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Flora Gouzerh

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THÈSE POUR OBTENIR LE GRADE DE DOCTEUR DE L'UNIVERSITÉ DE MONTPELLIER

Filière BDI - Biologie des Interactions

École doctorale Gaia

Unité de recherche Mivegec/CEFE

CONSEQUENCES ECOLOGIQUES, EVOLUTIVES ET APPLIQUEES DES MODIFICATIONS D'ODEURS CORPORELLES ASSOCIEES AU CANCER

Présentée par Flora GOUZERH

Le 8 septembre 2022

Sous la direction de Frédéric THOMAS et Laurent DORMONT
Et co-encadrée par Guila GANEM

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UNIVERSITÉ
DE MONTPELLIER

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*« La vie mettra des pierres sur ta route,
à toi de décider si tu en feras un
mur où un pont »*

Coluche.

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~ Liste des publications et communications ~

Liste des publications :

Acceptées et en cours de préparation

- **Article 1 : Gouzerh, F.**, Bessière, J. M., Ujvari, B., Thomas, F., Dujon, A. M., & Dormont, L. (2022). Odors and cancer: Current status and future directions. *Biochimica et Biophysica Acta (BBA)-Reviews on Cancer*, 1877(1), 188644.
- **Article 2 : Gouzerh, F.**, Pichevin, A., Dormont, L., Ganem, G., Thomas, F., (**In preparation**). Ability of animals to detect cancer.
- **Article 3 : Gouzerh, F.**, Buatois, B., Hervé, M. R., Mancini, M., Maraver, A., Dormont, L., Thomas, F. & Ganem, G. (2022). Odours of cancerous mouse congeners: detection and attractiveness. *Biology Open*, 11(4), bio059208.
- **Article 4: Gouzerh, F.**, Buatois, B., Hervé, M. R., Mancini, M., Maraver, A., Dormont, L., Thomas, F. & Ganem, G., (**In preparation**) Detection of volatile organic compounds from preclinical lung cancer mouse models, *Journal of Chemical Ecology*.
- **Article 5: Gouzerh, F.**, Buatois, B., Hervé, M. R., Mancini, M., Maraver, A., Dormont, L., Thomas, F. & Ganem, G., (**In preparation**). Urinary VOCs as Biomarkers of lung cancer in Mice.

Articles publiés en parallèle de la thèse :

- Proffit, M., Lapeyre, B., Buatois, B., Deng, X., Arnal, P., **Gouzerh, F.**, ... & Hossaert-McKey, M. (2020). Chemical signal is in the blend: bases of plant pollinator encounter in a highly specialized interaction. *Scientific Report*. <https://doi.org/10.1038/s41598-020-66655-w>
- Thomas, F., Giraudeau, M., **Gouzerh, F.**, Boutry, J., ... & Ujvari, B. (2019). The evolution of resistance and tolerance as cancer defences. *Parasitology* 1–8. <https://doi.org/10.1017/S0031182019001501>
- Thomas, F., Giraudeau, M., Dheilly, N.M., **Gouzerh, F.**, ... & Ujvari, B. (2020). Rare and unique adaptations to cancer in domesticated species: An untapped resource? *Evolutionary applications*. <https://doi.org/10.1111/eva.12920>

Communications orales :

- Poster intitulé « Odours of cancerous mouse congeners: detection and attractiveness » lors de la « 6th international mouse meeting » le 21-23 juin 2022 à Montpellier
- « Detection and preference of mice for smells of cancerous congeners » 15th Meeting on Chemical Signals in Vertebrates 5 Novembre 2021. On line Conference
- « Conséquences écologiques, évolutives et appliquées des modifications d'odeurs corporelles associées au cancer » 9 janvier 2019. Séminaire MIVEGEC.
- « Recherche de COVs biomarqueurs précoces de cancer ». GDR d'écologie chimique MEDIATEC 2018. Rennes, France.

Enseignement et vulgarisation scientifique :

- Mission complémentaire d'enseignement au sein de l'université Paul Valéry, TD de biologie à des étudiants en licence 1^{er} année de psychologie (2019, 24h de TD).
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~ Liste des abréviations ~

NC : Non Cancéreuses

CC : Cancéreuses

COVs : Composés Organiques Volatils

SCLC : carcinomes pulmonaires à petites cellules

NSCLC : carcinome pulmonaire non à petites cellules

SPME : Solid Phase MicroExtraction

GC-MS: Chromatographie Gazeuse –Spectrometrie de Masse

MUP : Protéine Urinaire Majeure

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~ Introduction ~

I) Les composés organiques volatils dans la recherche scientifique

1) Généralités sur les COVs

Les composés organiques volatils, aussi appelés COVs, sont des molécules caractérisées par une grande volatilité qui leur permet de se répandre aisément dans l'environnement. Ils représentent toutes les substances organiques ayant une pression de vapeur égale ou supérieure à 0,01kPa à une température de 20°C. Ils concernent tous les composés autres que le méthane qui contiennent au moins un atome de carbone et un ou plusieurs autres éléments tels que l'hydrogène, l'oxygène, les halogènes, le soufre ou encore le phosphore. Ces composés représentent une multitude de substances qui peuvent être d'origine biogénique (naturels) ou anthropique (humaine). Ces derniers sont issus de toutes les activités humaines tels que le trafic routier, l'industrie chimique ou encore l'agriculture. Les COVs d'origine biogénique, quant à eux, proviennent des surfaces marines, des superficies terrestres, de la végétation ou encore des animaux (Cicolella, 2008).

Tous les animaux émettent des COVs et l'Homme n'y échappe pas. De nombreuses études se sont penchées sur les différentes odeurs corporelles émises par l'Homme (Shirasu & Touhara, 2011; Amann et al., 2014; Costello et al., 2014). Ces odeurs corporelles sont le résultat de la combinaison de centaines de COVs odorants émis et qui sont sécrétés à la fois par nos propres cellules et par les cellules de notre microbiote¹, composé en grandes parties de bactéries (Taylor et al., 2003; James et al., 2004; Natsch et al., 2006; Verhulst et al., 2010). Les principales sources de COVs sont la respiration, la peau, les fèces, l'urine, la salive ou encore le sang (Shirasu & Touhara, 2011; Amann et al., 2014). En considérant toutes ces sources possibles d'émanation de COVs, plus de 2000 composés ont été identifiés, émanant d'individus sains (Costello et al., 2014). La quantité de composés émis par ces différentes sources d'odeurs n'est pas équivalente d'une source à l'autre (Broza et al., 2015) (Figure 1).

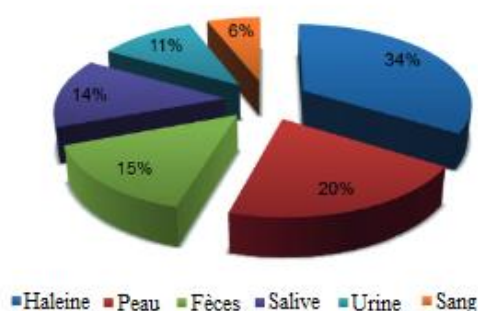


Figure 1: Graphique adapté de Broza et al 2015 sur le pourcentage de COVs dans les différents fluides corporels d'Homme en bonne santé. Au total, 2577 composés ont été mesurés.

¹ Le microbiote est l'ensemble des micro-organismes, bactéries, virus, parasites et champignons non pathogènes qui vivent au sein d'un organisme dit hôte, tel que l'Homme. Il existe différents microbiotes ; au niveau de la peau, de la bouche, intestinaux, du poumon ...

D'autre part, il existe un grand nombre de familles différentes de composés. Ces composés ne proviennent pas tous des mêmes familles en fonction de la source d'émission considérée (Figure 2). On observe une forte variabilité interindividuelle de ces composés (Amann et al., 2014) que ce soit en comparant une source d'émission telle que le souffle entre individus mais aussi entre toutes ces sources (Costello et al., 2014). Pour chaque source d'émission, on retrouve les mêmes familles de composés tels que des hydrocarbures, des alcools, des cétones ou encore des aldéhydes qui sont caractérisés par des groupements de molécules différentes (Tableau1). Différentes familles de composés se retrouvent aussi bien chez l'Homme que chez les autres animaux.

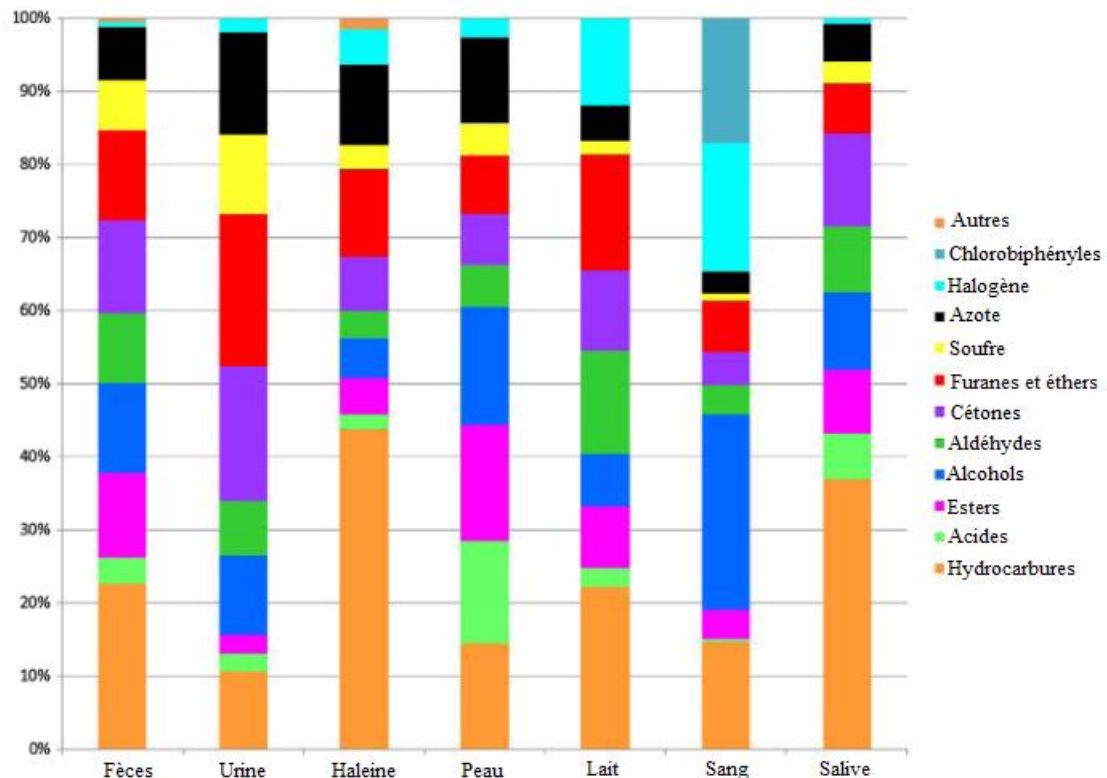


Figure 2: Graphique d'Amann et al 2014 représentant le nombre relatif de composés de chaque famille de molécules détectées chez l'Homme dans différents compartiments.

Tableau 1: Formule chimique des familles de composés organiques.

Classe	Formules
Aldéhydes	R-CHO
Cétones	R-CO-R'
Acides	R-COOH
Alcools	$C_nH_{2n+1}OH$
Halogènes	R-X avec X soit F, Cl, Br ou I
Azote	N
Soufre	S
Furanes	C_4H_4O
Esters	R-COO-R'
Hydrocarbure	C_nH_m , n et m étant deux nombres entiers naturels non nuls.

Tous ces composés sont issus de 3 grandes familles de COVs qui correspondent à 3 grandes voies de biosynthèse :

- **Les terpènes** : cette famille de composés, dits terpénoïdes ou isoprénoïdes, est formée à partir de l'association d'au moins deux unités à 5 atomes de carbones. Les terpènes sont essentiellement produits par le métabolisme végétal, et sont souvent impliqués dans les propriétés odorantes des plantes. Il existe très peu d'exemples de sources de terpènes d'origine animale.
- **Les composés shikimiques ou aromatiques** : ce sont des molécules formées à partir d'un cycle benzénique et dérivées de l'acide shikimique. Ces composés sont présents uniquement chez les plantes, les organismes marins et les micro-organismes.
- **Les dérivés d'acides gras** : Ce sont des acides carboxyliques à longue chaîne carbonée. On trouve les acides gras saturés qui n'ont pas de double liaison. Ils sont produits aussi bien par les végétaux, les animaux, que les champignons et bactéries. Concernant les acides gras insaturés ils possèdent une ou plusieurs doubles liaisons. Ils sont rencontrés en grande quantité chez les végétaux ainsi que chez les poissons.

On retrouve donc chez les odeurs issues d'animaux en grande majorité des dérivés lipidiques et dans de très rares cas des composés terpéniques. Tous ces composés varient entre chaque individu mais aussi au sein même d'un individu, au cours du temps en fonction de différents paramètres telle que l'âge, la nourriture ou encore l'état de santé des individus (Shirasu & Touhara, 2011).

2) Technique de prélèvement et d'analyse des COVs

a) Technique de prélèvement d'odeurs

Il existe différentes techniques de piégeage des COVs. L'une des techniques couramment utilisées, est une technique de thermodésorption, la micro-extraction en phase solide, ou SPME. Elle a été développée à l'origine pour la surveillance des polluants atmosphériques. Elle a ensuite été développée pour l'analyse d'échantillons environnementaux, alimentaires, aromatiques ou encore dans le domaine médical (Pawliszyn, 1999).

La SPME, (solid phase micro extraction) a été développée en 1995 par Zhang et Pawliszyn (Zhang & Pawliszyn, 1995). Cette technique peut être utilisée de manière directe dans le contexte des analyses de l'air, des gaz ou encore des liquides, ainsi qu'à partir d'un espace de tête². La SPME est une technique dite passive, ce qui signifie qu'elle n'a pas besoin de systèmes dynamiques comme une pompe par exemple pour entraîner les composés dans un flux d'air. D'autre part, elle ne nécessite pas d'extraction par solvant avant d'être analysée par chromatographie en phase gazeuse. Elle permet ainsi des échantillonnages et une extraction facile sur site. De ce fait, cette procédure d'échantillonnage provoque des perturbations minimales pour l'échantillon analysé, car aucun liquide n'interfère avec les composés (Pawliszyn, 2003). Pour toutes ces raisons, la technique des fibres SPME est considérée simple d'utilisation, efficace, sensible, et idéalement adaptée à l'étude des COVs émis par les organismes vivants (Musteata & Pawliszyn, 2007; Hashemi, Zohrabi, & Shamsipur, 2018). Seule cette technique a été utilisée au cours de cette thèse, pour toutes les raisons d'ordre pratique et d'efficacité évoquées précédemment, ainsi que par son utilisation généralisée dans le domaine des odeurs humaines liées aux maladies, ce qui permet une meilleure comparaison des composés détectés entre publications.

La technique des fibres SPME consiste en l'extraction de COVs à l'aide d'une fibre ayant un revêtement de polymère adsorbant (couche de quelques dizaines de micromètres d'épaisseur) qui coulisse à l'intérieur d'une aiguille creuse à la manière d'une seringue. Lorsque la fibre est au repos (non utilisée ou entre deux utilisations), elle est rentrée à l'intérieur de l'aiguille (Figure 3). Différentes phases d'adsorption des COVs sont commercialisées ; dans notre étude nous utilisons exclusivement des fibres ayant une phase composée de polydiméthylsiloxane –divinylbenzène (PDMS-DVB), qui est la plus polyvalente et possède un spectre d'adsorption très large.

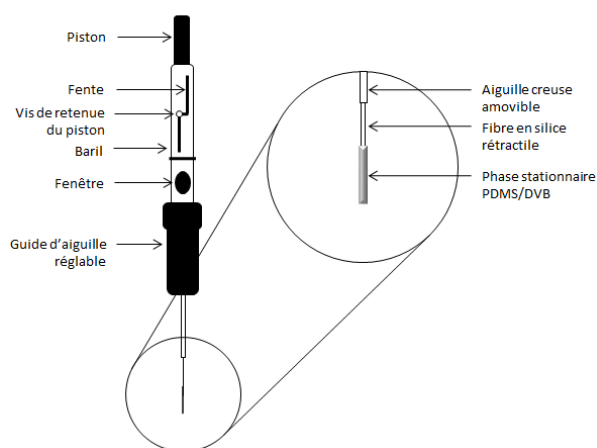


Figure 3: Représentation d'une fibre SPME.

² L'espace de tête (headspace) est l'espace de gaz qui contient les émissions volatiles à proximité immédiate de l'échantillon liquide ou solide dans un flacon.

Lors d'un prélèvement d'odeur, la fibre va soit être mise directement au contact de l'échantillon ou bien en espace de tête.

b) Technique d'analyse des COVs

La GC-MS, chromatographie en phase gazeuse couplée à un spectromètre de masse, est la technique la plus utilisée dans les analyses d'odeurs humaine ou animale. C'est la raison pour laquelle dans notre étude nous avons décidé d'utiliser uniquement cette technique. La GC-MS consiste d'une part à séparer les différents composés contenus dans l'échantillon avec le GC. Pour cela, à l'aide d'un gaz inerte (hélium par exemple), les composés vont être transportés à l'intérieur d'une colonne capillaire tandis que la température du four dans laquelle se trouve la colonne, va augmenter. Les composés les plus volatils vont atteindre plus rapidement l'extrémité de la colonne tandis que les composés ayant un plus gros poids moléculaire vont prendre plus de temps à parcourir la colonne. La GC va donc séparer les composés du mélange chimique et ensuite grâce à la MS, spectrométrie de masse couplée au GC, nous allons pouvoir identifier les composés. Ces derniers vont être fragmentés par ionisation et en traversant le champ électrique, ils vont pouvoir être analysés par leur charge et leur masse en fonction de leur sortie sur la colonne. Nous pourrons ensuite visualiser ce qu'on appelle un chromatogramme (Figure 4), qui nous permet de visualiser chaque molécule présente dans un échantillon ainsi que son abondance relative dans le mélange. Pour chaque molécule présente (chaque pic sur le chromatogramme), un spectre de masse est associé, qui permet de reconstituer l'identité de la molécule à partir des fragments présents.

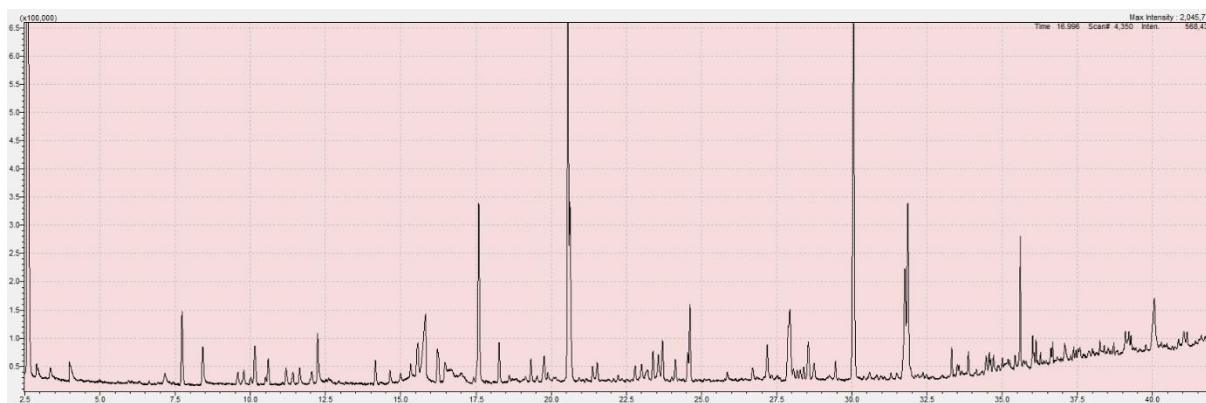


Figure 4: Chromatogramme de souris. Les différents pics correspondent chacun à un composé différent, avec en abscisse le temps de rétention qui correspond au temps de sortie du composé le long de la colonne, et en ordonnée son abondance relative.

3) *Utilisation des COVs dans le contexte des maladies*

La première utilisation connue des odeurs pour identifier des maladies remonte à l'antiquité. La première personne à avoir établi un lien entre les maladies et des différences de COV émis fut le médecin grec Hippocrate. Il se basait sur l'haleine humaine pour établir un diagnostic de l'état de santé des patients. Il avait alors décrit le fotor oris « mauvaise » haleine, qu'il décrivait chez les patients en mauvaise santé et parmi ces malades, les maladies hépatiques reconnaissables grâce à ce qu'il appelait le fotor hepaticus (Buljubasic & Buchbauer, 2015).

Aujourd'hui, l'utilisation des COVs comme biomarqueurs est classique, l'exemple le plus connu est sans doute l'utilisation des éthylotests pour quantifier le taux d'alcool dans l'haleine. Au cours de ce test, on mesure le taux d'éthanol qui réagit avec d'autres composés pour indiquer le taux d'alcool. L'éthanol est un composé volatil dans l'haleine ce qui permet de le détecter facilement. L'intérêt des COVs pour détecter des maladies a augmenté depuis le début du 20ème siècle, avec de nombreuses études sur diverses pathologies (Shirasu & Touhara, 2011), comme par exemple des maladies d'origines infectieuses telles que le paludisme (Lacroix et al., 2005) ou la tuberculose (Syhre & Chambers, 2008). Dans ces situations, les modifications d'odeurs peuvent s'expliquer par deux phénomènes non mutuellement exclusifs : i) l'émission directe de signaux émis par les agents infectieux présents dans l'organisme ou bien ii) un effet indirect, par le biais des modifications du métabolisme ou encore de la composition microbienne de l'hôte, entraînant des changements dans le profil olfactif de l'hôte. Les maladies chroniques inflammatoires comme l'asthme (Ibrahim et al., 2011), la polyarthrite rhumatoïde (Humad et al., 2009) ou encore les maladies inflammatoires de l'intestin (Kokoszka et al., 1993) peuvent aussi amener à des modifications d'odeur. D'autres maladies chroniques telles que le diabète (Phillips et al., 2004) peuvent aussi modifier les COVs émis par l'individu malade. Dans le cas du diabète, les patients ne sont plus capables de synthétiser l'insuline qui est une hormone fabriquée par le pancréas et qui signale aux cellules d'absorber le glucose. Les patients atteints de diabète de type 1 ne pouvant donc pas synthétiser l'insuline, voient leur glucose s'accumuler dans le sang et être excrété dans l'urine. Dans ces conditions, les cellules vont utiliser des acides gras plutôt que du glucose comme source d'énergie. Le catabolisme des acides gras va créer des composés cétoniques, tels que l'acétoacétate, le 3-hydroxybutyrate et l'acétone. Ces cétones en excès vont être sécrétées dans l'urine et l'haleine et vont ainsi modifier l'odeur émise par l'urine et par l'haleine du patient (Rooth & Ostenson, 1966). Pour les maladies liées à des troubles métaboliques, telles que des carences enzymatiques ou des défauts de transport entraînant l'accumulation de métabolites particuliers dans les fluides corporels (en raison d'une altération des voies biochimiques), on observe souvent des odeurs spécifiques liées à l'accumulation de certains métabolites dans le corps (Shirasu & Touhara, 2011). Parmi les maladies modifiant les odeurs émises par un individu malade, on retrouve les maladies cancéreuses.

II) Généralités sur le cancer

1) Définition et origine du cancer

Le terme cancer, désigne une famille de maladies propres aux organismes multicellulaires depuis leur apparition, il y a environ un demi-milliard d'années, caractérisée par la prolifération anarchique de certaines cellules constitutives du corps suite à des mutations génétiques et/ou épigénétiques. Les cellules normales et cancéreuses, diffèrent par plusieurs critères qui correspondent à l'acquisition successive d'au moins dix étapes caractérisant la malignité (nommées hallmarks) décrites par Hanahan et Weinberg 2011 (Hanahan & Weinberg, 2011). L'accumulation et la structuration de ces cellules mutées conduisent à des amas fonctionnels de cellules que l'on appelle des tumeurs. Dans le cas des tumeurs malignes, les cellules tumorales envahissent les tissus environnants et se disséminent via la circulation sanguine ou lymphatique. Ces cellules tumorales circulantes peuvent établir des foyers cancéreux secondaires, appelés métastases, dans d'autres organes du corps. Les métastases sont généralement la cause de 90% de la mortalité liée au cancer (Chaffer & Weinberg, 2011; Dillekås, Rogers, & Straume, 2019). La tumorigenèse³, depuis les lésions précancéreuses jusqu'aux stades métastatiques (i.e. cancer généralisé), ainsi que ses multiples manifestations (néo-angiogenèse, évasion immunitaire, résistance aux thérapies) suivent un processus Darwinien (Cairns, 1975; Ujvari Beata, Roche Benjamin, & Thomas Frédéric, 2017). Les processus cancéreux concernent l'ensemble de l'arbre du vivant multicellulaire actuel ou passé, des hydres aux baleines (Albuquerque et al., 2018), en passant par les dinosaures du Jurassique (Rothschild et al., 1998; DumbrĂ et al., 2016). Chez l'Homme le cancer fait partie des maladies les plus courantes et les plus mortelles. De ce fait, de nombreuses études voient le jour chaque année, que ce soit pour soigner la maladie mais aussi dans un cadre de prévention et de détection précoce du cancer.

2) Prévention et détection précoce du cancer

Les maladies cancéreuses, figurent en deuxième place après les maladies cardiovasculaires comme causes de morbidité et de mortalité dans le monde. En 2018, les cancers représentaient 18,1% des décès chez l'Homme soit 9,6 millions de décès avec environ 14 millions de nouveaux cas (Bray et al., 2018; Sung et al., 2021). Le cancer au sens large fait référence à plus de 200 types différents de maladies cancéreuses chez l'Homme (Stratton, Campbell, & Futreal, 2009; Gatta et al., 2011; Hassanpour & Dehghani, 2017). Elles peuvent être classées en 2 catégories, les tumeurs dites solides

³ La tumorigenèse englobe l'ensemble des étapes menant à la formation des tumeurs.

qui vont toucher un tissu/un organe, elles sont capables d'évoluer en cancers métastatiques. Elles vont disséminer via la circulation sanguine des cellules cancéreuses qui vont ensuite coloniser d'autres organes/tissus, ce qui est le cas du cancer du poumon, du sein ou encore du cancer colorectal pour les cancers les plus connus. A l'opposé, on retrouve des tumeurs dites liquides dont l'origine est hématopoïétique⁴ et pour lesquelles les cellules cancéreuses circulent dans l'organisme via le système sanguin et lymphatique, on peut citer les leucémies (cancer dans le sang et la moelle osseuse), les lymphomes (cancers dans les organes lymphoïdes) ou encore les myélomes (cancers de la moelle osseuse).

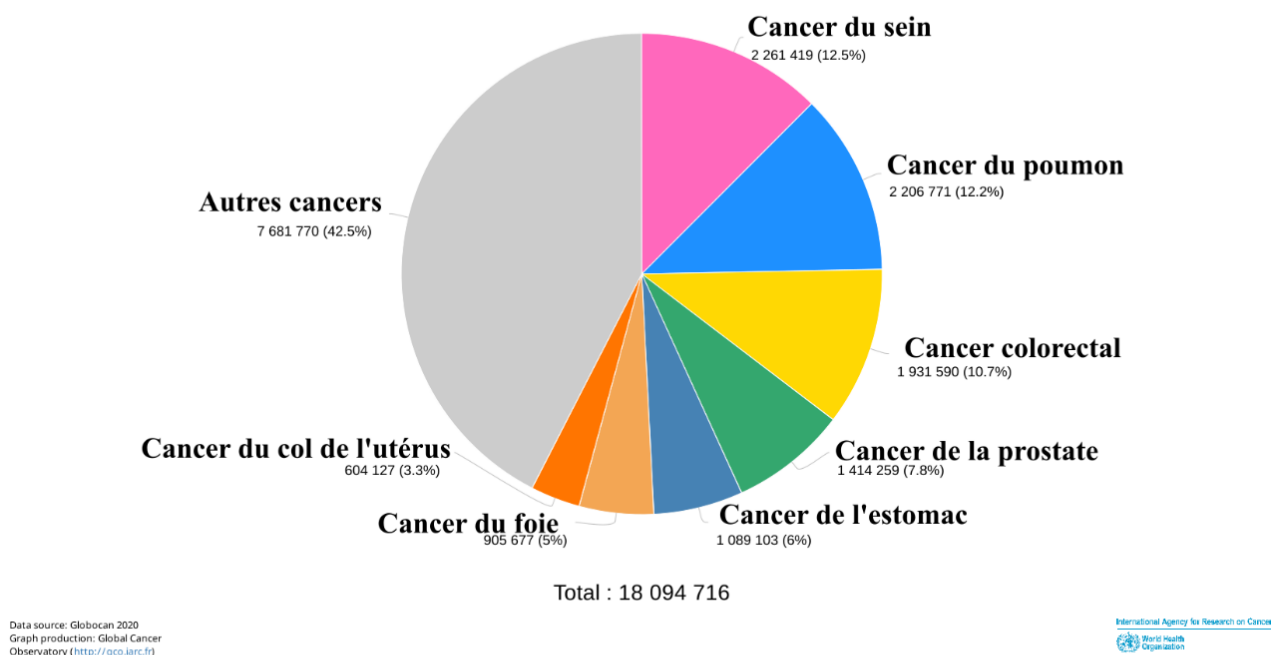
Ces différents cancers ont des origines et des facteurs de risques divers. 90% d'entre eux sont considérés comme attribuables à des facteurs de risques évitables (Islami et al., 2018; Morze et al., 2020), avec le tabagisme (Alexandrov et al., 2016) comme facteur premier. Vient ensuite l'obésité, suivi de la consommation d'alcool (Connor, 2017) et l'exposition au soleil (Nair-Shalliker et al., 2012). Les agents pathogènes, comme pour le cancer du col de l'utérus (Olusola et al., 2019) représentent à eux seuls presque 20% des cancers (de Martel et al., 2012). Les causes peuvent aussi être dans une moindre proportion d'ordre génétique (Joo et al., 2018). Parmi les maladies cancéreuses, celle touchant le poumon, est l'une des principales causes de décès en Europe et dans le monde occidental. Cette forte mortalité est en partie due à un diagnostic souvent trop tardif, du fait de mauvaises méthodes de diagnostic qui ne sont pas assez sensibles et spécifiques (Bajtarevic et al., 2009).

⁴ L'hématopoïèse est le processus physiologique de production des cellules sanguines dans l'organisme.

- **Cas du cancer du poumon**

Le cancer du poumon est le second cancer le plus fréquent après le cancer du sein, sur l'ensemble de la population mondiale, mais il représente le plus grand nombre de décès en 2020 avec plus de 2,2 million de nouveaux cas et 1,8 millions de morts dans le monde en 2020 (Sung et al., 2021; Ferlay et al., 2021) (Figure 5).

Estimation du nombre de cas en 2020 dans le monde (age et sexes ensemble)



Estimation du nombre de morts en 2020 dans le monde (age et sexes ensemble)

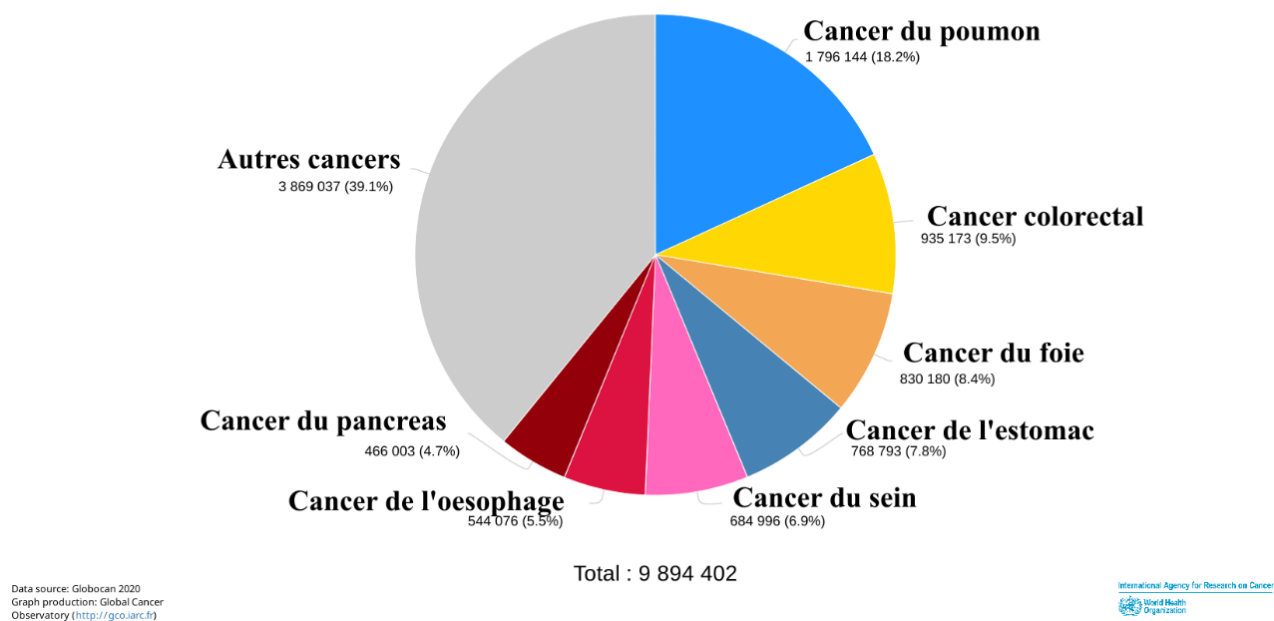
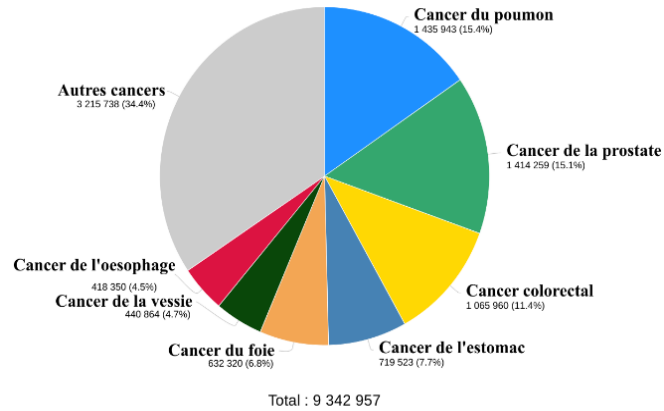


Figure 5: Figure adaptée des données de Globocan 2020 sur le taux d'incidence et le taux de mortalité des cancers les plus courants sur l'ensemble de la population mondiale.

A

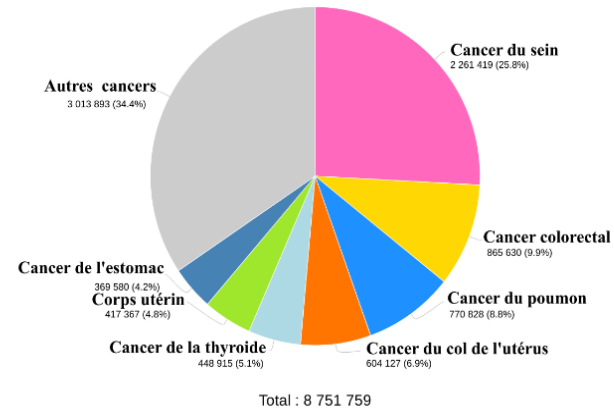
Estimation du nombre de nouveaux cas en 2020 dans le monde, chez les hommes



Data source: Globocan 2020
Graph production: Global Cancer Observatory (<http://gco.iarc.fr>)

B

Estimation du nombre de nouveaux cas en 2020 dans le monde, chez les femmes

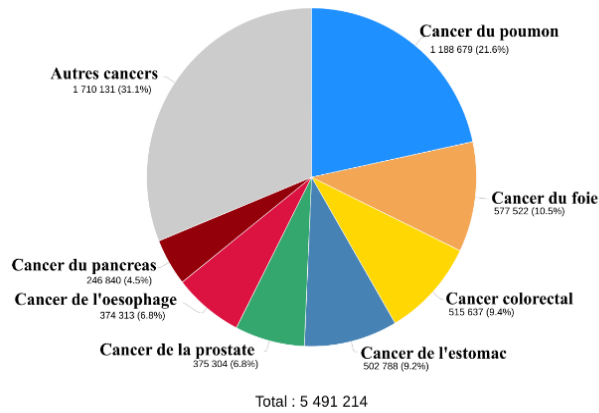


International Agency for Research on Cancer
World Health Organization

Data source: Globocan 2020
Graph production: Global Cancer Observatory (<http://gco.iarc.fr>)

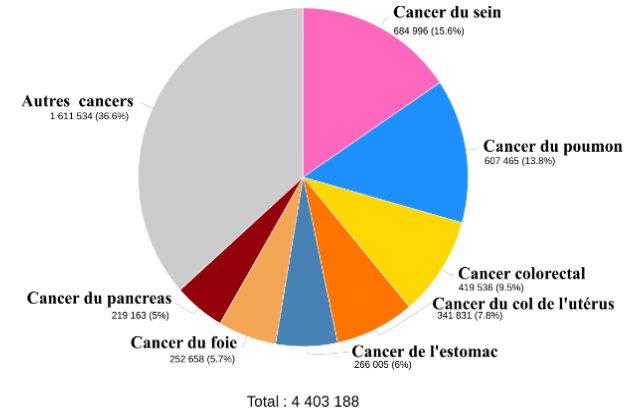
International Agency for Research on Cancer
World Health Organization

Estimation du nombre de morts en 2020 dans le monde, chez les hommes



Data source: Globocan 2020
Graph production: Global Cancer Observatory (<http://gco.iarc.fr>)

Estimation du nombre de morts en 2020 dans le monde, chez les femmes



International Agency for Research on Cancer
World Health Organization

Data source: Globocan 2020
Graph production: Global Cancer Observatory (<http://gco.iarc.fr>)

International Agency for Research on Cancer
World Health Organization

Figure 6: Figure adaptée des données de Globocan 2020 sur les taux d'incidence des cancers et leurs taux de mortalité associée dans le monde chez A) l'homme et B) la femme.

Chez l'homme il représente la 1^{ère} cause de morbidité et de mortalité, alors que chez la femme il se classe 3^{ème} pour l'incidence après le cancer du sein et le cancer colorectal, et au deuxième rang pour la mortalité après le cancer du sein (Figure 6). Actuellement la survie à 5 ans des patients atteints d'un cancer du poumon n'est que de 10 à 20%.

Le cancer du poumon comprend différents types cliniques de pathologies. Les principaux types de cancer du poumon sont le carcinome pulmonaire à petites cellules (SCLC) qui représente 15% des cancers du poumon et le carcinome pulmonaire non à petites cellules (NSCLC) qui en représente 85%. Ce deuxième type de cancer est divisé en trois grands groupes histologiques :

- l'adénocarcinome représentant 40 à 60% des cas
- le carcinome épidermoïde représentant 30-40% des cas
- le carcinome à grandes cellules représentant 10% des cas

Ces différents types de cancers du poumon sont liés à différentes mutations dans le génome lesquelles conduisent à l'activation de gènes suppresseurs de tumeurs et à des défauts au niveau des gènes de régulation. En fonction du type de cancer, ce ne sont pas nécessairement les mêmes gènes qui sont mutés et qui entraînent la tumorigenèse. Dans notre étude nous nous intéresserons au cancer du poumon qui touche le plus fréquemment l'Homme, l'adénocarcinome pulmonaire.

3) Utilisation des odeurs dans la détection précoce du cancer chez l'Homme

Comme indiqué précédemment, les maladies cancéreuses font aussi l'objet d'études utilisant les odeurs dans le but d'identifier des biomarqueurs précoces de la tumorigenèse. Plusieurs hypothèses ont été émises pour expliquer comment le développement des tumeurs peut modifier les émissions d'odeurs corporelles.

- i) Les modifications génétiques qui sont associées à la tumeur peuvent conduire à des changements qualitatifs ou quantitatifs des COVs émis dans le micro-environnement de la cellule, et par conséquent des fluides corporels émis par l'individu (Davies et al., 2014; Ruzsanyi et al., 2014). Les dommages au niveau de l'ADN chez les cellules cancéreuses peuvent changer l'information génétique et entraîner une absence de fabrication de certaines protéines. Une protéine conduisant à la formation de COVs, altérée ou produite dans des concentrations différentes, peut entraîner un changement dans la composition ou dans la concentration de ces COVs émis (Broza et al., 2015) (Figure 7).

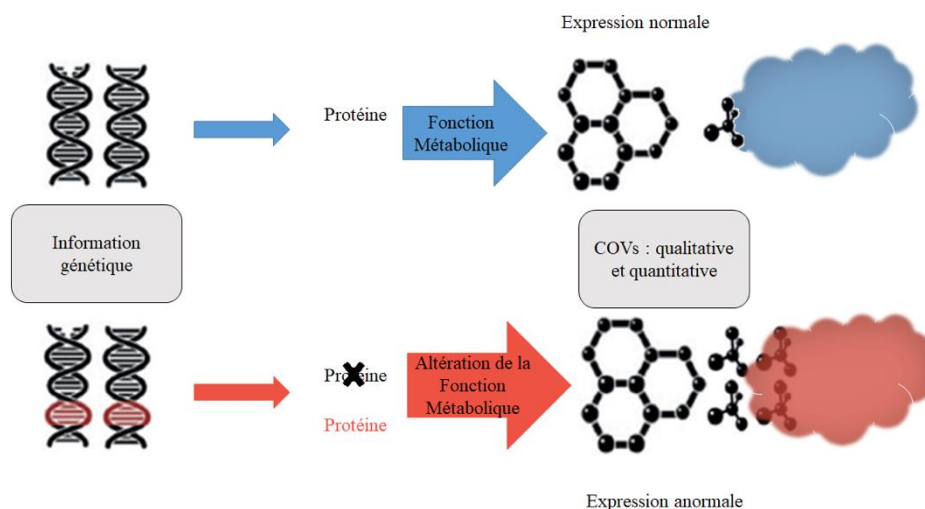


Figure 7: Figure tirée de Broza et al., 2015 représentant le processus en aval de l'altération génétique qui peut affecter la production de COV.

- ii) L'une des principales causes du cancer est le stress oxydant appelé aussi stress oxydatif (Gill, Piskounova, & Morrison, 2016; Hayes, Dinkova-Kostova, & Tew, 2020). Ce stress est lié à un déséquilibre entre la quantité excessive de radicaux libres et des antioxydants. Lors de ce stress oxydatif les formes réactives de l'oxygène vont sortir des mitochondries, et les acides gras polysaturés peroxydés présents dans les membranes cellulaires vont quant à elle générer des alcanes volatils et des alcanes méthylés qui vont être excrétés dans l'haleine (López-Jaén, Codoñer-Franch, & Valls-Bellés, 2013). Ainsi, lors de la formation d'un cancer induit par un stress oxydatif, les modifications d'odeurs peuvent provenir de nouveaux COVs générés par le stress oxydatif, ce qui va modifier la composition chimique de l'haleine. Ce stress peut être induit par divers stress cellulaires biotiques et abiotiques tels que des agents pathogènes, des rayonnements UV, la pollution, les pesticides etc...
- iii) De nombreux COVs sont émis par des bactéries symbiotiques provenant de l'intérieur et de la surface de notre corps. Elles sont susceptibles de modifier chimiquement des composés produits par notre métabolisme. Lorsque la prolifération de cellules cancéreuses entraîne des modifications du microbiome (Cullin et al., 2021), il peut alors se mettre en place une relation (indirecte) entre la présence de tumeurs et les émissions d'odeurs corporelles.
- iv) Dans le cadre des cancers ayant une origine infectieuse, la modification du microbiome (et des odeurs corporelles) peut aussi être due à la présence du pathogène oncogène (Cullin et al., 2021). Il est admis qu'au moins 15 à 20% de cancers ont une origine infectieuse (de Martel et al., 2012). On peut citer le virus d'Epstein-Barr (EBV) qui peut provoquer l'apparition de lymphomes et de cancer du nasopharynx, le virus du papillome humain (VPH, sous types 16 et 18) qui peut

induire le cancer du col de l'utérus, ou encore la bactérie *Helicobacter pylori* qui est responsable d'un nombre significatif de cancers de l'estomac (IARC, 2009; Cullin et al., 2021).

Au vu des études déjà menées sur le sujet, il semblerait que ces différentes hypothèses sont susceptibles d'être impliquées dans les modifications d'odeurs liées au cancer. Par exemples, certaines études ont montré que des cultures cellulaires de cellules cancéreuses présentent des changements d'odeurs spécifiques comparés aux cellules saines, ce qui validerait l'hypothèse que ce sont les cellules cancéreuses elles-mêmes qui sont à l'origine de signatures olfactives particulières des cancers (Peled et al., 2013; Davies et al., 2014; Silva et al., 2017). Toutefois, les modifications d'odeurs chez un patient cancéreux ne se limitent pas aux COVs directement émis par les cellules cancéreuses, car d'une part ces derniers sont noyés parmi d'autres COVs émis par l'activité normale de l'organisme, et d'autre part les cancers, comme évoqué précédemment, influencent les odeurs corporelles via différents processus. Ainsi, l'analyse globale des odeurs émis par un individu en utilisant des prélèvements d'urine, de fèces ou encore d'air expiré semble la plus appropriée pour prendre en compte toutes les voies de modification possibles induites par l'apparition du cancer.

