or 4-methylhept-6-en-3-one, pentan-2-one and 3-
237 methylbutan-2-one).

238 The metabolic pathways of the molecules identified in this study have never been explored.
239 Pentan-2-one is the only compound present in the KEGG database, but not as part of *Mus*
240 *musculus* metabolic pathway. The involvement of amino acids such as isoleucine or cysteine
241 in the biosynthesis of the 2-*sec*-butyl-4,5-dihydro-thiazole was proposed by Pickett et al.
242 (2014) [35].

243 **4. Discussion**

244 The use of urinary volatile biomarkers to diagnose a disease such as lung cancer is very promising: it
245 is both minimally invasive and a rapid way to obtain a diagnosis. In this study, a lung cancer model
246 was used to investigate the impact of tumour development on mouse urine VOC profiles and to
247 identify potential candidate molecules that could help diagnose a cancer tumour at an early
248 developmental stage, i.e., when cancer is not detected by small imaging techniques such as computed
249 tomography. Lung cancer can have several clinical forms; we chose a mouse model that develops non-
250 small cell lung adenocarcinoma (NSCLC), which is a good way to simulate a human lung cancer [19].
251 The two groups of mice, NC and CC, only differed by the presence of two transgenes, and this slight
252 difference in their genotype did not seem to influence their urinary VOC profiles at T0, although it
253 influenced other odour sources [36]. Our experimental design allowed us to distinguish between two
254 distinct effects of doxycycline on mouse VOC profiles: its influence on microbiota, impacting odour
255 production in both CC and NC mice, versus its induction of tumour development, which impacted the
256 metabolism and odour production only of CC mice. Interestingly, the VOC profiles of the two CC
257 mice that did not develop a lung tumour following doxycycline treatment were not different from the

258 profiles of NC mice, strongly reinforcing our findings that cancer impacts VOC variations from an
259 early stage of development (Table S1). We identified at least five compounds that could help diagnose
260 lung cancer at all developmental stages, including when only small lesions are observed (stage T2).

261 The doxycycline treatment was continuous and lasted for 12 weeks. Doxycycline-driven alterations
262 (independent of cancer development) were observed for three compounds, butan-2-one, 3-
263 methylcyclopentane, and 3-ethylcyclopentanone. Butan-2-one was found both in mouse faeces [37]
264 and urine [38,39] and was shown to be a potential biomarker of epilepsy, with higher relative
265 proportions in epileptic compared to control mice [38]. Consistent with our findings, this compound
266 was often found in human cancer literature as a biomarker for cancer (Table S2). It was sometimes
267 found in higher proportions [40–47] and other times in lower proportions [48–51] in patients with
268 cancer. Butan-2-one is known to be produced by bacteria such as *Pseudomonas*, *Staphylococcus*,
269 *Escherichia coli*, or *Saccharomyces cerevisiae* [52–56]. The lower relative proportion of this
270 compound in mouse urine during antibiotic treatment suggests a negative impact of doxycycline on
271 mouse microbiota. On the contrary, we observed that the relative proportions of 3-
272 methylcyclopentanone and 3-ethylcyclopentanone significantly increased during doxycycline
273 treatment. While these compounds were not reported in other mouse studies, they were found in all
274 mice at all experimental conditions in the present study, albeit in low proportions (1–2%).
275 Interestingly, these two compounds have been shown to be involved in territorial marking and to be
276 emitted in high proportions in carnivores such as wolves, lions, the Siberian tiger, and badgers [57–
277 60]. Whether higher excretion of these compounds during the doxycycline treatment in our study
278 relates to changes in mouse microbiota—favouring some convergence with carnivore microbiota—is a
279 speculation that could be tested.

280 Tumour development impacted the VOC profile of cancerous mice at all stages of lung cancer
281 development (from T2 to T12). Earlier studies indicated that two to three weeks of doxycycline
282 treatment of CC mice resulted in alveoli lesions only visible with a histological approach, and the first
283 lesions that could be revealed with small imaging techniques were visible only following five to six
284 weeks of doxycycline treatment [19]. Urinary VOC analyses hence offer a relatively simple non-

285 invasive method to diagnose early stage mouse lung cancer that imaging techniques could not detect.
286 Here we identified five compounds indicative of cancer development from an early to a more evolved
287 stage. Phenylacetone was a minor peak in the mouse VOC profile (less than 1%) whose relative
288 proportion increased significantly in cancerous mice starting from T2. This amphetamine metabolite is
289 often reported to be present in mice urine [39,61,62]; it was shown that its excretion tended to
290 decrease with aging [63] although an increase in phenylacetone was also reported in mice showing
291 Alzheimer's disease [64]. Higher levels of phenylacetone in the urine of cancerous mice might be an
292 indication of metabolic modifications during cancer development, which has often been reported in
293 cancer research [65].