III) L'olfaction et les animaux

1) L'olfaction dans le règne animal

L'olfaction est un des sens les plus utilisés dans le règne animal. Il est utilisé par de nombreuses espèces des bactéries aux vertébrés (Ache & Young, 2005). Les composés impliqués dans l'olfaction vont être émis soit dans le cadre de la communication intra et interindividuelle, comme par exemple certaines phéromones⁵ (Wyatt, 2010), ou bien émis via le métabolisme des organismes, sans qu'il y ait une valeur adaptative particulière. Ces composés peuvent être des COVs ou bien des composés non volatils comme des protéines, peptides, etc... Ces deux types de molécules contribuent à la communication olfactive. En général, la communication à longue distance va nécessiter des COVs alors que la communication à courte distance ou de contact va plutôt nécessiter un contact direct avec la source de l'odeur (Muller-Schwarze, 2006). L'olfaction chez les animaux est utilisée à de nombreuses occasions au cours de leur vie, que ce soit dans les relations intra ou interspécifiques (Blaustein, 1981; Symonds & Elgar, 2008; Caro & Balthazart, 2010; Woodley, 2010), comme par exemple dans le cadre de la reproduction pour repérer son ou sa partenaire, pour la recherche de nourriture (Provecho & Josens, 2009), ou encore pour délimiter son territoire (Mota-Rojas et al., 2018), etc... Ces capacités sont partagées par un grand nombre d'animaux, que ce soit chez les vertébrés ou les invertébrés (Hildebrand and Shepherd, 1997).

⁵ Les phéromones sont des composés qui sont produits et secrétés par certains animaux et qui entraînent à distance une réponse comportementale d'autres individus de la même espèce.

Les composés olfactifs sont détectés par des structures spéciales, telles que la cavité nasale pour les vertébrés, ou les antennes pour les invertébrés comme les insectes. À la surface de ces structures, les composés olfactifs (tels que les COVs) vont être détectés par des neurones qu'on appelle des récepteurs olfactifs. Ces récepteurs olfactifs vont déclencher des potentiels d'action en réponse à un composé volatil, qui vont permettre de transmettre l'information au système nerveux central de l'individu. Avant de pénétrer dans les centres cérébraux supérieurs, l'information va passer par des glomérules, qui sont les points de contact entre les neurones périphériques et centraux. Chez les insectes, les glomérules se trouvent dans le lobe antennaire, tandis que chez les vertébrés, ils se trouvent dans le bulbe olfactif. Les récepteurs olfactifs peuvent être très spécifiques, comme ceux utilisés pour la détection des phéromones.

Ces capacités olfactives, partagées par un grand nombre d'espèces, peuvent servir également à reconnaître des congénères malades suite à des infections parasitaires, telles que chez les poissons (guppies) (Kennedy et al., 1987), les singes (mandrills) (Poirotte et al., 2017) ou encore chez les souris (Kavaliers and Colwell, 1995 ; Ehman and Scott, 2001; Kavaliers et al., 2003). Cependant cette capacité à détecter et éviter des congénères malades n'a jamais été évaluée dans le domaine du cancer. Le cancer a pour le moment été très souvent étudié sous un prisme médical, mais très peu d'un point de vue évolutif et encore moins sur ses conséquences écologiques, que ce soit dans les relations intra ou interspécifiques. Les quelques études sur le sujet ont tout de même révélé des résultats interpellants. Par exemple, les hydres (cnidaires d'eau douce) porteuses de tumeurs, comparées à des hydres saines, sont d'avantage sujettes à la prédation par des poissons chassant à vue, a priori du fait de leurs tumeurs les rendant plus détectables (Boutry et al., 2022). Les hydres tumorales sont également des hôtes préférentiellement choisis par des ciliés commensaux (e.g. *Kerona pediculus*), lesquels ont une dynamique de prolifération supérieure sur les hôtes hébergeant des tumeurs comparées aux hôtes sains. Concernant les relations intra-spécifiques, des études ont montré que les drosophiles rejoignaient de façon différentielle des congénères sains ou tumoraux, en fonction de leur propre statut de santé (Dawson et al., 2018) . Ces choix influencent en retour la croissance tumorale, celle-ci est en effet diminuée lorsqu'une drosophile tumorale est en présence de conspécifiques tumoraux, et à l'inverse augmentée lorsqu'ils sont sains. En dehors de ces études, aucune à notre connaissance ne s'est penchée sur l'impact du cancer d'un point de vue écologique et notamment l'impact que cela peut avoir d'un point de vue de la reconnaissance olfactive.

2) *Utilisation par l'Homme de l'odorat des animaux.*

Les capacités olfactives poussées de certaines espèces ont à plusieurs reprises été utilisées par l'Homme. Les premières à avoir été utilisées sont les chiens qui comptent plus de 800 récepteurs olfactifs (Niimura & Nei, 2007). Les premières utilisations des chiens l'ont été dans un cadre militaire pour détecter des objets tels que des explosifs, des mines lors de la seconde guerre mondiale ou encore des cadavres (Rebmann, David, & H.Sorg, 2000). D'autres animaux ont été entraînés à détecter des

explosifs, notamment les rats et les souris qui présentent aussi un système olfactif très sensible avec plus de 1300 récepteurs olfactifs (Buck & Axel, 1991; Sakano, 2010). Les rongeurs, pouvant plus facilement que les chiens se faufiler dans des petites zones sans déclencher les explosifs, présentent ainsi des avantages pour détecter des mines ou des explosifs (Marshall et al., 1981; Poling et al., 2011). En plus des explosifs, les chiens et les rongeurs ont été entraînés à détecter des substances telles que des médicaments ou des drogues (cocaïne, héroïne etc.). D'autres animaux tels que les insectes (abeilles, guêpes, drosophiles) ont aussi fait l'objet d'études sur leur capacité à détecter des narcotiques ou des mines (Bindokas, Gauger, & Greenberg, 1988; Bromenshenk et al., 2003; Marshall, Warr, & de Bruyne, 2010; Olson & Rains, 2014). L'aide à la recherche et au sauvetage humain (Fenton, 1992), ainsi que la chasse et l'aide à la conservation d'espèces difficilement détectables dans la nature (Cablk et al., 2008), font aussi partie des utilisations possibles du chien. Depuis les années 90, l'utilisation des animaux a été élargie au domaine médical, pas essentiellement pour détecter des médicaments, mais aussi pour détecter des maladies. Comme mentionné précédemment, de nombreuses études ont permis de montrer qu'un grand nombre de maladies pouvaient modifier les odeurs. A partir de ce constat, des chiens ont été entraînés à détecter des maladies humaines et animales. Chez l'Homme, des maladies chroniques telles que l'épilepsie (Strong & Brown, 2000) ou le diabète (Rooth & Ostenson, 1966), ou des maladies parasitaires (Angle et al., 2016) telles que la malaria (Guest et al., 2019) ou plus récemment le COVID-19 (Jendry et al., 2020; Grandjean et al., 2022) ont fait l'objet d'études convaincantes en utilisant l'odorat du chien. Des maladies animales peuvent aussi être détectées par les chiens, comme par exemple la gale sarcoptique due à un ectoparasite, *Sarcoptes scabiei*, qui infecte les mammifères (Alasaad et al., 2012). Comme expliqué plus haut, les maladies cancéreuses font aussi partie des maladies qui modifient le profil d'odeurs des personnes malades, des études ont donc été menées sur la capacité de certains animaux à pouvoir discriminer les individus cancéreux (voir **Manuscrit II**).

3) Olfaction chez la souris

Chez les mammifères, les composés olfactifs sont détectés grâce à la cavité nasale qui est composée de neurones sensoriels olfactifs. Au sein de la cavité nasale on retrouve deux organes sensoriels distincts, la muqueuse olfactive qui est composée en partie de neurones sensoriels olfactifs qui expriment des récepteurs olfactifs. Le deuxième organe est l'organe voméronasal qui est composé en partie de neurones sensoriels voméronasaux. Ces neurones expriment principalement des récepteurs spécifiques aux phéromones (Rodriguez, 2004). Ces deux organes sensoriels sont connectés à une structure appelée le bulbe olfactif (Figure 8). Ces organes olfactifs permettent une capacité de discrimination vis-à-vis de milliers de produits chimiques volatils, alors perçus comme des odeurs distinctes. Un neurone olfactif est capable de reconnaître une multitude de composés odorants et un composé peut être reconnu par de multiples neurones olfactifs (Malnic et al., 1999). Les souris font

partie des mammifères ayant l'un des systèmes olfactifs les plus développés, avec la capacité de détecter plus de 100 000 composés odorants, grâce à plus de 1300 gènes récepteurs olfactifs (Buck & Axel, 1991; Sakano, 2010). Ce grand nombre de gènes leur permet un codage complexe et quasi illimité, compte tenu de la grande variété de récepteurs olfactifs et d'odeurs existantes. Ainsi leur odorat est doté d'une grande sélectivité⁶ et d'une très grande sensibilité⁷.

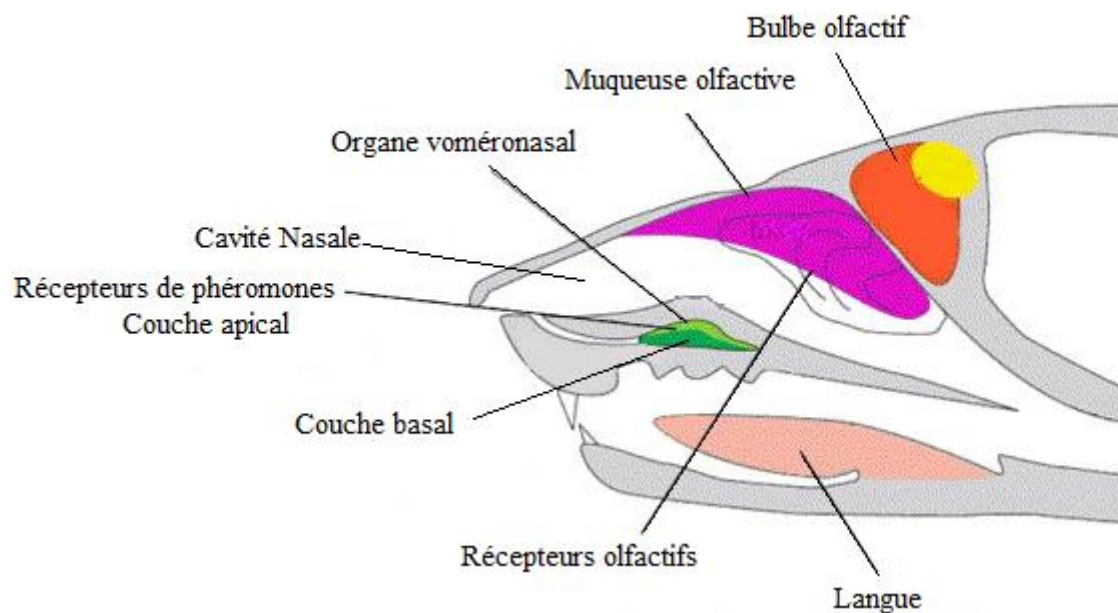


Figure 8: Représentation générale du système olfactif de la souris en vue sagittale adapté de Rouquier et al.2007.

Cette capacité olfactive, chez les rongeurs et notamment la souris, joue un rôle primordial dans les relations intra-spécifiques, que ce soit dans les relations parentales (Noirot, 1969) ou dans la communication entre adultes (Mota-Rojas et al., 2018). La plupart des sources d'odeurs utilisées dans la communication entre les souris proviennent des urines, des glandes préputiales, de la glande coagulante et des glandes plantaires. De nombreuses études ont montré le rôle des signaux chimiques émis par les souris sur la physiologie ou le comportement de reproduction. Parmi ces composés, on retrouve des phéromones qui peuvent jouer un rôle sur le cycle d'œstrus de la femelle, tel que le farnesene (Novotny et al., 1999) ou encore le 2-sec-butyl-dihydrothiazole et le 3,4-dihydro-*exo*-brevicomine qui sont attractifs pour les femelles adultes et augmentent l'agressivité des mâles (Novotny et al., 1985). Cette communication olfactive sert aussi à détecter des congénères malades pour éviter la

⁶ Capacité de bien distinguer et séparer les différents composés.

⁷ Aptitude à réagir à différents stimuli.

contamination, ainsi qu'éviter de se reproduire avec des congénères ayant une faible valeur sélective (Clayton, 1991; Kavaliers & Colwell, 1995; Scott & Ehman, 2002; Hurst, 2009). Comme expliqué précédemment, très peu d'études ont pour le moment étudié l'impact du cancer d'un point de vue écologique et évolutif, par exemple si le développement d'un cancer chez les animaux et notamment la souris, peut avoir un impact sur le comportement des mâles et/ou des femelles. Le cancer est présent dans la nature chez la souris (Andervont & Dunn, 1962; Prejean et al., 1973). Cependant, celui-ci étant très peu étudié en conditions naturelles, il est difficile de savoir quelle est sa prévalence exacte. Le peu d'études ayant été réalisées sur le sujet ont montré que la plupart des cancers chez la souris apparaissent souvent après 1 an. Comme le cancer survient le plus souvent après la phase reproductive, contrairement à de nombreuses maladies parasitaires qui peuvent arriver tout au long de la vie d'un organisme, nous pouvons nous demander si les processus oncogéniques constituent une force de sélection significative sur leurs hôtes pouvant influencer le choix du partenaire sexuel. Les souris présentent-elles des comportements d'évitement vis-à-vis de partenaires cancéreux ? Si oui, est-ce une réponse spécifique au cancer ou bien aux pathologies en général ? En dehors des questions évolutives, les souris ont été utilisées tout comme les chiens pour leur capacité olfactive. Quelques études ont été réalisées sur les souris pour savoir si elles étaient capables de discriminer des individus cancéreux après un apprentissage (voir **Manuscrit II**). Cela a été réalisé sur différents cancers : le mélanome (Kokocinska-kusiak et al., 2020), le cancer du poumon (Matsumura et al., 2010), ou encore le cancer de la vessie (Sato et al., 2017). Sur la base de ces observations préliminaires il est légitime de se demander si les capacités olfactives des souris pourraient significativement aider à la détection des cancers, en les utilisant comme des bio-détecteurs.

IV) Enjeux et objectifs

Le présent travail se place dans un contexte de recherche fondamentale et appliquée. S'il n'est plus à démontrer que le cancer a un impact sur les modifications d'odeurs corporelles, il y a en revanche moins de connaissances quant au moment précis de la tumorigenèse où ces modifications deviennent perceptibles par les animaux. Ces informations sont pourtant cruciales pour déterminer si l'odorat des souris peut servir au diagnostic des cancers dès l'apparition des lésions précancéreuses ou des premiers stades de tumeurs, ainsi que pour préciser l'identité des COVs impliqués. A côté de ce volet à vocation appliquée, ce travail a aussi pour ambition d'apporter des éléments d'informations nouveaux quant au rôle des processus cancéreux dans les interactions inter-individuelles.

L'objectif global de cette thèse est donc de mieux connaître l'impact du cancer sur les modifications d'odeurs corporelles en déterminant le stade à partir duquel on peut détecter la maladie, ainsi que l'influence de ces changements sur les relations intra-spécifiques. En utilisant des souris (*Mus musculus*) comme modèle biologique, j'ai tenté de savoir 1) si nous pouvions utiliser des souris comme

nez pour discriminer des individus cancéreux sans méthode d'apprentissage ou de conditionnement au préalable, 2) si ces odeurs pouvaient influencer les préférences sexuelles des femelles, ainsi que le comportement des mâles. Enfin, dans un cadre médical nous avons voulu savoir 3) à quel stade de développement du cancer nous pouvions observer des modifications de COV, et quelle est l'identité des possibles biomarqueurs potentiellement utilisables chez l'Homme. Les liens entre les différents objectifs de la thèse sont illustrés Figure 9.

Les deux premiers chapitres correspondent à des synthèses bibliographiques sur, 1) l'ensemble des études réalisées sur les modifications d'odeurs induites par le cancer chez l'Homme et 2) les capacités des animaux à détecter le cancer. Le 3^{ème} chapitre présentera brièvement les différents modèles d'études ; le cancer et les souris utilisées dans cette thèse ainsi que les méthodes générales. Le chapitre 4 répond à la fois aux objectifs 1 et 2. Il présente l'utilisation des souris comme nez pour discriminer des individus sains d'individus cancéreux ainsi que l'impact de ces odeurs sur leurs comportements. Je consacrerai le chapitre 5 à l'analyse des litières qui ont servi pour les tests de comportement présentés dans le chapitre 4. Le dernier chapitre répond quant à lui à l'objectif 3 de cette thèse. Il sera consacré à la détermination du moment dans la tumorigenèse où nous pouvons identifier des biomarqueurs de la maladie dans les urines. Pour finir, nous discuterons des perspectives possibles d'un point de vue médical ainsi que des perspectives écologiques et évolutives liées à l'impact des modifications d'odeurs induites par le cancer sur les interactions intra et interspécifiques en milieu naturel.

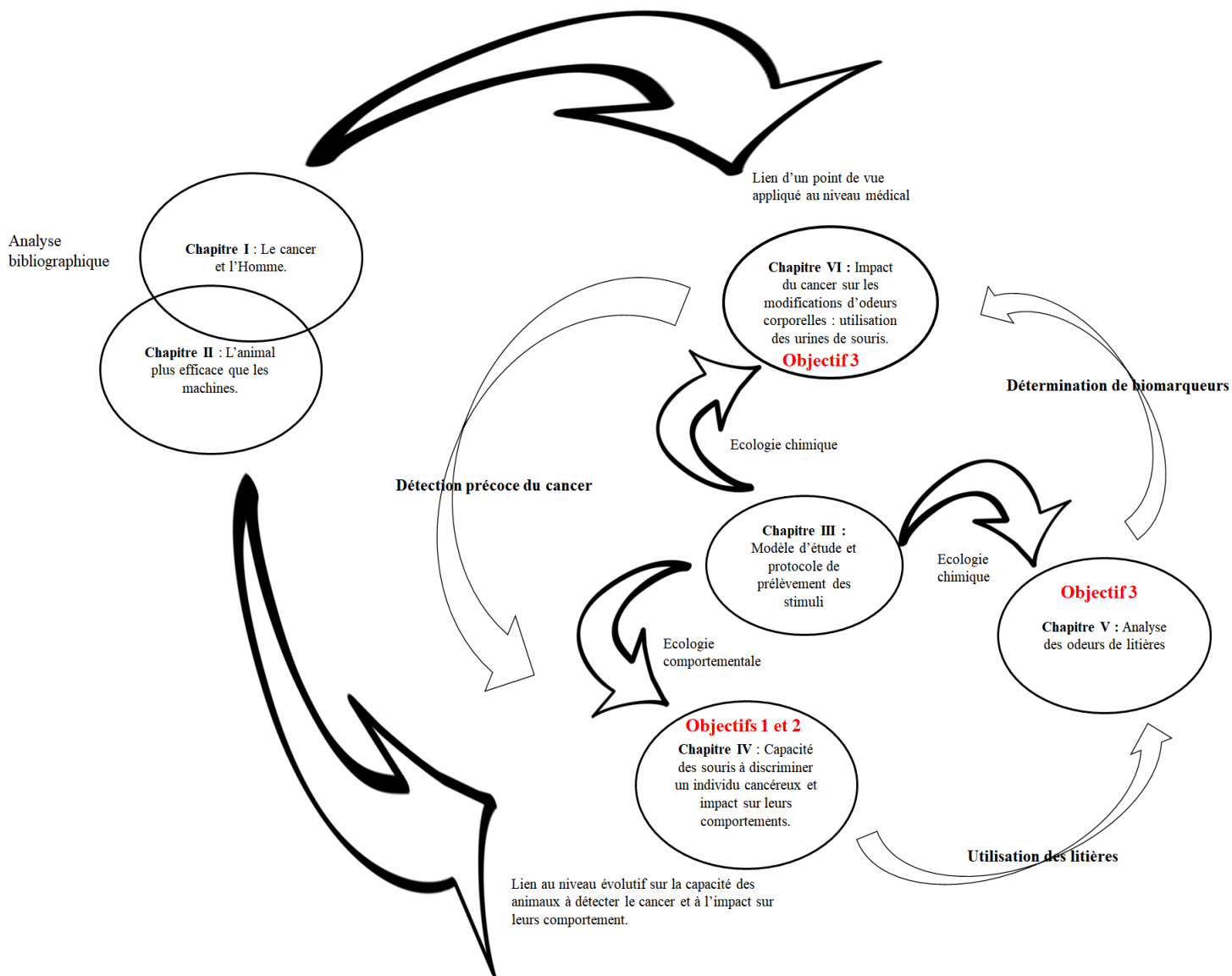


Figure 9: Schéma du plan de déroulement de cette thèse. Chaque cercle représente un chapitre avec les flèches représentant les liens entre chaque chapitre ainsi que les différents objectifs auxquels chaque chapitre a voulu répondre.

~ Chapitre I ~

Les odeurs du cancer chez l'Homme

Contexte et objectifs

Le cancer est la deuxième cause de décès dans le monde comme présenté dans l'introduction. Le traitement et le diagnostic précoce des cancers est un enjeu mondial, d'autant plus que nous savons que les tumeurs détectées à un stade précoce sont plus faciles à traiter. De ce fait, la recherche de biomarqueurs - notamment non invasifs - permettant une détection précoce des tumeurs malignes reste un objectif central pour réduire la mortalité due au cancer. L'utilisation des odeurs pour trouver des biomarqueurs précoces du cancer a fait l'objet de multiples études ces 30 dernières années. L'objectif de ce premier chapitre a été de faire un état des lieux de ce qui avait déjà été réalisé sur l'Homme, et d'essayer de déterminer un ensemble de composés pouvant être retrouvés dans un grand nombre d'études et pouvant potentiellement servir de biomarqueurs du cancer.

Résumé de l'article

Cette synthèse regroupe l'ensemble des publications ayant fait l'objet d'une étude sur des modifications d'odeurs liées au cancer. Au total, 208 publications entre 1984 et 2020 ont été réalisées. Nous avons analysé les principaux résultats de chacune de ces études, en listant et en classant les COVs liés à différents types de cancer tout en considérant à la fois les méthodes d'échantillonnage et les techniques d'analyse. En considérant cette synthèse, nous discutons des défis et des perspectives les plus prometteuses de cette direction de recherche dans la lutte contre le cancer.

Sur l'ensemble de ces publications, 18 cancers différents ont été étudiés en utilisant 9 sources différentes d'émission d'odeurs. Sur les 208 études répertoriées, 142 publications ont identifié des molécules discriminant les patients cancéreux. Plus de 700 composés différents ont été ainsi identifiés, même si un grand nombre de composés ont été identifiés et décrits dans une seule publication. Cette synthèse permet de mettre en évidence les avancées dans la recherche de biomarqueurs en utilisant les COVs mais aussi les lacunes à combler. En effet, malgré les recherches approfondies qui se sont concentrées sur l'identification des COVs en tant que signatures potentielles des cancers, ce sujet n'en est qu'à ses débuts et aucune tendance nette n'existe actuellement. Même si le nombre de composés identifiés ne semble pas converger vers un seul ou un groupe de composés qui permettrait d'identifier à coup sûr le cancer, nous avons pu établir une liste de 28 composés qui sont communs à la plupart des études. Ces molécules se sont avérées être fréquemment présentes dans les émissions volatiles de divers échantillons appartenant à des individus atteints de cancer. Cependant, la nature de ces 28 composés associés au cancer est parfois intrigante. En effet, 12 parmi ces 28 molécules ne sont pas des composés naturels, car il s'agit de composés d'origine exogène qui ne peuvent être produits par aucun organisme vivant. Leur présence dans des échantillons biologiques soulève des questions quant à leur origine, et des hypothèses ou des explications doivent être proposées avant que ces composés puissent être

considérés comme des biomarqueurs volatils potentiellement pertinents. D'autre part, nous avons tout de même pu isoler des composés retrouvés uniquement dans un seul type de cancer. C'est le cas du 1-propanol et du pentane retrouvés uniquement dans les études sur le cancer du poumon et du p-crésol identifié dans deux cancers digestifs, le cancer colorectal et gastrique. Il faudra certainement d'autres études pour confirmer la valeur spécifique potentielle de ces composés en tant que biomarqueurs du cancer.

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Review

Odors and cancer: Current status and future directions

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ABSTRACT

Cancer is the second leading cause of death in the world. Because tumors detected at early stages are easier to treat, the search for biomarkers—especially non-invasive ones—that allow early detection of malignancies remains a central goal to reduce cancer mortality. Cancer, like other pathologies, often alters body odors, and much has been done by scientists over the last few decades to assess the value of volatile organic compounds (VOCs) as signatures of cancers. We present here a quantitative review of 208 studies carried out between 1984 and 2020 that explore VOCs as potential biomarkers of cancers. We analyzed the main findings of these studies, listing and classifying VOCs related to different cancer types while considering both sampling methods and analysis techniques. Considering this synthesis, we discuss several of the challenges and the most promising prospects of this research direction in the war against cancer.

1. Introduction

Cancer is the second leading cause of death in the world, after heart disease, with an estimated nine million deaths in 2020 [1,2]. In 90% of cases, cancer-related deaths are attributable to metastasis, not to primary tumors [3,4]. To reduce mortality in cancer patients, it is therefore important to detect malignancies as early as possible [5], before metastasis and establishment of tumor cells at distant sites. Early diagnosis of cancer has indeed been shown to greatly increase the effectiveness of treatments, which in return reduces mortality and morbidity [6]. However, early cancer detection remains a difficult task for several reasons, a major one being that tumor progression is often an elusive process: patients may display symptoms such as fatigue, weight loss [7], or pain only at already advanced stages of the disease. Thus more than ever, the development of tools and biomarkers for early cancer detection holds tremendous hope for early treatment.

Cancer diagnosis today relies on a variety of clinical settings, including radiography, computerized tomography, magnetic resonance imaging, positron emission tomography, endoscopy, ultrasound, thermography, cytology, and biopsy (reviewed in [8,9]). Apart from these

clinical techniques, there are molecular tools based on both genomic and proteomic signatures of cancer [10]. All of these existing technologies and methods are effective; however, several of them are invasive, expensive, time-consuming, or limited to facilities in large hospitals [11–13]. Thus, there is a great interest in finding biomarkers linked to cancer that could be detected with less invasive and less expensive measures. Since the mid-1980s, increasing attention has been devoted to exploring the relevance of volatile organic compounds (VOCs) emitted by the body as potential biomarkers of cancers.

The study of human smell for detecting diseases is not new. Indeed, the first to have used human scent as sign of disease was Hippocrates, around 400 BCE, who asked his students to smell the breath of their patients as a potential indicator of disease [14]. With advances in analytical techniques such as gas chromatography, many studies on VOCs in humans [15,16] have been carried out with great potential for the detection of biomarkers in many diseases. Volatile organic compounds are emitted from the whole body and can be analyzed from exhaled air, feces, saliva [15], skin [15,17–19], urine [15,20], and blood [21]. Since the beginning of the 20th century, the number of studies linked to variations in VOCs emitted by humans to detect diseases has

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been steadily increasing, whether they concern parasitic diseases such as malaria [22] or chronic diseases [23] such as asthma [24], diabetes [25], or tuberculosis [26], or even to the recently unfolding COVID 19 pandemic [27]. The study of VOCs can also be used in the context of disease diagnosis in both wild animals, e.g., *Mycobacterium bovis* infection in badgers and cattle [28] and domesticated animals, such as respiratory infections of bovine [29]. This allows a less invasive approach in animals, where medical examinations can be highly stressful and where disease detection is mostly done post mortem [30].

Concerning malignant pathologies, several studies published over the last few decades have been conducted on volatile organic compounds emitted by cancer patients in the hope of developing a non-invasive way to detect early cancers [31–33]. These studies yielded several publications for different types of cancers, in particular for lung cancer (for reviews, see [31,34,35]. Different sources of VOCs emission have been considered, such as exhaled air [36,37], urine [38], or even cell culture [39]. Various analysis techniques have been employed, including gas chromatography–mass selective detection (GC-MS) [40,41], proton transfer reaction–mass spectrometry (PTR-MS) [42], and electronic nose techniques [43]. However, most review papers that focused on the qualitative analysis of the topic have not been able to reach a consensus on the number and identity of VOCs that would allow (early) cancer diagnosis [36,44,45]. We therefore decided to conduct an in-depth review of the various approaches and techniques that have so far been employed across the fields of cancer and chemical ecology. By conducting a comprehensive review and meta-analysis of the literature, we quantify the number of compounds that may have a role in cancer detection, taking into account the different sampling and analysis techniques. Finally, we provide some recommendations on how to improve our capacity at identifying relevant VOCs biomarkers for cancer.

2. Materials and methods

2.1. Literature review

Our systematic database review was conducted by searching published articles on PubMed (<https://pubmed.ncbi.nlm.nih.gov/>), Web of Science (www.webofknowledge.com), and Google Scholar (<https://scholar.google.com/>) that contained the following terms: (“cancer” AND “VOC” OR “volatile organic” OR “odor detection”). Much of the information on the generalities of different cancers was found on the American Cancer Society site (<https://www.cancer.org/>). We took into account all the studies that have analyzed the chemical composition of odor profiles from human cancerous patients. The literature included articles published in English from 1984 and until August 2020, excluding systematic reviews, meta-analyses, case report studies such as experimental treatments designed to modify odors (e.g. induced oxidative stress), studies performed on animals, as well as studies that investigated the ability of diverse animals to smell cancer.

For each study, we extracted the following data: type of cancer studied, sample type (e.g., exhaled air, urine...), year of publication, analytical methods, numbers of compounds detected, and the name of the volatile compounds that were shown to be specific to cancer odors. Each compound found in the literature was verified using the National Institute of Standards and Technology (NIST) (<https://www.nist.gov/>) and Pubchem databases (<https://pubchem.ncbi.nlm.nih.gov/>) to ensure that compound annotations were consistent. We categorized publications based on the analytical method used. Gas chromatography–mass

spectrometry (GC-MS) included all the analyses conducted with TD-GC-MS (thermal desorption–gas chromatography–mass spectrometry) and/or GC-TOF-MS (gas chromatography/time-of-flight mass spectrometry). Studies dealing with electronic nose techniques were grouped under the term e-nose [46]. We grouped rarely used techniques in a category called “other”: FT-ICR-MS (nanoelectrospray Fourier transform ion cyclotron resonance mass spectrometry) [47], TD-SPI-MS (thermal desorption–single photon ionization–mass spectrometry) [48], TDLS (tunable diode laser spectrometer) [49], and H-NMR (proton nuclear magnetic resonance) [50].

2.2. Statistical analyses

We used multiple correspondence analysis to investigate the underlying structure of the dataset, which was fully categorical. The aim was to investigate whether the various chemical compounds reported in the literature formed groups based on some categorical variables. This would help to identify experimental conditions in which they would be more likely to be detected. We included the year of publication (expecting that certain molecules would only be detected in recent years due to technological improvement in detection methods), the reference of each paper (to test for potential effects of publications reporting a large number of molecules), the cancer type (expecting different molecules to be observed for different cancers), and the detection methods (expecting different molecules would be detected with different methods). All analyses were carried out with R software v. 4.0.3 [51], and the multiple correspondence analysis was computed using the FactoMineR [52] and factoextra [53] packages.

3. Results

A total of 208 publications were identified as being studies on the characterization of cancer using VOCs (Table 1).

Studies that used e-nose do not include chemical identification of VOCs, so lists of potential compounds that may discriminate between healthy persons and cancer patients were available in 142 of these studies. Among the 208 publications, 13 had not only studied a single cancer but had also compared patients with different types of cancers to healthy patients [89,99,202,205,210,113,139,162,169,180,184,185,187]. Eight other publications used different sampling techniques or different analysis techniques in the same study [44,55,56,58,86,102,107,187].

3.1. Diversity of cancers and methodologies

We found 208 studies that were carried out on different types of cancers with different sampling techniques and different analysis methods. Altogether, 142 publications (70% of the total publications referenced) investigated VOCs emission from different body parts. The first study exploring the potential of VOCs as cancer biomarkers was published in the mid-80s [171]. However, this study focused only on a single compound (emission of methane) in the exhaled air of patients displaying a colorectal cancer. The first investigation that carried out a comprehensive study on a large spectrum of VOCs between healthy individuals and individuals with lung cancer was published in 1985 [76]. There was an increase in the number of publications on the topic during the 2000s, thanks to advances in new technologies for odor sampling and analyses (Fig. 1), yielding an average of 15 publications per year since 2010.

Table 1

Studies on cancer volatiles according to cancer type, sample type, and analysis technique. The numbers in brackets refer to the number of publications for each category.

Type of cancer	Sample	Analysis technique	Reference	
Lung (118)	blood (4)	GC-MS (3)	[54–58]	
		LC-MS (1)	[54]	
	cell culture (17)	GC-MS (11)	[59,60,69,61–68]	
		e-nose (2)	[70,71]	
		PTR-MS (1)	[72]	
	exhaled breath (93)	SIFT-MS (3)	[73–75]	
		GC-MS (48)	[21,40,80–89,44,90–99,56,100–109,57,110–117,58,76–79]	
		e-nose (43)	[32,34,119–128,83,129–138,89,139–148,94,149–151,99,106,108,113,118]	
		GC-IMS (4)	[152–156]	
		Other (3)	[47,49,157,158]	
	pleural effusion sample (2)	PTR-MS (3)	[90,101,159]	
		GC-MS (2)	[160,161]	
	sweat patches (1)	GC-MS (1)	[162]	
tissue (4)	GC-MS (4)	[44,86,102,107]		
urine (4)	GC-MS (4)	[163–166]		
Colorectal (26)	exhaled breath (11)	e-nose (6)	[89,113,167–170]	
		GC-MS (8)	[41,89,113,171–175]	
	fecal (5)	e-nose (1)	[176]	
		GC-MS (4)	[174,177–179]	
	saliva (1)	GC-MS (1)	[180]	
	sweat patches (1)	GC-MS(1)	[162]	
		e-nose(1)	[181]	
	urine (7)	FAIMS (1)	[182]	
		GC-MS (6)	[5,50,182–185]	
		H-NMR (1)	[50]	
	Breast (21)	blood (1)	GC-MS (1)	[186]
			GC-MS (2)	[187,188]
		cell culture (5)	IMS (1)	[189]
SIFT-MS (2)			[190,191]	
exhaled breath (11)		GC-MS (9)	[89,192–199]	
		e-nose (4)	[89,195,200,201]	
urine (4)		LC-MS (1)	[202]	
		GC-MS (4)	[184,185,202,203]	
saliva (1)		GC-MS (1)	[204]	
		exhaled breath (2)	e-nose (2)	[113,169]
urine (6)			GC-MS (1)	[113]
		e-nose (3)	[205–207]	
GC-MS (2)		[208,209]		
SIFT-MS (1)	[210]			
Gastric (11)	exhaled breath (10)	GC-MS-MS(6)	[113,162,175,211–213]	
		e-nose(4)	[113,175,214,215]	
	TD-SPI-MS (1)	[48]		
	SIFT-MS (2)	[216,217]		
	sweat patches (1)	GC-MS (1)	[162]	
Gastro-esophageal (2)	exhaled breath (1)	SIFT-MS (1)	[218]	
	urine (1)	SIFT-MS (1)	[187]	
Head and neck (11)	exhaled breath (9)	e-nose (7)	[99,113,139,169,219–221]	
		GC-MS (4)	[99,113,219,222]	
	sweat patches (1)	GC-MS (1)	[162]	
	saliva (1)	GC-MS (1)	[223]	
Kidney (2)	exhaled breath (1)	e-nose (1)	[113]	
		GC-MS (1)	[113]	
Leukemia (3)	sweat patches (1)	GC-MS (1)	[162]	
	cell culture (2)	GC-MS (2)	[224,225]	
Liver (4)	urine (1)	GC-MS (1)	[184]	
		GC-MS (1)	[226]	
	cell culture (2)	GC-MS (2)	[227,228]	
Lymphoma (2)	exhaled breath (1)	e-nose (1)	[227]	
		GC-MS (1)	[229]	
	sweat patches (1)	GC-MS (1)	[162]	
	urine (1)	GC-MS (1)	[184]	
Malignant Pleural Mesothelioma (6)	exhaled breath (5)	e-nose (2)	[230,231]	
		GC-MS (2)	[232,233]	
Melanoma (3)	cell culture (1)	IMS (1)	[156]	
		GC-MS (1)	[234]	
	skin (2)	e-nose (1)	[235]	
Ovarian (5)	exhaled breath (4)	GC-MS (2)	[235,236]	
		e-nose (4)	[113,237–239]	
	urine (1)	GC-MS (2)	[113,237]	
Pancreas (1)	sweat patches (1)	LC-MS (1)	[202]	
		GC-MS (1)	[202]	
Prostate (11)	exhaled breath (4)	GC-MS (1)	[162]	
		e-nose (4)	[89,113,240,241]	

(continued on next page)

Table 1 (continued)

Type of cancer	Sample	Analysis technique	Reference
	sweat patches (1) urine (6)	GC-MS (2)	[89,113]
		GC-MS (1)	[162]
		e-nose (3)	[205,242,243]
		GC-MS (2)	[244,245]
		SIFT-MS (1)	[210]
Stomach (2)	exhaled breath (1) saliva (1) tissue (1)	GC-MS (1)	[246]
		GC-MS (1)	[180]
		GC-MS (1)	[246]
		GC-MS (1)	[247]
Thyroid (1)	exhaled breath (1)	GC-MS (1)	[247]

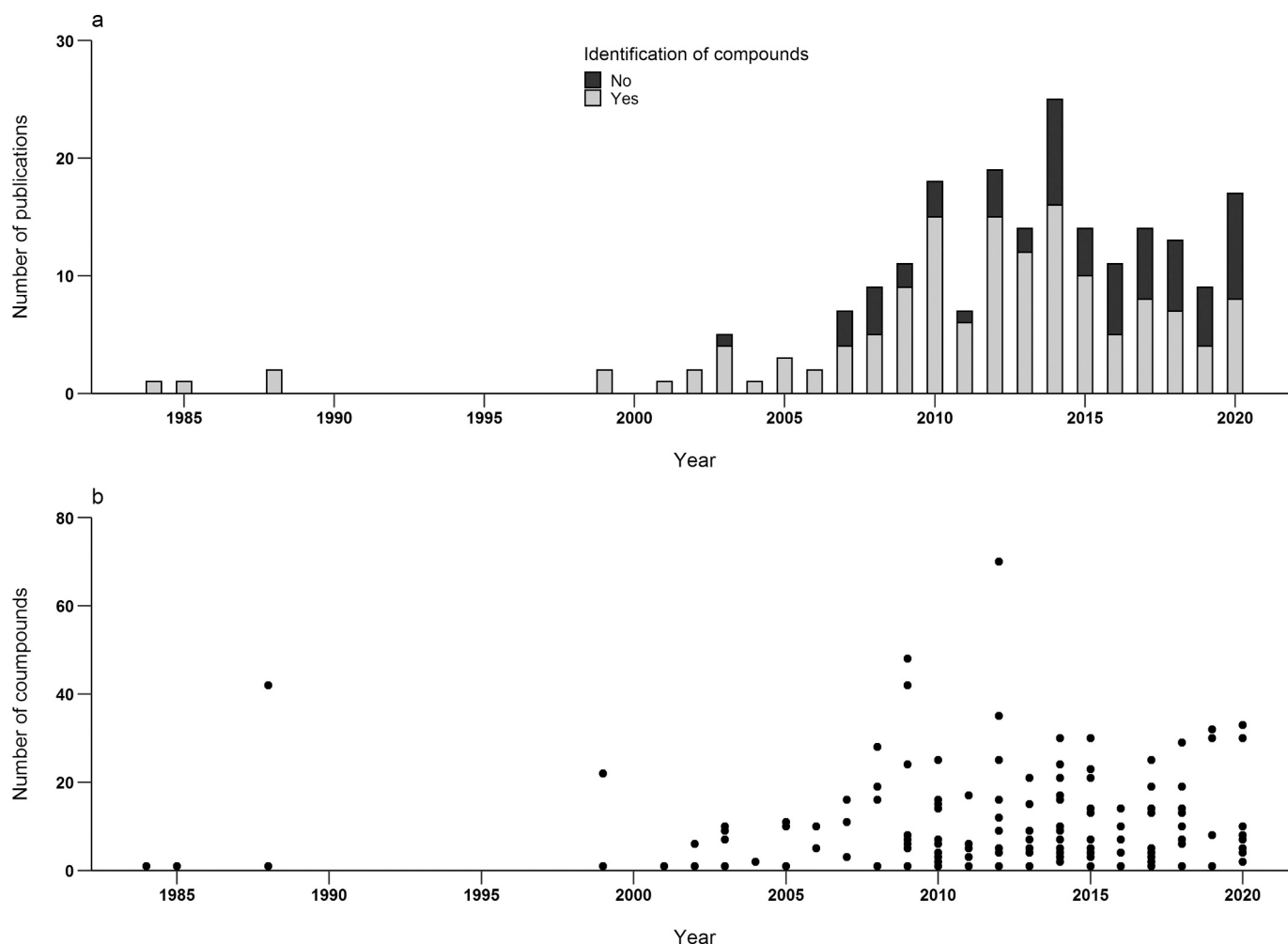


Fig. 1. a) Evolution of the yearly number of papers published from 1984 through August 2020 that investigated volatile organic compounds (VOCs) released by cancerous samples. Gray bars: compounds were identified; black bars: compounds were not identified. b) Number of compounds identified per study according to the year of publication.

There was also an increase in the number of publications that did not carry out compound identifications. Studies that did not identify compounds were generally those that used the electronic nose technique or those that simply aimed at differentiating between healthy people and those with cancer without identifying the compounds involved. The *E*-nose technique has become more frequently used since 2003. The first publication was on lung cancer [118], after which an increasing proportion of publications used this technology. As a consequence, there was a decrease in the number of studies attempting to identify cancer-related compounds into the mid-2010s. Among the publications that identified discriminating compounds, there was great heterogeneity in

the number of compounds detected. Some detected only 10 compounds or fewer, while others reported up to 70 compounds [66]. In this latter publication, the large number of compounds detected was due to the fact that authors collected odor samples from cell cultures at different incubation times and observed different new compounds through time. The large variation in the number of compounds isolated may be because some studies used different analysis techniques, for example GC-MS (Gas Chromatography–Mass Spectrometry) and LC-MS (Liquid Chromatography–Mass Spectrometry), or different samples, such as exhaled air and cell culture, which increases the number of compounds that can be detected.

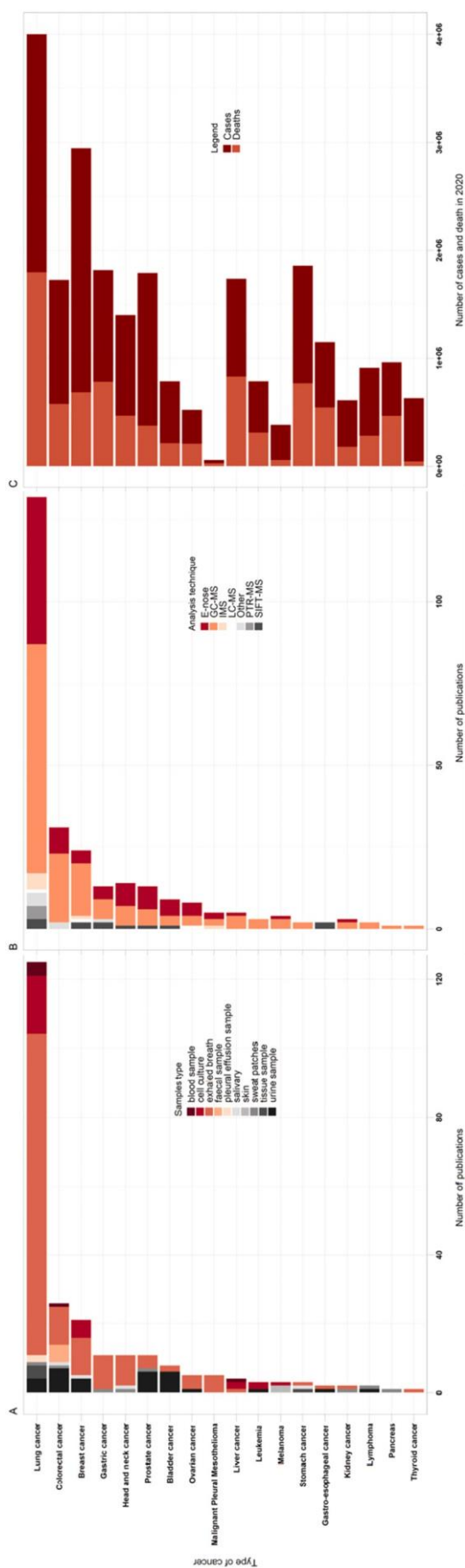


Fig. 2. Number of publications per type of cancer according to the sample source examined (A) the chemical analyses used (B). The graph C gives the number of cases and deaths for each cancer in 2020 [1].

These 208 publications included 18 different types of cancers, with a predominance for lung cancer (Fig. 2). Since the seminal publication on lung cancer [76], 118 studies have been published on this cancer, accounting for almost 60% of the studies. AfSeven hundred eighty four different studied cancers were colorectal cancer, with 26 papers (12,5% of publications), and breast cancer, with 21 papers (10% of publications). The other types of cancer studied represent together 72 papers (35% of publications). The second most common cancer affecting only men [1], prostate cancer, accounts for only 11 articles (5% of the publications). Among the cancers affecting only women, apart from breast cancer, only ovarian cancer has been studied (five papers, 2,5% of publications). Three other cancers were studied, gastric cancer (11 publications), head and neck cancer (11 publications), and bladder cancer (8 publications), which represent 4–5% of publications. Finally, leukemia, melanoma, and kidney cancer, were covered in fewer than 1% of publications, with only one or two articles focusing on the specific cancer. Altogether, the 208 publications finally represented 237 “studies”, when considering the different types of cancer in these publications.

Samples were from nine different areas, with the chemical analysis of exhaled breath being largely dominant compared to other body parts. Exhaled air sampling has been used to detect almost all the cancers listed in our review, except for leukemia, lymphoma, melanoma, and liver and pancreatic cancer. Exhaled breath analysis is a non-invasive technique that allows the observation of by-products from biochemical processes in the body [248]. It is a rapid and easy method with many advantages compared to other diagnostic techniques, such as bronchoscopy for lung cancer or other medical imaging [39]. It is non-invasive and painless—the patient is just asked to blow into a machine, similar to a stress test with a pulmonologist. The method can be used as often as necessary without restriction, allowing frequent patient follow-ups.

Following exhaled air, the two most often used samples were cell cultures and urine samples, with 27 and 25 papers (13% and 12% of publications), respectively. Becker [38] suggested that urine has more advantages than breath for the search of volatile biomarkers, and thus must be considered as a better method for non-invasive cancer detection. The other seven types of samples represent fewer than 10% of publications (only 1 to 2.5% for each sampling technique), indicating that these body extracts, e.g. tissue samples or sweat patches, were largely under-represented in the literature. Skin samples (melanoma) and saliva samples (colorectal and stomach cancer) were used in fewer than 1% of publications. Pleural effusion samples are used exclusively for the study of lung cancer, and this technique was used in only one publication [160]. This technique involves the removal by thoracentesis (needle aspiration) of fluid from a pleural effusion.

To carry out odor sampling on the different VOCs emission routes that we referenced above, different techniques were used. The most common, SPME (solid-phase microextraction) was developed in 1995 [249]. The first publication using this technique dates from 2003 [119]; it coupled SPME to a SAW (surface acoustic wave) sensor array, a type of electronic nose. SPME fiber is more frequently coupled to GC-MS. Apart from SPME fiber, studies used sorbent traps, glass tubes, or Tenax sorbent tubes. To analyze these samples, different analysis techniques using various high-performance equipment were employed, with GC-MS being the most frequently used technique (128 papers, 61.5% of publications) (Fig. 2). The GC-MS technique has been used on all types of cancer except gastroesophageal cancer, which was studied only by selected ion flow tube mass spectrometry (SIFT-MS). The e-nose was used in 75 publications (36%). This method was designed to imitate the mammalian olfactory system with an instrument designed to obtain repeatable measurements. This technique does not necessarily require the identification of VOCs to characterize people as being healthy or with cancer [46]. The electronic nose technique has been used with cancers that had been studied with other techniques; the seven least-studied cancers (kidney, leukemia, gastroesophageal, lymphoma, stomach, pancreatic, and thyroid cancer) have never been studied with this technique (Fig. 2). The ion flow tube mass spectrometry (SIFT-MS) [250] and proton

Table 2

The 28 most common compounds cited in the 142 studies on cancer odors. Only volatile compounds that were reported in more than 10 studies are shown. Origin (natural or not natural) and occurrence of compounds in the studies are given.

Compound	Number of publications N (%)	Compound origin ¹	Possible contamination source	Cancer Type / Number of publications (N)			
				Lung (83)	Colorectal (18)	Breast (16)	Other cancer (46)
Hexanal	27(19)	Natural: fatty acid derivative		19	3	1	11
Acetone	25(17.6)	Natural: fatty acid derivative		15	5	3	11
Styrene	20(14.1)	Not natural: industrial origin (manufacture of polymers and copolymers)	Ambient air, cigarette smoke, food packaging	16	1	1	9
Ethanol	20(14)	Natural: fatty acid derivative		11	4	1	11
Xylene	19(13.4)	Not natural: solvent used in industry and medical technology	Ambient air, cigarette smoke. Printing, rubber, paint, and leather materials	11	4	1	14
Toluene	19(13.4)	Not natural: industrial solvent, gasoline, chemical products	Ambient air, cigarette smoke, vehicle exhaust, consumer products	16	3	1	14
2-Butanone	19(13.4)	Natural: shikimate pathway		14	1	1	4
Isoprene	18(12.7)	Natural: mevalonate or methylerythritol 4-phosphate pathway		14	1	1	7
Ethylbenzene	18(12.7)	Not natural: petrochemical industry (polystyrene, gasoline additive, rubber, paints...)	Ambient air, water, cigarette smoke, consumer products	14	3	1	13
2-ethyl-1-hexanol	18(12.7)	Not natural: industrial origin (production of plasticizer phthalate, fragrance for cosmetics)	Ambient air, plastics, cosmetics	13	2	3	14
Nonanal	18(12.7)	Natural: fatty acid derivative		9	5	1	17
Phenol	18(12.7)	Not natural: plastic manufacture, antiseptic	Indoor air, water	5	3	5	13
Benzene	15(10.6)	Not natural: industrial chemical, chemical precursor	Gasoline vapors, vehicle emissions, cigarette smoke, food packaging	13	0	1	1
2-Propanol	15(10.6.0)	Not natural ² : solvent for coatings or industrial processes, pharmaceutical applications, rubbing alcohol...	Indoor air, household and commercial products (cleaners, disinfectants, cosmetics, solvents...)	11	2	1	2
Cyclohexanone	14(9.9)	Not natural: industrial chemical precursor to polyamides (nylon), solvent for plastics, rubbers, waxes	Indoor air, cigarette smoke	8	1	2	3
Heptanal	13(9.2)	Natural: fatty acid derivative		8	2	2	8
Decanal	13(9.2)	Natural: fatty acid derivative		7	4	0	9
Benzaldehyde	13(9.2)	Natural: shikimate pathway		9	1	1	2
Decane	13(9.2)	Natural: fatty acid derivative		9	1	1	2
Undecane	12(8.5)	Natural: fatty acid derivative		8	4	1	13
Dodecane	12(8.5)	Natural: fatty acid derivative		5	3	2	2
Acetaldehyde	12(8.5)	Natural: fatty acid derivative		10	1	0	2
1-Propanol	12(8.5)	Natural: fatty acid derivative		10	0	0	1
Hexane	11(7.7)	Natural: fatty acid derivative		9	0	1	1
2-Methylpentane (isohexane)	10(7.0)	Not natural: industrial solvent, gasoline, chemical intermediate	Gasoline vapors, indoor air	10	1	0	6
Pentane	10(7.0)	Not natural: solvent. Possible endogenous origin (lipid peroxidation)	Indoor air	10	0	0	0
p-Cresol (4-methylphenol)	10(7.0)	Natural: by-product of tyrosine metabolism		1	5	2	3
Cyclohexane	10(7.0)	Not natural: industrial solvent	Air / water contaminant	5	2	1	2

¹ Origin of compounds: the name of the biosynthetic pathway is given for compounds naturally produced by living organisms while details for industrial origin are given for compounds that are not natural.

² Possible natural origin of isopropanol: [255, 256]

transfer reaction mass spectrometry (PTR-MS) [251] techniques were used in 11 (5.3%) and in four (2%) publications, respectively. Because these techniques are based on chemical ionization and only molecular ions can be generated, compounds with identical molecular masses after ionization cannot be distinguished from each other with this technique. Another technique, ion mobility spectrometry IMS [252,253], was used in seven (3.4%) publications. Finally, the LC-MS technique [254], which involves liquid chromatography (LC) coupled to mass spectrometry (MS), was only mentioned in 1% of the publications: one on lung cancer from a blood sample [54] and another on urine from two cancers, breast and ovarian [202].

3.2. Volatile compounds associated with cancer

A total of 784 different compounds were isolated and identified

across the 142 references—all cancers combined—as being compounds that distinguish between healthy people and those with cancer. Of these compounds, 190 compounds were detected in more than one study. Table 2 lists the 28 most commonly occurring compounds from these studies: they occurred in at least 10 studies out of the 142. Fig. 3 indicates whether the concentration of those compounds decreases or increases / appears or disappears as cancer develops.

In total, 16 of the 28 compounds are of natural origin, including nonanal, hexanal, acetone, and ethanol. Compounds such as styrene, xylene, toluene, and ethylbenzene are pollutants found in the ambient air or cigarette smoke; these are not produced by the body but are still found in certain types of samples, such as exhaled air [113], cell culture [65,66], sweat patches [162], and urine samples [209]. We found all these compounds in at least three different odor source samples and at least nine of the 18 cancer studies (Fig. 4). In some studies, certain

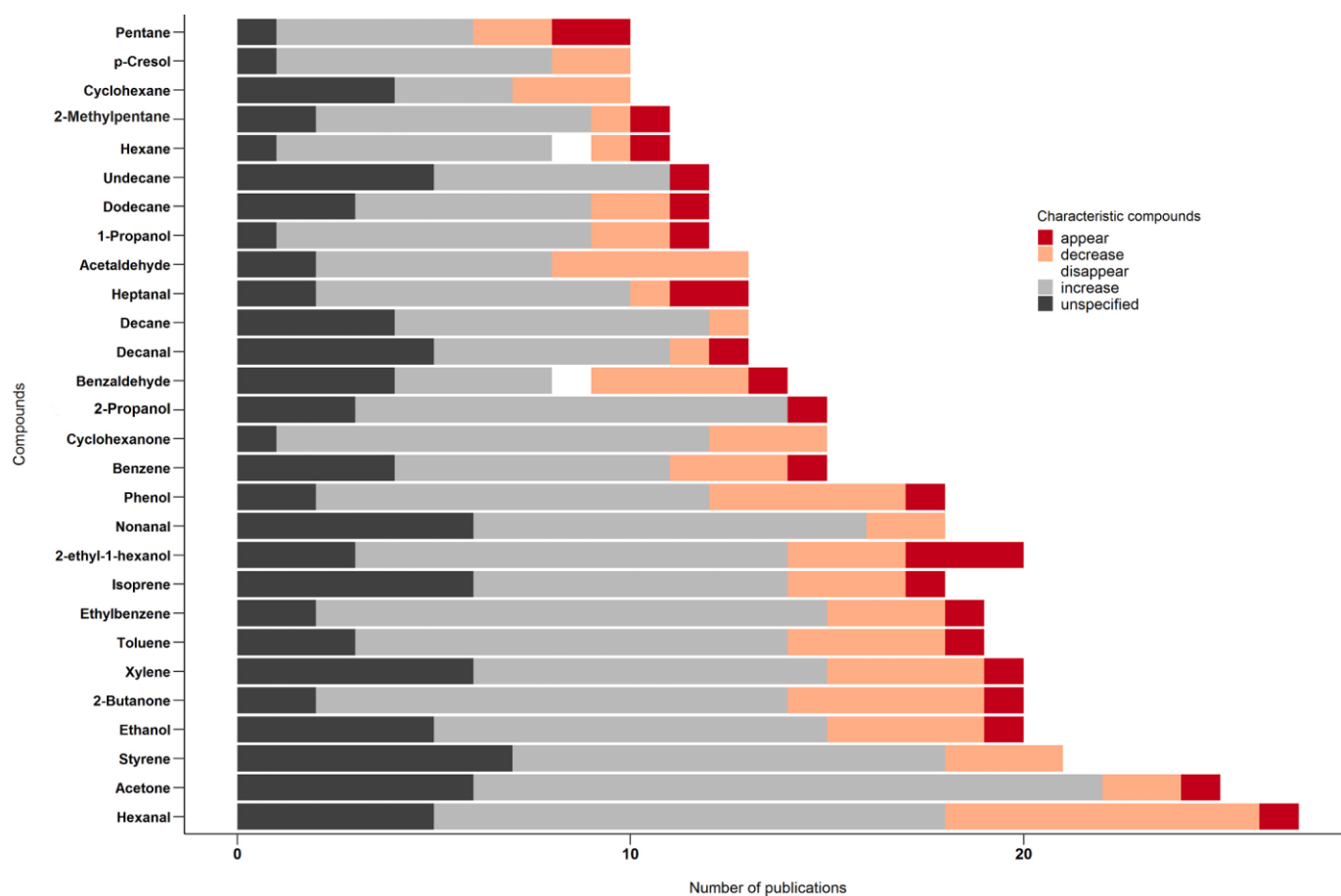


Fig. 3. Occurrence and variation characteristics of the 28 most common volatile compounds found to be associated with cancer.

compounds were specific to cancer patients, while in others they were present in both healthy and sick individuals but at different levels. Full details for these two situations and for each cancer types are given below. In some cases, the compounds were mentioned to allow differentiation between the healthy and cancer group, without indicating whether their concentration increased or decreased. In most cases, the 28 compounds tended to increase, or were sometimes only detected in cancer patients.

a) Lung cancer

Lung cancer is the third most common cancer in humans, but it is the most prevalent cause of cancer death worldwide [257]. Lung cancer includes different clinical types of pathology, with the main types of lung cancer being small-cell lung carcinoma (SCLC) and non-small-cell lung carcinoma (NSCLC). NSCLC is divided into three major histologic types: adenocarcinoma, squamous cell carcinoma, and large-cell carcinoma [258]. Different types of lung cancers are linked to different mutations that lead to the activation of oncogenes, the inactivation of tumor suppressors, and defects in the maintenance of chromosomal integrity. Depending on the type of lung cancer, it is not necessarily the same mutated genes that cause the tumor. For example, K-RAS mutations are detected almost exclusively in adenocarcinoma [259]. Because different types of lung cancers exist, it could be expected that different volatile profiles may occur. However, most studies did not consider the stage of cancer development or the type of cancer.

Analyses of volatiles from lung cancer represent 60% of the publications on cancer odors, with a total of 118 studies, of which 79 carried out individual compound identifications. A total of 458 different compounds were identified, but only 116 compounds have been recorded in

more than a single publication. Five compounds were found in at least 15 publications (i.e., 10%): hexanal, toluene, styrene, ethylbenzene and acetone. These compounds are not just found in lung cancer, e.g., hexanal was also detected in breast cancer [198] and colorectal cancer [162]. Forty four studies found that these five compounds may help distinguish between cancer patients and healthy individuals; the differentiating effect in 30 studies is associated with increased concentrations of these compounds in cancer patients while 11 studies found decreased concentrations. These five compounds are found in different types of samples (Table 1), such as exhaled breath, urine, tissue, blood, cell culture, and sweat patches. For three compounds—hexanal, styrene and ethylbenzene—decreased levels were observed in four publications studying cell cultures [59,63,65,66], but increased levels were found in 22 publication in other types of samples. Among 28 compounds identified, none was specific to lung cancer, except 1-propanol and pentane. 1-propanol has been found exhaled breath samples (10 studies) and from cell cultures odors (1 study) of lung cancer patients. In 9/10 publications, these compounds were found to increase or appear in cancer patients. Eighteen percent (10 out of 55 studies) of all publications on lung cancer that sampled exhaled breath have detected 1-propanol. Pentane has been found in 10 publications, 9 on exhaled breath and one on cell culture. Such as 1-propanol this compound was observed to increase or appear in most publications (8 out of 10 studies).

b) Cancer of the digestive system

Cancers of the digestive system include colorectal, stomach, gastric, gastroesophageal, pancreatic, and liver cancer. Among these cancers, colorectal is the third most common cancer in men and the second in women, and it represents 9% of all cancer deaths [257]. As previously

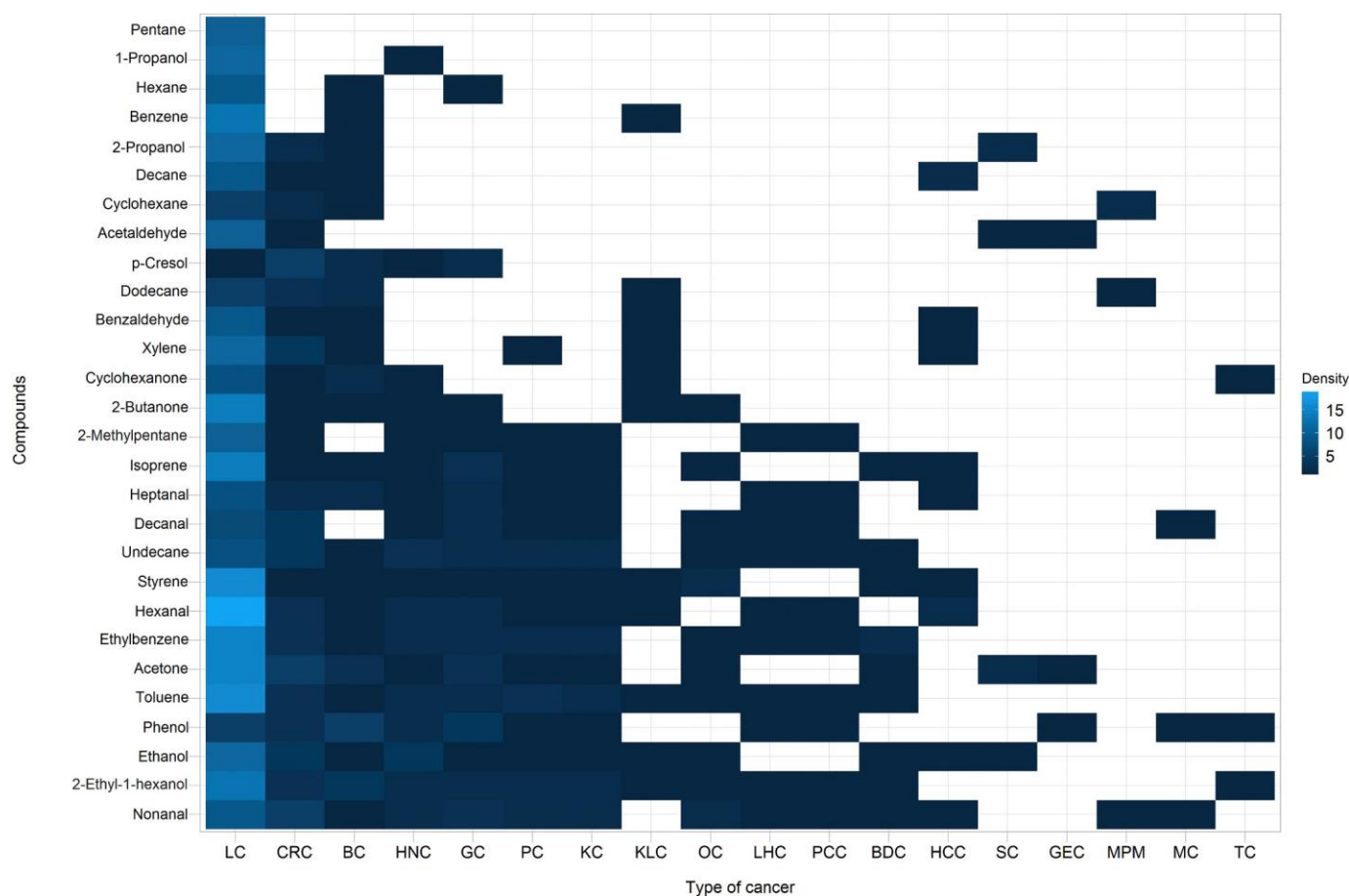


Fig. 4. Occurrence of the 28 most common volatile compounds associated with cancer for each type of cancer and the number of studies by cancer type for which each compound was found. LC: lung cancer; CRC: colorectal cancer; BC: breast cancer; HNC: Head and neck cancer; GC: gastric cancer; PC: prostate cancer; KC: kidney cancer; KLC: Leukemia; OC: ovarian cancer; LHC: Lymphoma; PCC: Pancreas cancer; BDC: Bladder cancer; HCC: Liver cancer (Hepatocellular carcinoma); SC: stomach cancer; GEC: gastro-esophageal cancer; MPM: malignant pleural mesothelioma; MC: melanoma; TC: thyroid cancer.

indicated, colorectal cancer is also the second most studied cancer for the identification of VOCs. With colorectal cancer, 95% of cases are adenocarcinomas and usually begin with adenomatous colon polyps. These polyps may then develop into malignant tumors following a series of genetic mutations [260]. These developments can be prevented if an intervention is made to excise polyps. It is therefore critical to find a technique to detect polyps and lesions before they become malignant.

Analyses of volatiles from individuals with colorectal cancer represent almost 10% of the publications; this includes 26 studies, 20 of which performed VOCs identification. Two hundred compounds were isolated and identified, but 170 were detected only once. The 200 compounds were isolated from six different types of samples: fecal, exhaled breath, urine, sweat patches, blood and saliva. Of the 28 compounds appearing in Fig. 4, 24 compounds were found in studies on colorectal cancer. Nine compounds (styrene, xylene, ethylbenzene, nonanal, dodecane, undecane, 2-propanol, 2-ethyl-1-hexanol and decanal) increase in cancer patients, other compounds increased or decreased depending on the publication. All these 24 compounds have been found in other types of cancer and are not unique to colorectal cancer (Fig. 4). The exception was p-cresol, which was found only in the urine: increased levels were observed in three publications [50,183,261] and decreased levels were reported in other publications [5]. This compound was found in four out of five publications for this cancer;

analyses were performed on urine.

For gastric cancer, the second-most studied digestive cancer, nine publications identified 62 compounds by investigating two types of samples, exhaled breath and sweat patches. Among the list of 25 compounds, sixteen were found in gastric cancer but only in two or three publications. Only phenol common to colorectal cancer which increases in cancer patients was found in four publications studying exhaled air [48,216,217] and sweat patches [162]. Two studies [187,216] found increased levels of p-cresol in the exhaled breath of gastric cancer patients.

Four studies investigated odor emissions linked to liver cancer, and no common compound was found among the four studies. Two discriminant compounds were found in two publications: decane was found to increase in cancer patients [226,229] while hexanal was found to decrease [228]. Of the 25 compounds most reported across publications, only nine were found in liver cancer (Fig. 4).

Very few studies have been carried out for other digestive cancers: two for gastroesophageal and stomach and one for pancreas. In gastroesophageal cancer, only one compound, hydrogen sulfide, was found in the two publications. Compounds identified in other cancers such as acetone or phenol were also identified in these studies [187,218]. These two publications did not use the same techniques and sampled urine [187] and exhaled air [218], which may explain the differences in the

compounds found. In total, 14 compounds were found in these two publications. For stomach cancer, we found only five compounds in two publications [180,246], and only two compounds were identified in both publications, acetone and 2-propanol. Two studies on stomach cancer were conducted on different sample types, exhaled breath and tissue [246] and saliva [180]. Acetone and 2-propanol were found with these three sample types; concentrations increased in exhaled breath and tissue, but concentrations were not specified in the article using saliva. The only publication on the pancreas was published in 2020 [162], but this study considered only two patients with pancreatic cancer and also included other cancers. The identified compounds were common to other cancers, such as colorectal or lung cancers.

c) Breast cancer

Breast cancer is the most frequently diagnosed cancer in women worldwide, with more than two million new cases per year, and it is the first cause of cancer death in women, with 626,700 deaths world-wide (data from 2018, [257]). Despite current diagnostic techniques based on mammograms and biopsy when mammograms shows breast change, breast cancer is often fatal when detected too late [262]. Despite the importance of finding early breast cancer biomarkers, only 8% of studies looking at odors linked to cancer have studied breast cancer. Among the 19 studies that investigated volatile emissions associated with breast cancer, 113 compounds were isolated and identified. Only four types of samples were used to study breast cancer odors: exhaled breath, urine, salivary and cell culture. Twelve compounds are common among these 16 studies, and only five of the 28 common compounds were not found for breast cancer (1-propanol, decanal, acetaldehyde, 2-methylpentane, pentane). Among the compounds identified in this cancer, only phenol was found in five publications [185,188,203,204,263] and 2-ethyl-1-hexanol in four publications [188,195,203,264] while all other compounds were only common to one or two publications.

d) Reproductive cancers: ovarian and prostate cancer

Ovarian cancer is one of the most lethal cancers in women (184,000 deaths in 2018 [257,265]), three out of four cases are diagnosed in advanced stages [266]. The principal cause of ovarian cancer lethality is the absence of valid screening programs and its rapid spread through the peritoneal surface. There is currently no screening test for the early detection of ovarian cancer, and new means of detection that could be more precocious and less invasive—such as VOCs analyses—would be of great value. Nevertheless, only five studies explored this direction for this cancer. Of these, only three identified compounds since the two other studies used the electronic nose. Two of the studies were carried out on exhaled air [113,237] and the other on urine [202]. Only nine of the 25 common compounds were cited as compounds detecting patients with ovarian cancer. Among these nine compounds, styrene and nonanal were common to both publications on exhaled breath. Different compounds were found in urine: androstenedione, 3-methyluridine, and 1-methyladenosine.

Prostate cancer is the second most frequently diagnosed cancer in men, with an estimated 1,3 million new cases and 395,000 deaths in 2018; it is the fifth leading cause of cancer death in men worldwide [257]. Like ovarian cancer, prostate cancer has been poorly investigated for VOCs: only 11 publications, of which only five attempted to identify specific VOCs. Two publications have examined exhaled breath [89,113], two considered urine samples [210,245] and one was based on sweat patches [162]. Of these five publications, 45 compounds were identified, 15 of which were in the list of 28 compounds. Toluene is the only compound found in three publications [89,113,162], and was

shown either to increase in cancerous samples [89,113] or to decrease [162].

e) Other cancers

• Bladder

Bladder cancer mainly occurs in older people. Most people (9/10) with this cancer are over 55, and the average age at diagnosis is around 73 years of age. Most bladder cancers start in the innermost lining of the bladder, which is called the urothelium or transitional epithelium. The more the cancer grows inside or through the layers of the bladder wall, the more difficult it is to treat as it invades the tissues of the bladder. To detect this cancer, urinary cytology and cystoscopy imaging are carried out, and the definitive pathological diagnosis is made by the transurethral route with a biopsy of the bladder. These examinations are highly invasive [267,268]. Finding a less invasive diagnostic technique, such as VOCs from urine, is thus highly sought. Eight studies were published on bladder cancer, six using urine samples, but two also examined exhaled air. Among these eight publications, only three focused on the identifying compounds, while the others used the electronic nose. Two publications looked for biomarkers in urine [209,210], and the third [113] used exhaled breath and considered different cancers. Another study [210] also investigated several cancers, including prostate and bladder cancers, and looked only at changes in the levels of a single compound, formaldehyde, which seems to increase in people with cancer. In two other publications [113,209], only one compound, ethylbenzene, was found in common.

• Head and neck squamous cell carcinoma

The term head-and-neck cancer (HNC) refers to a group of various types of tumors originating from various anatomical structures, including soft tissues, craniofacial bone, salivary glands, mucous membranes, and skin [269]. Most head and neck cancer cases (90%) are squamous cell carcinomas (HNSCC). Despite advanced diagnostics and treatment methods, this cancer family is often fatal; it is the sixth most common cancer worldwide, killing 450,000 people in 2018 [257,270]. The diagnostic technique for HNSCC is invasive panendoscopy, which consists of rigid esophagoscopy, rigid tracheobronchoscopy, direct laryngoscopy, hypopharyngoscopy, and inspection and palpation of the oral cavity and oropharynx with subsequent biopsy for histopathology examination under general anesthesia [220]. Only 11 publications investigated VOCs in this cancer, and only seven identified compounds. Only three samples were tested, exhaled breath, salivary and sweat patches. Eighty seven compounds were identified in these studies, but only two compounds were found in common, ethanol [113,219,222,223] and undecane [107,217,220].

• Kidney

Kidney cancer starts in the kidney, a pair of bean-shaped organs that remove excess water, salt, and wastes from blood in the renal arteries [271]. This cancer, like pancreatic cancer and lymphoma, has appeared in publications that studied several cancers at the same time [113,162]. These two publications have examined exhaled breath [113] and sweat patches [162]. Five compounds were in common in these two studies, which belong to the list of 28 compounds (Table 2): undecane, toluene, nonanal, ethylbenzene and 2-ethyl-1-hexanol.

• Leukemia

Leukemia is a group of cancers that usually starts in the bone marrow and leads to high numbers of abnormal and dysfunctional white blood cells. Only three studies were carried out on this type of cancer, one on human leukemia cell lines (THP1) [225] that detected seven potential biomarker compounds, including styrene and 2-ethyl-1-hexanol, which increased in cancer patients, and 2-butanone, which decreased. The other publication focused on myeloid leukemia [224], which is one of the most common hematologic malignancies. This study explored two cell lines (lymphoma cell line JEKO and acute mononuclear leukemia cell line SHI-1) that differed from the cell lines studied in the other publication [225]. The two cell lines do not have the same compounds: nine compounds were found for cell line JEKO and eight for cell line SHI-1. These include compounds already identified in other cancers, such as toluene, xylene, ethanol, and hexanal. All the compounds mentioned were found in other publications on other cancers except dodecane, 3,7-dimethyldodecane. One study [184] was conducted on the urine of patients with different types of cancers, including leukemia, colorectal cancer, and lymphoma. Four compounds were found to differentiate between healthy individuals and cancer patients. Among these three publications, no compounds were found in common.

- Lymphoma

Lymphoma, like leukemia, is a cancer of the blood. It includes cancer of the lymphatic system, which is characterized by the excessive proliferation of lymphocytes in the ganglia. The main symptom is an increase in node size, and diagnosis is based on biopsy of the affected lymph nodes. Lymphoma is one of the ten most common cancers in the world [265]. Lymphoma has been investigated for VOCs in two publications that also studied other types of cancers [162,184]. Investigations were conducted on sweat patches [162] and on urine samples [184]. In urine samples, two compounds, 2-methyl-3-phenyl-2-propenal and 3-heptanone, were observed to increase significantly in the cancerous group [184].

- Malignant pleural mesothelioma

Malignant pleural mesothelioma is an aggressive tumor originating from the pleural lining of the thorax. It is causally associated with previous asbestos exposure [272]. Despite the ban on asbestos in 2005 across the European Union, it remains present in certain buildings, and the latency period for the cancer to appear following asbestos exposure is 40–50 years [272]. Thus, incidence is increasing despite the ban on asbestos. In addition to its high incidence rate, this cancer has a five-year survival rate of less than 5%. This very poor prognosis highlights the importance of early detection [273]. Five publications investigated volatiles from malignant pleural mesothelioma. Two studies identified 10 compounds from the exhaled air of patients [156,232], while three others [156,230,231] used the electronic nose. No compound was common to the two publications [156,232].

- Melanoma

Melanoma is the deadliest form of skin cancer because it can quickly spread to other parts of the body. Only two out of three publications identified compounds associated with melanoma [234,236], and it was only studied in cell culture. Twenty-three compounds were identified in these two publications, but no compound was common to both publications.

- Thyroid

Thyroid cancer starts in the thyroid gland, which makes hormones that help regulate metabolism, heart rate, blood pressure, and body temperature. The prevalence of this cancer has seemed to increase compared to other types of cancer, mostly because the use of high-

resolution ultrasound allows better detection of thyroid nodules [265]. Although most nodules are benign, 5–10% can become malignant [274]. To evaluate their level of malignancy, the most common diagnostic method is fine-needle aspiration biopsy, which has the advantage of direct characterization, but it remains an invasive operation [275]. Only one study focused on thyroid cancer by examining the composition of exhaled breath between normal persons and patients with thyroid disease [247]. Seven volatile compounds were found characteristics of thyroid cancer. Considering the list of 28 compounds (Table 2), only 2-ethyl-1-hexanol and phenol were identified in thyroid cancer.

3.3. Multiple correspondence analysis

The multiple correspondence analysis only explained 4.1% of the variance of the dataset. This indicates that, overall, there are very few compounds in the dataset that are specific to certain publications, cancer types, year of publication, or detection method. This also suggests that many VOCs can be detected using multiple methods. If this is the case, it could lead to the discovery of potential biomarkers that can be relatively easily detected using the wide range of machines available in pathology laboratories and hospitals.

4. Discussion

Our review demonstrates that, despite extensive research focused on identifying VOCs as potential signatures of cancers in recent years, this topic remains in its infancy and no definitive answers currently exist. Two important results also emerge from this review. First, even though many similar studies on the same cancer type found distinct volatile patterns and showed highly variable discriminant compounds, we could still establish a list of 28 compounds (Table 2) that were detected in most studies. These molecules were shown to be frequently present in the volatile emissions of various samples belonging to individuals with cancer. Second, the nature of the 28 compounds commonly associated with cancer is intriguing. Twelve of the 28 molecules are not natural compounds, i.e., they are compounds of exogenous origin that cannot be produced by any living organism, and the presence of these industrial compounds in biological samples inevitably raises questions concerning their origin. Several hypotheses can be proposed to explain this finding, ranging from body contamination, pollution associated with sampling, and/or misidentification. The other compounds of natural origin in this list are all common compounds regularly found (and even systemically found for a few, such as nonanal) in odor samples from animals or plants, or from other headspace sampling [276–279]. No particular or specific volatile compound(s), whenever considering quantitative or qualitative changes associated with cancer, emerged from all the studies reviewed here.

Regarding these molecules, several important questions remain unanswered: i) To what extent are changes in compound profiles linked to the tumor growth itself or to other metabolic disorders associated with tumor formation? ii) What are the biochemical pathways involved in the changes of volatile emissions—at least for natural compounds—that are associated with cancer? iii) What is the origin of the exogenous industrial chemical compounds in biological samples? Given that compounds such as styrene, xylene, toluene, and ethylbenzene are all classified as strong human carcinogens [280,281], is their presence in the volatile emissions of cancer individuals a consequence or cause of cancer?

Although many of the studies that investigated cancer volatiles have found promising discriminating compounds, none of the 28 most common compounds identified in these studies can be hypothesized to be produced and emitted by tumor growth itself. It currently remains uncertain what kind of metabolic disorders, such as those already recorded [282] can be involved in generating particular components that will finally emanate from the body as volatile compounds associated with cancer. Some authors have proposed biochemical processes, such as

accelerated oxidative stress linked to tumor growth, may result in the production of particular compounds [283,284].

The presence of exogenous compounds in odor profiles has often been validated by multiple authors without providing an explanation of their origin; conversely, other authors have eliminated these apparently aberrant data [90]. A few authors tentatively proposed reasons for their presence as potentially relevant volatile biomarkers [264,283]. Compounds such as benzene, toluene, ethylbenzene, and xylene (so-called BTEX compounds) are ubiquitous volatiles, almost always present in GC profiles from any kind of odor collection [285,286]. Their presence in the human body, for example in fatty tissues, may originate from multiple sources, such as regular exposure to cigarette smoke, indoor air, occupational environment, and pollution [287–289]. It seems reasonable to think that patients with lung cancer may eventually display higher levels of BTEX compounds than healthy patients in their breath because such compounds have likely accumulated in their body over time and also have known carcinogenic effects [290].

Another volatile compound often isolated from cancerous samples is 2-ethyl-1-hexanol, a potential candidate for a cancer biomarker [160,188,195]. Even though some explanations for an endogenous origin have been tentatively proposed in these studies, it remains to be demonstrated how a ubiquitous phthalate metabolite, known to be a common pollutant [291,292], may have a biomarker value.

For all these reasons, it is important to consider with caution the compounds with exogenous origin (Table 2) before assigning them as potential disease biomarkers. There is an urgent need to explore the biochemical processes that may explain why such contaminants can be emitted differently between cancer and healthy patients.

Of the 28 compounds recorded here, only two have been found in a single type of cancer, i.e., lung cancer: 1-propanol and pentane. 1-propanol is an endogenous and primary alcohol, usually detected in the exhaled air or even organ tissues [102]. Given the apparent specificity of this compound to lung cancer, more investigation would be necessary, with particular focus on the molecular pathways that contribute to its production. Regarding pentane, its endogenous presence in exhaled breath has been hypothesized to result from an increase peroxidation of fatty acids in patients with severe lung disease [293,294]. Further studies will have to confirm whether these compounds are biomarkers exclusive of lung cancer.

Apart from the 28 compounds found in the majority of cancers, other compounds were also detected in particular families of cancers affecting related organs; for example, p-cresol was only found in colorectal cancer [5,50,183,184] and gastric cancer [48,162,216,217], which are both digestive cancers. These compounds are endogenous, and higher levels are found mainly in urine samples of cancer patients. The compound p-cresol (4-methyl phenol) is a shikimic malodorous product with a typical fecal odor, and it has been isolated in volatile emissions from diverse mammal feces [295] and particularly human feces [296,297]. The modification of compounds emitted by the microbiome could be a sign of the impact of cancer on bacteria in our bodies. In the case of digestive cancers, this could result from an alteration in the important microbiome community present in this compartment of the body. However, it is surprising that p-cresol was identified mostly in the urine and was not found in studies using feces. In any case, the elevated concentration of this compound in the urine of patients with digestive cancers might be a good indicator of this type of malignancies. In summary, among the many compounds studied so far, 28 were found in most studies investigating VOCs and cancer. Two compounds stood out: 1-propanol was only associated with lung cancer while p-cresol could be of interest in the detection of digestive cancer (colorectal and gastric).

Despite these first promising advances, major problems need to be resolved before generalizations can be made and VOCs routinely used as a diagnostic tool of cancers by clinicians. First, we need to understand the reasons for the high heterogeneity between the results from different studies, namely whether such differences are biologically relevant or are merely artifacts, and whether better standardization of the

methodologies could be achieved. For example, in lung cancer, three compounds (hexanal, ethylbenzene, and styrene) were shown to vary differently when distinct samples were used. The concentrations of these compounds were observed to decrease in cell culture studies, but their levels increased for samples originating from breath, urine, tissues, or blood. This observation suggests that VOCs emitted by cancer cells do not behave in the same way when they originate from living organisms or are recovered from cell culture. A critical review [298] explained differences in the volatile patterns between breath samples and *in vitro* cell cultures by the age of the patient, environmental temperature, medication, disease stage, or comorbidities of each patient. Their review only focused on lung cancer and included two sampling techniques, but the possible variation due to such parameters could be generalized to most studies on cancer and odors. A standardized protocol for each cancer type should be proposed and applied in clinical settings. For instance, in the light of previous works, it seems that the most relevant sample for lung cancer is exhaled breath since lung cancer directly affects the respiratory system; in addition, the technique has proven its effectiveness, is currently used, and is not invasive. For other cancers, exhaled air might be less relevant since it may not be indicative of the physiological status of the impacted organ. Different sample types can be more applicable than others for different types of cancers, e.g., tissue for melanoma, feces for digestive cancer, urine for the kidneys, and blood for leukemia and lymphoma. VOCs measured from cell cultures may also have diagnostic value; however, because of the potential differences between analyses performed on cells versus patients, it is likely necessary to consider both sources of information.

The present synthesis also reveals that some cancers are more studied than others, for instance, lung cancers are over-represented while others like thyroid, pancreas, kidney, and lymphoma are underrepresented and/or never studied alone. Other cancers, such as bone or cervical cancers, have never been studied. For example, since cervical cancer is induced by papillomaviruses, it would be interesting to explore if the mere presence of the virus modifies odors and how these VOCs change when cells become cancerous. It is all the more surprising that no study has been done on this cancer because research has shown that dogs were able to detect other cancers, e.g. cervical cancer, with a sensitivity and specificity greater than 90% [299].

Animal models are particularly promising for studies of cancer-associated VOCs, since they offer the possibility of controlling numerous variables that add noise (e.g., age of individuals, age at which cancer starts its growth, sex, alimentation, reproductive status), and hence theoretically could help to isolate appropriate biomarkers [300]. These kinds of studies, especially those using genetically modified models for which cancer induction is controlled by the investigators, will also help to determine precisely how changes in VOCs co-vary in magnitude with tumor growth as well as the extent to which VOCs are altered before cancer can be detected. Such longitudinal studies are lacking at the moment. Studies using a mouse model mostly investigated the terminal stage of lung cancer [66,301,302]. Only two studies [303,304] examined changes in VOCs during cancer development in a mouse model. Experiments on mouse models [303,304] generally inject cancer cells into the animals, thus limiting the possibility of observing the natural evolution of cells within an organ as well as the precise timing odor changes over tumor progression. The mouse model seems a good way to study these variations and better understand them, although because mice do not emit the same compounds as humans, it is impossible to extrapolate the results even with genetically modified mouse models that develop human cancers. However, this approach may also allow to estimate at what cancer growth stage different variations of compounds can be observable. Furthermore, standardized rigorous protocols can be developed to assess the best sampling techniques for each cancer. Finally, the mouse model approach could allow the study of mechanisms that generate these modifications as well as ways to decipher the biochemical pathways involved in all cancer development and progression. Indeed, as long as the mechanism

controlling VOCs modification is poorly understood, it will be difficult to know how it occurs within a patient and to predict how VOCs change with disease progression.

The mechanistic and biochemical links between VOCs variation and cancer progression are not currently understood. Different research directions have been proposed and further explorations are needed. For instance, it has been suggested [305] that aberrant protein synthesis associated with melanoma growth may have produced a specific odor (to which a dog responded). Regarding breath, other authors [79] considered that oxygen free-radical activity may increase in cancerous cells. This process would degrade cell membranes by lipid peroxidation and convert polyunsaturated fatty acids to volatile alkanes that may be the main odorous cancer markers in exhaled breath. More recently, it has been hypothesized that cancer odor is a product of alterations in the major histocompatibility complex (MHC) [306].

Finally, an important point to consider for the future of this discipline is the need for comparative analyses that consider not only different cancers, i.e., on different organs, but also other (non-malignant) pathologies. Identifying VOCs as biomarkers of pathologies is not feasible for early cancer diagnosis. Given that most—if not all—pathologies are to some extent associated with body odor modifications, this point is crucial. Nakhleh et al. (2017)[113] studied 17 different diseases using an artificially intelligent nanoarray and detected 13 compounds, including some that were also found in other cancer studies (e.g., ethylbenzene, nonanal, styrene, toluene, and undecane). None of these compounds can be solely considered as a biomarker, but variations in volatile patterns may help to identifying specific diseases. Our review showed that no particular group of compounds emerged for any type of cancer. With artificially intelligent techniques, such as electronic nose, it is possible to examine small variations within a set of compounds. The sensitivity and specificity (i.e., the proportion of correctly identified healthy and sick people) are reported to be greater than 90% in some publications [94,99,122,140]. The use of noses, whether animal or electronic, can be considered as a very promising technique for the detection of odor variation linked to cancer. With appropriate learning, animals such as dogs and mice can detect with close to 100% sensitivity and specificity individuals with lung, breast [307], colorectal [308], and bladder cancer [309]. In addition to dogs and mice, the use of insects as a disease detector seems also possible. The antennal responses (i.e. without associative training possibility) of *Drosophila* to mammary epithelial cells from different types of breast cancer cells has been tested [310].

Other authors [303] showed that the nematode *Caenorhabditis elegans* was able to discriminate between human odor samples from cancerous and healthy patients. The response of *C.elegans* olfactory neurons to urine from cancer patients was significantly stronger to cancerous odors than to controlled odors, with a sensitivity and specificity of 95%. Furthermore, animals seem to be able to detect cancer at early stages [304,311], which would indicate the promising potential of odor analysis for early diagnosis of cancer. A very promising aspect of

odor analysis in the context of cancer diagnosis could be to develop and establish an individual monitoring system, similar to the concept of regular mammography in breast cancer. For instance, we suggest long-term monitoring of odor profiles throughout an individual's life while carrying out imaging or other clinical examinations in parallel. This would allow the retrospective establishment of links between odor alterations with cancer initiation or progression.

5. Conclusion

1) Despite an increasing number of publications in the field of cancer-related odors over the last 20 years, with 208 publications referenced, this subject is still in its infancy and no definitive answer currently exists. The increasing enthusiasm in the field observed in the recent years gives hope that these techniques will be able to diagnose cancers earlier. The use of new techniques, such as electronic

noses in compelling compound identification seems to be increasingly developed. At the moment most studies have focused on a few cancers and mainly with the same sampling method (exhaled breath).

- 2) More than 700 different compounds have been isolated and identified in these publications. Here, we propose a detailed list of the 28 most common compounds. Among these most cited compounds, 12 are exogenous compounds of industrial origin. Their presence in biological samples raises questions concerning their origin, and hypotheses or explanations should be proposed before such compounds could be considered as potentially relevant volatile biomarkers.
- 3) While many compounds were isolated in several types of cancer, a few compounds were found in one type of cancer, e.g. 1-propanol and pentane for lung cancer and p-cresol for 2 types of digestive cancer, colorectal and gastric cancer. However, further studies are needed to confirm the specific value of such compounds as a cancer biomarker.
- 4) Finally, the search for VOCs as the signature of cancer is more than ever a stimulating and promising research direction, but it requires a much larger global research effort to go beyond the descriptive phase. In particular, biologists need the assistance of qualified chemists to avoid compound misidentification or error of interpretation. An effort should also be made to extend the list of cancers explored with this approach, using a variety of techniques.

Author's contribution

FG collected the data in the 208 publications, analyzed them with AMD and wrote the paper. LD, FT and BU conceived the original idea, supervised the project, discussed the results and contributed to the final manuscript. JM supervised the chemical ecology aspects.

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.

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~ Chapitre II ~

L'animal plus efficace que les machines

Contexte et objectifs

L'animal a depuis longtemps été utilisé par l'Homme que ce soit pour l'aide agricole, la chasse, le déplacement ou encore dans le domaine médical etc. L'animal a aussi très vite été utilisé pour ses capacités de détection olfactive, notamment le chien, que ce soit dans le cadre de la recherche de personnes, de narcotiques et depuis plusieurs années dans la détection de maladies. En effet, comme expliqué dans l'introduction, de nombreuses maladies modifient les odeurs corporelles, et le conditionnement à ces odeurs permet d'entraîner des chiens ou des souris à reconnaître des maladies telle qu'Alzheimer, le diabète etc. Ce chapitre a pour but de faire le point sur les études réalisées à l'aide de l'odorat d'animaux pour détecter le cancer, de référencer les animaux qui ont déjà fait l'objet d'études sur le sujet, ainsi que le recensement des techniques d'entraînement, d'apprentissage, et les avantages et inconvénients de l'utilisation de chaque organisme dans la détection du cancer.