294 The relative proportions of hept-4-en-2-one, another candidate biomarker of early-stage tumour
295 development, showed a significant decrease in CC profiles, with a significant drop from T0 to T2 and
296 a second drop from T8 to T12. This compound was identified in the urine of healthy mice [38,66],
297 while epilepsy did not induce changes in its concentration [38]. Two other compounds, thiazoline and
298 brevicomine—both testosterone-dependent mouse pheromones [67]—showed marked changes in
299 association with tumour development starting at T2. These two pheromones were shown to be
300 involved in advertising dominance [68] and their variation in cancerous mice might indicate that
301 cancer development affected mouse metabolic paths involving testosterone, although to date the
302 metabolic pathway of these two molecules remains unknown. This suggests that targeting
303 testosterone-related compounds in humans might be of interest when tentatively diagnosing cancer
304 with scent at an early stage of tumour development.

305 Two other compounds, 4-methylhept-6-en-3-one and 6-methylheptan-3-one, were found in higher
306 proportions in CC compared NC mice two weeks after doxycycline treatment began, although the
307 differences between CC and NC for 6-methylheptan-3-one proportions were not significant at T4 and
308 T8. Both compounds have been described in other studies involving mice [69,70] and were identified
309 as markers of different diseases. For example, 6-methyl-heptan-3-one was found in higher relative
310 proportions in the presence of mouse melanoma [17,71] and mouse lung cancer [72] at advanced
311 stages of cancer development. 4-methyl-6-hepten-3-one was found to increase in the profiles of mice

312 with melanoma and to decrease in those with lung cancer [72]. Other studies found an increase in 4-
313 methyl-6-hepten-3-one linked to Alzheimer's disease [70]. 6-methyl-heptan-3-one was also reported to
314 decrease in association with anxiety and depression in mice [63,69,73]. Finally, these two molecules
315 are likely not specific of lung cancer but may constitute indicators of non-specific changes affecting an
316 organism at the beginning of cancer tumour development.

317 Two molecules, pentan-2-one and 3-methylbutan-2-one, were affected at a late-stage of tumour
318 development: their relative proportions decreased 10 weeks after the start of the doxycycline treatment
319 in CC mice. These compounds have been reported in other mouse cancers, i.e., melanoma [71] and
320 breast cancer [74]. However, earlier studies reported an increase in pentan-2-one, while a human lung
321 cancer study [40] reported a decrease in the relative proportion of this molecule, a pattern consistent
322 with our results. Furthermore, Liu and collaborators [75] reported the same pattern of reduction in the
323 relative proportion of this compound in human colorectal cancer cell culture. Pentan-2-one and 3-
324 methylbutan-2-one are ketones resulting from fat metabolism, and the reduction in their relative
325 proportions in mouse urine may indicate a reduction in fatty acids oxidation rate. In addition, lower
326 levels of pentan-2-one in CC mouse urine might be attributed to the downregulations of protein
327 metabolism and of the ketogenic pathway caused by cancer [76]. Finally, 3-methylbutan-2-one, an
328 indicator of late-stage of development of mouse lung tumour, revealed in our study, was reported to be
329 linked to cancer in a publication analysing human saliva VOC profiles of patients affected by head and
330 neck cancer [77].

331 The analysis of the correlation matrix suggests that the candidate molecules pointed out in this study
332 may not be considered as inter-related, as some of them are negatively, positively, or even not
333 correlated. Moreover, some molecules that show a positive correlation of their relative ratios did not
334 respond in the same way to cancer induction. These candidate biomarkers might be considered as
335 signals of metabolic changes induced by cancer development. Unfortunately, we were not able to
336 identify any of the metabolic pathways in which these molecules may be involved. Future research
337 will have to explore the metabolic pathways that may produce such molecules.

338 **Conclusion**

339 Cancer induced by doxycycline impact the mouse urine VOC profiles. Remarkably, we identified, for
340 the first time, five VOCs associated with an early stage of cancer development (two weeks). These
341 VOCs could result from cancer inducing metabolic changes and/or the tumour *per se* producing some
342 of the VOCs. Interestingly, some of the biomarker candidates identified in this study were also
343 described in cancer development in human patients. This observation encourages us to propose more
344 translational studies on humans. Moreover, our findings may encourage research focusing on VOCs, a
345 non-invasive and inexpensive diagnostic tool, that could be promising. Different types of cancers have
346 different VOC signatures, although most of the literature cited in our discussion suggests that some
347 convergence and similarities in the influence of different types of cancer on VOCs may exist. Finally,
348 the specific pathways impacted by cancer and inducing VOC changes may be worth a thorough
349 investigation to better understand the general impact of cancer on the whole body; a research path that
350 could help to develop new treatments or increase the efficiency of existing ones.

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