Résumé de l'article

Cette synthèse a permis de mettre en évidence que le chien n'est pas le seul à être capable de détecter le cancer chez des patients cancéreux. L'analyse de la littérature a montré que d'autres vertébrés tels la souris et le rat en sont également capables, ainsi que des invertébrés, tels que les fourmis, les drosophiles ou encore les nématodes. 49 publications ont été réalisées sur le sujet depuis 1989 jusqu'à nos jours. La première publication est un « case report » décrivant le cas d'un chien qui fut capable de détecter un mélanome chez une personne de 44 ans. Après cette publication, deux autres cas de chien de propriétaires reniflant ou léchant une lésion sur leur propriétaire ont été rapportés, permettant de soigner à temps un mélanome. Depuis ces exemples pionniers, 34 publications sur les chiens ont été réalisées. Concernant les autres animaux étudiés, 4 études ont été menées sur les souris et 1 sur les rats et ont montré qu'avec un apprentissage par conditionnement, en assimilant de la nourriture ou de l'eau sucrée à une odeur spécifique, ces rongeurs étaient capables avec un taux de réussite supérieur à 80% d'identifier des échantillons d'individus cancéreux. Concernant les invertébrés, seulement trois études, sur la fourmi, la drosophile et l'abeille ont été réalisées. Concernant la fourmi et la drosophile, il a été montré que ces insectes sont capables sans conditionnement de discriminer des individus cancéreux vis-à-vis d'individus sains. Quant à l'abeille, il semble que suite à un conditionnement en assimilant une odeur cancéreuse à un stimulus tel que le sucre, elle ne soit pas capable de faire la différence entre un stimulus cancéreux d'un stimulus sain. 7 études ont été publiées par une équipe japonaise entre 2015 à 2021 en utilisant un nématode, *Caenorhabditis elegans*. Ces études ont montré que ces organismes invertébrés étaient capables de discriminer un organisme cancéreux. Ceux-ci évitent les individus sains mais s'approchent des stimuli issus des individus cancéreux.

Notre synthèse, en plus de référencer toutes les études utilisant des animaux pour détecter des individus cancéreux, a permis de réaliser une analyse critique des publications en décrivant les avantages et inconvénients de chaque organisme ainsi que les futures voies de recherche que ce soit d'un point de vue médical ou d'un point de vue écologique et/ou évolutif.

Manuscrit associé à ce chapitre :

Manuscrit II :

- ~ Gouzerh, F., Pichevin, A., Dormont, L & Thomas, F. Ability of animals to detect cancer odours
(*In prep*)

Ability of animals to detect cancer odors

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Abstract:

The olfactory capacity of animals has long been used by humans to help with various activities, e.g., hunting, detecting mines, locating people, and to diagnose diseases. Cancer is among the leading diseases causing death worldwide. Several recent studies have underscored the benefit of using scent to detect cancer, and this paper will review the studies using animals to detect tumor scents. A large variety of animals have been used for this purpose—dogs, rodents, insects, and nematodes—and have shown their capacity to detect cancer, with a success rate close to 90%. Here we discuss these studies, their methodologies, and the animal models used. Finally, we discuss the medical perspectives for cancer diagnosis using odors.

Keywords: Cancer odor, olfactory sensor, odor discrimination, volatile organic compounds, behavioral response.

Introduction

Cancer is the second leading cause of death in the world, with an estimated 9.9 million deaths in 2020 (Sung et al., 2021). For most cancers, it is widely acknowledged that the early determination of a malignant tumor is crucial for increasing the chance of survival. Indeed, most cancer deaths are linked to a delay in the diagnosis, attributable to the fact early stage cancers are rarely associated with clear symptoms. Early diagnosis usually increases the effectiveness of therapies by treating the tumor at a stage at which it has not yet caused significant tissue damage or transferred metastases to other organs (Athey et al., 2012). Thus, any tool allowing early tumor detection would be particularly valuable in the war against cancer.

For the last 20 years, a new approach for early cancer detection has been explored that is based on the hypothesis that the presence of a tumor—for various reasons, e.g., the altered metabolism of cancer cells—could result in the production of specific volatile substances, called volatile organic compounds (VOCs) that are detectable by chemical analysis techniques (Gouzerh et al., 2021). In fact, many diseases can be detected because of the modifications they induce in body odors (Prugnonle et al., 2009; Sethi et al., 2013; Buljubasic and Buchbauer, 2015; Pereira et al., 2015). Among these diseases are infectious ones such as malaria (Lacroix et al., 2005) and tuberculosis (Syhre and Chambers, 2008), and chronic diseases such as mucoviscidose (Barker et al., 2006), asthma (Ibrahim et al., 2011), and diabetes (Phillips et al., 2004). Cancer is no exception: several studies have shown that it was possible to detect differences in VOC emission between healthy people and cancer patients, especially for lung, breast, and prostate cancers (Gouzerh et al., 2021).

For many animal species, olfaction plays an essential role in both intra- and interspecific interactions (Blaustein, 1981; Symonds and Elgar, 2008; Caro and Balthazart, 2010; Woodley, 2010). For example, scent is involved in the identification of conspecifics, sex, social rank, or reproductive status (Desjardins et al., 1973; Restrepo et al., 2006). There is also some evidence that olfactory capacities are used to recognize congeners that are sick following infections by pathogens (Clayton, 1991; Kavaliers and Colwell, 1995; Kennedy et al., 1987; Ehman and Scott, 2001; O'Shea et al., 2002; Poirotte et al., 2017).

Because of their olfactory acuity, animals—especially dogs— have been used by humans for various tasks, from hunting to scent detection. The first use of animals for their sense of smell was dogs in a military context to detect objects such as explosives, mines, and dead bodies during the Second World War (Rebmann et al., 2000). Later, in the 1980s, rodents were used for the same reason. Because of their small size, mice could more easily enter small areas without triggering the explosives (Marshall et al., 1981; Poling et al., 2011). The dog's sense of smell is regularly used by police to detect explosives (Gazit and Terkel, 2003; Furton and Myers, 2001) and drugs (Fattah and el Abdel-Hamid, 2020), and to

search for cadavers (Rebmann et al., 2000). For human diseases, it has been established that dogs, trained or not, can detect the imminence of an epilepsy crisis based on changes in their master's body odor (Francis et al., 2019; Strong and Brown, 2000). More recently, dogs have been used to detect COVID-19 (Jendry et al., 2020; Grandjean et al., 2022). In addition to dogs, other animal species have been found that can detect body odors, notably rodents (Edwards et al., 2015), bees (Bromenshenk et al., 2003), or drosophila (Marshall et al., 2010). Other insects such as wasps have also been trained to detect substances such as cocaine (Olson and Rains, 2014).

Recent advances in cancer research have shown that the ability of dogs to detect tumor-associated odors could offer a relevant tool for cancer detection (Brooks et al., 2015; Jezierski et al., 2015; Pirrone and Albertini, 2017). The aim of this paper is to review current knowledge concerning the use of various animals to detect cancerous processes in humans. We examined 42 publications on this topic between 1989—the year when the first publication in this field was reported—and 2022. We listed the species capable of detecting cancers and analyzed the different methods to test scent discrimination by detailing the advantages and inconveniences of each species. Finally, we explored the potential interest of each animal group for early detection of cancer in humans.

Material and methods

Our exhaustive review was conducted by searching for published articles on PubMed (<https://pubmed.ncbi.nlm.nih.gov/>), Web of Science (www.webofknowledge.com), and Google Scholar (<https://scholar.google.com/>) that contained the following keywords: “animal smells” or “animal olfaction”, or “animal nose” AND “cancer” or “cancer detection” or “cancer odors “ or “cancer scent.” The literature included articles published in English from 1989 and until 2022, excluding systematic reviews or meta-analyses. For each study, in addition to publication year, we extracted the following information: species, type of cancer, odor source (e.g., exhaled air, urine), if animals were naive or not before being included in the test, specificity, and sensitivity of the test. All graphical representations were performed with the software R version 4.0.3 in Rstudio (R Core Team, 2020) and required the following packages: ggplot2 (Wickham, 2014), GGally (Schloerke et al., 2018), and cowplot (Wilke et al., 2019).

Results

1) History of the use of animals in cancer detection

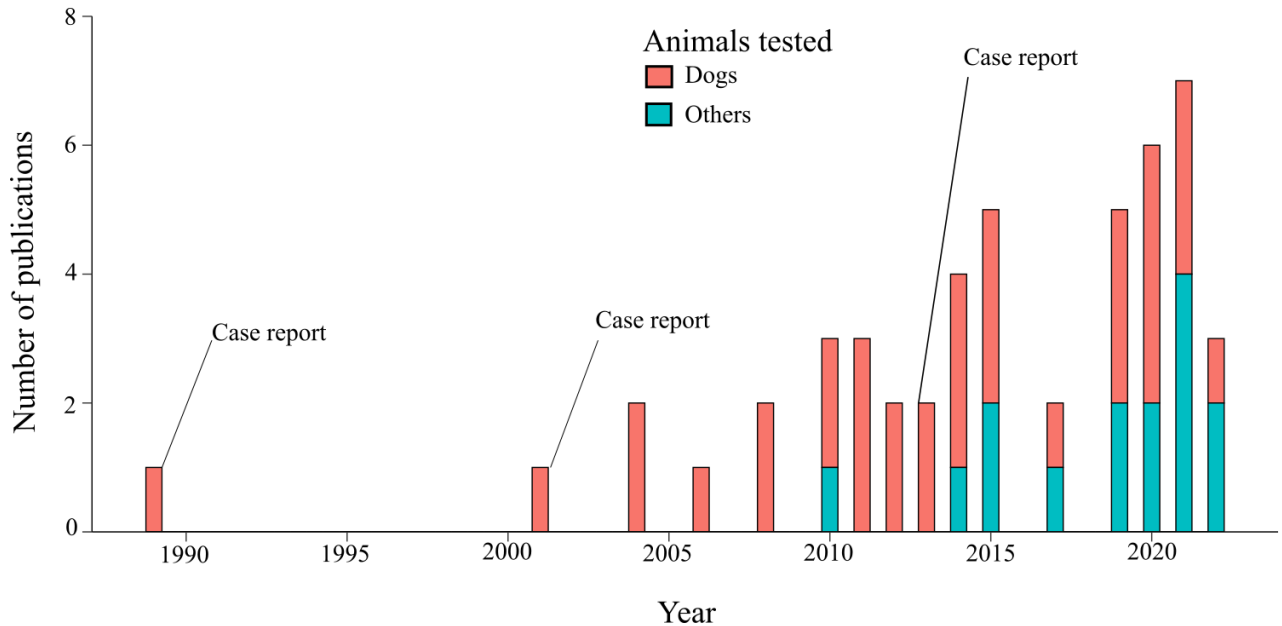


Figure 1: Yearly number of papers published between 1989 and 2022 that addressed the ability of animals to detect cancer odors. Studied animals were divided into dogs and other species, which included nematodes, bees, drosophila, ants, mice, and rats.

Despite knowledge of the olfactory abilities of animals and their use in different fields, it was not investigated until anecdotes from the late 1980s about particular dog behaviors toward people harboring tumors that cancer research in this area began to surface. The first study on the subject is a case report in 1989 (Williams and Pembroke, 1989). This note refers to a 44-year-old woman who presented lesions, which were subsequently diagnosed as malignant melanoma. This patient sought medical care because her two dogs, a Border collie and a Doberman, had detected a lesion by sniffing it. Following this initial observation, no study was launched on the subject. It was not until a second case report in 2001 (Church and Williams, 2001), which described the same scenario, that research was undertaken on the subject. In this second observation of spontaneous identification of cancer by a dog, the owner of a male Labrador retriever was diagnosed with melanoma following his dog's incessant reaction to the lesion (licking and sniffing). Following this second case report, animal studies were undertaken. In parallel, in the 2000s, research showed that it was possible to obtain differences in odor profiles between healthy and cancerous individuals (Gouzerh et al., 2021).

Since these first case reports, 46 studies were conducted between 2004 and the present on the ability of animals to detect cancer (**Fig. 1, Table S1 and S2**). Most of these publications (i.e., 31)

concerned dogs, following the case studies that highlighted their ability to detect cancer. Because dogs are already common subjects of research based on their sense of smell, many training protocols had already been developed. However, new species began to be studied from 2010 onwards, although much less frequently compared to studies with dogs (**Fig. 1 & 2**).

Among the other species studied are mouse and rat; these species are widely used in medical research, including oncology (Meuwissen and Berns, 2005; Carver and Pandolfi, 2006). Through the use of genetically modified murine models, it is possible to induce cancers that are very similar to those occurring in humans (e.g., Li et al., 2007; Jiao et al., 2019) and then explore how tumorigenesis induces modifications in mouse odor bouquet (Matsumura et al., 2010; Hanai et al., 2012). It is also known that olfaction is one of the most used senses in rodents (Malnic et al., 1999; Sakano, 2010). Only four publications involved mice between 2010 and 2022 (Matsumura et al., 2010; Sato et al., 2017; Kokocinska-Kusiak et al., 2020; Gouzerh et al., 2022) and only one involved rats (Oh et al., 2021).

More recently—in 2014—invertebrates began to be used in this research field (Strauch et al., 2014). The emergence of new study models is certainly linked to the constraint of experimentation on vertebrate animals, when researchers were encouraged to reduce the use of vertebrates and replace them with invertebrates. Olfaction in insects is a highly developed, among the most sensitive of the animal kingdom (Gadenne et al., 2016). In the context of cancer, only three studies have so far tested the olfactory capacity of insects. In 2014, a first publication on drosophila (*Drosophila melanogaster*) (Strauch et al., 2014), another in 2015 on bees (*Apis mellifera*) (Schallschmidt et al., 2015), and one in 2022 on ants (*Formica fusca*) (Piqueret et al., 2022).

In 2015, a team in Japan began studying the ability of the nematode *Caenorhabditis elegans* to detect cancer (Hirotsu et al., 2015; Kusumoto et al., 2019; Ueda et al., 2019; Kusumoto et al., 2020; Asai et al., 2021; Inaba et al., 2021; Kobayashi et al., 2021). *C. elegans* is used as a study model for sensory neurobiology. This organism offers many advantages as a model system: it has a small and transparent body, which allows the imaging of neuronal activity in real time (Rengarajan and Hallem, 2016). Japanese team has published seven publications supporting the hypothesis that nematodes can detect and distinguish odors emitted by scent sources from cancerous and healthy humans.

Figure 2 illustrates the percentage of publications made on each animal species. Many different breeds of dogs were used. Certain breeds were selected that excel in olfaction-based tasks. For instance, dogs such as Labrador retrievers, German shepherds, and Belgian shepherds are often used in scenting work in the military field because of their drive and high trainability (Polgár et al., 2016). However, at least 28 other dog breeds of various sizes have been tested for their ability to detect cancer-related odors. More than half of the publications used medium-sized dogs (30–60 cm); 14 different breeds of these sized were tested, especially Labrador retrievers.

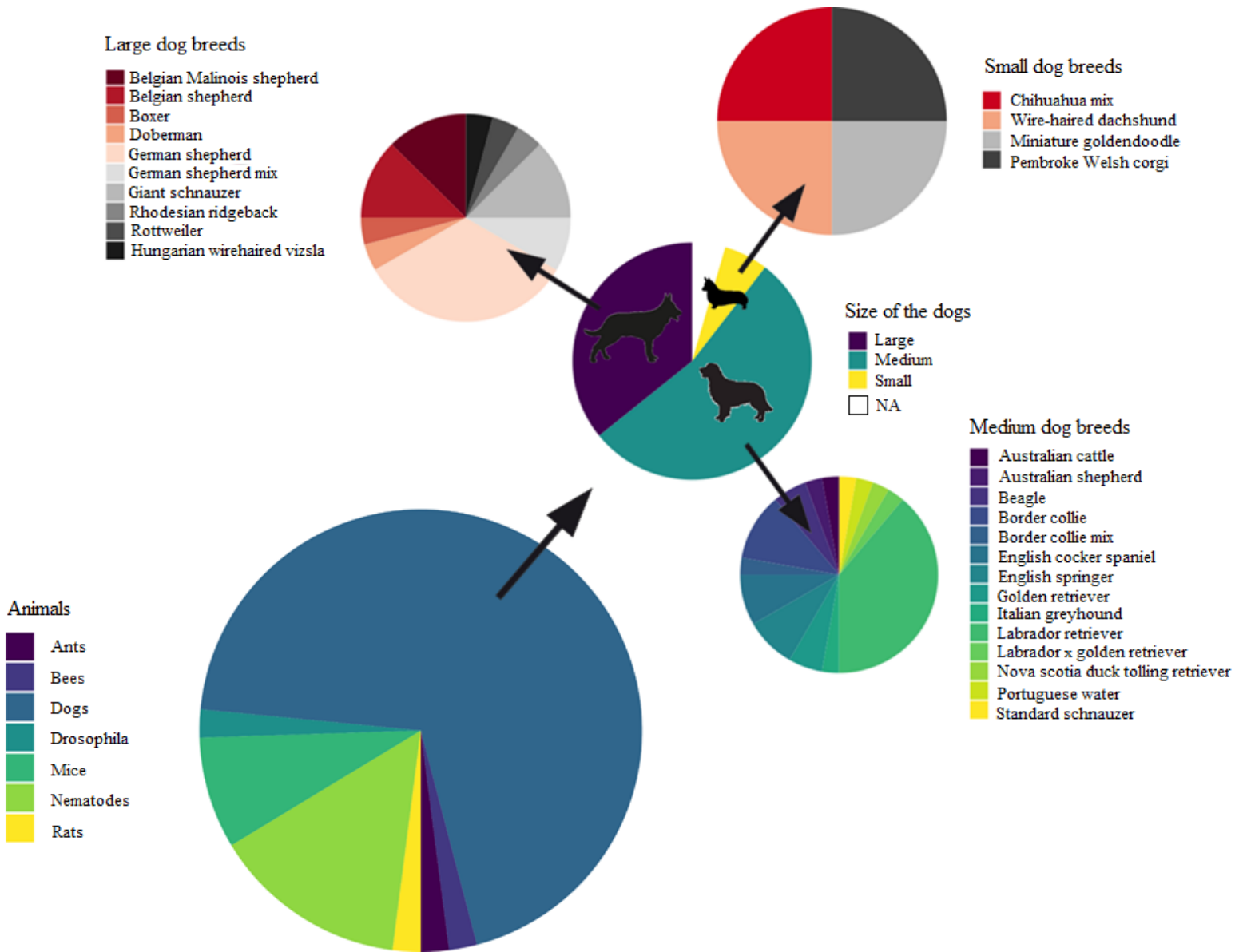


Figure 2: Proportions of different animals used for odor detection. The dog breeds used (28 different breeds in referenced publications) were classified into three size categories: small, medium, large. Some dogs were crosses between two known breeds, so we were unable to identify the size category to which they belonged and were therefore classified in the size category "NA."

2) Diversity of cancers

The sense of smell of these different species was used to discriminate different cancers from non-cancer samples. A total of 12 cancers were studied, with 10 different odor sources (**Fig. 3**). Lung cancer was the focus of the most studies, with 14 publications, followed by breast cancer (10 publications), ovarian cancer (seven publications), and colorectal, melanoma, and prostate cancers, with five publications each. The greater number of studies on lung and breast cancers are because lung cancer is the most prevalent cause of cancer deaths worldwide and breast cancer is the most frequently diagnosed cancer in women worldwide (Sung et al., 2021). Studies on lung cancer have been done mainly with dogs, comprising 75% of the publications. Lung cancer and breast cancer are also the two cancers that have been studied using the largest number of different organisms. In addition to dogs, lung cancer detection has been tested in mice, rats, and bees. Breast cancer has been tested with dogs, ants, drosophila, and nematodes. Some cancers or stimuli have only been tested using one species, e.g., prostate cancer (five dog studies), cervical cancer (one dog study), lymphoma (one nematode study), leukemia (one nematode study), pancreatic cancer (four nematode studies), and gastrointestinal cancer (three nematode studies). The detection abilities of dogs were tested on a larger number of cancers than for any of the other organisms, eight in all: prostate, ovarian, melanoma, lung, colorectal, cervical, breast, and bladder cancers. Studies using nematodes have included six different cancers (pancreatic, lymphoma, leukemia, gastrointestinal, colorectal, and breast). Among studies conducted using mice, two were on lung cancer (Matsumura et al., 2010; Gouzerh et al., 2022), one on melanoma (Kokocinska-Kusiak et al., 2020), and one on bladder cancer (Sato et al., 2017). Only one publication on lung cancer tested the ability rats (Oh et al., 2021).

Animals are presented with different sources of stimuli to see if they can discriminate the odors of cancerous individuals. The most commonly used source is urine. Dogs were tested with odors derived from human urine (11 publications) while human or mouse urine was used on mice (two publications with mouse urine and one with human) and nematodes (one publication with mouse urine and six with human). The second most used source is exhaled air from humans, with nine publications on dogs and one on rats. Breath sample collection is not invasive to humans—the person only needs to blow into a machine, similar to a stress test by a pulmonologist. Both urine and exhaled breath are simple ways to quickly obtain odor samples. There are other noninvasive methods, such as stool samples or soiled mouse bedding, from which odors emitted by mouse urine, feces, and glands can be found. These techniques are not yet widely used, with only two publications using stool samples and one using soiled bedding. These could be further developed, particularly in the context of colorectal and digestive cancers.

The most common odor sources—urine and air—are not the ones that have been tested on the most organisms: cell cultures are used in studies with insects. The three publications on bees, ants, or drosophila all used odor stimuli from cell cultures. This stimulus was also tested in three publications with dogs and one with nematodes. The other samples presented to the animals are more invasive and require either a blood sample or biopsies for tissue or skin samples. Dogs were tested with a wide spectrum of samples, with eight different stimuli used. Nematodes were also tested for different cancers and different stimuli, although the vast majority of the Japan team were made using human urine (Hirotsu et al., 2015; Kusumoto et al., 2019; Kusumoto et al., 2020; Asai et al., 2021; Kobayashi et al., 2021; Inaba et al., 2021).

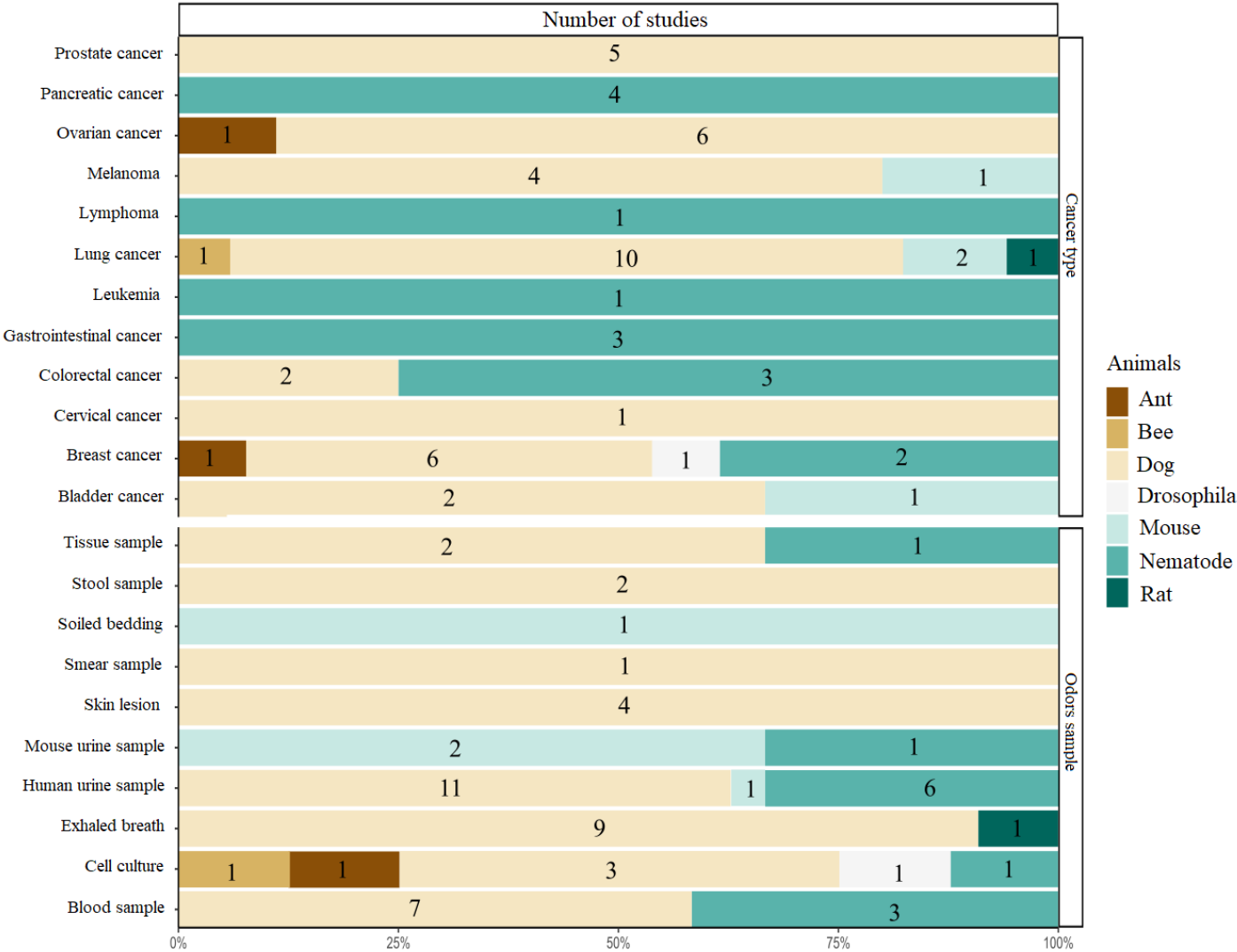


Figure 3: Summary of the cancers and odor sources used with each animal, including the number of publications realized for each cancer and each stimulus.

3) Experimental setup

The animals tested for cancer odor discrimination have different olfactory abilities and behavioral profiles, which necessitates adapting the experimental set-up to the chosen animal model. Some methods are exclusive to one organism, while others can be generalized and used on several organisms by adapting the method to the study model. The method varies depending on the organism used—it will not be the same if used on a dog or a mouse, let alone an insect measuring a few millimeters. Three main techniques were identified: tests resulting from conditioning training, behavioral tests carried out without conditioning, and finally electrophysiology tests (**Table 1 and Experimental setup**). These tests showed similar overall capacities of the animals, except that tests using bees were inconclusive.

Table 1: Summary of the advantages and disadvantages of techniques using each species to detect odors. Test details are given in Supplementary material.

Test technique	Species	Advantages	Disadvantages
Conditioning training	Dogs	<ul style="list-style-type: none"> • Good sensitivity and specificity • A single trained individual is useful for several years • Many samples tested • Animal not killed 	<ul style="list-style-type: none"> • Long training time required (a few months) • Few individuals • Ethical documents are needed for the experiment
	Bees		<ul style="list-style-type: none"> • No discrimination of cancerous individuals • Many individuals are necessary • Insects killed after tests
	Ants	<ul style="list-style-type: none"> • Good sensitivity and specificity • No ethical documents needed for experimentation • Large number of individuals available • Short testing time (a few minutes) • The individual can be killed or returned to the colony after the test 	<ul style="list-style-type: none"> • Many individuals are necessary
	Rats	<ul style="list-style-type: none"> • Good sensitivity and specificity • Short training time (a few days) 	<ul style="list-style-type: none"> • Many individuals are necessary • Ethical documents are needed for the experiment
	Mice	<ul style="list-style-type: none"> • Good sensitivity and specificity • Short training time (a few days) 	<ul style="list-style-type: none"> • Many individuals are necessary • Ethical documents are needed for the experiment

Behavior test: habituation/generalization	Mice	<ul style="list-style-type: none"> • Good sensitivity and specificity • Short test time (a few minutes) 	<ul style="list-style-type: none"> • Many individuals are necessary • Ethical documents are needed for the experiment • Limited number of tests per individuals
Behavior test: preference	Mice		<ul style="list-style-type: none"> • No preference for cancerous or healthy stimuli
	Nematodes	<ul style="list-style-type: none"> • Preference for cancerous stimuli • Good sensitivity and specificity • No ethical documents needed for experimentation • Large numbers of individuals are available • Reduced total duration of the experiment (a few minutes) • No training 	<ul style="list-style-type: none"> • Many individuals are needed for one test
Electrophysiology test (calcium imaging)	Drosophila	<ul style="list-style-type: none"> • Good sensitivity and specificity • No ethical documents needed for experimentation • Large number of individuals are available • Reduced total duration of the experiment (a few minutes) • No training 	<ul style="list-style-type: none"> • Many individuals are needed for one test • Insects killed after tests

Conditioning tests consist of learning by a system of reward or punishment (**see Experimental setup**). This type of test can be applied to dogs, insects, and rodents. To estimate the success rate of conditioning tests, we can calculate the sensitivity and specificity as follows:

$$\text{Sensitivity} = \text{CP} / (\text{CP} + \text{MI})$$

$$\text{Specificity} = \text{CN} / (\text{CN} + \text{FA})$$

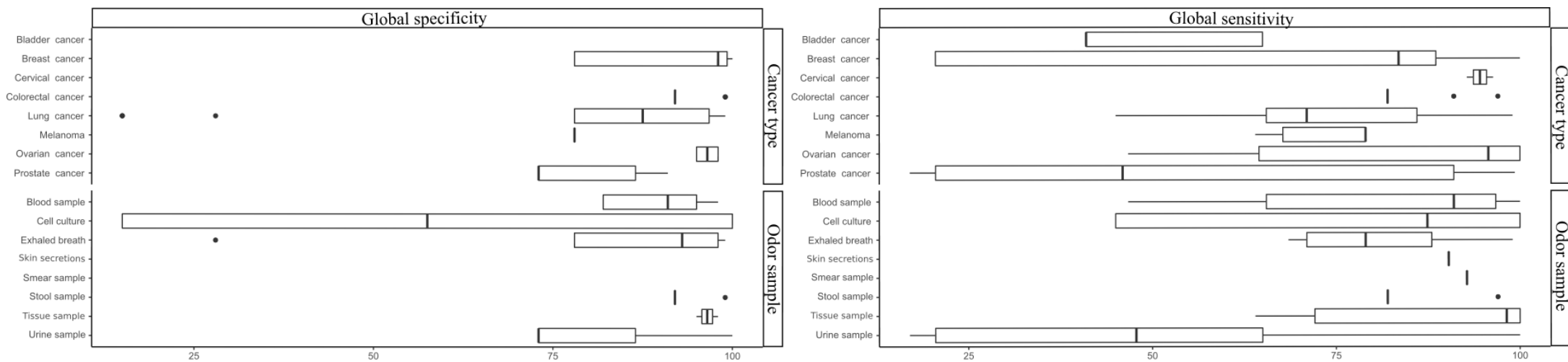
where CP indicates a correct positive response, when animals indicated the target sample; MI indicates a false negative response, when animals did not indicate the target sample; CN indicates a correct negative response, when animals refrained from indicating a control sample; and FA indicates a false positive alert, when animals indicated a blank or control sample.

Dogs on average display good sensitivity, i.e., 71 ± 24 , as well as a good specificity, i.e., 85 ± 18 . Some studies obtained scores higher than 90% or even 100% success; for example, beagles using blood serum had a sensitivity of 96.7% and a specificity of 97.5% (Junqueira et al., 2019). On the other hand, other studies obtained much less conclusive results, with success rates below 50% (Willis et al., 2004; Gordon et al., 2008; Elliker et al., 2014). Sensitivity and specificity vary among studies, and we noted that they vary depending on the cancers tested and the stimuli presented (**Fig. 4**). Sensitivity varies more than specificity; this means that dogs are more apt to make false positives—indicating a sick individual as healthy—than false negatives. Urine showed the lowest sensitivity among the stimuli, yet it is the most common stimulus tested on the dogs. Regarding the types of cancers tested, prostate and bladder cancers had average sensitivities close to 50%, making them the least well detected cancers by dogs. This poor detection level may be because only urine samples were used with these cancers.

Of the other animals tested with by conditioning, mice showed success rates between 80 and 100% in the three studies using this type of test. In another study, mice were tested using mouse urine. It seems that, unlike dogs, they are better able to discriminate cancerous conspecifics using this stimulus. The rat study used exhaled breath samples to detect lung cancer, resulting in a sensitivity of 82% and a specificity of 81%. The two studies conducted on insects using conditioning tests gave mixed results. Bees did not seem to be capable of discrimination between healthy and cancerous cell cultures (Schallschmidt et al., 2015). The study using ants (Piqueret et al., 2022) showed that when they are conditioned with sugar water to the odor of healthy cell cultures, they can discriminate the odor of cancerous individuals by returning to the odor to which they were conditioned, i.e., the healthy cultures. However, when they are conditioned to the odors of cancer cells, they fail to discriminate and do not return to the odors of the cancer culture. It is as if these cell cultures, although both cancerous, do not emit exactly the same odor profiles, thus the ant does not generalize the learned smell and recognize two cancerous cell cultures. Conditioning has advantages and disadvantages, depending on the animal tested. For all species tested other than bees, this method resulted in sensitivities and specificities above 80%.

Conditioning tests require a learning period that can be variable in length. This training time can be a disadvantage with dogs. The average age that dogs started training was 10 ± 4.8 months, with training periods varying greatly among studies (average 41 ± 34 weeks). Some dogs were trained for only 3–4 weeks prior to testing (McCulloch et al., 2006; Junqueira et al., 2019), and others were trained for seven to 24 months (Sonoda et al., 2011; Horvath, Andersson, & Nemes, 2013; Mazzola et al., 2020). This makes the dog the organism that takes the longest to provide conclusive results. For all other species tested, the learning time was much shorter, from a few minutes to a few days.

Figure 4: Global sensitivity and specificity for all experiments with dogs as a function of cancer type and odor samples. Boxplots represent the first, median and third quartiles on the 95% extent. The dots represent the outliers and when there is only one data a line is represented.



However, the dog's training can then last for its lifetime and thus be used for a large number of tests, unlike insects or rodents, which are usually used only once. The possibility of using trained dogs throughout their lives makes them a good study model. With other species, a sufficient number of individuals is needed to be statistically reliable: discrimination is not done at the individual level but is based on all the analyses done on several individuals. Typically, between 10 and 20 mice/rats and 30 or so ants were involved in a given experiment in contrast to dog studies, where only one to 10 dogs were involved. This results in a large number of organisms to be tested, which in the case of rodents requires ethical documents for the right to experiment on animals. This is not the case for invertebrates, which have fewer restrictions for experimentation.

These advantages and disadvantages including the number of individuals required and the ethical issues are common to all three testing methods—the other two being behavioral tests without prior learning or electrophysiology tests. Behavioral tests were carried out on mice and nematodes. Two types of tests were performed on mice. The first was a habituation/generalization test (Gouzerh et al., 2022), where the mouse was habituated for 14 minutes to a scent stimulus (cancerous or healthy) and then presented with two new stimuli. The mouse will typically spend more time smelling the stimulus that is not the first one smelled. In this way, it can be determined whether mice can differentiate between the smells of cancerous and healthy people. This study showed that mice were able to discriminate between cancerous conspecifics but—as with ants—this depended on the stimuli presented at the start of the experiment. Mice were able to differentiate one cancerous individual among two healthy individuals, even early in the development of cancer. But when stimuli in habituation is cancerous mice, mice may have difficulty discriminating among either two individuals or two different cancerous cell lines. This may be due to possible differences among cancerous stimuli.

The second behavioral test consists of a preference test where two different odors are placed opposite each other either in a Y-tube for mice or in a Petri dish for nematodes, and the time spent on each side is noted. Despite their ability to detect the odors, mice did not show a preference for healthy or cancerous samples. This was not the case for nematodes, which were attracted to the odors of the cancer patients. Between 50 and 100 individuals were placed in a Petri dish with two odors placed on opposite sides. The number of nematodes on each side of Petri dish was then used to calculate the chemotaxis index as follows:

$$(A - B) / (A + B)$$

where A is the number of nematodes on the side of the Petri dish with the first odor and B is the number of nematodes on the opposite side that contains the odor to be tested. Success was close to 80%. This detection technique was called N-NOSE for nematode nose by the authors, which makes reference to

the e-nose (electronic-nose). These studies show that *C. elegans* can detect cancer regardless of the cancer type or stage.

Behavioral tests with mice or nematodes give good sensitivity and specificity, and they reduce testing time by eliminating the need to learn by positive or negative reinforcement. Nevertheless, as mentioned above, the number of organisms required is much larger compared to conditioning with dogs. This disadvantage is not an issue for nematodes since there are no ethical requirements for experimentation, which allows an unlimited number of individuals to be tested.

The last technique involves electrophysiology. As of now, it has only been tested on *Drosophila melanogaster*, with calcium imaging. For this method, the neural response of drosophila to olfactory stimuli was examined using calcium imaging on the fly's antennae. This work showed that drosophila have a network of natural chemosensors that seem to be very sensitive to odors of medical interest. The antenna displayed distinct olfactory responses depending on the samples presented (healthy or cancerous cells' lines).

4) Medical perspectives

Cancer detection by animals—from dogs or mice to insects or nematodes—seems to be a promising avenue. Indeed, they seem to be able to detect cancer with only slight variations in VOCs in the body. This capacity to detect cancer could allow a much more accurate diagnosis than current cancer screening tests, such as MRI. However, there are different experimental setups depending on which animal is used and different behavioral responses and measures, such as digging, rolling over, sitting, rearing, and barking with dogs; physiological measurements such as electroantennography (EAG) or calcium imaging with insects; and attraction or repulsion responses with mice or nematodes. Furthermore, a number of different stimuli can be presented to animals. The optimization of these samples for each type of cancer and for each animal must be considered. Some stimuli are currently little used even though they are potentially reliable. Other stimuli, such as urine, are often used even though they do not necessarily give the best results (in studies with dogs). Furthermore, given the detection capacity of insects, it is important to test stimuli other than cell cultures to see if this capacity could be extended to other commonly used stimuli, e.g., urine or exhaled air.

Twelve different cancers have been studied using animals as cancer detectors. Human cancers (18 in total) have already been studied by chemical analysis of their VOCs to identify differences between cancerous and healthy patients (see the review by Gouzerh et al., 2021). It is interesting to note that the cancers most studied by chemical analysis of human VOCs—apart from lung cancer—are not always those that have been investigated by animal detection. Conversely, changes in human odors

associated with cervical cancer have never been studied by chemical analysis, although research has been carried out using dogs (Guerrero-Flores et al., 2017).

To make the best use of animals in the field of disease detection and especially for cancer, it is essential to choose an experimental set-up that is easily duplicated and can be extended to a large number of cancers. From previous studies, it appears that animals can detect cancer at very early stages of precancerous lesions. This could be useful for initial diagnoses or for cases of relapse, which are sometimes difficult to detect. Early detection is critical to more effectively treat patients. In addition, cancer detection methods must be affordable, fast, simple, and accurate. In this respect, animals that are well trained, or for which a reliable protocol can be established, can differentiate cancerous individuals using choice tests or electrophysiology for example. This could have enormous potential for primary and noninvasive screening. While the different animals used all have advantages and disadvantages, all offer a non-invasive rapid diagnostic tool for human cancers.

The use of animals is ethically problematic, especially the use of dogs and mice, so in this context it seems preferable to use organisms such as insects or nematodes. Another alternative is chemical analysis. Techniques such as GC-MS (gas chromatography – mass spectrometry), LC-MS (liquid chromatography coupled to mass spectrometry), and PTRMS (proton transfer reaction mass spectrometry) show promise in the identification of VOCs that can differentiate cancerous individuals. However, these analyses are time-consuming and tedious because we do not know which compounds the cancer modifies, thus many samples and analyses would have to be done to identify potential cancer biomarkers. Many VOCs have already been identified, but there is no consensus on one or more potential biomarkers, although some compounds such as hexanal, acetone, ethanol, or nonanal have been identified in several publications (Gouzerh et al., 2021). The use of animals avoids the identification of compounds, which can be complicated.

Discussion

The use of animals in the fight against cancer, although effective, is still in its infancy. Indeed, the potential of animals to detect cancer at an early stage has only been explored in the last 20 years. Different species seem promising, with success rates above 80% for most. All species tested have advantages and disadvantages, although from an ethical point of view the development of experiments using invertebrates such as nematodes, ants, or drosophila should be given special attention. This ability to detect cancer—with or without learning—opens the door to new questions about how and why animals are able to discriminate these odors.

Many species have evolved highly developed olfactory systems, whether to find their prey or food, to avoid predators and toxic resources, or to socially interact (Blaustein, 1981; Ache and Young, 2005). The need to know one's environment has led animals to develop a highly efficient olfactory system that—with or without training—is able to discriminate against cancerous individuals. The possibility of detection without prior learning, as seen in mice, ants, drosophila and nematodes as well as with some dogs with their owners, implies that cancerous odors are part of their environment and that they are familiar with them. Does this imply that cancer in nature has underestimated role? We know about the impact of infectious parasites on the social relationships of several animal species, but we know little about cancer. Very few studies have looked at the ability of conspecifics in the wild to avoid cancerous individuals. Documentation of cancer in natural populations is limited because most wild animals live and die anonymously. The exact prevalence of cancer in a population requires detailed knowledge of the organism under study (e.g., demography, distribution, migration) as well as extensive monitoring and sampling. However, the relative paucity of cancer research in natural environments should not be confused with a lack of its importance in determining the survival of an organism and in shaping ecological dynamics. Indeed, we know that many species—from hydra to humans—can develop cancer (Albuquerque et al., 2018). Although there is little information on the prevalence of cancer in nature, some studies have been able to show that cancer is significant cause of death in nature (Ratcliffe, 1933; Andervont and Dunn, 1962; McAloose and Newton, 2009; Madsen et al., 2017), although population monitoring to investigate the prevalence of cancer is very difficult to set up in the wild. Only Tasmanian devils and beluga (Martineau et al., 2002; McCallum et al., 2007) have thus far been extensively studied for cancer.

Concerning the impact of cancer on wild animals, i.e., whether their olfactory capacity allows them identify a cancerous congener, no study to our knowledge has asked this question. The only study carried out on the impact of cancer on intra- or interspecies relationships was done on tumoral hydras and showed that tumorous polyps are more predated by fish (Boutry et al., 2022). Is this only due to their larger size, or do they emit different odors that allow fish to differentiate them? For other predators such as the wolf, the dog's ancestor, do they use their sense of smell to detect sick individuals, and can they distinguish cancerous individuals? Are cancerous individuals, which may carry more pathogens following cancer-induced immune suppression (Dujon et al., 2021), excluded from the group or less frequently chosen as sexual partners (e.g., Vittecoq et al., 2015). These questions are even more relevant for species harboring transmissible tumors, such as Tasmanian devils (McAloose and Newton, 2009). In this species, the extent to which individuals avoid each other to avoid contamination is poorly understood. There are many questions about how cancer is perceived in nature by animals and especially how animals perceive cancerous animals using their sense of smell. For species with post-reproductive cancer, the impact on conspecifics (repulsion, non-preference) should not be felt ; selection should not

play a role since the cancer will not affect the fitness of the healthy conspecifics. However, cancer may appear at a younger age in some species, or in the case of transmissible cancers, does selection play a role in the olfactory capacities of animals to enable them to avoid sick conspecifics ?

Oncogenic agents such as UV radiation, oncogenic contaminants, and hormones released into waterways are increasingly present in the environment due to human encroachment and habitat degradation. Because many of these carcinogens are new, exposed organisms may not have developed effective defense mechanisms and may not have the capacity to avoid them, which makes wildlife particularly vulnerable to carcinogenesis (Fernandez and Bowser, 2010; Giraudeau et al 2018; Sepp et al 2019). Exploring the extent to which chemical ecology will be central in these novel ecological contexts will be fascinating and will require the integration of concepts and techniques from different disciplines in addition to chemical ecology (e.g., behavioral ecology, physiology, molecular biology, oncology). We believe that answers to many current and future questions on these topics could come from this starting convergence between disciplines.

Concluding remarks

Animals can use their sense of smell to detect objects, drugs, people, or even diseases such as cancer. This ability is shared by many animals that use olfaction as a means of communication in the wild. Their sense of smell allows them to obtain information about the individuals around them, including age, sex, and health status. Using their ability to identify individuals with parasitic, chronic, or genetic diseases such as cancer is a promising avenue for the early detection of many diseases.

Different experimental techniques could be used on different animals. This review covered olfactory studies that used dogs as well as other species and highlighted the advantages and disadvantages of each species. Analyzing the neural electrophysiological responses (e.g., electroantennography) of species capable of detecting cancer and thus identifying the compounds that are different between cancer patients and healthy ones would be invaluable. However, this involves a vast number of individuals who are destined to be euthanized. In this respect, insects or other invertebrates may be an interesting avenue of research: they have highly developed olfactory capacities and their use in experimentation is not at this time subjected to any ethical constraints. The ability of animals to detect cancer raises many interesting ecological and evolutionary questions.

AUTHOR'S CONTRIBUTION

FG collected and analyzed data and wrote the paper. LD, FT, and GG supervised the project and contributed to the final manuscript.

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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.

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SUPPLEMENTARY MATERIAL:

Table S1: List of studies that examined the ability of dogs to discriminate cancer odors, including details on dog breed, sex, age, training duration, and previous experience; cancer type; sample type; and the main results of each study.

Study type	Authors	Cancer type	Number of dogs tested	Breed	Sex	Age	Length of time trained	Previous experience	Odor samples	Sensitivity for each dog	Specificity for each dog	Global detection sensitivity %	Global detection specificity %
Case reports	Williams and Pembroke, 1989	Melanoma	1	Border collie and Doberman	NA	NA		Naive	Skin lesion				
	Church and Williams, 2001	Melanoma	1	Labrador retriever	Male	NA		Naive	Skin lesion				
	Campbell et al., 2013	Melanoma	1	Alsatian	Female	NA		Naive	Skin lesion				
Prospective study	Pickel et al., 2004	Melanoma	2	Standard schnauzer	Male	4 years		Different distinctions. Served 2.5 years in police; certified bomb-detection dog; served in research efforts by the US Department of Defense	Tissue	NA	NA	86%	
				Golden retriever	Female	6 years	Different distinctions	75%					
	Willis et al., 2004	Bladder cancer	6	Mixed breed	Male	6 years	7 months	Obedience	Urine	11%	NA	41%	NA
Labrador retrievers	Female	7 years	33%										

				Cocker spaniel	Male	1.5 years				55.50%			
					Female	2 years				55.50%			
					Female	5 years				44%			
					Female	7 years				44%			
McCulloch et al., 2006	Breast cancer and lung cancer	5	2–3 weeks	Labrador retriever	Male	7–18 months	Recruited dogs were screened for their level of eagerness to sniff objects and respond to commands	Exhaled breath	NA	NA	Lung cancer: 99% Breast cancer: 88%	Lung cancer: 99% Breast cancer: 98%	
				Labrador retriever	Male								
				Labrador retriever	Female								
				Portuguese water dog	Male								
				Portuguese water dog	Female								
Gordon et al., 2008	Breast cancer	6	12–14 months	Aussie cocker mix	Male	2 years	NA	Urine	28%	NA	20.5%	NA	
				Collie mix	Females	8 years	NA						28%
				German shepherd		4 years	Tracking, agility, obedience						22%
				Rhodesian ridgeback		5 years	Agility, obedience, rally						28%
				Boxer		6.5 years	Agility, obedience, rally						17%
	Italian greyhound	Male	5 years	Agility	11%								
	Prostate cancer	4			Chihuahua mix	Females	4 years	Service: therapy	6%				
					Miniature goldendoodle		2.5 years	Service: therapy	15%				
					Pembroke Welsh corgi		6 years	Therapy: obedience	22%				
					Border collie		2 years	NA	28%				

Horvath et al., 2008	Ovarian cancer	1	Giant schnauzer	NA	4 years	12 months		Human sample tumor bank	100%	100%	100%	97.5%
									100%	91%		
Horvath et al., 2010	Ovarian cancer	2	Giant schnauzer	Females	7 years	9 months	Trained to detect ovarian carcinoma	Blood and tissue test			Blood: 100%	Blood: 98%
			Giant schnauzer		3 years	9 months	Naive				Tissue: 100 %	Tissue: 95 %
Cornu et al., 2011	Prostate cancer	1	Belgian Malinois shepherd	NA	NA	16 months	Chosen among young dogs destined for explosives detection training. No previous training	Urine and blood	91%			
Ehmann et al., 2012	Lung cancer	4	German shepherd	2 Males and 2 Females	2.5–3 years	NA	Family dogs	Exhaled breath	NA	NA	71%	93%
			German shepher									
			Labrador retriever									
			Australian shepher									
Sonoda et al., 2011	Colorectal cancer	1	Labrador retriever	Female	8 years	6 months (November 2008 through June 2009)	Trained for water rescue beginning in 2003 and then began training as a cancer detection dog in 2005	Human watery stool	91%	99%	91%	99%
								Exhaled breath	97%	99%	97%	99%

Willis et al., 2010	Bladder cancer	4	Cocker spaniel	Males	6 and 8 years	NA	Obedience commands	Urine	NA	NA	57–73%	92–56 %
			Springer spaniel	Male	5 years							
			Labrador retriever	Female	6 years							
Buszewski et al., 2012	Lung cancer	NA	German shepherd mix	Male	20–22 months		NA	Exhaled breath	NA	NA	82.2%	82.4 %
Walczak et al., 2012	Breast and lung cancer, melanoma	6	German shepherd	Males	3 dogs \geq 20–22 months; 3 dogs \leq 6 months	NA	Naive	Exhaled breath	NA	NA	79%	78%
			Labrador retriever									
Horvath et al., 2013	Ovarian cancer	2	Giant schnauzer	NA	10 years	2 years		Blood	Series I: 97%; Series II (3 months after chemo): 50%; 6 months after chemo: 60 %	Series I: 99%; Series II (3 months after chemo): 96%; 6 months after chemo: 90%	Serie I: 97%; Serie II: 70% and 80%	Serie I: 99%; Serie II: 95% and 92%
			Giant schnauzer	NA	6 years				Series I: 97%; Series II	Series I: 100% Series II (3 months		

										(3 months after chemo): 80%; 6 months after chemo: 100%	after chemo): 94%; 6 months after chemo: 94%		
Amundsen et al., 2014	Lung cancer	4	Belgian shepherd	Male	8 years		Scent-tracking missing people, environmental pollutants	Exhaled breath, Urine	Exhaled breath: 76%; urine: 66%	Exhaled breath: 33.3%; urine: 41.7%	66–76%	41.7–33.0%	
			Border collie	Male	4 years	Scent tracking, environmental pollutants							
			Wire-haired dachshund	Male	8 years	Scent tracking							
			Rottweiler	Female	3 years	Scent tracking							
Elliker et al., 2014	Prostate cancer	2	Labrador	NA	9 years			Urine	13%	71%	17%	73%	
			Border collie	NA	3 years		25%		75%				
Rudnicka et al., 2014	Lung cancer	2	German shepherd mix	Male	NA	NA	NA	Exhaled breath	NA	NA	86%	28%	
Yoel et al., 2015	Breast cancer	2	Belgian shepherd, Groenendael type	Female	5 years	6 months	Detect explosives and identify people who need to be singled out in a group	Cell culture	NA	NA	100%	100%	
			Belgian shepherd, Malinois type	Female	2.5 years								
Taverna et al., 2015	Prostate cancer	2	German shepherd	Females	2–3 years	4 months	Explosives detection	Urine	Dog 1: 100% Dog 2: 98.6%	Dog 1: 98.7% Dog 2: 97.6%	98.7–100%	98.7–97.6%	
Schallschmidt et al., 2015	Lung cancer	2	Labrador retriever	Female	3 years	8 months	Both dogs had basic obedience	2 lines of cell culture	40%	50%	45%	15%	

				Mixed breed	Male			training and were experienced in scent discrimination		20%	10%		
Guerrero-Flores et al., 2017	Cervical cancer	1		Beagle	Male	3 years		Trained for drug detection	Human fresh biopsies; smear sample	Smears: 92.78%; Surgical bandages: 96.36%	Smears: 99.1%; Surgical bandages: 99.55%	Smears: 92.78; Surgical bandages: 96.36	Smears: 99.1; Surgical bandages: 99.55
Junqueira et al., 2019	Lung cancer	3	Beagle 1	NA	2 years	4 weeks	Naive		Blood serum	91%	100%	96.7%	97.5%
			Beagle 2							100%	100%		
			Beagle 3							82%	97%		
Murarka et al., 2019	Ovarian cancer	1(4 started training)	German shepherd	Female	33 months	NA	Naive		Cell culture	87.40%			
			German shepherd	Female	12 months								
			Labrador retriever	Male	49 months								
			German shepherd	Female	50 months								
Thuleau et al., 2019	Breast cancer	2	Belgian shepherd	Males	18 months	5 months	Naive		Skin secretions	100%	100%	90.3%	90.3%
			Belgian shepherd							88.9%	88.9%		
Essler et al., 2020	Ovarian cancer	4	German shepherd	3 Females / 1 Male	2-7 years	NA	NA		Blood plasma	False alarm: 14-41%; hesitated: 42-7%	NA	24-56%	NA
			Labrador retriever										
			English springer spaniel										
Mazzola et al., 2020	Lung cancer	3		Belgian Malinois	Female	5 years	1 year	NA	Urine	First trial 45%;	First trial 89%;	54.60%	90.30%

				Belgian Malinois	Female	3 years				second trial 55%	second trial 91%		
				Mixed breed	Female	3 years				First trial 55%; second trial 36%	First trial 91%; second trial 87%		
										first trial 73%; second trial 64%	First trial 91%; second trial 93%		
Kybert et al., 2020	Ovarian cancer	3	English springer spaniel	NA	12–20 months	6 months	NA	Blood plasma	95.1 %	NA	95.70%	NA	
			German shepherd						98.7 %				
			Labrador retriever, yellow						93.3 %				
Schoon et al., 2020	Colorectal cancer	8	Nova Scotia duck tolling retriever	Female	2 years	NA	Privately owned	Stool	74%	94%	82%	92%	
			Labrador × golden retriever	Female	2 years		Rejected as guide dog		88%	91%			
			English cocker spaniel	Female	1 years		Working dog line		88%	87%			
			English cocker spaniel	Female	1 years		Working dog line		79%	90%			
			Labrador × golden retriever	Female	2 years		Rejected as guide dog		83%	90%			
			Labrador × golden retriever	Female	1 years		Rejected as guide dog		80%	91%			
			English cocker spaniel	Male	1 years		Working dog line		80%	95%			
			Belgian Malinois	Female			Working dog line		84%	96%			

Guest et al., 2021	Prostate cancer	2	Labrador retriever	Female	4 years	3 years	NA	Urine	71.4%	76.2%	71.4%	73
			Wire-haired Hungarian Vizsla	Female	7 years				71.4%	70.0%		
Kure et al., 2021	Breast cancer	1	Labrador retriever	Female	9 years	12 months	Water rescue dog, and then, because of her high ability of sniffing out and eagerness, she was recruited to have a cancer detection training	Urine	100%	100%		
Riedlova et al., 2022	Lung cancer	2	Labrador retriever		4 years	21 months	Naive	Blood, Exhaled breath	blood: 69% breath: 62%	blood: 67% breath: 71%		
			Australian cattle dog		5 years	15 months	Naive		blood: 62% breath: 75%	blood: 97% breath: 90%		
Feil et al., 2021	Lung cancer	1	Golden retriever		7 years	1 year	Raised to discriminate different smells e.g., chamomile tea, cinnamon, or coffee	Exhaled breath	78%			
								Urine	87.80%			

Table S2: List of studies that examined the ability of rodents and invertebrates to discriminate cancer odors, including details on cancer type; animal type, sex, and age; type and duration of training; odor source; and results.

Animal	Authors	Cancer type	Sex	Age (weeks)	Number of animals tested	Training	Training duration	Odor samples	Success or p-value
Mouse	Matsumura et al., 2010	Lung	Males		6	Y training maze with water recompense	14 days	Mouse urine	Discrimination success > 80%
			Female		1				
	Sato et al., 2017	Bladder	Males	3 weeks	23	Y-maze behavioral assay with water recompense	1–2 days	Human urine	100%
	Kokocinska-Kusiak et al., 2020	Melanoma	Females	9 weeks	11	Spontaneous preference/avoidance in the open fields and Y-maze tests	few days	Mouse urine	Non-significant preference for conspecifics with melanoma
			Males		15				
			Females		20	Trained mice tested in Y maze with treats as a recompense			
			Males		20				
	Gouzerh et al., 2022	Lung	Females	12 weeks	18	Spontaneous preference/avoidance in Y-maze tests	10 min test	Soiled mouse bedding	Non-significant preference for conspecifics with melanoma
Males			12 weeks	11–16	Habituation/generalization tests	21 min test	Soiled mouse bedding	p<0.01; mice discriminate cancerous mice at early and late stages of cancer development	

Rats	Oh et al., 2021	Lung	Males	4 weeks	12	Trained rats tested to discrimination reinforcement / reward / punishment	30 min once a day	Exhaled breath	Sensitivity: 82%; Specificity: 81%
<i>Drosophila melanogaster</i>	Strauch et al., 2014	Breast	Females	1–5 days old	NA	Calcium imagery	A few minutes	Cell culture	Significant difference
Honeybee (<i>Apis mellifera</i>)	Schallschmidt et al., 2015	Lung	Females	NA	20 in total, 14 tested	Test of proboscis extension (PER)	1 h	Cell culture	No discrimination (p = 0.062)
Ant (<i>Formica fusca</i>)	Piqueret et al., 2022	Ovarian and breast	Females (foragers)	A few days	36	Conditioning test with reward sugar solution + memory tests	A few minutes	Cell culture	Discrimination between healthy and cancerous, but not between two cell culture lines
Nematode (<i>Caenorhabditis elegans</i>)	Hirotsu et al., 2015	Colorectal, gastric, breast		Young adults	50–100	Preference tests in Petri dish with one stimulus (healthy or cancerous) on each side	A few minutes	Cell culture, tissue, blood, urine	Sensitivity: 95.8%; Specificity: 95.00%
	Kusumoto et al., 2019	Colorectal, gastric			50–100			Urine	61.1–82.1%
		Pancreatic						Mouse urine	

Ueda et al., 2019				50				Sensitivity: 71.40%; Specificity: 83.30%
Kusumoto et al., 2020	Gastrointestinal			50			Urine, blood	80–90%
Kobayashi et al., 2021	Pancreatic			50–100			Urine	Sensitivity: 84.6%; Specificity: 60%
Inaba et al., 2021	Breast, esophageal, gastric, colon, gallbladder, bile duct, pancreatic, malignant lymphoma, acute myeloid leukemia			100			Urine	Sensitivity: 87.50%; Specificity: 90.20%
Asai et al., 2021	Pancreatic cancer			50–100			Blood, urine	P < 0.001

Experimental setup:

I) Conditioning training

Two types of conditioning training can be used in animals: classical conditioning and operant conditioning (Oh et al., 2014).

- **Classical conditioning**

Classical conditioning, which is based on the Pavlov reflex, implies that certain stimuli automatically provoke certain responses (Rescorla, 1988). The first step in classical conditioning is to associate a neutral stimulus that initially has no effect on the animal—for example, the smell of a cancerous individual—with an unconditioned stimulus, such as sugar, which provokes an unconditioned response. As the neutral stimulus is closely followed by an unconditioned stimulus that naturally triggers an unconditioned response, the neutral stimulus is associated with an unconditioned stimulus, so that the association between the two becomes stronger and the unconditioned response develops. Based on Pavlov's principle, procedures have been adapted to insects that are more commonly called differential conditioning of the proboscis extension response (PER) (Bitterman et al., 1983; Matsumoto et al., 2012).

- **Operant conditioning**

The other type of learning is operant conditioning, which refers to the fact that a behavior can be controlled by its consequence (reward, called reinforcement, or punishment). The animal's behavioral response to a stimulus will be followed by a reinforcement, i.e., the response has a favorable results such as food (Schapiro et al., 2010). In the case of negative reinforcement, the animal will be subjected to an aversive stimulus such as an electric shock, or bitter or unpleasant food (Iwata, 1987). This learning technique is used to train dogs and rats, and for some studies on mice. With conditioning, two parameters are calculated to evaluate detection capacity—sensitivity and specificity.

II) Behavioral tests

- **Habituation/ generalization tests**

The concept of these tests is to habituate an individual to an odor for a given period of time and then present him two new odors; it is assumed that the odor to which the organism will go more is the one that is more different (Todrank and Heth, 2003).

- **Preference tests / choice tests**

The choice tests take place in a Y-shaped enclosure or another container such as a Petri dish for small organisms. These containers allow the individual to move towards one of the two stimuli presented, which are placed at each end of the olfactometer branch or on opposite sides of the Petri dish. The time spent in each position indicates the preferred stimulus.

III) Electrophysiology test

The last type of test used to determine if animals detect cancer consists of analyzing the neuronal response of an insect's antenna to an odorant stimulus. For these tests, insects must be killed before antennae are removed from the animal to perform the experiments.

- **Electroantennographie (EAG)**

Electroantennographie (EAG) is a bioassay widely used in experimental entomology for the detection of volatile compounds. This technique consists of analyzing what the insect's antennal olfactory receptor detects. A neurological response recorded from the antenna indicates whether the compound is detected by the organism. For this purpose, the antenna of an insect is connected to two electrodes. The antennae are exposed to an odor (a single compound or mixture of volatile components) and the electric response of the antenna is recorded by the electrodes via an amplifier (Schneider, 1957; Weissbecker et al., 2000). This technique has never been used to determine whether an insect is able to detect the smell of a cancerous individual.

- **Calcium imaging**

This technique allows an even finer level of detection compared to EAG. It consists in visualizing the activated areas of the olfactory lobes of an insect during olfactory perception (Grienberger and Konnerth, 2012).

~ Chapitre III ~

Modèle d'étude et protocole de prélèvement des stimuli

I) Modèle d'étude

Pour répondre à mes différents objectifs présentés dans l'introduction, j'ai réalisé des tests de comportement sur des souris ainsi que des analyses chimiques à partir de différents stimuli présentés par la suite.

1) *Modèle de souris cancéreuses*

Le modèle de souris cancéreuses utilisé pour cette thèse provient de l'IRCM, Institut de Recherche en Cancérologie de Montpellier. Il s'agit d'un modèle de souris transgéniques CCSP/EGFRTL. Il a été généré en croisant une souche de souris transgénique Tet-ON-EGFR^{T790M/L858R} qu'on appellera par la suite model EGFR, avec la souche CCSP-rtTA portant le promoteur rtTA qui est un élément sensible à la tétracycline⁸, sous le contrôle d'un promoteur spécifique du poumon. Chez les souris CCSP/EGFR, le gène EGFR contient à la fois la mutation oncogène L858R et la mutation T790M. Ce système permet l'expression du gène EGFR spécifiquement dans le poumon, cette expression se fait uniquement lors de l'ingestion de la doxycycline (antibiotique), ce qui conduit au développement d'adénocarcinomes pulmonaires périphériques avec des caractéristiques bronchioalvéolaires dans les alvéoles ainsi que des adénocarcinomes papillaires (Figure 10) (Li et al., 2007; Mur et al., 2020). Les croisements d'accouplement ont été maintenus en hétérozygotie, de sorte que la portée résultante pouvait avoir l'un des quatre génotypes possibles : WT/WT, WT/EGFRTLtg, CCSPtg/WT et CCSPtg/EGFRTLtg. Dans notre cadre expérimental, nous avons utilisé des souris WT/WT, dépourvues des deux transgènes CCSP et EGFRTL, et donc incapables de développer un cancer lors de l'induction par la doxycycline, ci-après appelées souris non cancéreuses (NC), et des souris CCSPtg/EGFRTLtg portant les deux transgènes qui développent une tumeur pulmonaire lors de l'induction à la doxycycline, ci-après appelées souris cancéreuses (CC).

⁸ La tétracycline est une méthode d'expression génique inductible dans laquelle la transcription est activée ou désactivée de manière réversible en présence de l'antibiotique tétracycline ou un de ses dérivés telle que la doxycycline.

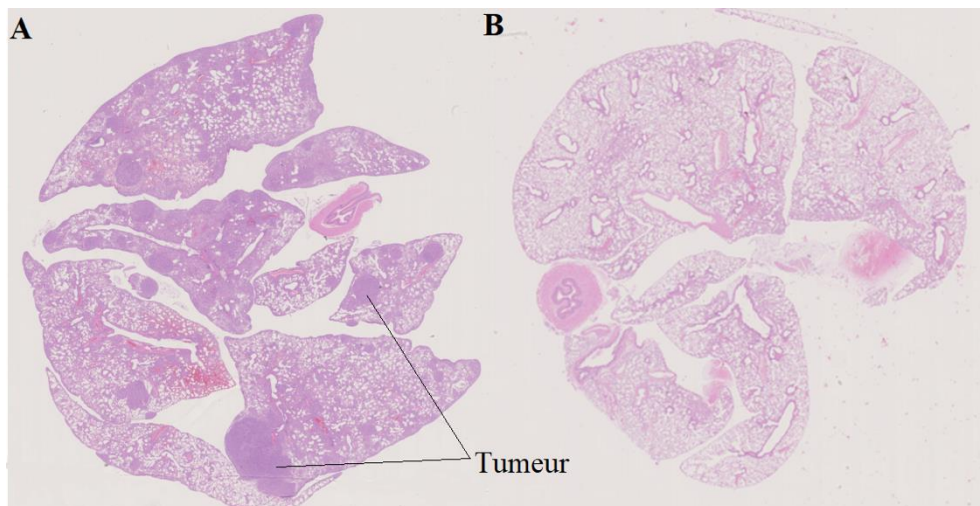


Figure 10: Coupe histologique de poumon de souris. A) souris de génotype CC porteuse de tumeur, B) souris de génotype NC saine.

24 souris mâles, utilisées comme stimuli odorants, ont été fournies par l'IRCM (Institut de Recherche en Cancérologie de Montpellier) avec lequel nous collaborons. Parmi ces souris, 11 étaient mutées sur les deux gènes, et sont devenues par la suite cancéreuses (CC), et 13 souris non mutées sur les deux gènes ont constitué des souris témoins (NC). Elles ont été transférées à l'âge de 6 semaines, pour les expérimentations, à l'animalerie de l'IRD de Montpellier. Elles ont été maintenues par groupe de 2 ou 4 souris frères dans des cages de (26.8cmL*21.5cmL*14.1cmH) agrémentées chacune de carrés de cellulose, de foin et de tunnels en carton. Elles ont ensuite été isolées à l'âge de 10 semaines pour s'habituer à l'isolement avant le début des expérimentations à l'âge de 13 semaines. Les souris ont toutes été individualisées pour éviter tout effet de dominant/dominé entre les mâles. À partir du moment où les souris ont été isolées elles ont été maintenues dans des cages avec uniquement un carré de cellulose pour éviter toute autre odeur qui pourrait interférer avec les odeurs des souris, tels que du foin ou des jeux en plastique ou en carton. Chaque semaine, leur nourriture ainsi que leur poids ont été contrôlés pour surveiller leur état de santé (euthanasie si perte de 10% de masse). De plus, elles ont été nourries de sorte qu'elles aient toutes dans leur mangeoire une quantité de 80g de croquettes, ce qui correspond à la quantité nécessaire pour qu'elles puissent s'alimenter correctement durant 1 semaine. Les litières ont été changées toutes les deux semaines, et la quantité mise à chaque fois (130g) correspondait, après calcul, à la quantité nécessaire pour réaliser les tests de comportement que nous envisageons de réaliser par la suite.

Pour induire le cancer, nous avons ajouté le traitement à la doxycycline dans leur nourriture lorsque toutes les souris avaient 13 semaines, et ce pendant une durée de 12 semaines, à l'issue de laquelle, pour des raisons d'éthique, nous les avons euthanasiées. Les expérimentations sur les souris EGFR se sont étalées sur une période allant de février 2019 à octobre 2019. Les souris EGFR étant issues de 4 couples différents il a fallu attendre différentes portées et étaler les expérimentations dans le

temps. Il a également fallu attendre 2 mois pour obtenir les résultats des prélèvements de poumon pour vérifier que nos souris avaient bien déclenché le cancer, et connaître ainsi les prélèvements utilisables pour les expériences de comportement.

2) *Modèle de souris Nez*

Les tests comportementaux ont été réalisés sur des souris sauvages mâles et femelles *Mus musculus domesticus* originaires du Danemark, hébergées à l'université de Montpellier (RAM-CECEMA). Elles ont été logées dans des cages en plastique transparent (20cm L x35cm l x14cm H) contenant une litière faite de sciure de bois, de foin, et d'un morceau de carton. Un igloo en plastique rouge équipé d'une roue était également présent dans chaque cage. Des croisements ont été réalisés au cours de la 1^{ère} année de thèse pour obtenir une génération de souris sur laquelle les expérimentations ont été réalisées. 37 couples différents ont été formés pour obtenir au moins 37 portées, d'où ont été issus les mâles et les femelles utilisés pour les tests comportementaux. Les descendants issus de ces croisements (2 mâles et 2 femelles gardés pour chaque portée) ont été séparés des parents à l'âge de 3 semaines et maintenus en couples de frères et sœurs, ce jusqu'à 2 semaines avant le début des tests vers l'âge de 10 semaines. A partir de 10 semaines les mâles ont été maintenus isolés et ce durant toute la durée des expérimentations. Les femelles, une fois séparées de leurs frères, ont soit été mises directement par 2, soit isolées si celles-ci étaient gestantes ou allaitantes puis placées par 2 une fois le sevrage effectué. Toutes les souris ont suivi un rythme 12h jour / 12h nuit.

II) **Mise en place du protocole expérimental**

1) *Expérience préliminaire*

Avant le début de ma thèse, j'ai pu participer lors de mon master 2 aux études préliminaires mises en place par Frédéric Thomas et Laurent Dormont pour étudier les odeurs de souris cancéreuses.

- **Mise en place des prélèvements d'odeur sur souris cancéreuses**

Des expériences préliminaires avec des souris entières vivantes placées dans des bocaux en verre de 500ml, de 10cm de diamètre sur 15cm de hauteur (Legallais ®, Montferriez sur Lez), munis d'orifices en haut et en bas pour que les souris puissent respirer, ont été réalisées. Une première série d'expériences a été conduite pour mettre en place le dispositif expérimental et étudier certains paramètres tel que le temps optimal de prélèvement. Le dispositif final était constitué d'un bocal fermé à l'aide d'un film plastique inerte transparent Nalophan ® (Kalle Nalo GmbH, Wursthüllen, Germany), fixé hermétiquement autour du bocal. Un grillage en dessous de la souris été placé pour éviter que la souris ne soit en contact direct avec ses urines et ses excréments. Les prélèvements d'odeurs ont été réalisés à l'aide de deux méthodes différentes d'extraction : une méthode passive, celle des fibres SPME, et

l'utilisation des ChromatoProbes, qui est une méthode dynamique nécessitant une pompe pour aspirer les COVs en continu, et dont le principe d'extraction des COVs repose sur des polymères absorbants différents de la SPME (mélange de Tenax et Porapak). La durée de prélèvement pour les deux méthodes a été fixée à 4h, ce temps correspondant à la durée optimale de prélèvement pour capter un maximum de composés tout en préservant des conditions expérimentales acceptables pour l'organisme vivant. Pour chaque bocal contenant une souris, une fibre SPME et un ChromatoProbe ont été simultanément insérés dans le bocal après avoir réalisé une fine entaille dans le Nalophan. Pour les ChromatoProbes ceux-ci ont été connectés à un tuyau relié à un débitmètre, lui-même relié à une pompe pour aspirer en continu les COVs sur la phase d'adsorption des ChromatoProbes (Figure 11).

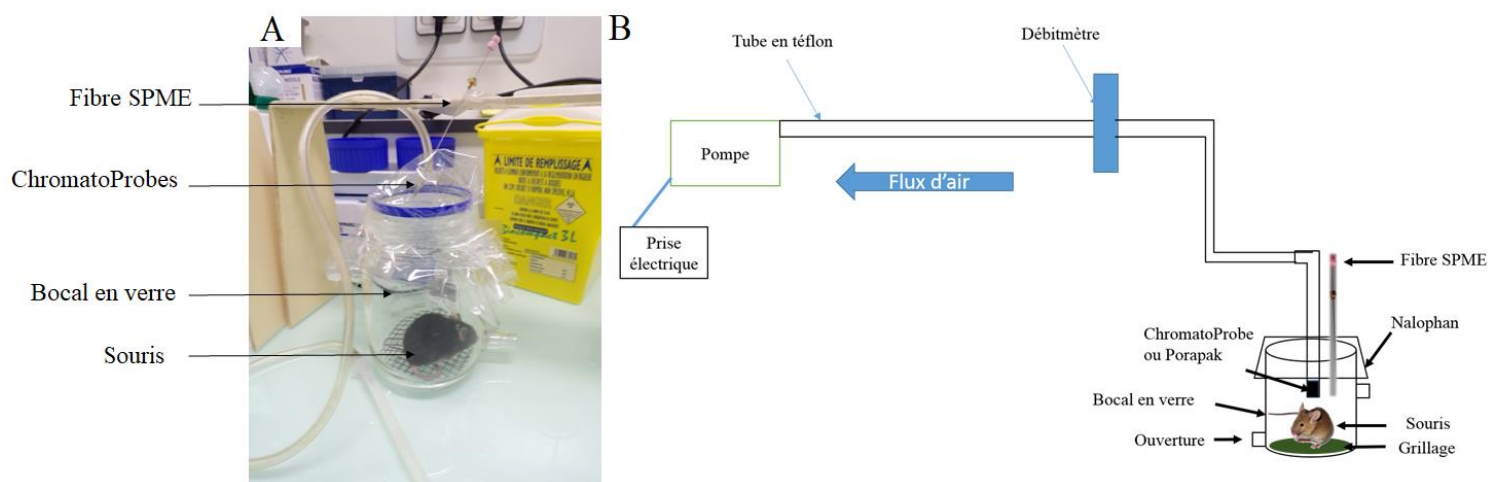


Figure 11: Photo A) et schéma B) représentant le dispositif expérimental utilisé pour prélever les odeurs de souris.

Les débitmètres étaient réglés pour avoir un débit d'extraction de 60mL/sec. Ces prélèvements ont été réalisés toutes les deux semaines au cours du développement du cancer. Les échantillons issus des fibres SPME et ChromatoProbes, ont ensuite été analysés à la plateforme d'analyse chimique en écologie (PACE), labellisée au sein du « Labex CEMEB » et localisée au CEFÉ (Centre d'Ecologie Fonctionnelle et Evolutive) sur le campus du CNRS (Centre National de la Recherche Scientifique) à Montpellier. Ils ont été analysés par chromatographie en phase gazeuse couplée à un spectre de masse (GC-MS). Deux GC-MS différents ont été utilisés, car ces deux techniques de prélèvement ne peuvent être analysées sur la même machine. Le Shimadzu QP2010 Plus (Shimadzu, Kyoto, Japon) a été utilisé pour les fibres SPME et le GC Trace 1310 couplé à un spectromètre de masse ISQ QD Single Quadrupole (Thermo-Electron, Milan, Italie) pour les ChromatoProbes. Pour identifier les composés et analyser les profils d'odeurs après passage au GC-MS, deux logiciels ont été utilisés pour chacune des deux techniques. Les profils chromatographiques des fibres SPME ont été analysés grâce au logiciel Shimadzu software, GCMS solution v.4.11. et les ChromatoProbes avec le logiciel Xcalibur (Thermo-Electron). Pour les deux techniques de prélèvement, la même procédure pour analyser les profils chromatographiques a été mise en place. Pour chacune des souris, une liste des différents COVs présents a été établie. Les analyses issues de ces prélèvements n'ont pas permis d'obtenir des résultats concluants

du fait d'un trop grand bruit de fond et d'une trop grande variabilité entre nos échantillons. En effet, lors des prélèvements, certaines souris urinaient et d'autres non, de plus une grande quantité de composés provenant de la pièce a été prélevée en même temps que nos odeurs de souris, à cause d'une ouverture au niveau des bouchons pour que les souris puissent respirer. Ces deux paramètres ont engendré la capture d'un grand nombre de composés, ce qui a rendu difficilement exploitables les chromatogrammes. Parmi les composés identifiés comme pouvant être émis par les souris nous avons pu voir qu'une grande variation des profils d'odeur était induite par le fait que les souris urinent ou non dans le flacon. N'ayant qu'une dizaine de souris CC et NC lors de ces expérimentations préliminaires, nous n'avons pas pu séparer les souris en deux groupes (uriné /non uriné) pour faire les analyses. En effet, cela nous aurait donné un échantillon trop faible pour réaliser des analyses statistiques qui soient valables. Nous avons pu constater qu'avec ce dispositif, il existait une trop grande variation de COVs indépendamment du paramètre recherché, le cancer. Cela ne nous a donc pas permis de conclure sur l'impact du cancer sur les COVs de souris. Pour optimiser ce dispositif, il faudrait pouvoir réduire les entrées d'air au niveau des deux extrémités du bocal (ou filtrer l'air entrant dans le bocal avec du charbon actif). Il faudrait donc réfléchir à améliorer le dispositif pour garder un volume de contenant raisonnable (pour concentrer les COVs) tout en permettant aux souris de respirer correctement pendant les 4h d'expérience. Souhaitant être le moins invasif et stressant pour la souris, nous avons donc décidé de modifier notre approche et de réaliser à la fois des prélèvements d'urines de souris et de leurs litières souillées, pour ainsi avoir ainsi l'ensemble des odeurs des fluides émis par les souris (féces, urines, air expiré, glandes) et ainsi pouvoir mieux calibrer la quantité exacte d'urine ou de litières prélevées et ainsi obtenir un profil d'odeur le plus comparable possible entre individus.

- **Mise en place des prélèvements d'urine de souris**

Souhaitant réaliser des prélèvements d'urine sur des souris cancéreuses pour étudier l'impact du cancer sur les COVs émis, nous avons utilisé aux cours de cette thèse, le protocole mis en place par Guila Ganem et son équipe lors d'études préliminaires sur de l'urine de souris sauvages. Une durée de 1h a été fixée comme temps nécessaire pour récolter la majorité des COVs et refléter la composition des échantillons, notamment à la vue de la chronologie de la libération des COVs par les MUP (Protéine urinaire majeure) (Cavaggioni et al., 2006). Lors de ces prélèvements des fibres SPME de type PDMS/DVB Polydiméthylsiloxane/Divinylbenzène ont été utilisées, ayant un large spectre d'affinité avec les COVs pouvant être émis par les organismes vivants. Le dispositif mis en place et utilisé par la suite au cours de ma thèse en l'adaptant à mes échantillons est le suivant. Un flacon en verre de 5ml a été placé dans une étuve à 22°C avec une quantité d'urine mise au fond du flacon, généralement entre 40-50µl. Une fois le flacon placé dans l'étuve et après un temps d'équilibrage, le septum (partie supérieure du bouchon constituée à l'extérieur de silicone) a été percé à l'aide d'une aiguille propre pour

ensuite pouvoir insérer la fibre SPME dans le flacon (Figure 12). La fibre a ensuite été laissée 1h puis rétractée et retirée du flacon pour ensuite être injectée dans le GC-MS.

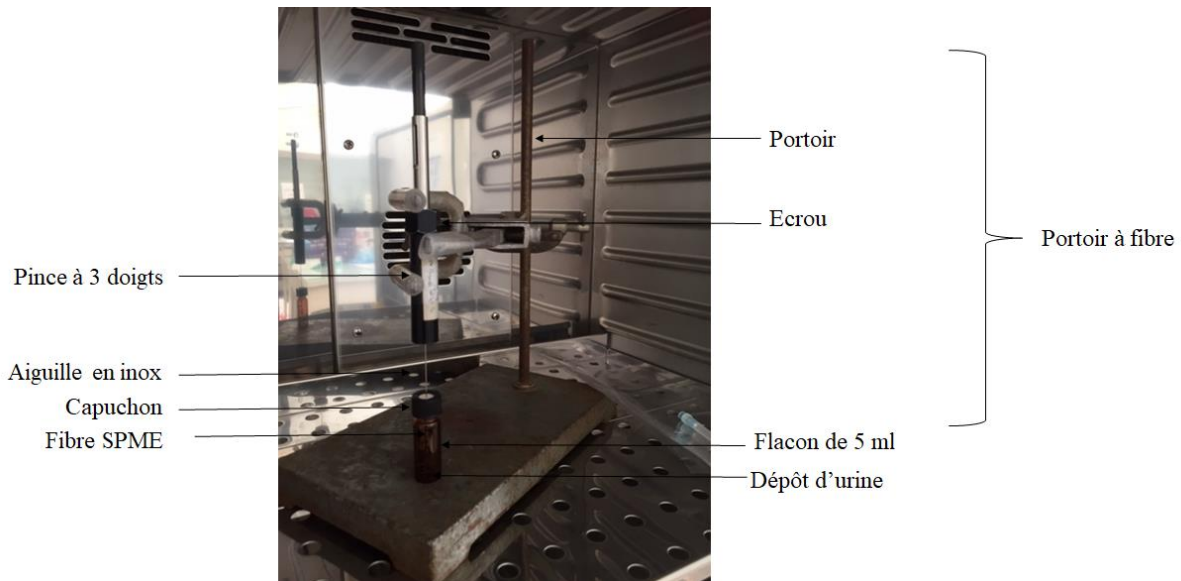


Figure 12: Photo du dispositif expérimental de prélèvement d'odeur en « espace de tête » à l'aide de fibre SPME

2) Protocole des prélèvements d'odeur

Au vu des analyses préliminaires réalisées au cours de mon Master 2, j'ai donc effectué des prélèvements à la fois de litières pour avoir l'ensemble des odeurs émises par les souris (fèces, urines, air expiré, glandes) et d'urine, l'un des prélèvements les plus étudiés sur les souris car non invasif et permettant d'obtenir une grande quantité de composés émis par les souris. Ces prélèvements ont été réalisés durant l'année 2019 sur les souris mâles EGFR âgées de 13 semaines au début des prélèvements. Toutes les deux semaines, la litière souillée a été récupérée et mise au congélateur (-20°C) pour ensuite être utilisée pour les tests de comportement. Un premier prélèvement appelé T0 a été réalisé avant l'ajout de doxycycline dans la nourriture des souris, puis un prélèvement toutes les 2 semaines pendant 12 semaines, soit 7 prélèvements réalisés, notés de T0 à T12 (Figure 13).

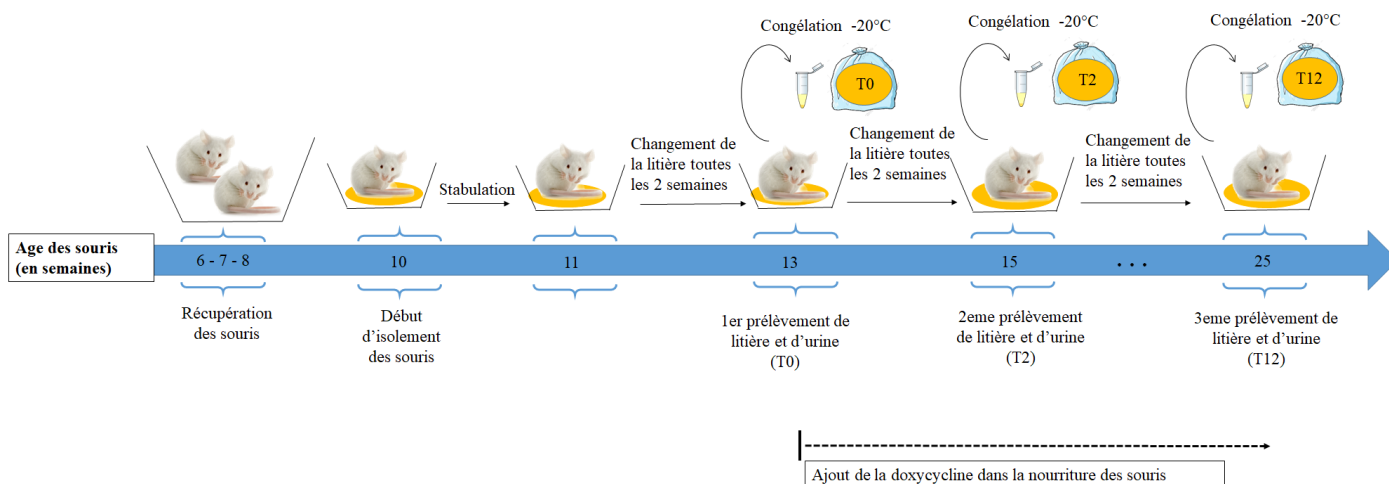


Figure 13: Schéma décrivant le protocole d'échantillonnage des urines et des litières utilisées pour les analyses GC-MS et les tests comportementaux.

Des prélèvements d'urine ont également été réalisés toutes les 2 semaines. Sur chaque individu, nous avons prélevé 1 fois/jour les urines durant 3 jours consécutifs, à différents moments de la journée matin et après-midi, pour capturer une plus grande part de l'hétérogénéité de la sécrétion journalière. Le protocole d'échantillonnage de l'urine était le suivant : chaque souris a été placée seule dans un contenant propre jusqu'à ce qu'elles urinent. L'urine a ensuite été collectée au moyen d'une micropipette, et conservée dans un tube eppendorf maintenu dans de la glace avant congélation à -20°C . Des témoins techniques ont également été réalisés en parallèle. Il s'agissait de mettre des gouttes d'eau distillée dans le contenant propre et de le pipeter dans les mêmes conditions que les échantillons biologiques et de les conserver dans le même type de tubes à -20°C . Sur l'ensemble des souris, trois souris NC n'ont jamais voulu uriner.

Pour réaliser les tests de comportement des pools de litières ont été réalisés. Nous avons mélangé la litière souillée de plusieurs souris non apparentées pour atténuer l'effet des odeurs individuelles sur le bouquet odorant. Pour les tests d'habituation / discrimination, présentés ci-après, nous avons réalisé : 2 pools distincts contenant chacun 3 souris CC et 2 pools distincts contenant la litière de 3 souris NC. Les 3 souris de chaque pool n'étaient pas apparentées, mais entre les 4 pools il s'agissait de souris frères (Tableau 1). Pour les tests de préférence, 1 pool contenant 4 souris CC et 1 pool contenant 4 souris NC ont été réalisés. Comme pour l'habituation les 4 souris d'un même pool n'étaient pas apparentées, mais entre les 2 pools CC et NC il s'agissait d'individus frères. Ces pools ont été réalisés pour les trois stades de progression du cancer, à T0 avant le début du traitement, à T2, deux semaines après le début du traitement et enfin à T12 ce qui correspond à 12 semaines de traitement avant que les souris soient euthanasiées.

Tableau 2 : Caractéristiques des stimuli utilisés lors des tests comportementaux.

Chaque stimulus a été obtenu par la mise en commun de la litière souillée de plusieurs individus. Les donneurs étaient la progéniture de quatre parents distincts. CC : souris cancéreuses ; et NC : souris non cancéreuses.

Test	Statut des souris	Pool	Parents 1	Parents 2	Parents 3	Parents 4
Habituation/discrimination	CC	Pool A	Souris 1	Souris 1	Souris 1	
	CC	Pool B	Souris 2	Souris 2	Souris 2	
	NC	Pool C	Souris 3	Souris 3	Souris 3	
	NC	Pool D	Souris 4	Souris 4	Souris 4	
Préférence	CC	Pool E	Souris 5	Souris 5	Souris 5	Souris 1
	NC	Pool F	Souris 6	Souris 6	Souris 6	Souris 2

3) Test comportementaux

- Test habituation/ discrimination

Les tests d'habituation / discrimination (Todrank & Heth, 2003) consistent à habituer une souris à une odeur A. On considère qu'une souris est habituée lorsque la durée passée à renifler le stimulus diminue avec le temps (Figure 14). Une fois l'habituation réalisée nous effectuons une phase dite de discrimination ou généralisation en lui présentant soit l'odeur A et une nouvelle odeur B dans le cas de la discrimination, ou bien deux odeurs B et C dans le cas de la généralisation. Le temps passé à renifler chaque odeur est mesuré, et cela nous informe sur la différence potentielle entre les odeurs. L'odeur la plus reniflée est considérée comme la plus éloignée de l'odeur A. Dans le cas de la discrimination cela nous permet de voir si la souris est capable de discriminer l'odeur A et B. Dans le cas de la généralisation, cela nous permet de savoir qui de B et C est le plus proche de A d'un point de vue odorant. Dans notre cas, les stimuli présentés sont des pools de litières réalisés à partir des litières de souris CC et NC. Nous avons réalisé des premiers tests pour vérifier la capacité de nos souris à discriminer des odeurs NC et CC. Pour cela, nous avons réalisé des tests habituation/discrimination avec comme odeurs A soit un pool NC ou CC, puis l'odeur A vis-à-vis d'une odeur différente soit NC ou CC. Nous avons ensuite réalisé des tests d'habituation/ généralisation pour savoir si les souris faisaient une différence entre des individus CC et NC. Dans ce cas l'odeur d'habituation A était soit une odeur issue d'un pool (CC) soit une odeur d'un pool (NC), puis en généralisation un autre pool (CC) était testé vis-à-vis d'un autre pool (NC). Nous avons émis l'hypothèse que lorsque le cancer était à un stade avancé avec les prélèvements T12 les souris devraient aller plus renifler l'odeur (NC) lorsqu'on les aura soumises à l'odeur (CC) en habituation, et inversement lorsqu'on leur aura présenté l'odeur (NC) en

habituation. La durée des tests après une phase de tests préliminaire a été fixée à 14 min pour la phase d'habituation puis de 7 min pour la phase de discrimination/généralisation. En début de test, la souris est placée dans une boîte de départ pendant deux minutes et pendant ce temps 4g de litière est placée dans la boîte d'habituation. Au bout de ces 2 min, la trappe est ouverte pour laisser la souris accéder à la boîte d'habituation. 3 min avant la fin du test, les litières utilisées pour la phase de discrimination ou de généralisation ont été pesées et mises dans une nouvelle boîte. Au bout des 14 min la souris a de nouveau été isolée dans la boîte de départ. Nous avons retiré la boîte d'habituation et tout de suite remplacé par une boîte contenant 4g de deux litières différentes pour que la souris ne reste pas plus de 30 secondes dans la boîte de départ et se déshabitue à l'odeur présentée. Nous lui avons ensuite ouvert et l'avons laissée 14min dans le compartiment, temps durant lequel nous avons mesuré le temps passé à renifler chaque stimulus.

Le dispositif expérimental est composé de 3 parties, une boîte de départ : de 15cm L x 15cm l x 10cm h, un couloir : cylindre de 25 cm de long sur 5 cm de diamètre, et une boîte contenant les stimuli (30cm L x 30cm l x 10cm h) (Figure 14).

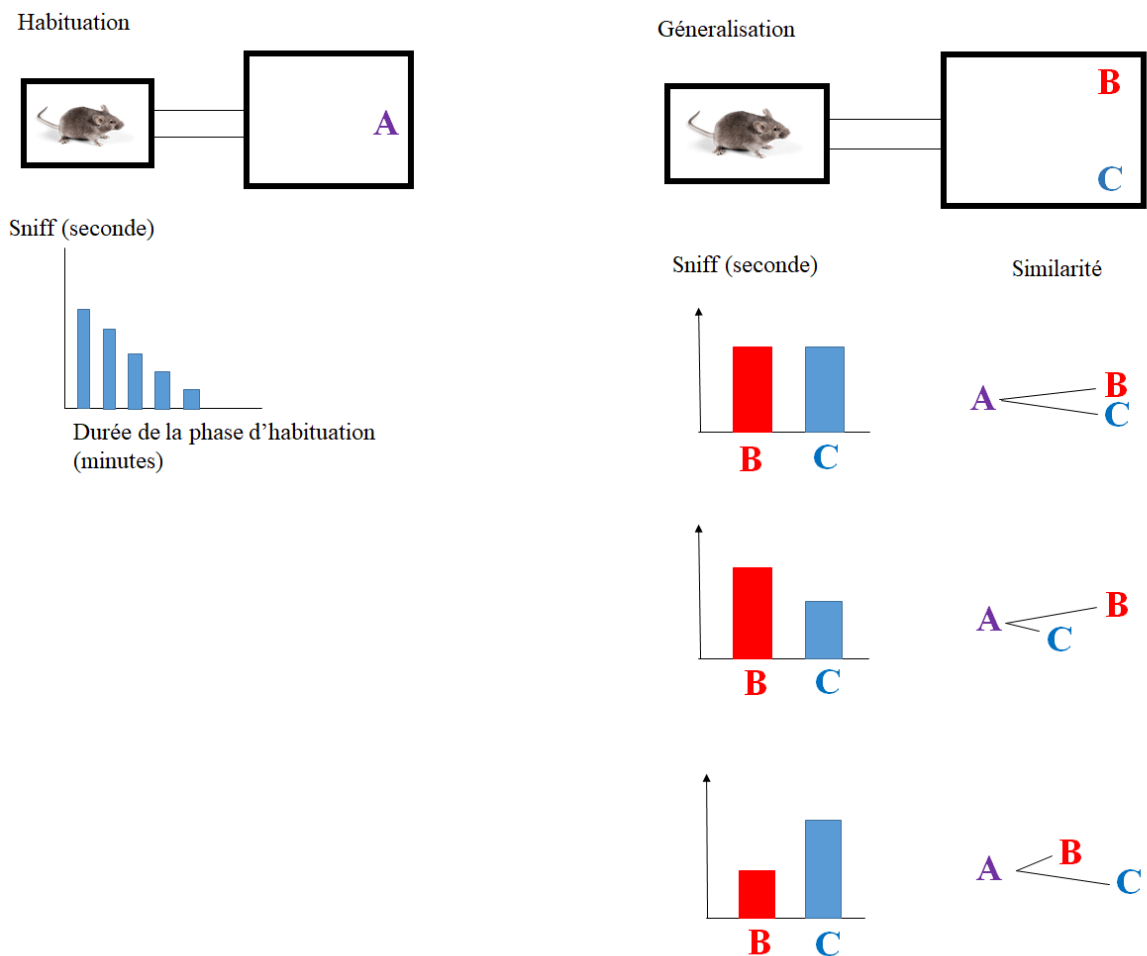


Figure 14: Schéma décrivant la procédure d'habituation/généralisation et les prédictions.

Pour éviter un biais de latéralité, les pools lors de la phase de discrimination/ généralisation ont été alternés de manière aléatoire. La mise en place des dispositifs, exceptée la boîte de départ et la mise en place des caméras a été effectuée avant de placer la souris. Cela a permis de limiter au maximum le stress imposé à la souris en subissant des déplacements humains ou d'objets. Les litières ont été pesées à l'aide d'une balance mesurant les grammes au dixième près. Les tests ont été filmés puis analysés à l'aide du logiciel The Observer.

Lors de la phase de discrimination, j'ai comparé le temps relatif passé au contact avec les deux stimuli ce qui nous a permis d'évaluer si la souris pouvait discriminer les différents pools de litières. Lorsque l'un des stimuli mis lors de la phase de discrimination/ généralisation est le même que celui d'habituation, on s'attend à ce qu'il soit le stimulus le moins reniflé. La différence du temps passé en contact avec le stimuli (CC) et (NC) permet d'évaluer leur degré de différence. Le temps de latence que mettait chaque souris pour renifler la litière d'habituation ainsi que le temps passé à renifler cette litière pour chaque prélèvement a aussi été pris en compte, pour voir si en fonction du prélèvement, la souris passait plus ou moins de temps dans la boîte.

- Test de préférence

Les tests de préférences ont consisté à placer dans 2 compartiments différents des odeurs différentes, et à laisser la souris renifler ces deux odeurs durant un temps déterminé, puis analyser vers quel stimulus elle est allée le plus. Ces tests ont été réalisés à l'aide d'un labyrinthe en Y relié à 3 boîtes. Le dispositif expérimental est constitué d'un compartiment de départ de 15cm L x 15cm l x 10cm h, et de deux autres boîtes placées aux extrémités du Y, compartiment dans lequel se trouve les odeurs. Ces derniers mesurent tous les deux 15cm L x 20cm l x 10cm h (Figure 15). Ces tests ont été réalisés sur des souris femelles sauvages âgées de 12 à 18 semaines. Pour les tests sur les femelles, de la litière de mâle a été placée dans leurs cage 2 jours avant les tests chaque semaine pour synchroniser leur cycle et induire l'œstrus ou pro-œstrus chez chacune d'elles. Cela a permis d'avoir des femelles réceptives aux odeurs de mâle lors des tests. Pour vérifier que les souris étaient bien synchronisées et en chaleur, des frottis ont été réalisés sur chaque souris après les tests de préférence. Les souris ont été placées dans le compartiment de départ pendant 2 minutes. Pendant ce temps, 4g de litière issue des deux pools CC et NC à différents stades T0, T2 et T12 ont été placés dans les deux autres compartiments. L'emplacement des litières a été alterné aléatoirement. Le chronomètre a été déclenché à partir du moment où les deux pattes de la souris dépassaient de la boîte de départ. A partir du déclenchement du chronomètre le test durait 10 minutes. Si au bout de 5 minutes la souris n'était pas entrée dans au moins un des compartiments contenant les litières, le test pour cette souris été arrêté.

Tous les tests ont été filmés, et les analyses ont été faites à partir de l'analyse des vidéos, et les observations enregistrées à l'aide du logiciel The Observer. Pour chaque vidéo, le temps passé dans la boîte de départ, du côté gauche ou droit ainsi que le temps à renifler l'odeur à gauche et à droite ont été

pris en compte, ainsi que la latence pour aller dans chaque compartiment et le temps de latence que les souris ont mis à renifler les deux odeurs.

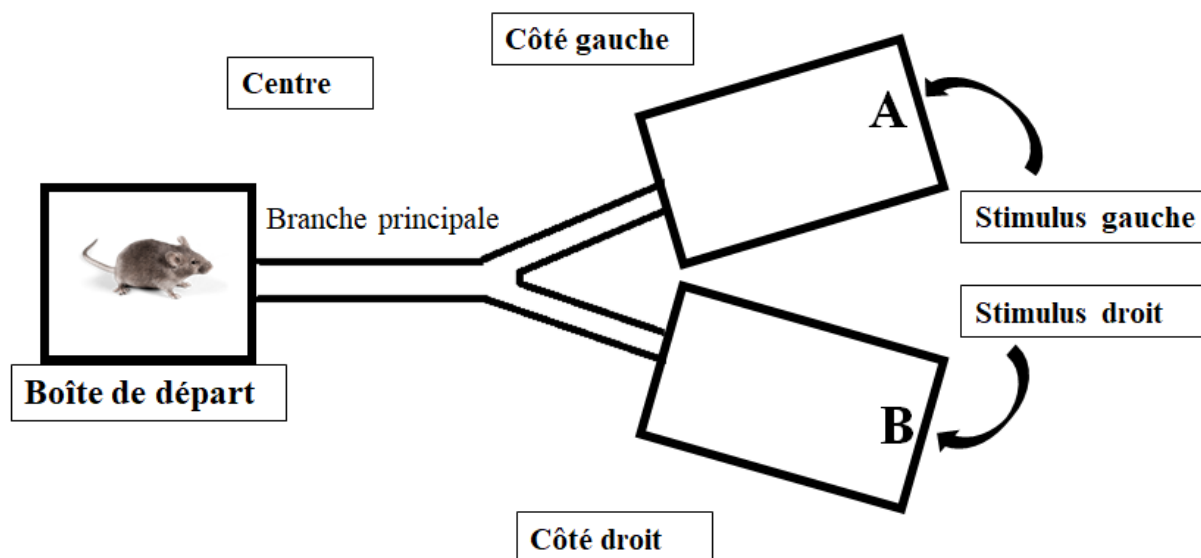


Figure 15: Représentation du dispositif du test de préférences

La préférence a été évaluée en comparant 1) le temps que les souris femelles sauvages passent au contact (i.e. renifler, goûter) des deux stimuli, 2) le temps passé dans les deux compartiments et dans les deux couloirs contenant ces stimuli, et 3) les temps de latence mis pour rentrer dans chaque compartiment et le temps pour renifler la litière.

4) Analyse des stimuli

Les analyses chimiques ont été effectuées à la PACE. Au vu des analyses préliminaires réalisées avant ma thèse, nous avons donc décidé de prélever les COVs présents dans les urines et dans les litières uniquement en espace de tête par la technique des fibres SPME. La fibre utilisée avait un revêtement PDMS/DVB. Pour les deux types d'échantillons nous avons utilisé le protocole mis en place par Guila Ganem et son équipe comme expliqué plus haut. Les échantillons, après avoir été décongelés et gardés dans la glace pour réduire les risques d'évaporation, ont été mis dans un flacon en verre, dans lequel la fibre SPME a été insérée. Un temps d'équilibrage de quelques minutes a été réalisé, puis l'aiguille de la fibre a été insérée durant une période de 60 minutes (Figure 16), qui est le temps déterminé comme nécessaire pour récolter la majorité des COVs. Les prélèvements ont été réalisés sur un volume de 40 μ l d'urine ou de 3g de litières. Toute la phase d'extraction a été réalisée dans une étuve à 22°C. La fibre a ensuite été retirée et désorbée en GC-MS avec le même protocole que celui utilisé lors des expérimentations préliminaires avec un programme d'analyse de 30 min.

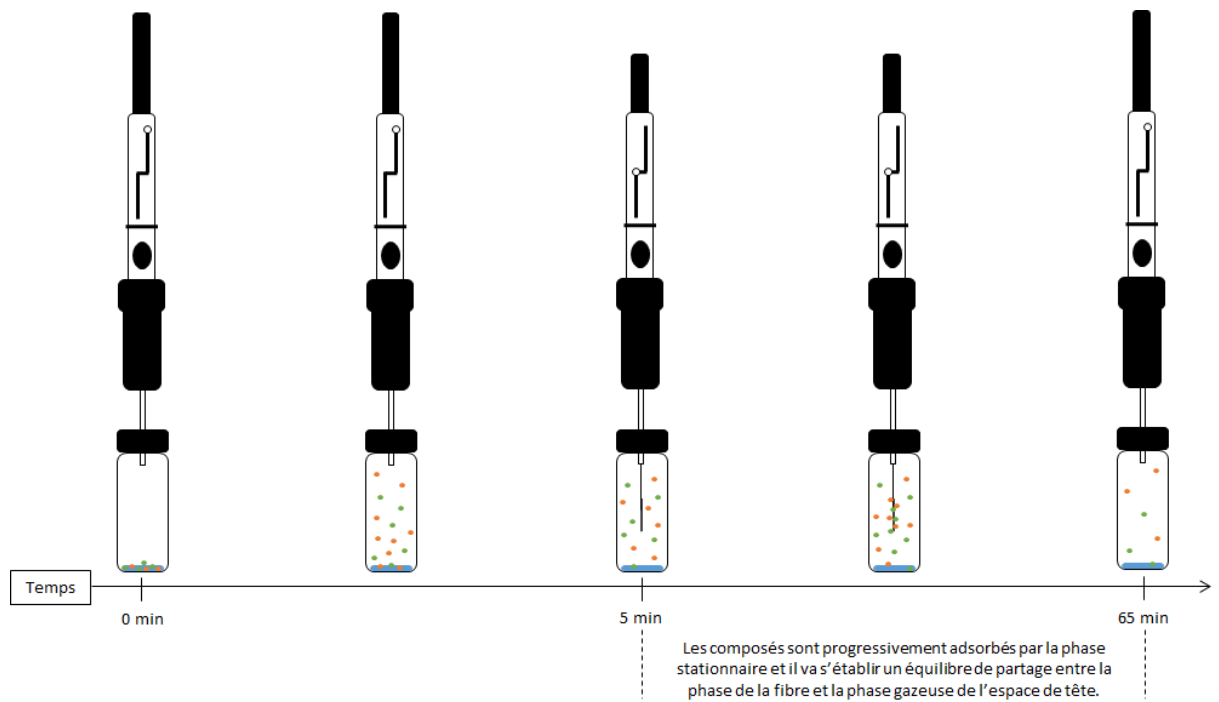


Figure 16: Schéma décrivant le protocole expérimental de prélèvement d'odeur. Les flacons contiennent soit 3g de litière ou 40 μ l d'urine déposés au fond d'un flacon en verre, et les composés (points verts et oranges) sont prélevés à l'aide d'une fibre SPME.

~ Chapitre IV ~

**Capacité des souris à discriminer un individu cancéreux et
conséquences comportementales.**

Contexte et objectifs

Comme expliqué dans l'introduction, la communication chimique joue un rôle majeur dans les interactions sociales chez de nombreuses espèces. Le cancer, en induisant des changements dans les odeurs corporelles, peut par la même occasion modifier les interactions entre les individus. Pour le moment aucune étude ne s'est vraiment penchée sur les conséquences du cancer sur les interactions sociales, notamment dans un cadre de sélection sexuelle. D'autre part, dans la perspective de trouver des méthodes non invasives pour détecter les stades précoces du développement du cancer, cette étude a cherché à savoir si des souris non entraînées pouvaient détecter les changements d'odeur chez des congénères cancéreux. Grâce à l'article de synthèse sur la capacité des animaux à détecter le cancer (**Manuscrit II**), nous avons vu que les souris, avec un conditionnement associé à une récompense, étaient comme les chiens, capables de discriminer des individus cancéreux. Notre étude, avait pour but de déterminer si sans apprentissage les souris en étaient capables aussi, et si cette détection pourrait influencer leur comportement. Pour répondre à ces questions, des tests d'habituation/discrimination sur des souris mâles et des tests de préférence sur des souris femelles ont été réalisés. Nous avons utilisé le modèle de souris cancéreuse EGFR, sur lequel nous avons prélevé les litières pour faire les tests, et des souris *Mus musculus domesticus* issues du milieu sauvage pour les tests de comportement. Les litières ont été utilisées comme stimuli pour les tests de comportement à 3 temps de prélèvements différents ; T0, avant le début du traitement, T2 deux semaines après le début du traitement et enfin T12 en fin de traitement. Deux groupes de 20 mâles *Mus musculus domesticus* ont réalisé des tests d'habituation / discrimination. Nous avons présenté au premier groupe des odeurs de souris NC en habituation et des odeurs de souris CC au deuxième groupe. Les deux groupes ont ensuite eu à discriminer les odeurs de souris CC et NC. Nous avons en plus de leur capacité de discrimination, observé le temps passé à renifler la litière souillée ainsi que le temps de latence de chaque souris passé en présence du stimuli NC ou CC lors de l'habituation, pour voir si leur comportement était modifié en fonction du stimulus présenté. D'autres part, nous avons voulu savoir si la présence du cancer pouvait influencer le choix de partenaire sexuel. Pour cela, nous avons réalisé des tests de préférence avec un dispositif en Y sur 18 femelles *Mus musculus domesticus*. Nous leur avons présenté des stimuli issus des mêmes stades de développement du cancer que pour les tests d'habituation/ discrimination soit T0, T2 et T12.

Résumé de l'article

L'utilisation des souris pour discriminer des individus cancéreux nous a permis de montrer qu'elles étaient capables de faire la différence entre des individus cancéreux et sains sans avoir été entraînées au préalable. Cependant cette discrimination dépend du stimulus présenté au départ, les souris mâles sont capables de discriminer une souris cancéreuse parmi 2 individus sains, et ce dès le

prélèvement T2, mais ne sont pas capables de discriminer une souris saine parmi deux cancéreuses. Pour les souris mâles, les 3 groupes de souris (2 CC et 1NC) sont autant différents l'un de l'autre, ce qui peut sous-entendre qu'il existe une plus grande hétérogénéité entre les souris CC qu'entre des souris NC. Cela nous indiquerait que le cancer modifie bien les profils d'odeurs des souris, et cela à un stade précoce de développement, cependant cette modification n'est pas la même entre tous les individus. D'autre part, nous avons pu voir que les souris mâles étaient plus attirées par les stimuli CC que NC. Elles ont en effet passé plus de temps et la latence était plus faible, lors du test d'habituation à renifler les odeurs de souris CC, ce qui suggère qu'elles sont capables de discriminer des congénères malades mais sans que cela ne génère de comportement d'évitement, contrairement au cas des maladies parasitaires. Nos résultats indiquent également que les souris mâles ont moins reniflé les stimulus issus du prélèvement T12 par rapport au stimuli T0 et T2. Ce désintérêt pour les souris ayant reçu un traitement durant 12 semaines peut être lié à l'impact d'un long régime à la doxycycline, ou bien à l'âge avancé des souris, puisqu'elles passent de 13 semaines en T0 à 25 semaines. Concernant les femelles, nous avons pu montrer qu'elles ne présentent pas de préférence ou de répulsion pour les souris CC. Le cancer ne semble pas influencer le choix des femelles, quel que soit le prélèvement présenté T0, T2 et T12.

Manuscrit associé à ce chapitre :

Manuscrit III :

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Detection of volatile organic compounds from preclinical lung cancer mouse models

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Abstract

Volatile organic compounds (VOCs) may help detect cancer tumour. This study addressed this question, as well as two methodological issues: 1) repeatability, comparing VOCs profiles obtained with two Solid Phase Micro Extraction (SPME) fibers used simultaneously; 2) detectability of cancer VOCs biomarkers, comparing profiles obtained following 1h versus 24h exposure of SPME fibers. We analyzed VOCs composition of soiled bedding obtained from a lung adenocarcinoma mouse model in which cancer was induced by doxycycline ingestion. We compared the VOCs profile of soiled bedding of cancerous (CC) and non-cancerous (NC) mice, before (T0), after two-weeks (T2) and after twelve weeks (T12) doxycycline ingestion. The results indicate : 1) qualitative and quantitative consistency in VOCs detection by two distinct SPME fibers ; 2) although more VOCs were detected following a 24h compared to 1h SPME exposure, none of the former molecules were related to cancer; 3) doxycycline impacted VOCs emissions in both CC and NC mice; 4) cancer impacted four VOCs at T12 only : the benzaldehyde which showed higher levels in CC mice and the hexan-1-ol and two mice pheromones, the 2-sec-butyl-4,5-dihydrothiazole and the 3,4-dehydro-*exo*-brevicomine, which showed lower levels in CC mice. Our study points out that the use of two SPME fibers and an extraction duration of 1h may be considered a good compromise allowing detection of cancer biomarkers while easing bench constraints.

Key Worlds- SPME, repeatability , odour signature, cancer biomarkers, EFGR cancer, *Mus musculus*.

Introduction

Volatile organic compounds (VOCs) are carbon-based small molecules, which are characterized by their volatility at ambient temperature. During the last decades, increasing attention has been devoted to exploring the relevance of VOCs emitted by the body as potential biomarkers of pathologies. VOCs are emitted and can be analysed from breath, skin, saliva, sweat, blood, urine and faeces. Nowadays, VOCs are commonly used as biomarkers of various diseases (Sethi et al., 2013; Shirasu and Touhara, 2011) such as fibrosis (Barker et al., 2006; Jalali et al., 2016), asthma (Ibrahim et al., 2011), Alzheimer disease (Mazzatenta et al., 2015), diabetes and tuberculosis (Phillips et al., 2004; Phillips et al., 2007), and an increasing attention has been given at using them for cancer detection (Gouzerh et al., 2021).

VOCs extraction involves the use of effective trapping methods, of which Solid-Phase Micro-Extraction (SPME) is the most commonly used (Zhang and Pawliszyn, 1995). For instance, this technique has been successfully used to detect VOC biomarkers in the context of diseases such as gastrointestinal disorders (Garner et al., 2007), cholera (Garner et al., 2009), tuberculosis (Syhre and Chambers, 2008) and cancers (Liu et al., 2014; Poli et al., 2010; Rudnicka et al., 2011). Accuracy and repeatability of VOC extraction strongly rely on the characteristics of the SPME fiber used (Dixon et al., 2011) and on extraction conditions such as temperature and duration (Lord and Pawliszyn, 2000; Miekisch et al., 2001; Živković Semren et al., 2018). Some journals recommend using a single SPME in comparative studies, arguing that the use of more than one fiber in such studies may result in biased interpretations. However, when a study includes a large number of samples, it may be unpractical to use a single fiber for all experiment, and multiple successive use of the same fiber may also induce measurements biases due, for example, to wear. Another methodological issue is sampling duration. Emission of VOCs from biological substrate can be dynamic (Cavaggioni et al., 2006) and, in order to account for emission variations, comparative studies need to homogenize sampling duration. A short sampling duration may not allow capturing the diversity of VOCs emitted by the substrate nor detecting components present in very low concentrations, partly because competition between molecules present in the headspace may disadvantage the adsorption of components present in relatively small proportions (Gó et al., 1997; Song et al., 1997; Stashenko and Martínez, 2007).

In the context of investigations of variation in VOCs composition induced by lung cancer in mice, the present study addressed repeatability of results obtained when sampling involved two different SPME fibers, and the impact of sampling duration on the detection of changes in the VOCs composition of mice soiled bedding during the course of tumour development. We also assessed whether VOCs induced by lung cancer were better detected after a 24h as compared to a 1h SPME exposure, and sought to identify murine lung adenocarcinoma VOC biomarkers. The odour sources were soiled beddings

obtained from a genetically modified mouse model suffering from lung cancer, and considered an early and a late stage of tumour development.

Material and methods

1. Ethical clearance

All the precautions for animal welfare were followed during our experiments, and our study received ethic clearance from the Ethical Committee for Animal Experimentation (French Ministry of Higher Education, Research and Innovation) number 1645-22123.

2. Animals

Two genotype lines were involved in this study: WT and CCSP-rtTA / EGFR^{T790M/L858R} (Li et al., 2007; Mur et al., 2020). The WT mice, lacking both CCSP-rtTA and EGFR^{T790M/L858R} transgenes, do not develop any tumour upon doxycycline (antibiotic) induction, hereafter non-cancerous (NC) mice, and the CCSP-rtTA / EGFR^{T790M/L858R} mice, carrying both transgenes, develop lung adenocarcinoma upon doxycycline induction, hereafter cancerous (CC) mice. To induce the EGFR^{T790M/L858R} in a lung specific manner, the mice received a doxycycline supplemented food (1 mg/kg). The treatment lasted 12 weeks starting when the mice were 13 weeks old.

NC and CC male mice were obtained from the IRCM (Montpellier Cancer Research Institute) at the age of 6 weeks and maintained at the breeding facilities of the IRD (Institute of Research and Development) in Montpellier. Before the start of the experiment they were maintained in groups of 2 to 4 mice in transparent plastic cages (26.8cmW*21.5cmL*14.1cmH). Each cage contained sawdust, a cellulose square, hay and a cardboard tunnel. The mice were observed and weighted once a week during the entire experimental period to monitor their health. All mice were euthanized at the end of the experiment at the IRCM at 25 weeks-of age. The non-cancerous status of all NC and the cancerous status of all CC mice were confirmed by histopathological analysis of haematoxylin & eosin stain of whole lung sections obtained after necropsy.

3. Odour source sampling

Odour sources were two-week old soiled bedding of CC and NC mice. Each mouse was isolated at the age of 10 weeks in a cage containing 130g sawdust and a cellulose square. The housing conditions of all donors were homogenized as much as possible, and all mice were given the same quantity of food. Every two weeks, all the soiled bedding of every cage was collected in a plastic bag and kept at -20 ° C

and replaced by cleaned bedding (i.e. 130g sawdust). Soiled bedding analyzed in this study were pools of bedding obtained from 3 to 4 different mice. A sample of clean sawdust was also collected in a plastic bag and frozen to serve as a control for the VOCs analyses.

4. VOCs extraction

PMDS-DVB (polydimethylsiloxane and divinylbenzene) SPME fibers, recently purchased, and which had never been used before, were used in this study (thickness 65 μ m, Stable Flex 24Ga, Sigma-Aldrich, Bellefonte, USA). The odour samples were processed as follows. A frozen soiled bedding was thawed while being maintained on ice during the entire procedure. Then a 3g aliquot was transferred into a 125 ml glass vial, which was then sealed with a rubber septum. The VOCs sampling phase took place in an oven maintained at 22°C. The SPME fiber was introduced into the vial after piercing the septum with a needle after a 3min equilibration time, its position was always the same, ~2cm above the stimulus (**Fig. 1**).

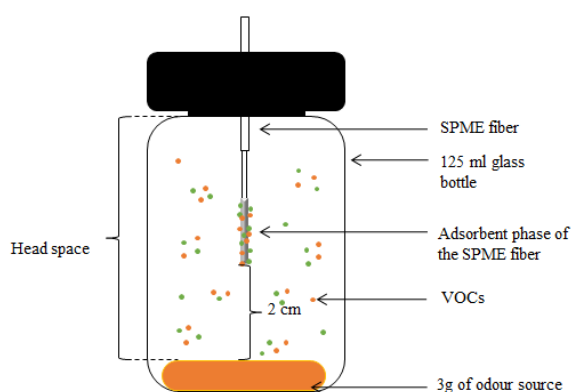
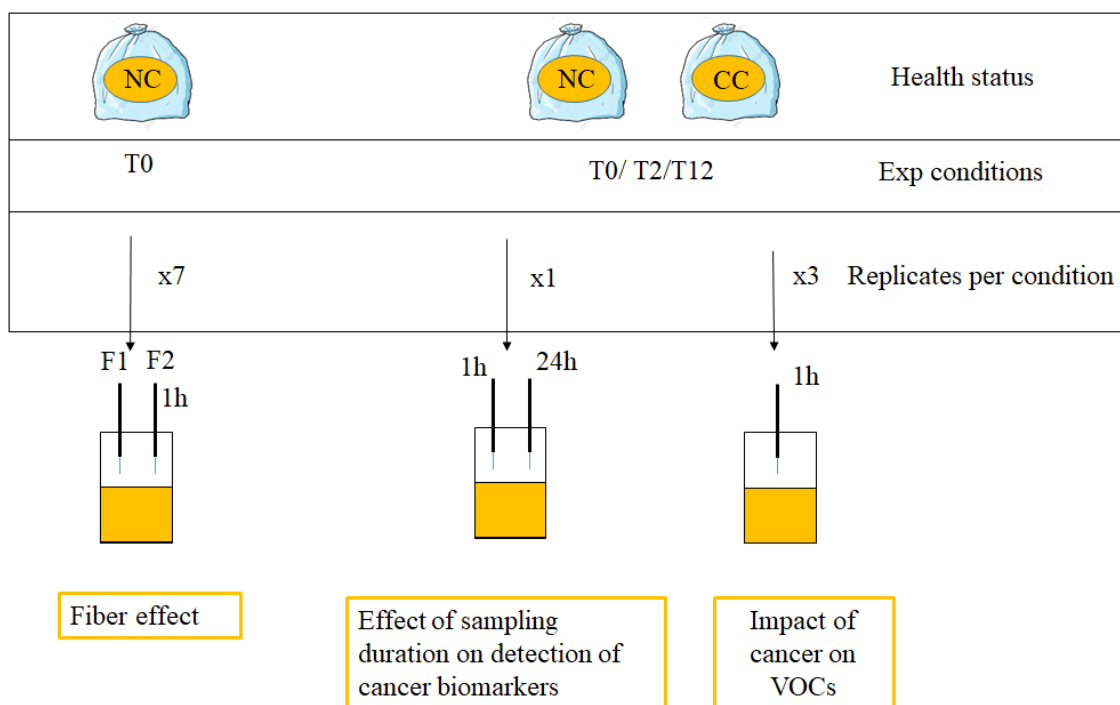


Fig. 1 : A schematic of the experimental protocol. Each bag contained a mixture of bedding obtained from 3 to 4 mice, at different experimental conditions (T0: before treatment, T2: after 2-weeks doxycycline diet; and T12: after 12 weeks doxycycline diet). Three different types of tests were performed: - 1h VOCs sampling using two different SPME fibers exposed simultaneously to the head space and involving T0 non-cancerous (NC) soiled bedding (testing “Fiber effect”); - Two fibers exposed simultaneously, one for 1-h the other for 24h and involving soiled bedding of NC and CC (cancerous) soiled bedding sampled at T0, T2 and T12 (testing “Effect of sampling time”); and – A single fiber exposed for 1h and involving 3 replicates of the same source of NC and CC soiled bedding at T0, T2 and T12 (“Cancer influence”).

5. VOCs characterisation by Gas Chromatography- Mass Spectrometry

The SPME extract was injected into a Gas Chromatography - Mass Spectrometry (GC-MS), quadrupole mass spectrometer Shimadzu QP2010-SE (Shimadzu, Kyoto, 282 Japan) for further identification and relative quantification of its chemical content. Desorption was achieved by inserting the SPME fiber into the Ultra Inlet liner (for SPME/Purge and trap, 0.75mmID, Agilent CrossLab, Santa-Clara, USA) placed in Split/Splitless GC-MS injector heated at 250°C. The injection was made using a split ratio of 1:4 to allow a Gaussian form of low boiling point compounds and so, a better separation and further integration. The GC was equipped with an Optima 5-MS fused silica capillary column (30 m x 0.25 mm x 0.25 µm film thickness, Macherey-Nagel, Düren, Germany). Helium was used as the carrier gas (1 ml.min⁻¹). The GC temperature was maintained at 40 °C for 2 min, after which the temperature increased by 5°C every minute till it reached 175 °C, and then by 12°C/min. till it reached 220°C.

6. Identification of the VOCs present in the extracts

Spectra were analysed with the resident software (GCMS Solution, Shimadzu, Kyoto, Japan). Identification of compounds was based on chromatograms analysis of peak retention time (RT) and examination of their mass spectra. Retention indices (RI) were calculated using as reference the RI of a series of n-alkanes injected in the same apparatus (Alkanes standard solution, 04070, Sigma-Aldrich). Identification of compounds was made by both spectral and RI comparison with reference databases (NIST 2011, Wiley 292 Registry Ninth; e.g. Adams, 2007, Pubchem, 293 <https://pubchem.ncbi.nlm.nih.gov/>). Synthetic compounds were also used as a reference to confirm the identity of some VOCs. The peak surface area of each compound was based on the Total Ion Current Chromatogram (TICC). To be conservative, we excluded from the analyses compounds that were present in both our controls (clean bedding) and our biological samples, even when they were in higher proportion in the biological samples.

Compounds identified with their mass spectra but present in the form of traces could not be quantified with the above described method and were given an arbitrary value corresponding to 10% of the surface area calculated for the smallest peak identified in the entire dataset. Peak areas for all compounds were then summed per chromatogram, and their relative proportion calculated as the ratio of the compound surface area divided by the sum of the surface areas of all other compounds identified in that chromatogram.

7. Testing the repeatability of odour sampling

We tested the repeatability of odour sampling among two different SPME fibers (F1 and F2) pertaining to the same new pack of 3 fibers (Sigma-Aldrich). The fibers were conditioned following the manufacturer's recommendations, i.e. 30 minutes at 250°C. They were simultaneously introduced into a glass bottle containing the odour sample (3g of soiled bedding of 13-weeks old NC mice, Fig.1) and were exposed at the same time to the bottle headspace for 1h. The distance from the odour sample was controlled to be the same for the two fibers (~2cm), and the distance between the two fibers was ~1.5 cm. The same procedure was repeated at seven occasions with the same fibers and the aliquots extracted were obtained from the same odour source, maintained at -20° between samplings. The seven occasions corresponded to the first, 8th, 15th, 22th, 30th, 39th and 45th use of each fiber.

8. Testing the influence of sampling duration

We analyzed the qualitative and quantitative variations of VOCs obtained from 18 samples of soiled bedding sampled with two fibers simultaneously, one exposed to the headspace for 1h the other for 24h. The odour sources were obtained from CC and NC mice at three experimental conditions: T0, i.e. before ingestion of doxycycline, T2, i.e. after two weeks' doxycycline treatment, and T12, i.e. after 12 weeks' doxycycline treatment. (Fig. 1). We proceeded as follows, two SPME fibers were inserted into the same glass bottle containing soiled bedding and were exposed simultaneously to an odour source. The two fibers were placed at the same distance from this source and at a 1cm-distance from each other. After 1h exposure, one of the fibers was removed, while the other was left for another 23h. The procedure for the 18 samples lasted three days, and each day an additional glass bottle containing clean bedding was also sampled following the same protocol and served as a control.

9. Identifying VOCs biomarkers of murine lung cancer

The same 18 odour sources as the above were processed. However, here each of the sources was represented by three replicates to control for sampling bias (i.e. collection of 3g aliquots for VOCs extraction). All together we analyzed 54 biological samples, In addition, a sample of clean bedding was extracted every day to serve as a control. Two SPME fibers were used alternatively for the purpose of this analysis, randomizing their use among NC and CC.

10. Statistical analyses

All statistical analyses were performed with Rstudio version 3.4.4 (R Core Team, 2020). We used the following packages: ade4 (Thioulouse et al., 2018), vegan (Oksanen et al., 2020), mixOmics (Rohart et al., 2017), ggplot2 (Wickham, 2014), RVAideMemoire (Hervé, 2022), Hotelling (Curran, 2021).

Variation of the relative proportions of VOCs was assessed with a multivariate approach using redundancy analyses (RDAs) followed by permutation F tests (Hervé et al., 2018) and the R package *vegan* (Oksanen et al., 2020). Relative proportions were CLR-transformed prior to RDAs, and as our data included zeroes, a small constant of an order of magnitude smaller than the smallest non-zero value was added to all values prior to the transformation (i.e. 0.01 if the smallest non-zero value is 0.1). Factors included in RDAs depended on the questions tested. The first model questioned the consistency of extraction by the two SPME fibers used simultaneously at 7 sampling occasions, and included “fiber identity” and “sampling occasions” as factors. The second RDA model addressed the impact of sampling/extraction duration on detection of cancer biomarker VOCs. Two models were considered. Because the number of VOCs detected after 24h extraction was higher than that after 1h extraction, the first model analysed only the data obtained after 24h and sought to identify presence of VOCs induced by cancer tumour, not detected after 1h extraction. The model included experimental conditions (T0, T2, T12), health status (CC, NC) and their interactions as factors. The second model considered the VOCs identified both after 1h and 24h sampling and addressed whether quantitative differences existed. This model included duration (1h/24h), experimental conditions (T0, T2, T12), and health status (CC, NC) and their interactions as factors.

Finally, the entire data set (56 samples corresponding to 3 replicates of each of 18 odour sources) obtained after 1h extraction was analysed using four RDA models to identify candidate cancer VOCs. In the two first models we addressed the impact of experimental condition (T0, T2 and T12) on VOCs composition for each health status separately. This model included experimental conditions as a fixed factor and replicates (3 per sample) and fiber identity (2 fibers used) as random factors (condition). The third model asked whether the VOCs composition of NC and CC at T0 was similar and included health status (NC/CC) as a fixed factor and replicates (3 per sample), and fiber identity (2 fibers used) as random factors (condition). The last model excluded the VOCs identified as different between NC and CC before the doxycycline treatment (at T0) and addressed whether the rest of the VOCs differed between health status, experimental conditions and their interactions (with replicate and fiber identity as random factors). When relevant, we identified and listed the VOCs showing an absolute correlation coefficient > 0.8 with the RDA constrained axis associated with significant effects in a given model.

Results

1. Consistency of VOCs sampling across fibers and impact of multiple samplings

We detected 89 compounds (**Table.S1**) in the technical controls (clean bedding). When compounds from this list were also detected in the biological samples at similar amounts (soiled bedding), they were

not considered in the final list of compounds. The composition of the volatile profiles specific to the biological samples is given in **Table.1**. We identified the same 17 compounds with the two fibers (**Table.1**). Quantitative variation was assessed with a RDA analysis including the 17 compounds with “fiber identity” (2 modalities) and “sampling occasions” (7 modalities) as factors (**model 1: Table 3**). VOCs composition did not vary significantly with fiber identity ($F= 0.957$, $P = 0.395$), while it did between sampling occasions ($F= 34.185$, $P < 0.001$) (**model 1 : Table.2**). Almost all compounds, except two, showed variations in proportions over time (Figure S1). However, these variations were not linear and did not seem to relate to fiber wear. Although the same quantity of soiled bedding from the same odour source was analysed each time, variation in the quantity and quality of urine or faeces present in each aliquot might be the source of VOCs variation. These results further suggested that it might be important to use multiple replicates of every sample when comparing relatively small quantities (here 3g) of a complex substrate such as soiled bedding.

Table 1 : List of the 17 VOCs identified after simultaneous exposure of two SPME fibers to soiled bedding of non-cancerous mice at T0. Only the components not present in the technical controls (clean bedding) were retained in this list (Table S1).

Compounds	RT ^a (min)	RI ^b	F1	F2	F1 ^d	F2 ^d
			n=7 ^c		%	
Propanoic acid	4,31	707	7	7	12.8 ± 0.7	12.9 ± 0.6
3-methyl-Butan-1-ol	4,89	733	6	6	0.9±0.2	0.8± 0.1
Butanoic acid	6,47	802	7	7	24.1± 2.6	25.2± 2.3
(x)-2-ethyl-Hex-2-en-1-al*	6,68	809	7	7	10. 6± 0.7	9.9 ± 0.7
3-methylButanoic acid	7,98	853	7	7	8.5 ± 0.6	8.3± 0.6
2-methylButanoic acid	8,26	862	7	7	2.2 ± 0.1	2.1± 0.1
Hexan-1-ol	8,45	869	7	7	7.9± 0.3	7.5± 0.2
(x)-2,4,4-trimethyl-pent-2-en-1-al*	8,59	873	7	7	3.2 ± 0.3	3.5 ± 0.5
Benzaldehyde	11,19	956	5	5	2.4± 0.6	2.7± 0.7
2,3-dehydro- <i>exo</i> -Brevicomine*	13,8	1039	7	7	3.5 ± 0.4	3.9± 0.209
3,4-dehydro- <i>exo</i> -Brevicomine	14,06	1047	7	7	9.3± 0.3	9.1 ± 0.4
Fenchone	15,36	1089	2	2	0.5± 0.4	0.6± 0.4
2- <i>sec</i> -butyl-4,5-Dihydrothiazole	15,82	1103	7	7	6.9 ± 0.4	6.1± 0.5
Nopinone	16,79	1135	4	4	0.9± 0.3	1± 0.3
Camphor	17,06	1144	7	7	3.5± 0.2	3.5 ± 0.1
Camphene hydrate	17,25	1150	7	7	1.6 ± 0.1	1.6± 0.2
(<i>E</i>)-3-Pinocampnone	17,53	1160	6	6	1.4 ± 0.3	1.4 ± 0.3

*: attempt of identification when the exact configuration could not be determined.

^a retention time

^b calculated retention indices

^c Maximum number of occurrences.

^d relative proportion of the compounds (% , mean +/- SE)

2. Influence of sampling duration on VOCs' detection: 1h versus 24h

The same 17 compounds as in the first experiment were detected after 1h extraction and the 17 VOCs plus 11 others after 24h extraction (**Table.S2, Fig. 2**). A RDA was first performed to address if among the 11 VOCs only found after 24h extraction any or all were related to cancer tumour development. We designed a model considering as fixed factors experimental conditions (T0, T2, T12), health status (CC, NC) and their interaction (**model 2: Table.2**). VOCs detected only after a 24-h extraction did not differ between NC and CC nor interactively with experimental conditions. Variation in VOCs composition differed significantly only between experimental conditions ($F=3.910, P < 0.001$), pointing out significant differences in the VOCs profiles at T0 compared to T2 and T12 (**model 2: Table.2**). We then analysed the 17 VOCs common to the 1h and 24h extractions, and tested the same model with an additional fixed factor, sampling duration, and its interactions with the other two factors mentioned above (**model 3 : Table 2**). The results indicated a significant influence of the three main factors, but no interaction between sampling duration and health status (**Fig.3**) further suggesting that 24h extraction was not necessary to address the impact of cancer on the mice VOCs profile.

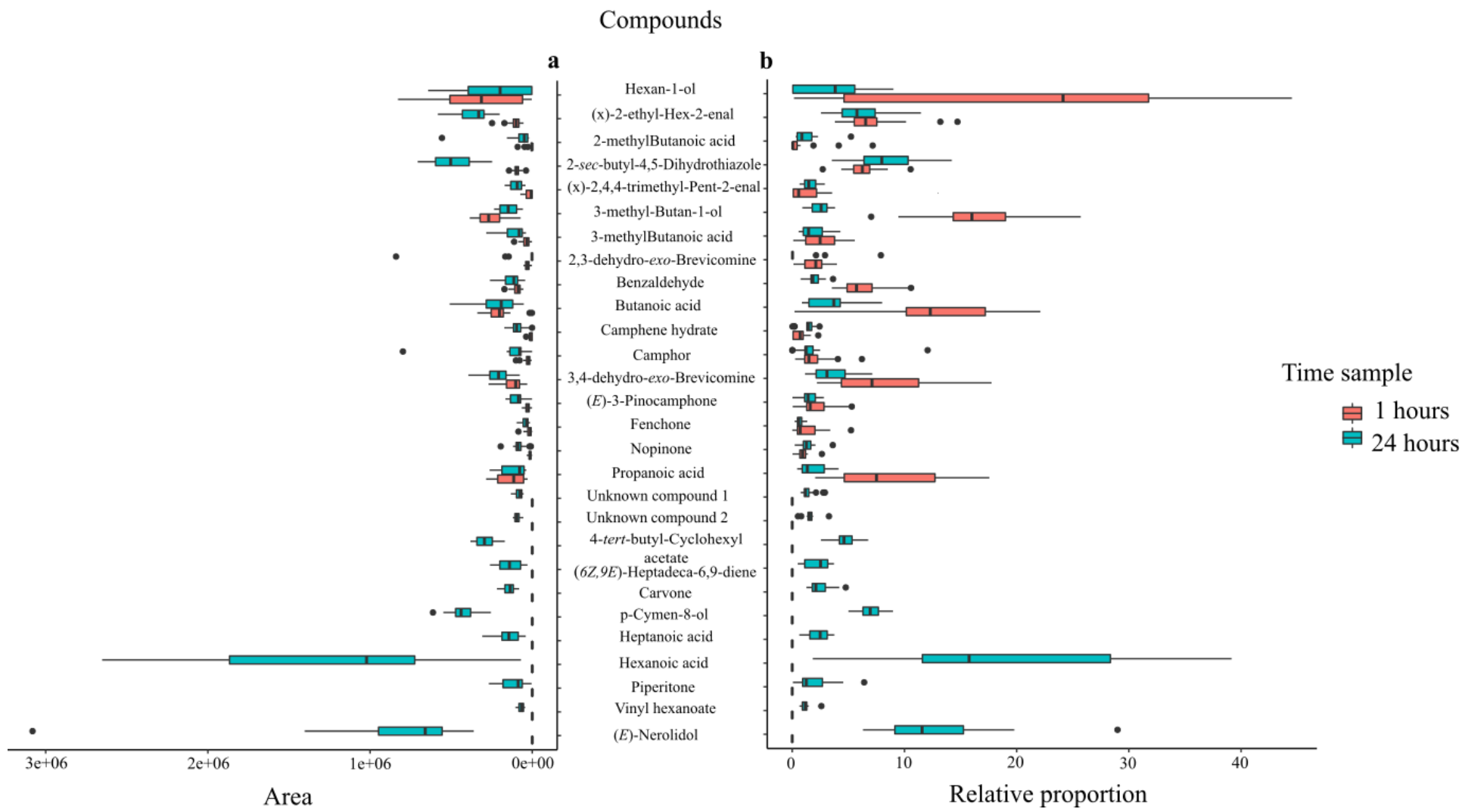


Fig. 2 : Pic surface areas (a), and relative proportions (b) of compounds identified in mice soiled bedding samples after 1h versus 24h SPME extractions.

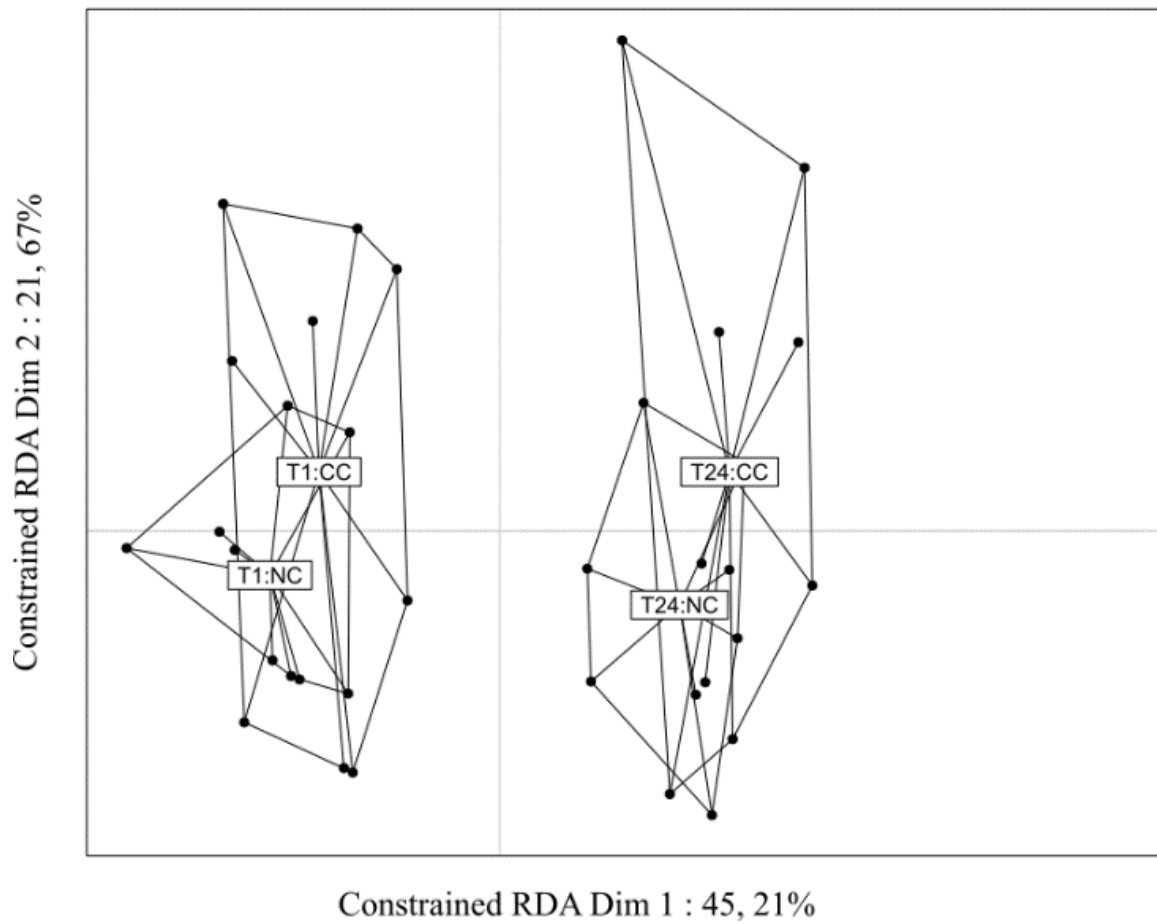


Fig. 3 : Score plots of the redundancy analysis (RDA) comparing variation of 17 VOCs identified in the 1h extraction profiles (T1) versus the 24h extraction profiles (T24) of non-cancerous (NC) and cancerous (CC) mice soiled bedding.

Table 2 : Results of the permutation *F* tests based on redundancy analyses linking VOCs composition to health status (NC non-cancerous mice versus CC potentially cancerous mice).

	random factors	variance explained	explanatory factors	variance explained	F	P
Model 1 : analysis of fibers F1 and F2			Fibers (F1/F2)	97,17%	0.957	0.395
			Time (7samples)		34.185	<0.001
Model 2 : analysis of 24h sample			Exp condition(T0,T2,T12)	45,70%	2.82	<0,001
			Status (NC/CC)		1.39	0.47
			Interactions		1.53	0.08
Model 3 : analysis of 1h and 24h sample			Time (1h/ 24h)	57.4%	14.277	<0.001
			Exp condition((T0,T2,T12)		3.91	<0.001
			Status (NC/CC)		2.037	0.0461
			Time : exp condition (T0,T2,T12)		0.769	0.734
			Time : status		1.075	0.356
			Exp condition : status		1.86	0.027
			Time :exp condition : status		0.939	0.512

Model 4 : analysis of difference of NC sample at 1h	Fibers + replicates	9.60%	NC	24.87%	4,174	<0,001
Model 5 : analysis of difference of CC sample at 1h	Fibers + replicates	2.60%	CC	57.85%	16,085	<0,001
Model 6 : analysis of NC and CC at T0	Fibers + replicates	10.20%	T0 : NC-CC	21.64%	4,174	0,002
Model 7 : analysis of NC and CC for 3 experimental condition	Fibers + replicates	4.30%	Exp condition (T0,T2,T12)		17,0065	
			Status (NC –CC)	46.31%	3,545	<0.001
			Exp condition : Status		2,23	

3. Cancer influence on VOCs production

We did not detect qualitative differences in VOCs composition between CC and NC mice (**Table.3**). To address possible quantitative differences, we proceeded in several steps. First we tested the impact of experimental conditions within the CC and within the NC samples (**Fig.4**). The two RDA analyses comprising replicates (3 per sample) and fiber identity (2 fibers used) as random factors, and experimental conditions (T0, T2 and T12) as fixed factor, indicated that the random factors explained a relatively negligible part of the total variation (9.60 % and 2.60% respectively for NC and for CC). The experimental conditions explained 24.87 % (NC) and 57.85% (CC) of the total variance. The VOCs composition varied significantly between the three experimental conditions, both for NC and CC mice (NC: $F = 4.174$; CC : $F = 16.085$; $P < 0.001$; post hoc tests all $P < 0.001$; **model 4 & 5 : Table.2**), further indicating the influence of experimental conditions on the VOCs profiles of both NC and CC mice.

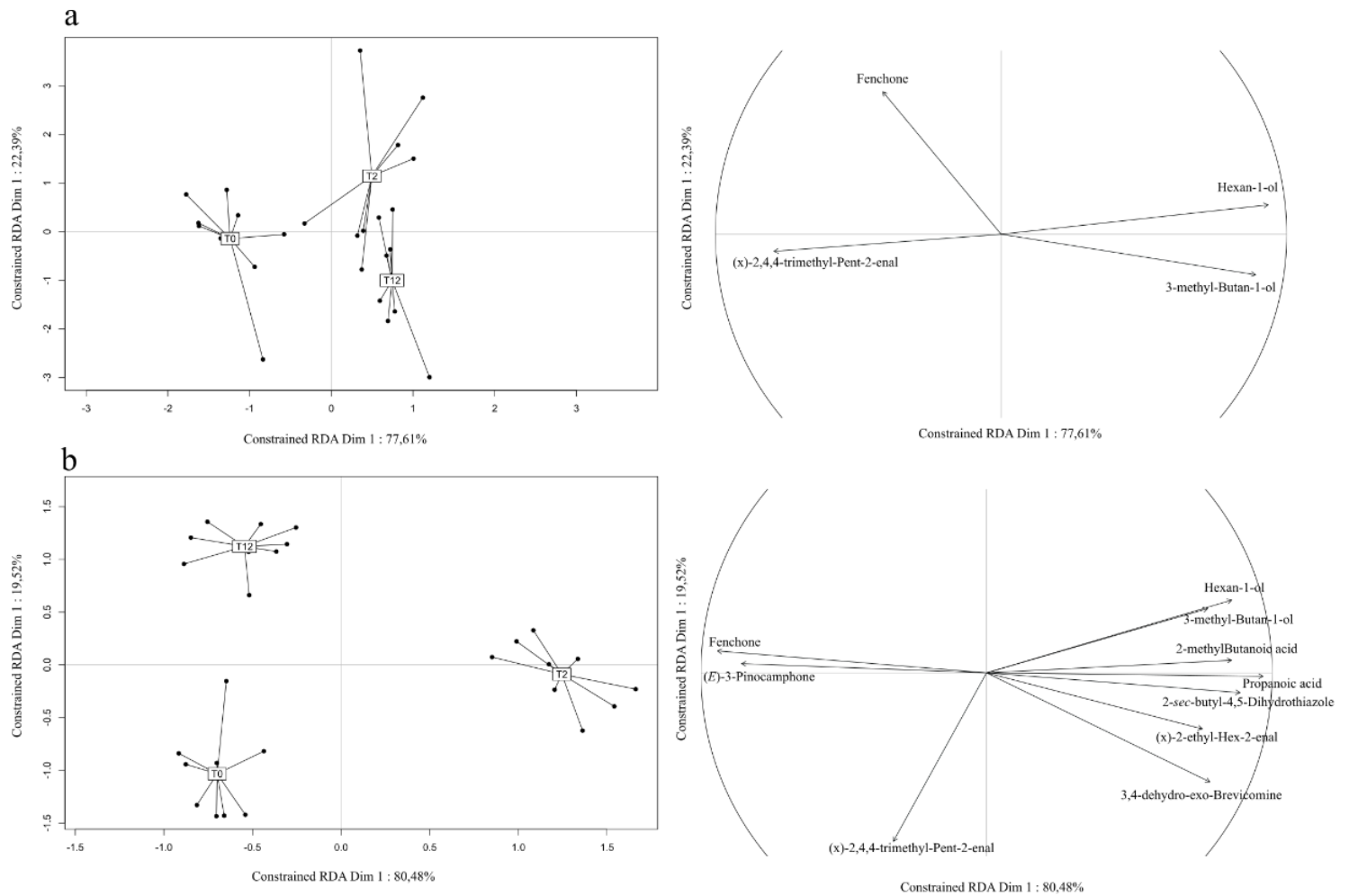


Fig. 4 : Score plots of the redundancy analyses (RDAs) comparing variation of 17 VOCs identified following 1h SPME extraction of non-cancerous soiled bedding (A) and cancerous soiled bedding (B) at three experimental condition (T0: before treatment, T2: after 2 weeks doxycycline diet; and T12: after 12 weeks doxycycline diet). Correlation circles represent the position of VOCs showing an absolute correlation coefficient > 0.8 .

The second step consisted in evaluating the impact of cancer. To address this question, we first verified if the VOCs composition of CC and NC mice differed significantly before the start of the doxycycline diet, at T0, which was the case (**model 6 : Table.2**) indicating that the two genotypes had a different VOCs profile at the start of the experiment. Seven compounds had an absolute correlation > 0.8 with the RDA main axes, and hence were identified to be involved in the differences between CC and NC at T0 (**Table. 3**). To further address the impact of cancer we removed these molecules from the data set and performed a RDA analysis including only the 10 VOCs that did not differ between CC and NC mice at T0 (**model 7 : Table.2 and Table.4**). The two random factors, replicates and fiber identity, accounted for a negligible part of the total variance, respectively, 3.32% and 1.05%. Both experimental conditions (T0, T2 and T12), health status (NC and CC) and their interaction were found to be significant (all $P < 0.005$; **Table.2**). As expected, we did not detect a significant difference in VOCs' composition between CC and NC mice at T0 ($P=0.622$) (**Table.4**). At T2 the two mice types (CC, NC) were also characterised by the same VOCs profile ($P=0.495$) but different from T0 ($P<0.001$), suggesting that at T2 only the doxycycline diet had altered the VOCs composition and that it influenced similarly CC and NC mice. The VOCs profiles of NC and CC were significantly different at T12 ($P< 0.001$; **Table.4**), and the CC profile at T12 was significantly different from that of CC at T2 and from that of NC both at T2 and T12, indicating that lung adenocarcinoma development influenced the VOCs' composition of CC mice (**Fig. 5**). The doxycycline treatment did not seem to have induced a change between T2 and T12 as far as NC mice were concerned (T2-T12 NC, $P = 0.259$). However, our analyses pointed out 4 compounds differing between the VOCs profiles of CC at T2 and at T12. Three of these compounds, 3,4-dehydro-*exo*-brevicomine, 2-sec-butyl-4,5-dihydrothiazole and hexan-1-ol, were present in lower relative proportions in CC mice at T12 as compared to both NC and CC individuals at T2. The fourth compound, benzaldehyde, was present in higher relative proportions in CC at T12 (**Table.3**). Our analyses pointed out 4 other compounds impacted by the doxycycline treatment: the hexan-1-ol, 3-methyl-butan-1-ol, (*x*)-2,4,4-trimethyl-pent-2-enal and the fenchone. The hexan-1-ol and the 3-methyl-butan-1-ol were either absent or present as a track before the start of the doxycycline diet (**Table.3**), while (*x*)-2,4,4-trimethyl-pent-2-enal disappeared from the VOCs profiles after tumour induction by doxycycline. Concerning the fenchone, it decreased over time in NC mice, while in CC mice it decreased at T2 and then increased again at T12 (**Table.3**).

Table 3 : Relative proportions (% , mean \pm se) of 17 VOCs identified in mice soiled bedding following 1-h SPME sampling, with reference to health status (non-cancerous, NC, cancerous, CC) at three experimental conditions (T0, T2 and T12). VOCs present both in the biological and the control samples are not listed here.

Compounds	T0		T2		T12		Reference	
	% CC	% NC	% CC	% NC	% CC	% NC	studies involving house mice	studies targeting cancer in house mice
Propanoic acid+	15.8 \pm 0.7	19.8 \pm 1.5	13.1 \pm 1.2	14.3 \pm 1.2	13.1 \pm 0.5	9.3 \pm 0.5	Faeces : (Goodrich et al., 1990a) Body : (Röck et al., 2006)	
3-methyl-Butan-1-ol+	1.3 \pm 0.2	0.2 \pm 0.1	6.5 \pm 0.9	2.9 \pm 0.5	3.1 \pm 0.3	3.7 \pm 0.2	Faeces : (Goodrich et al., 1990a)	
Butanoic acid	18 \pm 4.3	15.2 \pm 2.9	0.2 \pm 0.03	3.5 \pm 2.2	10.5 \pm 3	5.7 \pm 2.3	Urine and Body : (Röck et al., 2006)	
(x)-2-ethyl-Hex-2-en-1-al*	6.3 \pm 0.8	6. \pm 0.6	3 \pm 0.4	3.8 \pm 0.4	3.9 \pm 0.4	4.7 \pm 0.6		
3-methylButanoic acid	8.3 \pm 1.2	8. \pm 1.3	6.8 \pm 1.4	4.8 \pm 1.2	10.2 \pm 1.4	3.8 \pm 0.9	Urine : (Röck et al., 2006) Urine : (Fujita et al., 2019)	
2-methylButanoic acid	1.7 \pm 0.3	1.2 \pm 0.3	2.6 \pm 0.3	1.4 \pm 0.3	2.3 \pm 0.3	0.9 \pm 0.1	Urine : (Röck et al., 2006)	
Hexan-1-ol	10.8 \pm 4.4	6.2 \pm0..4	45.7\pm2.7	38.1\pm4.5	33. 9\pm3. 1	47.7\pm2.1	Body : (Röck et al., 2006)	
(x)-2,4,4-trimethyl-Pent-2-en-1-al*	2 \pm 0.5	1.1 \pm 0.6	0	0	0	0		
Benzaldehyde	1.4 \pm0.3	1.5\pm0.6	0.12\pm0.1	0.9\pm0.5	1.9\pm0.2	1\pm0.4	Urine and Body : (Röck et al., 2006) Urine : (Arnáiz et al., 2014; Fujita et al., 2019; Fujita et al., 2020; Jemiolo et al., 1987; Miyashita and Robinson, 1980; Osada et al., 2008; Schaefer et al., 2010; Schwende et al., 1986)	Breast cancer, urine : (Woollam et al., 2019) Melanoma, urine : (Sever et al., 2015) Lung cancer, urine : (Hanai et al., 2012)
2,3-dehydro- <i>exo</i> -Brevicomine*	3 \pm 0.5	2.9 \pm 0.6	2.7 \pm 0.7	0.7 \pm 0.2	1.9 \pm 0.3	2.2 \pm 0.2	Urine : (Mucignat-Caretta et al., 2010; Novotny et al., 2007; Röck et al., 2006)	
3,4-dehydro-<i>exo</i>-Brevicomine	15.5\pm2	11.6\pm 1.8	8.9\pm0.9	8.5\pm0.8	3.1 \pm0.3	5.5\pm0.4	Urine : (Fujita et al., 2019; Harvey et al., 1989; Jemiolo et al., 1987; Novotny et al., 1984; Osada et al., 2008; Schaefer et al., 2010; Schwende et al., 1986; Zhang et al., 2007)	

Fenchone +	1.6 ±0.2	4.5±0.5	0	3.3±0.6	2.1±0.5	1.6±0.2		
2-sec-butyl-4,5-Dihydrothiazole	8.5 ±1	8.5±0.7	8.3±1.4	7.9±0.5	5.7±0.6	7.4±0.8	Urine : (Arnáiz et al., 2014; Harvey et al., 1989; Kayali-Sayadi et al., 2003; Miyashita and Robinson, 1980; Mucignat-Caretta et al., 2010; Novotny et al., 2007; Osada et al., 2008; Röck et al., 2006; Schaefer et al., 2010; Schwende et al., 1986; Zhang et al., 2007) Body : (Röck et al., 2006)	Lung cancer, urine : (Matsumura et al., 2010)
Nopinone+	1. ±0.5	1.7± 0.2	0.8±0.3	1 ±0.2	1.5±0.4	0.9 ±0.2		
Camphor+	2.3 ±0.1	5.9 ± 0.5	0.9±0.3	3.7±0.6	3.5±0.7	2.8±0.2		Colorectal cancer, blood: (Liu et al., 2019)
Camphene hydrate+	0.7± 0.2	2.3± 0.3	0.2±0.1	1.2±0.3	1.5±0.3	1.3±0.1		
(E)-3-Pinocamphone+	1.9 ± 0.2	3.2± 0.2	0.2±0.2	2.1±0.3	1.9±0.4	1.5±0.3		

*: *attempt of identification; in some cases the configuration could not be determined.*

+VOCs differing between CC and NC mice at T0

Table 4 : Post-hoc tests following RDA model 7.

	T0_CC	T2_CC	T12_CC	T2_NC
T0_NC	0,622			< 0,001
T2_NC		0,4948		
T12_NC			< 0,001	0,2593
T2_CC	< 0,001			
T12_CC		< 0,001		

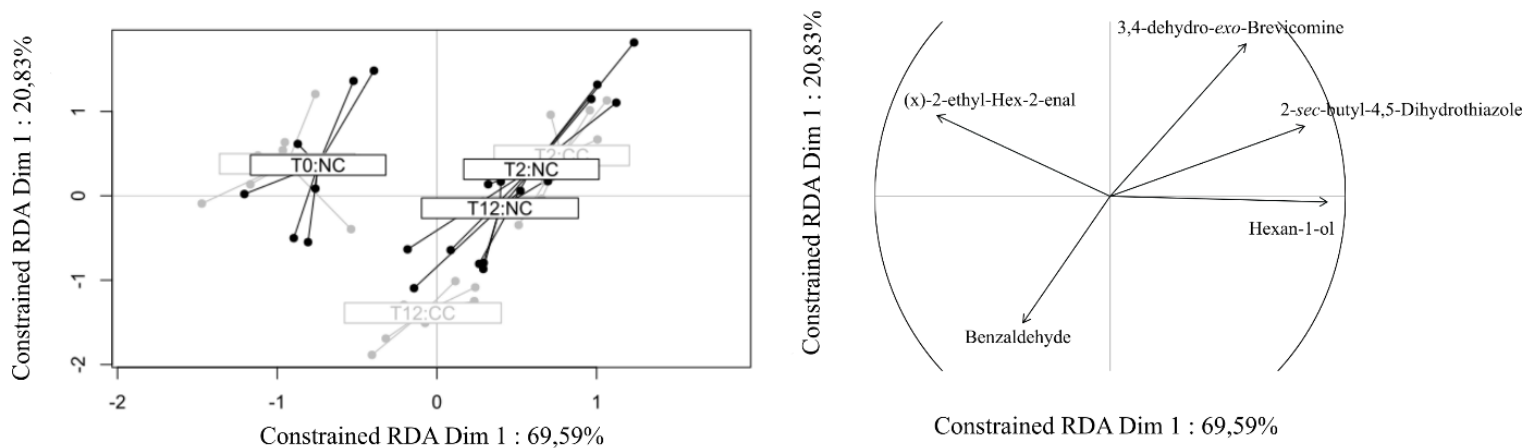


Fig. 5 : Score plots of the redundancy analysis (RDA) addressing variation of 10 VOCs present in the profiles obtained after 1h extraction of NC (non-cancerous) and CC (cancerous) soiled bedding at three experimental conditions; (T0: before treatment, T2: after 2 weeks doxycycline diet; and T12: after 12 weeks doxycycline diet). The correlation circle identifies VOCs showing an absolute correlation coefficient > 0.8.

Discussion

Characterization of VOCs composition can quickly become tedious when one needs to analyze a large number of samples with a single SPME fiber, and when fiber exposure duration for each sample is long. However, when several fibers are used and extraction duration per sample is reduced, the repeatability of the results and the detectability of the molecules may be questioned. This paper addressed these two methodological issues and sought to identify VOCs present in mice soiled bedding that could be considered as cancer biomarkers.

Repeatability when using multiple SPME fibers.

The use of several SPME fibers (with the same adsorption phase) to analyse a large number of samples might induce a bias due to inherent heterogeneity between fibers (Gómez-Ríos et al., 2015). However, our results showed that, when all other conditions were controlled (duration, temperature etc.), we did not detect significant qualitative and/or quantitative difference in the VOCs profiles obtained by two distinct SPME fibers exposed simultaneously to the same source of odour. Our results also indicated that when two different fibers were used in a comparative study and fiber identity was included in the model as a random factor, the percentage of variation caused by this factor was marginal (**models 4 and 5 : Table.2**).

Unexpectedly, our results also indicated strong variation in the VOCs profile of the same source of odour sampled at several occasions. The variation in the relative proportions of the different VOCs

was not linear and did not suggest an effect of fiber wear (between the first and the 45th use). A possible explanation of our results might be sample heterogeneity. Soiled bedding is a complex substrate, containing saw dust, food leftover, urine, faeces and other body secretions, difficult to homogenise. For the purpose of our study, we used pools of two-weeks old soiled bedding that we mixed as much as possible before sampling our aliquots (3g). Our results suggest that some of the aliquots used in this part of our study were not similar, further suggesting that to avoid such bias larger volumes than 3g should be used, and if not possible several replicates should be used to control for sampling heterogeneity, which we did to address cancer impact on VOCs (see below).

Influence of sampling duration on detection of VOCs related to cancer.

The release and adsorption of volatile compounds is dynamic (Liaud et al., 2016; Pawliszyn, 2012; Shirey, 2000). Some molecules will be released immediately after exposure while others will not, this may be due to their chemical property and/or their state in the odour source (e.g. bound to urinary proteins (Beynon and Hurst, 2004; Kwak et al., 2012) Low molecular weight VOCs will be quickly adsorbed by the SPME fiber while other molecules will take longer to be emitted and hence their detection could be delayed (Cavaggioni et al., 2006). Increasing sampling duration can increase the number of molecules likely to be adsorbed. By increasing the duration of VOCs extraction to 24h, we were able to detect 11 additional molecules compared to the 1h sampling. Compounds detected only after a 24h sampling were mostly relatively heavy compounds that came out at the end of the chromatogram profile. The lower volatility of such molecules may explain why they were only observed in the 24h samples (Gianelli et al., 2002; Marco et al., 2004). However, the increase in sampling duration did not necessarily result in an increase of the amount of compound trapped on the fiber for all compounds. For example, the relative proportions of some compounds such as 3,4 -dehydro-*exo*-brevicomine did not change between the 1h and 24h profiles while the proportion of thiazoline was higher in the 24h profiles compared to the 1h ones. This could be explained by the fact that while 3,4 -dehydro-*exo*-brevicomine is released during the first 20 minutes of sample exposure, thiazoline is emitted continuously for 24h (Cavaggioni et al., 2006). Among the 11 molecules exclusively detected after 24h extraction hexanoic acid and (*E*)-nerolidol were described in mice urine that were heated at different temperatures between 45°C and 65°C, enhancing their volatility (Goodrich et al., 1990b; Mucignat-Caretta et al., 2010; Röck et al., 2006; Schaefer et al., 2010). Some of the other compounds such as heptanoic acid and p-cymen -8 -ol are derived from microbial communities (Huang et al., 2019) and their emission was facilitated by heating or longer exposure to oxidative degradation as it is the case when extraction time lasts 24h.

Nonetheless, among the 11 compounds detected exclusively after a 24h extraction, none differed between CC and NC mice, further indicating that 1h sampling should suffice to address cancer impact

on mice VOC's profile (at least for such mouse model and such cancer type). Further, considering the cancer literature, none of the 11 molecules detected only in the 24h profiles was referenced as cancer biomarkers in mice. One compound was mentioned as a possible cancer biomarker in humans, the hexanoic acid, in gastric (Kumar et al., 2013; Kumar et al., 2015) and gastroesophageal cancer (Huang et al., 2013).

Cancer influence on VOCs production.

The use of soiled bedding allows to sample VOCs emitted by mice in their everyday activities in their cages, making the sampling non-invasive and non-stressful for the mice. It also allows collection of VOCs from a variety of sources simultaneously such as faeces, urine, saliva, preputial gland etc. Among the 17 compounds identified in this study, seven had not been reported in another publication on mice to our knowledge (**Table.3**). The other compounds, such as propanoic acid, hexan-1-ol or butanoic acid, were described before (Röck et al., 2006). The propanoic acid was reported to be emitted from the body or from faeces, while other compounds such as benzaldehyde was reported to be present in mice urine (Röck et al., 2006; Schaefer et al., 2010; Schwende et al., 1986). Some compounds that we found both in the control and in the biological samples, that we decided not to consider here, were reported as mouse compounds in the literature, such as acetic acid, pentan-1-ol, heptanal (Röck et al., 2006), nonanal (Röck et al., 2006; Soini et al., 2009) or again p-cymene (Soini et al., 2009).

Our results revealed significant quantitative differences in the VOCs profiles of the two mice lines (NC, CC) before doxycycline treatment (at T0). As these mice were kept in exactly the same conditions, and were all same age males and litter mates, the only difference that we see that could explain their different VOCs profile is their relatively slight different genetic background: presence versus lack of CCSP-rtTA and EGFR^{T790M/L858R}. Small differences in genetic background were already shown to impact the VOCs profile of mice, (Jemiolo et al., 1991; Schaefer et al., 2002; Yamazaki et al., 1990). Seven compounds differed between NC and CC at T0. Except for the 3-methylbutan-1-ol, these compounds were present in greater relative proportion in NC than in CC mice. Among the molecules described in the mouse VOCs literature, only the propanoic acid and the 3-methylbutan-1-ol were reported (Goodrich et al., 1990a), the other four compounds, nopinone, camphor, camphene hydrate and (*E*)-3-pinocamphone are terpenes also found in plants.

Our study revealed the effect of both doxycycline and cancer tumour development on the mice VOCs profile. Doxycycline diet seems to influence VOCs emissions both in NC and CC mice. One compounds disappeared from the profile (e.g. (x)-2,4,4-trimethyl-pent-2-enal) while two other compounds were present in higher proportions after the addition of doxycycline, i.e. hexan-1-ol and 3-methylbutanol, which are compounds notably emitted by bacteria (Korpi et al., 2009), indicating that the doxycycline treatment, by eliminating some bacteria, might have favoured others, impacting the

mice gut microbiome composition and thus inducing a change in the VOCs emitted by the mice (Zomer et al., 2008).

Our results indicated that the impact of cancer on VOCs composition was only detectable at T12, i.e. after 12 weeks of treatment when all mice harboured lung adenocarcinoma (Mur et al., 2020), and that this impact involved four compounds. Benzaldehyde was found in higher proportions in CC than NC mice at T12. It is a compound commonly found in mice urine (Arnáiz et al., 2014; Fujita et al., 2020; Kwak et al., 2008; Röck et al., 2006), and was already reported in studies using mice to study cancer (Hanai et al., 2012; Sever et al., 2015; Woollam et al., 2019). Benzaldehyde was also found in human cancers and might be considered as a relatively general biomarker of lung (Barash et al., 2012; Davies et al., 2014; Jia et al., 2018; Ligor et al., 2009), colorectal (Altomare et al., 2020) or breast cancer (Silva et al., 2017). Most of the time this compound was studied in cell cultures and was found to increase in cancerous cells, here we show that cancer impact the whole body emission. Benzaldehyde was also found to be discriminating in studies on Alzheimer's disease (Tian et al., 2021). The three other candidate VOCs identified in this study to be impacted by cancer development were found in smaller proportions in CC as compared to NC mice, namely, hexan-1-ol, 2-*sec*-butyl-4,5-dihydrothiazole (Thiazoline) and 3,4-dehydro-*exo*-brevicomine (Brevicomine). Of these three compounds, two are known mice pheromones described in male mice, brevicomine and thiazoline. These pheromones were shown to be involved in male dominance and male attractiveness to female mice (Novotny et al., 2003). Thiazoline was pointed as a candidate in another mouse lung cancer study (Matsumura et al., 2010). The onset of cancer therefore seems to impact pheromone emissions in sick mice, and hence is expected to impact their social life. The third candidate molecule is the hexan-1-ol also found in mice urine (Röck et al., 2006). Like in our study, hexan-1-ol was found in smaller proportions in cancerous as compared to non-cancerous individuals in investigations of human lung cancer (Hanai et al., 2012), melanoma (Kwak et al., 2013) and head and neck cancer (Shigeyama et al., 2019). Finally, among the compounds identified in similar proportions in both NC and CC sources in our study, the camphor was found to discriminate against colorectal cancer in mice (Liu et al., 2019), and the 3 methylbutanoic acid was found to discriminate melanoma (Kwak et al., 2013).

Conclusion

We were able to show that when used under strict controlled conditions, the use of different SPME fibers may have a marginal impact on the variance observed between samples. The use of two fibers allowed us to perform a larger number of samples in a relatively short time. In addition, our study validated the use of mouse soiled bedding to extract VOCs from mice without being invasive. A longer

sampling period may make it easier to identify compounds present in small amounts during a shorter sampling period. However, in our study, 1h extraction was sufficient to isolate the compounds that differentiated cancerous from non-cancerous mice. We did not detect cancer induced VOCs variation at an early stage of tumour development (T2), although behavioural investigations showed that mice discriminated between CC and NC soiled bedding at T2 (Gouzerh et al., 2022). The latter results suggest either, that our study method was not sensitive enough (compared to a mouse nose) to detect informative VOCs at T2, or most probably that other molecules, e.g. proteins, might be impacted by tumour development (Zhang et al., 2015). We identified four candidate compounds that discriminated CC from NC soiled bedding at T12: the benzaldehyde and the hexan-1-ol confirming earlier studies on mice and humans cancer, and two mice pheromones the Thiazoline and the Brevicomine, indicating that cancer could potentially impact social and sexual interactions in mice natural populations and, as such, might be selected against.

AUTHOR CONTRIBUTIONS

FG, GG, FT, LD developed the concept, FG realized the experiments, FG and GG performed the analytical measurements and analyzed the data with MH. BB supervised the chemical ecology aspects. MM and AM performed euthanasia of EGFR mice and verification of their cancer status. FG and GG prepared and edited the manuscript and all authors have reviewed the manuscript.

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COMPETING INTERESTS

The authors declare no competing financial interests.

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SUPPLEMENTARY MATERIAL:

Fig.S1: Representation of a) Score plots of the redundancy analysis (RDA) comparing variation of relative proportion of 17 volatile organic components sampled with fibers F1 and F2 at seven occasions; b) A correlation circle identifying compounds showing an absolute correlation coefficient > 0.8 and c) Variation of the relative proportion of the compounds pinpointed in the RDA analysis.

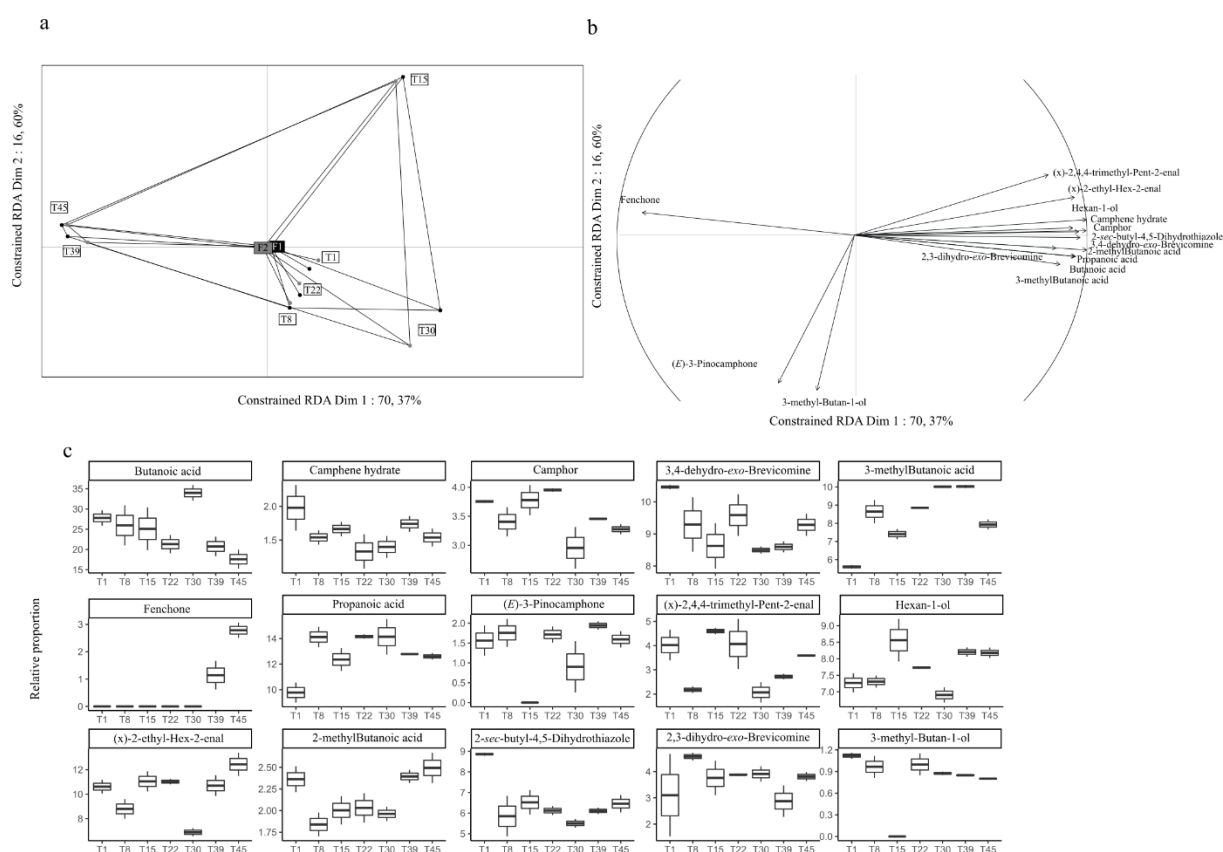


Table S1: List of VOCs identified both in the biological samples and the controls. In blue compounds found only in soiled bedding and retained for our analyses. In pink: the n-alkanes used as standards for the identification of the compounds.

Compounds	RT ^a	RI ^b
n-Hexane	2,79	600
Acetic acid	2,83	603
Pentanal	4,05	692
n-Heptane	4,16	700
Propanoic acid	4,31	707
3-methylButan-1-ol	4,89	733
NI	4,98	737
Toluene	5,55	762
Pentan-1-ol	5,62	765
Hexanal	6,35	798
n-Octane	6,40	800
Butanoic acid	6,47	802
2-ethyl-Hex-2-enal	6,68	809
3-methylButanoic acid	7,98	853
ethylBenzene	8,12	858
2-methylButanoic acid	8,26	862
nXylène	8,38	866
Hexan-1-ol	8,45	869
(x)-2,4,4-trimethyl-Pent-2-enal	8,59	873
NI	8,90	884
Styrene	9,00	887
Pentanoic acid	9,19	893
Heptanal	9,30	897
n-Nonane	9,39	900
NI	9,50	903
α -Pinene*	10,48	934
Hept-2-enal	11,06	952
Benzaldehyde*	11,19	956
Heptan-1-ol	11,60	969
β -Pinene*	11,84	977
Oct-1-en-3-ol*	11,88	978
6-methyl-Hept-5-en-2-one	12,03	983
2-pentylFuran	12,23	989
Hexanoic acid	12,44	996
Octanal	12,53	998
n-Decane	12,58	1000
δ -3-Carene	12,90	1010
<i>p</i> -Cymene*	13,32	1024
Limonene*	13,48	1029
NI	13,65	1034
2,3-dihydro- <i>exo</i> -Brevicomine	13,80	1039

6-methyl-Heptan-2-one	13,96	1044
3,4-dehydro- <i>exo</i> -Brevicomine	14,06	1047
Oct-2-enal	14,28	1054
vinylHexanoate	14,37	1057
Unknown compound 1	14,69	1067
Octan-1-ol	14,80	1071
Heptanoic acid	15,19	1083
Fenchone	15,36	1089
Cymenene	15,34	1088
n- Undecane	15,72	1100
Nonanal	15,73	1100
2- <i>sec</i> -butyl-4,5-Dihydrothiazole	15,82	1103
Fenchol	16,18	1115
NI	16,60	1129
Nopinone	16,79	1135
' <i>E</i> -Pinocarveol	16,95	1140
Camphor	17,06	1144
(<i>Z</i>)-Verbenol	17,13	1146
Camphene hydrate	17,25	1150
(<i>E</i>)-Non-2-enal	17,45	1157
(<i>E</i>)-3-Pinocamphone	17,53	1160
Pinocarvone	17,60	1162
Borneol	17,79	1168
(<i>Z</i>)-3-Pinocamphone	17,96	1174
4-Terpineol	18,12	1179
NI	18,19	1181
<i>p</i> -Cymen-8-ol	18,27	1184
α -Terpineol	18,37	1187
Unknown Monoterpene derivative 1	18,50	1191
Myrtenal	18,60	1195
Myrtenol	18,70	1198
n-Dodecane	18,76	1200
Decanal	18,80	1201
Verbenone	18,98	1208
2,4-Nonadienal	19,05	1210
Unknown compound 2	19,14	1213
Benzothiazole	19,40	1223
NI	19,63	1231
Cumin aldehyde	19,85	1238
Carvone	19,96	1242
Piperitone	20,27	1253
(<i>E</i>)-Dec-2-enal	20,43	1259
Undecan-2-one	21,31	1290
1-methyl-Naphthalene	21,43	1294
N,N-dibutyl-Formamide	21,60	1300

n-Tridecane	21,60	1300
NI	22,75	1343
α -Terpinyl acetate	22,94	1350
NI	23,22	1361
4- <i>tert</i> -butyl-Cyclohexyl acetate	23,53	1372
NI	23,76	1381
n-Tetradecane	24,27	1400
NI	24,57	1412
Geranyl acetone	25,53	1450
n-Pentadecane	26,81	1500
(<i>E</i>)-Nerolidol*	28,38	1572
n-Hexadecane	29,00	1600
Caryophyllene oxide	29,01	1600
Sesquiterpenol Inconnu	29,89	1641
(6 <i>Z</i> ,9 <i>E</i>)-Heptadeca-6,9-diene	30,60	1673
n-Heptadecane	31,13	1700

aCalculated retention indices

bRetention time

NI : non identified

Table S2: VOCs identified following 24h versus 1h extraction of soiled bedding. Only the components not present in the technical controls (clean bedding) are listed here.

Compounds	RT ^a	RI ^b	Frequency ^c		Relative proportion (%) ^d		Peak surface area ^e	
			1h	24h	1h	24h	1h	24h
Propanoic acid	4,31	707	18	18	8.8 ± 1.2	1.9 1 ± 0.3	138210±21251	113800±18072
3-methyl-Butanol	4,89	733	18	18	16.4 ± 1.1	2.4 ± 0.2	252880±20666	144730±14082
Butanoic acid	6,47	802	18	18	12.8 ± 1.4	3.6 ± 0.5	199270±20343	211310±29576
2-ethyl-Hex-2-enal	6,68	809	18	18	7.1 ± 0.7	6.1 ± 0.6	107310±11053	356570±26223
3-methylButanoic acid	7,98	853	18	18	2.5 ± 0.4	1.8 ± 0.3	38823± 6761	113740±18092
2-methylButanoic acid	8,26	862	18	18	0.8 ± 0.4	1.6 0.6	10771±5476	88525±29420
Hexan-1-ol	8,45	869	18	18	20.7 ± 3.6	3.5 ± 0.7	328740±65452	231950±51847
(x)-2,4,4-trimethyl-Pent-2-enal	8,59	873	18	18	1.5 ± 0.3	1.6 ± 0.2	21019±5820	98112±9508
Benzaldehyde	11,19	956	18	18	6.3 ± 0.5	2 ± 0.2	93406±7192	127530±14850
Hexanoic acid	12,44	996	/	18	/	19.2 ± 2.6	/	1243400±185210
2,3-dehydro- <i>exo</i> -Brevicomine*	13,8	1039	18	18	1.9± 0.3	0.7 ± 0.5	28640± 3800	65194±47046
3,4-dehydro- <i>exo</i> -Brevicomine	14,06	1047	18	18	8.06 ± 1.	3.6 ± 0.4	121530±16625	208840±19179
vinylHexanoate	14,37	1057	/	18	/	1.5 ± 0.1	/	69453±4453

Unknown compound 1	14,69	1067	/	18	/	1.4 ± 0.2	/	82456±5608
Heptanoic acid	15,19	1083	/	18	/	2.3 ± 0.2	/	144720±16808
Fenchone	15,36	1089	16	18	1.3 ± 0.3	/	21307±5286	41292 ± 4808
2-sec-butyl-4,5-Dihydrothiazole	15,82	1103	18	18	6.3 ± 0.4	8.4 ± 0.7	95078±6289	496870±32187
Nopinone	16,79	1135	18	18	1 ± 0.1	1.4 ± 0.2	14224± 1911	83731±9687
Camphor	17,06	1144	18	18	2 ± 0.3	1.9 ± 0.6	31815±5998	123530±41145
Camphene hydrate	17,25	1150	18	18	0.7 ± 0.2	1.7 ± 0.2		0.000145
(E)-3-Pinocamphone	17,53	1160	18	18	2. ± 0.4	1.4 ± 0.2	10905±2208	87533±10540
p-Cymen-8-ol	18,27	1184	/	18	/	7.1 ± 0.3	27784±4129	91493±12709
Unknown compound 2	19,14	1213	/	18	/	1.6 ± 0.1	/	433270±20811
Carvone	19,96	1242	/	18	/	2.4± 0.2	/	93592±4788
Piperitone	20,27	1253	/	18	/	1.9 ± 0.4	/	142380±9281
4-tert-butyl-Cyclohexyl acetate	23,53	1372	/	18	/	4.8 ± 0.3	/	111140±19920
(E)-nerolidol	28,38	1572	/	18	/	12.9 ± 1.3	/	290280±14621
(6Z,9E)-Heptadeca-6,9-diene	30,6	1673	/	18	/	2.2 ± 0.3	/	845700±147880

^a retention time

^b calculated retention indices

^c frequency of occurrence of the compounds.

^d relative proportion of the compounds (% , mean +/- SE)

^e relative quantity of the compounds (mean +/- SE)

~ Chapitre V ~

**Impact du cancer sur les modifications d'odeur corporelles :
analyse des litières de souris**

Contexte et objectifs

Comme nous avons pu le voir, les COVs peuvent aider à détecter les tumeurs cancéreuses. Dans cette étude nous avons voulu analyser les litières souillées de souris (utilisées pour les tests de comportement (**Manuscrit III**) pour tenter de détecter et d'identifier des différences de profils d'odeur entre souris saines et cancéreuses. En effet, la plupart des études réalisées sur le sujet ont utilisé des urines de souris. La litière souillée contient à la fois des urines et des fèces ainsi que des COVs émis par la respiration ou par la transpiration de la souris. Nous avons alors voulu déterminer quels composés ressortaient à partir de ce type de prélèvement, et tester si nous pouvions sur cette base identifier des COVs liés au cancer. A cela se sont ajoutées des questions d'ordre méthodologique liées à l'utilisation des fibres SPME pour le prélèvement de COVs émis par les litières. Pour être certains de la répétabilité des fibres SPME et écarter tout biais de prélèvement, nous avons comparé les profils de COVs obtenus avec deux fibres SPME distinctes et utilisées simultanément, prélevant en même temps le même échantillon de litière souillée. Nous avons ensuite comparé les profils d'odeur obtenus après 1h et 24h de prélèvement. Pour finir nous avons comparé des prélèvements de litières souillées durant 1h à différents temps de prélèvement de litières. Ces prélèvements ont été effectués à T0, c'est à dire avant le traitement induisant le cancer du poumon, puis à T2 soit 2 semaines après le début du traitement et à T12, soit 12 semaines après le début du traitement ce qui correspond au stade terminal du cancer.

Résumé de l'article

17 composés ont été identifiés comme présents uniquement dans les litières souillées par les souris et non présents dans nos contrôles (litière propre) lors des prélèvements d'1h. Concernant les prélèvements de 24h, 11 composés supplémentaires ont été identifiés. L'analyse sur la répétabilité des fibres SPME a montré que deux fibres prélevant en même temps un même échantillon adsorbent de la même manière les COVs émis par l'échantillon, que ce soit de manière qualitative ou quantitative. Cette observation nous a permis de réaliser tous nos prélèvements avec deux fibres SPME sans que le facteur « fibre » puisse induire un biais sur nos résultats. Concernant la différence de durée de prélèvement, nous avons identifié plus de composés à 24h par rapport à 1h. Cependant aucune des molécules prélevées à 24h ne semble liée au cancer. En effet, après analyse statistique comparant les proportions relatives des composés détectés à 24h chez les souris NC et CC, aucun des composés identifiés uniquement à 24h n'était impliqué dans la discrimination entre NC et CC. En revanche, parmi les 17 composés identifiés lors des prélèvements d'1h, nous avons pu montrer que le cancer impactait 4 d'entre eux en fin de traitement (T12), en augmentant les proportions relatives du benzaldéhyde qui est un composé fréquemment trouvé chez l'Homme, et en diminuant les proportions de l'hexan-1-ol et de deux phéromones de la souris le 2-*sec*-butyl-4,5-dihydrothiazole et la 3,4-dehydro-*exo*-brevicomine. Aucune différence n'a été observée en T0 et T2 entre les souris CC et NC, en revanche des différences ont été

observées entre le prélèvement T0 et T12 qui semblent être liées à l'impact de l'antibiotique sur les COVs émis par les souris quel que soit leur statut NC ou CC (Figure 17).

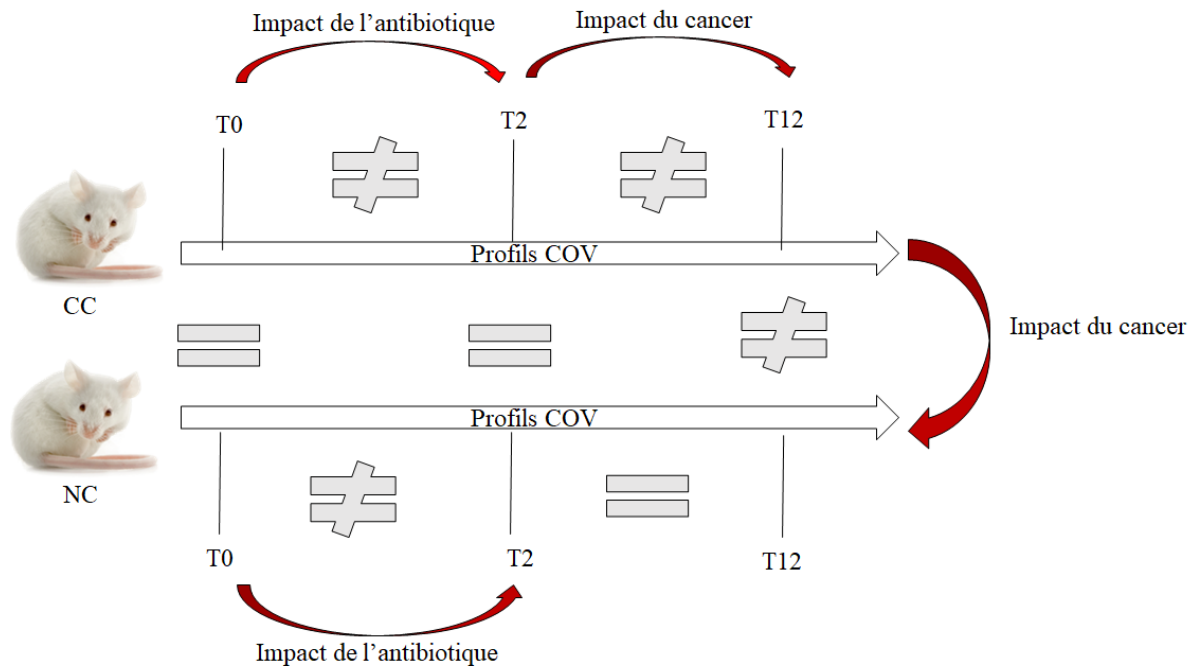


Figure 17: Figure résumant l'impact du cancer sur les COVs issus des échantillons de litière de souris. Avec les comparaisons des différences de profils d'odeur des souris NC (non cancéreuses) et CC (cancéreuses) au cours des différents prélèvements analysés, T0 avant le début du traitement, T2 au stade précoce de développement du cancer et T12 en stade terminal du cancer. Cette comparaison est faite entre les souris NC et CC et entre chaque groupe de souris. Les flèches rouges expliquent la différence entre les profils d'odeurs induite soit par la prise d'antibiotique soit par le cancer.

Manuscrit associé à ce chapitre :

Manuscrit IV :

- ~ Gouzerh, F., Buatois, B., Hervé, M. R., Mancini, M., Maraver, A., Dormont, L., Thomas, F & Ganem, G. Detection of volatile organic compounds from preclinical lung cancer mouse models (*In prep*).

RESEARCH ARTICLE

Odours of cancerous mouse congeners: detection and attractiveness

Flora Gouzerh^{1,2,*}, Bruno Buatois², Maxime R. Hervé³, Maicol Mancini⁴, Antonio Maraver⁴, Laurent Dormont², Frédéric Thomas¹ and Guila Ganem^{5,*}

ABSTRACT

Chemical communication plays a major role in social interactions. Cancer, by inducing changes in body odours, may alter interactions between individuals. In the framework of research targeting non-invasive methods to detect early stages of cancer development, this study asked whether untrained mice could detect odour changes in cancerous congeners. If yes, were they able to detect cancer at an early developmental stage? Did it influence female preference? Did variations in volatile organic components of the odour source paralleled mice behavioural responses? We used transgenic mice strains developing or not lung cancer upon antibiotic ingestion. We sampled soiled bedding of cancerous mice (CC) and not cancerous mice (NC), at three experimental conditions: before (T0), early stage (T2) and late stage (T12) of cancer development. Habituation/generalisation and two-way preference tests were performed where soiled beddings of CC and NC mice were presented to wild-derived mice. The composition and relative concentration of volatile organic components (VOC) in the two stimuli types were analysed. Females did not show directional preference at any of the experimental conditions, suggesting that cancer did not influence their choice behaviour. Males did not discriminate between CC and NC stimuli at T0 but did so at T2 and T12, indicating that wild-derived mice could detect cancer at an early stage of development. Finally, although the VOC bouquet differed between CC and NC it did not seem to parallel the observed behavioural response suggesting that other types of odorant components might be involved in behavioural discrimination between CC and NC mice.

KEY WORDS: Body odours, Odour discrimination, Female preference, Volatile organic compounds, Lung cancer, *Mus musculus domesticus*, EGFR oncogenic mutation

INTRODUCTION

Odour based communication influences interspecies relationships and plays an essential role in the social life of many taxa (Caro and

Balthazart, 2010; Mardon et al., 2010; Symonds and Elgar, 2008; Woodley, 2010; Wyatt, 2010). The information could be obtained via volatile (volatile organic compounds, VOCs) and non-volatile compounds (proteins, peptides, etc.). Both types of molecules contribute to olfactory communication in mammals. In general, long-distance communication requires volatile compounds while non-volatiles are involved in short-range communication and requires contact with the odour source (Muller-Schwarze, 2006). Odorant molecules are involved in signalling of, for example, species, sex, social rank, reproductive status and territory ownership (Ali et al., 2015; Célérier et al., 2010; 2011; Desjardins et al., 1973; Heth et al., 2001; Hurst and Beynon, 2004; Latour et al., 2014; Restrepo et al., 2006).

Olfaction, in addition to playing a key role in individual recognition for many species (Restrepo et al., 2006), also allows them to identify their congeners status in terms of stress and health. Such behaviour was observed in various vertebrate groups, such as fish, e.g. guppies *Poecilia reticulata* (Kennedy et al., 1987), and mammals including primates, e.g. *Mandrillus sphinx* (Poirotte et al., 2017), and rodents, e.g. *Cricetus cricetus* (O'Shea et al., 2002), or *Mus musculus domesticus* (Clayton, 1991; Ehman and Scott, 2001; Hurst, 2009; Kavaliers and Colwell, 1995). This ability allows individuals to reduce the risk of contagion, to avoid potentially costly aversive stimuli, or to ensure breeding with healthy partners (Thomas et al., 2005).


In view of the great olfactory capacity of some species, animals such as dogs or mice have been used as noses to detect various odour types. Dogs have been used in the detection of explosives and drugs (Gazit and Terkel, 2003; Jezierski et al., 2015), or human diseases such as epilepsy (Edwards et al., 2017; Strong and Brown, 2000) and more recently COVID-19 (Jendry et al., 2020). Among rodents, the house mouse has been an emblematic model in such research (Clayton, 1991; Ehman and Scott, 2001; Ehmann et al., 2012; Su et al., 2018). Mice olfactory capacity was further demonstrated in studies showing their ability to distinguish between odours of kin of other species, e.g. Baboons *Papio ursinus* (Célérier et al., 2010) and blue petrels *Halobaena caerulea* (Célérier et al., 2011).

Like illnesses such as malaria (de Moraes et al., 2018), asthma (Ibrahim et al., 2011) or diabetes (Phillips et al., 2004), cancer can induce changes in individual odours (Buljubasic and Buchbauer, 2015; Sever et al., 2015). In recent years, animal noses have been used to detect early stages of cancer development and hence increase the effectiveness of treatments and chances of survival (Athey et al., 2012). Animals involved in such studies were dogs (Jezierski et al., 2015; Pirrone and Albertini, 2017), mice (Kokocinska-kusiak et al., 2020; Matsumura et al., 2010; Sato et al., 2017) and *Drosophila sp.* (Strauch et al., 2014).

As far as rodent cancers are concerned, research on how cancer may induce odour changes has identified the involvement of several VOC, such as p-cresol (Qiu et al., 2010), isoprene (Woollam et al.,

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2020), hexanal, hexane or benzaldehyde (Kokocinska-kusiak et al., 2020). Changes in relative concentration of mouse volatile pheromones were also reported, involving elevation of relative concentration of exo-brevicomin and 2-sec-butyl-4,5-dihydrothiazole in lung cancerous mice (Hanai et al., 2012).

These molecules were shown to be present in higher relative concentrations in dominant as compared to subordinate males, and to induce female attraction (Hanai et al., 2012; Jemiolo et al., 1985).

In this study, we questioned whether cancer could alter female preference in mice and the ability of male to detect odour variation related to lung cancer at an early stage of development (i.e. not detected with IRM). As mentioned above, VOC were often pointed out as potential candidate molecules for cancer detection (Gouzerh et al., 2021), hence, we assessed whether changes in the mouse behaviour paralleled changes in the VOC composition of the stimuli presented to the mice during the behavioural tests. Specifically, we asked, (1) did the male's cancer status influence female preference for their odours? (2) Could untrained mouse detect the presence of cancer in odour sources of ill congeners? And if yes, were they able to detect cancer at an early developmental stage? (3) Did variations in VOC stimuli parallel mice behavioural responses to these stimuli?

Results

Female preference

We did not detect directional preference among wild-derived sexually receptive females at the three choice tests (T0: $N=16$, $V=73$, P -value=0.821, power=85%; T2: $N=12$, $V=39$, P -value=1, power=99%; T12: $N=13$, $V=64$, P -value=0.216, power=55%) (Fig. 1).

Moreover, duration of time spent smelling the two stimuli did not vary between experimental conditions [linear mixed model, (LMM) $F_{2,38}=0.873$, P -value=0.426].

Male discrimination

Male mice were either habituated to a cancerous (CC), series 1, stimulus or a non-cancerous (NC) one, series 2, (see Fig. S3 for the habituation results), then presented with two other stimuli, a NC and a CC, during the generalisation phase. Based on our second preliminary test we did not expect the mice to discriminate between T0 type stimuli, and the results confirmed our predictions (series 1: $N=11$, $V=26$, P -value=0.58, power=65%, series 2: $N=10$, $V=32$, P -value=0.7, power=75%) Fig. 2.

Two weeks after the antibiotic was added to the mice diet (T2), we expected that if development of lung tumour was initiated in CC mice, the NC and CC stimuli should be perceived as different. Hence, in the first series of tests, after habituation to CC we expected either no discrimination (if no tumour developed yet) or higher investigation of the NC stimulus. However, unlike predicted, mice investigated more the CC stimulus ($N=12$, $V=0$, P -value=1, power=100%; Fig. 2) suggesting that the difference between the two CC stimuli was higher than between CC and NC. We also expected that after habituation to the NC stimulus (series 2), if tumour development started in T2, the CC stimulus would be investigated more intensively than the NC one. This time our results confirmed the prediction ($N=11$, $V=6$, P -value=0.007; Fig. 2), indicating that the two NC stimuli were more similar to each other than the NC and the CC stimuli. Considering the tests involving discrimination between the T12 stimuli, for which presence of cancer tumour was confirmed for CC and excluded for NC, we expected the CC and NC smells to differ and hence be discriminated. For series 1, after habituation to CC, we expected

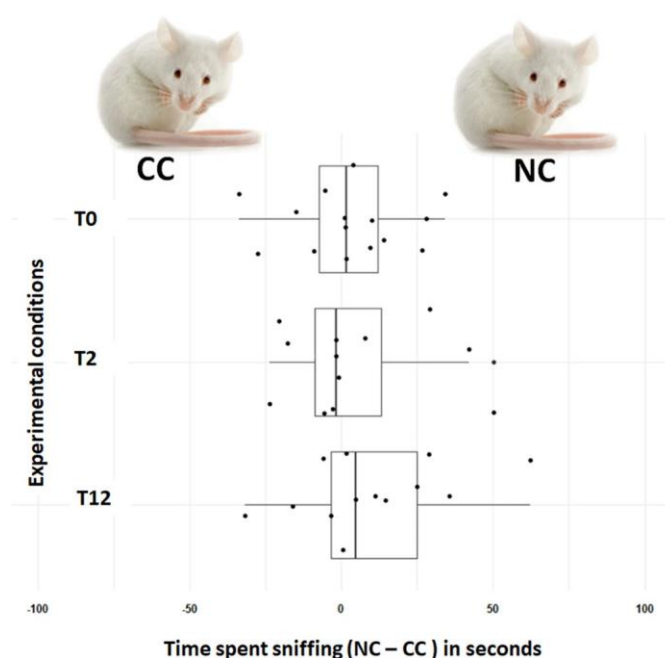


Fig. 1. Female preference for male stimuli. A comparison of time spent sniffing the two stimuli (CC/NC), sampled at three experimental conditions (T0, T2, T12) during the two-way choice tests. Number of female mice tested in each trial is $n=16$ for T0, $n=12$ for T2 and $n=13$ for T12. A positive value indicates that a mouse spent more time sniffing stimulus NC, and a negative value indicates that a mouse spent more time sniffing the CC stimulus. Each dot is an individual measurement, and the boxplots picture the median (vertical line), the first and the third quartiles, and whiskers represent the 95% confidence interval.

NC to be more investigated, nevertheless the mice did not show a significant discrimination between the stimuli ($N=16$, $V=75$, P -value=0.372, power=45%; Fig. 2). For series 2, after habituation to a NC stimulus, consistent with our prediction, mice discriminated significantly between NC and CC ($N=12$, $V=29$, P -value=0.010; Fig. 2), suggesting that NC and CC were more different than two NC stimuli.

Male general behaviour

To address attractiveness of CC and NC odours for the males we compared the total duration of time spent investigating the habituation odour as well as latency to approach this stimulus. The males spent significantly more time sniffing the CC stimulus as compared to the NC stimulus, when presented during the habituation phase, irrespective of the experimental conditions (LMM, 'health status': $F_{1,12}=3.923$, P -value=0.048), and the males latency to approach the CC stimulus was shorter than the latency to approach the NC stimulus ($F_{1,12}=5.129$, P -value=0.024), suggesting attractiveness to the CC stimulus. Finally, duration of sniffing was the highest at T0 and the lowest at T12 ('experimental conditions': $F_{2,12}=5.051$, P -value=0.006; post-hoc T0-T2: $P=1$; T0-T12: $P=0.02$; T2-T12: $P=0.3$) suggesting a reduction in attractiveness of all stimuli after 12 weeks of antibiotic diet (Table 1).

Variation of the VOC composition of the odour stimuli

Twenty-one compounds were identified in our biological samples after exclusion of molecules shared with the technical control. Three of the 21 compounds were excluded because of very low relative concentration (< 1%) and irregular detection in our samples

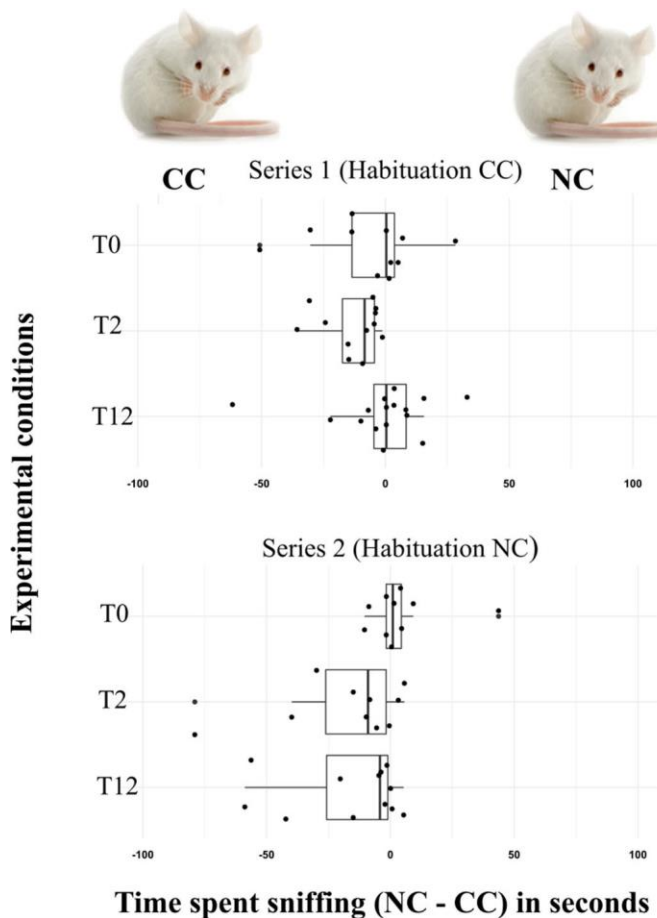


Fig. 2. Results of the generalisation tests. Difference between time spent sniffing stimuli NC versus CC (in seconds) during the generalisation phase for series 1 (habituation to stimulus CC) and series 2 (habituation to stimulus NC) for each experimental condition (T0, T2 and T12). Number of males tested for each trial is: for series 1: $n=11$ for T0, $n=12$ for T2 and $n=16$ for T12; and for series 2: $n=10$ for T0, $n=11$ for T2 and $n=12$ for T12. A positive difference indicates that the mice spent more time sniffing a NC stimulus and a negative one indicates that the mice spent more time sniffing a CC stimulus. Boxplots include the median (vertical line) and the first and third quartiles, the whiskers represent the 95% confidence interval. Each dot represents an individual measurement.

(Table S1). We did not detect qualitative differences between CC and NC stimuli (Table S1).

We compared the relative concentration of the 18 molecules composing the odour bouquets of the behavioural stimuli. First, we tested the hypothesis of a higher heterogeneity among the CC stimuli as compared to the NC stimuli following the antibiotic treatment (experimental conditions T2, T12). A test of multivariate dispersion revealed that for both experimental conditions, variation among CC pools did not differ significantly from that among NC pools (T2: $F=0.11$, $P=0.744$; T12: $F=0.001$, $P=0.971$). Then, we assessed differences in the relative concentrations of the 18 VOC in the different pools with reference to the health status at each of the experimental conditions (T0, T2, T12). Results of the RDAs indicated that the variance among replicates of each pool accounted for 2.22 to 13.29% of the total variance, while the variance explained by health status and stimuli pools (constrained variance) accounted for 49.29% to 62.18% of the total variance. The permutation F test indicated that CC and NC pools were significantly different at the three experimental conditions (T0,

Table 1. Variation of mice general behaviour during the habituation phase of the habituation/generalisation tests

		Df	F	P
i)	Status (NC/CC)	1,12	3.923	0.0476
	Experimental conditions (T0, T2, T12)	2,12	5.051	0.006
	Interactions	2,12	0.383	0.682
ii)	Status	1,12	5.129	0.024
	Experimental conditions	2,12	0.848	0.40
	Interactions	2,12	1.395	0.248

Results of two-way analyses of variance testing the influence of health status (CC/NC), experimental conditions (T0–T12) and their interaction on (i) time spent sniffing the habituation stimulus, (ii) latency to sniff the stimulus. Values in bold indicate significant effects.

T2, T12; $P<0.001$; Table 2), despite a significant heterogeneity between pools within status in T0 and T12 (Table 2, Fig. 3).

Discussion

In nature, olfaction plays a key role in identifying the social status, age and health condition of potential sexual partners (Ali et al., 2015; Célérier et al., 2010; Desjardins et al., 1973; Heth et al., 2001; Hurst and Beynon, 2004; Latour and Ganem, 2017; Restrepo et al., 2006). The choice of a sexual partner is crucial for the chooser's fitness. Olfactory mechanisms allow the detection of ill congeners, hence reducing the risk of contamination and of reproduction with congeners with lower fitness (Clayton, 1991; Ehman and Scott, 2001; Hurst, 2009; Kavaliers and Colwell, 1995; Poirotte et al., 2017; Thomas et al., 2005). Many studies have documented the ability of animals to discriminate against parasitized congeners (Ehman and Scott, 2002; Hurst, 2009; Kavaliers and Colwell, 1995; Kennedy et al., 1987; Poirotte et al., 2017). Concerning cancer, a pathology that can affect most metazoans (Leroi et al., 2003), consecutive modification of body odours has been largely described but very few studies have investigated the ability of individuals to detect a cancerous congener or examined whether and how this ability might impact intraspecific interactions. Cancer is relatively common in wild animals (Madsen et al., 2017). Our study reports that in a genetically engineered mouse model, lung cancer modifies the mouse odour signature and that these changes can be detected by wild-derived congeners at an early stage of the tumour development but does not influence female preference for the males' odours. Finally, although we identified quantitative differences in VOC composition of CC versus NC mice at the three stages of tumour development, these variations do not seem to parallel some of our behavioural results, suggesting that other odorant molecules, e.g. proteins, peptides, etc., present in the soiled bedding but not investigated in our study might be involved.

Female mice preference

Avoidance of ill sexual partners by females is expected to influence their fitness directly by reducing their own risk of contagion in case of infectious diseases, and/or indirectly by increasing the viability and attractiveness of their offspring (Ehman and Scott, 2002; Kavaliers and Colwell, 1995). This avoidance can be based on information contained in the male odour signature (Clayton, 1991; Ehman and Scott, 2002; Hurst, 2009; Kavaliers et al., 2005; Penn and Potts, 1998). In this study, wild-derived females investigated to the same extent CC and NC stimuli, at the three experimental conditions. Both variation in the VOC relative concentrations and evidence that males could discriminate between CC and NC (see below) suggest that the females were probably able to discriminate between the two odour

Table 2. Results of the permutation F tests based on redundancy analyses linking odour bouquets composition to health status (NC versus CC) and pool (soiled bedding stimuli)

		F	P
T0	Status	6.302	<0.001
	Status: pool	2.460	<0.001
T2	Status	10.980	<0.001
	Status: pool	1.612	0.057
T12	Status	6.043	<0.001
	Status: pool	2.371	0.007

The factor pool was nested in health status.

types, still discrimination did not induce a directional choice. Although absence of preference in T0 would indicate similar attractiveness of odours of males of the two lineages, absence of choice in T2 and T12 suggest that the odour of CC males was not repulsive, despite the fact that CC males carried full-blown cancer in T12. Consistent with our results, other studies reported that female mice may not be attracted to a healthy male when the alternative is a male carrying a cancerous melanoma (Kokocinska-kusiak et al., 2020).

This study results indicate that presence of a cancerous tumour may not influence female preference, unlike it does when the males carry parasites (Ehman and Scott, 2002; Kavaliers et al., 2005). Indeed, several studies have shown that chemical signals emitted by individuals carrying nematodes *Heligmosomoides polygyrus* (Ehman and Scott, 2001), protozoan *Eimeria vermiformis* (Kavaliers and Colwell, 1995) or murine louse *Polyplax serrata* (Kavaliers et al., 2003), were discriminated against by females, which usually chose an uninfected partner. However, unlike lung cancer or melanoma the parasites listed above could be transmitted to the females. Males at advanced stage of tumour development may not be able to defend a territory, protect a progeny, or keep a high social status, in natural conditions, and this should theoretically impact female preference (Hurst and Beynon, 2004). However, if in natural conditions cancer develops only at a late age, i.e. in non-reproductive animals, it should not impact reproduction and hence discrimination and avoidance of cancerous individuals may not be favoured by selection. Cancer in wild animals, in general, and more particularly among mice has been shown to be associated with aging. We know that rodents can develop cancer. Rats being the most tumour-prone rodent with a high tumour incidence at an early age but under laboratory conditions (Nakazawa et al., 2001). In other rodents such as laboratory gerbils (Vincent et al., 1979), laboratory mice (Frith et al., 1993; Greenacre, 2004; Ward, 1983), wild mice kept in laboratory conditions (Andervont and Dunn, 1962), pet chinchilla (Barbosa Lucena et al., 2012; Mans and Donnelly, 2013) or wild prairie dogs (Une et al., 1996), tumours were observed only in aging animals (Andervont and Dunn, 1962; Frith et al., 1993; Greenacre, 2004). Hence, cancer is probably rare in mice in the wild where longevity is estimated to be less than 1 year (Gerber et al., 2021), and the major causes of death in the wild are infectious diseases, predation or environmental perturbations (Vittecoq et al., 2013).

Ability of males to detect cancerous congeners

The males were more attracted to CC than to NC stimuli, i.e. shorter latency and higher investigation duration of CC stimuli, suggesting that, unlike congeners carrying contagious illnesses, those carrying a cancer tumour may not be avoided. Our results also indicate that stimuli of mice that were subjected to a 12-week antibiotic treatment

were less attractive than those of mice that did not receive an antibiotic treatment. Higher attractiveness of T0 than T12 odours may relate to the age difference of the two mouse types (Mucignat-Caretta et al., 2014) or to the impact of a long antibiotic diet. Further, wild-derived males were able to detect the tumour presence at an early stage of its development, i.e. two weeks after the antibiotic diet/lung oncogene induction. Surprisingly, our two series of experiments, differing by the health status of the stimulus presented during the habituation phase, yielded inconsistent results when the experiments involved T2 and T12 stimuli. Actually, when the habituation/generalisation test involved two CC stimuli (series 1), despite the fact that the CC donors were subjected to the same treatment, in T2, the two CC stimuli were treated as more distinct from each other than the CC and NC stimuli. It could be that the specific CC donors of the stimulus presented during the habituation phase did not develop a tumour in T2 and hence that the habituation stimulus was more similar to NC than to CC. Unfortunately, we cannot verify this hypothesis since the mice were euthanized only at T12.

We verified that at T12 all our CC mice stimuli donors developed lung cancer, and as expected none of the NC mice donors developed a tumour. Still, again, discrimination did not take place between CC and NC during series 1 (habituation to CC), suggesting that the CC stimulus presented during the habituation phase and the one presented during the generalization phase carried marked odour differences, equivalent to the differences between the habituation CC and the generalization NC. This intriguing result might be explained by a higher heterogeneity among CC stimuli, possibly linked to variations in the pattern of tumour development (Marjanovic et al., 2012), that would have induced greater heterogeneity in CC mouse chemical signature. The VOC are only part of the chemical signature of a mouse. Indeed the later also contains, e.g. proteins, peptides, sulphated steroids. Our results show differences in the VOC quantitative composition between CC and NC mice at all experimental conditions (T0 to T12), hence we could have expected discrimination between CC and NC in all our experiments. Further, the behavioural results suggest higher heterogeneity among the CC stimuli than among the NC ones, which are not reflected in the VOC composition of these stimuli. Research on mouse urine (Zhang et al., 2015) has shown that seven proteins could be potential biomarkers of lung cancer development, which may be also involved in our study, further analyses of the entire chemical signature of the CC and NC mice should help to clarify this issue.

Concluding remarks

The results presented here show that wild mice can discriminate a cancerous congener from a healthy one at an early stage of cancer development. This suggests that the chemical signature of an individual bearing a cancer can change at a very early stage of the tumorigenesis. It also opens up applied medical perspectives on the early detection of cancer using odorant sources, when imaging techniques, the gold-standard in lung cancer detection, cannot detect the tumour.

Our results indicate that wild-derived females do not discriminate against cancerous males, and that males do not avoid smells of cancerous congeners, consistent with the fact that such behaviour may not evolve when cancer does not impact individual fitness. Cancer has been present since the onset of multicellularity (Aktipis and Nesse, 2013). Still, very few studies address ecological implications of oncogenic processes, which, however, seem to have a theoretically significant impact on animal

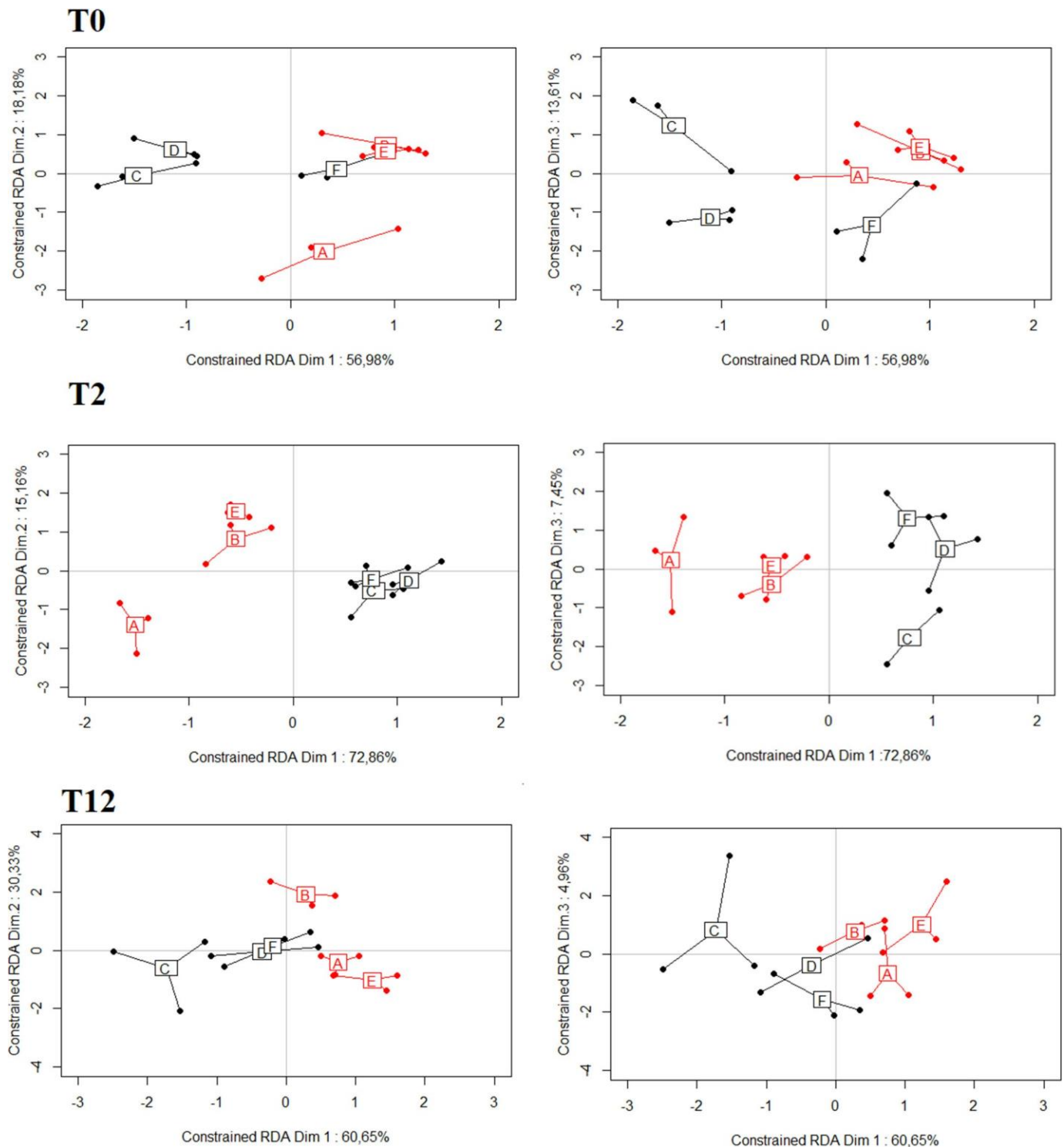


Fig. 3. Score plots of the redundancy analyses (RDAs) comparing variation of the relative concentrations of 18 VOCs with reference to health conditions (CC/NC) and the stimulus pools involved in the behavioural studies. The left panels correspond to the projection of scores on the two first axes of the constrained RDA, and the right panels correspond to the projections on axes 1 and 3. A, B and E correspond to the three CC pools (in red) and C, D and F correspond to the three NC pools (in black).

evolutionary ecology and ecosystems functioning (Thomas et al., 2017; Vittecoq et al., 2013). Such processes should be investigated in organisms in which cancer develops at an early life stage (Aktipis and Nesse, 2013; Thomas et al., 2017; Vittecoq et al., 2013).

MATERIALS AND METHODS

Ethical clearance

All the precautions for animal welfare were followed and all behavioural protocols received ethics clearance from the Ethical Committee for Animal Experimentation (French Ministry of Higher Education, Research and

Innovation) number E3417221 (for the behavioural experiments) and number 1645-22123 (for the transgenic mice).

Animals

Odour

Scent stimuli were obtained from CCSP/EGFRTL transgenic mice bred at the IRCM (Montpellier Cancer Research Institute). This mouse model was generated by crossing the Tet-ON-EGFR^{T790M/L858R} transgenic mouse strain (hereafter EGFRTL) with the CCSP-rtTA strain (hereafter CCSP) carrying rtTA, an inverse tetracycline responsive element, under the control of a lung specific promoter. In CCSP/EGFRTL mice the EGFR gene contains both the oncogenic L858R mutation (causing EGFR constitutive activation in the absence of ligand EGF) and T790M gate-keeper mutation (conferring resistance to the first generation of EGFR inhibitors, i.e. gefitinib/erlotinib). This system allows the expression of EGFRTL specifically in the lung but only upon doxycycline (antibiotic) exposure, thus leading to the development of peripheral adenocarcinomas with bronchioloalveolar features in alveoli as well as papillary adenocarcinomas (Li et al., 2007; Mur et al., 2020). Mating crosses were kept in heterozygosity, thus resulting littermate could have any of the four possible genotypes: WT/WT, WT/EGFRTLtg, CCSPtg/WT and CCSPtg/EGFRTLtg. In our experimental setting we used WT/WT mice, lacking both CCSP and EGFRTL transgenes, and hence not able to develop a cancer upon doxycycline induction, NC mice, and CCSPtg/EGFRTLtg mice carrying both transgenes that develop a lung tumour upon doxycycline induction, CC mice.

The stimuli donors, NC and CC mice were all males, and they were maintained at the breeding facilities of the IRD (Institute of Research and Development) in Montpellier, from the age of 6–8 weeks. They were kept in groups of two to four mice in plastic transparent cages (26.8 cm W*21.5 cm L*14.1 cm H). Each cage contained the same quantity of sawdust, a cellulose square, hay, and a cardboard tunnel. During all the experimentation period the mice mass was controlled (euthanasia if loss of $\geq 10\%$ of mass). Tumour development was induced by feeding the animals with doxycycline-containing food pellets (1 mg/kg) from the age of 13 weeks until the age of 25 weeks. All mice were euthanised at the age of 25 weeks after transfer to the IRCM where their lungs were inspected. Both the non-cancerous status of all NC and the cancerous status of all CC mice aged 25 weeks were confirmed by necropsy and with H&E (Haematoxylin and Eosin) staining on FFPE (formalin-fixed paraffin embedded) lung sections.

Mice involved in the behaviour tests

Behavioural tests were carried out with wild-derived male and female *Mus musculus domesticus*, which were part of an outbred colony founded with

mice trapped in southern Jutland (Denmark) in 2011. They were maintained in the animal facility of Montpellier University (RAM-CECEMA), under standardized conditions (photoperiod 12/12) and food and water were available *ad libitum*. They were housed in transparent plastic cages (20 cm L×35 cm W×14 cm H) containing bedding made of sawdust, hay, and a piece of cardboard box. A red plastic igloo equipped with a wheel was also present in each cage. After weaning at the age of 24 days the mice were kept in pairs (sister and brother) till the age of 8 weeks. Two weeks before the start of the experiments and during the entire experimental period, the males were kept isolated, while two unfamiliar females shared the same cage. Females involved in the choice tests were neither pregnant nor lactating but could have experienced pregnancy earlier in their life. Two days before being tested a mixture of male soiled bedding was added to the female cages to induce sexual receptivity (oestrus/prooestrus) and synchronize their cycle, nevertheless, vaginal smears were also performed at the end of each behavioural test to ascertain females' receptivity. Preliminary tests were also performed and involved 11–14, 4–5-month-old mice from the same breeding colony, kept under the same experimental conditions as the test animals.

Stimuli

The stimuli used for the behavioural tests were soiled bedding obtained from 17 males: eight CC and nine NC. Soiled bedding was a source of stimuli that contains a full range of odours emitted by the mouse. Each mouse was isolated at the age of 10 weeks in plastic transparent cages (26, 8 cm W×21, 5 cm L×14, 1 cm H) with 130 g sawdust and a cellulose square. The housing conditions of all odour donors were homogenized as much as possible. Each mouse was given the same quantity of bedding and food. Soiled bedding was collected every 2 weeks from each mouse and kept at -20°C . Clean bedding was also collected and kept in the same conditions. The first sample of soiled bedding was collected before feeding mice with doxycycline diet (hereafter T0), then 2 weeks (T2) and 12 weeks (T12) after starting the doxycycline diet (Fig. 4). Each scent stimulus was obtained by pooling soiled bedding of three to four mice to mitigate the effect of individual odours variation on the scent bouquet (details of pool composition for each test/experiment are given in Table 3).

Behavioural experiments

All the behavioural experiments were filmed to keep track of all tests (camera JVC Quad-Proof). We used the videos to record behaviour during the preference tests. Behaviour during the habituation/generalisation tests was live scored by an observer positioned 2 m away from the apparatus. The camera setup was placed before the start of any experiment. Data were recorded with the software Observer 5.0.31 (Noldus, 1991).

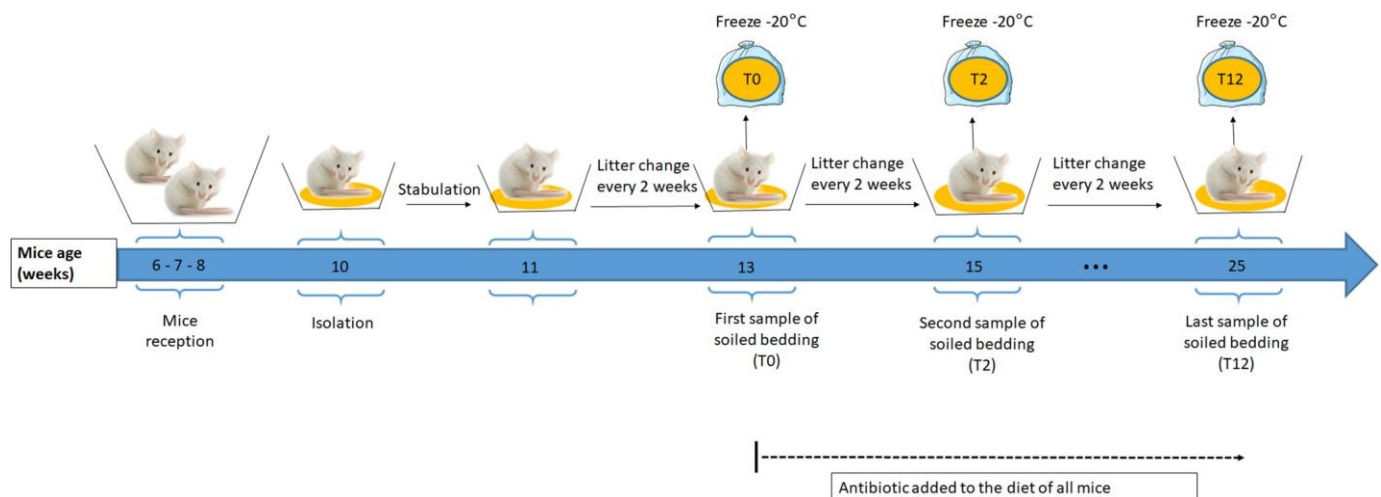


Fig. 4. Soiled bedding collection. The same quantity of fresh bedding was provided to all mice (NC and CC genotypes, see text) every 2 weeks and the soiled bedding was collected at week 13 (T0), at week 15 (T2) and at week 25 (T12) and kept in a plastic bag at -20°C . The mice diet was supplemented with antibiotic from the age of 13 weeks and for 12 weeks. All mice were euthanized at the age of 25 weeks and screened for presence of cancer tumours.

Table 3. Characteristics of the stimuli used during the behavioural tests

Test	Status of mice	Pool	Family 1	Family 2	Family 3	Family 4
Habituation/discrimination	CC	Pool A	Mouse 1	Mouse 1	Mouse 1	
	CC	Pool B	Mouse 2	Mouse 2	Mouse 2	
	NC	Pool C	Mouse 3	Mouse 3	Mouse 3	
	NC	Pool D	Mouse 4	Mouse 4	Mouse 4	
Preference	CC	Pool E	Mouse 5	Mouse 5	Mouse 5	Mouse 1
	NC	Pool F	Mouse 6	Mouse 6	Mouse 6	Mouse 2

Each stimulus was obtained by pooling soiled bedding of several individuals. The donors were progeny of four distinct families. CC, cancerous mice; NC, non-cancerous mice.

Experiment 1: female preference

Two-way choice tests were carried out using a Y-shaped maze connected to three boxes (15 cm L×15 cm W×10 cm H), a start box, and two peripheral boxes containing the stimuli. A female was placed in the start box for 2 min before the start of the experiment. During this time, two stimuli (4 g each) were placed at the far end of each of the peripheral boxes. The start box was connected to the Y maze just before the start of the experiment. Each test lasted 10 min starting when the mouse's two front paws and head were in the Y maze. If 5 min after the opening of the start box the mouse did not either enter the Y maze or visit one of the peripheral boxes, the test was not considered. The left and right position of the two stimuli was alternated between the tests to avoid biases due to lateralization.

A female was tested once a week and could have participated in up to three different tests each involving a choice between a pool of CC and a pool of NC collected during one of the three experimental conditions: T0, T2 or T12. The order of presentation of stimuli T0, T2 and T12 was randomized over the day and week. Out of 17 females, nine performed the three tests, the others, either because they were not in oestrus or because of long freezing during one of the tests, were involved either in only two tests (six) or a single test (two).

Preference for one of the two stimuli was inferred from comparisons of the relative time spent by a female in the box containing the NC stimulus versus the CC one.

Experiment 2: habituation/discrimination - habituation/generalisation

We used the habituation/generalisation test to assess differences in perception of similarities or of differences of CC versus NC odour sources (Fig. S1). The procedure started with a habituation phase during which a mouse was presented with a single stimulus until it got familiarized with it (reflected by the reduction of the time spent by the mouse investigating the stimulus over the course of the experiment). The second phase immediately followed the first, during which two new stimuli were presented to the mouse simultaneously (generalisation phase). During the generalisation phase, the mouse was expected to investigate less the stimulus most similar to the one presented during the habituation phase, unless the two stimuli were equally similar or different to the habituation stimulus (Todrank and Heth, 2003).

The apparatus was made of a Plexiglas transparent device comprising a starting box (15 cm L×15 cm W×10 cm H) connected to a test box (30 cm L×30 cm W×10 cm H) with a cylindrical tunnel (diameter 5 cm length 25 cm; Fig. S1). The mouse was introduced into the starting box, isolated from the rest of the apparatus by a sliding door, and left there for 1–2 min, while the habituation stimulus (4 g) was placed on the floor at the far end of the test box. The sliding door was then opened, and recording starts when the mouse two front paws were outside of the starting box. The habituation phase lasted 14 min. This phase was validated only if the mouse spent significantly more time investigating the stimulus during the first half than during the last half of the test. At the end of this phase, the mouse was isolated again in the starting box, and the habituation box was quickly replaced by a second test box containing two stimuli (4 g/stimulus let to thaw in the box) added 3 min before the end of the habituation phase and placed one on the left the other on the right side of the test box at 25 cm distance from each other. Not more than 30 s after the end of the habituation phase, the start box sliding door was opened again and the mouse was let to

investigate the discrimination box for 7 min. To avoid bias due to laterality, the left and right position of the two stimuli was alternated between tests.

Considering the heterogeneity of tumour development (Marjanovic et al., 2012; Melo et al., 2013) and the potential impact of this heterogeneity on the mice odour signature, we expected greater variability between the CC stimuli than between the NC ones. To address this issue, we carried out two series of habituation/generalisation tests, during which the mice were habituated either to a CC stimulus, and then presented to a second CC and a NC stimulus (series 1; 20 mice) or habituated to a NC stimulus and then presented to a second NC stimulus and a CC one (series 2; 22 mice). Each mouse was involved in a maximum of three different tests during which it was presented with stimuli sampled at the three experimental conditions: T0, T2, T12. Two weeks after doxycycline feeding started (at T2), we expected that if development of lung tumour was initiated in CC, the NC and CC stimuli should be perceived as different. Conversely, if the tumour did not develop at T2 we expected that the NC and CC stimuli would not be perceived as different.

Habituation took place for nine mice at the three tests type, for 16 mice at only two of the tests and for 17 mice at only one of the tests. At least 1 week separated involvement of the same mouse in two tests, and the order of tests to which a mouse participated was randomized.

During this experiment we recorded: (1) the duration of investigation of each stimulus; (2) total time spent by a mouse in each box; and, (3) latency to investigate each stimulus.

Preliminary tests

These tests involved stimuli collected before starting the doxycycline diet (T0).

Preliminary 1: validation of the habituation/generalisation procedure

We performed a series of tests during which one of the two stimuli presented during the discrimination phase was the same as the one presented during the habituation phase. We expected that if discrimination occurred the more familiar stimulus (i.e. the one presented both during the habituation and the discrimination phase) would be less investigated by the mouse.

Eleven mice participated to the habituation phase, but only nine showed clear habituation and hence participated to the discrimination phase (see Fig. S2 for the habituation results). Mice were habituated either to a CC (four tests) or a NC (five tests) stimulus. The mice spent significantly more time investigating the less familiar stimulus during the discrimination phase (Mann–Wilcoxon test for matched samples, unilateral test, $N=9$, $V=4$, $P=0.014$) indicating that discrimination took place.

Preliminary 2: did the smells of CC and NC brothers differ?

To address this question, we used individual stimuli. Soiled bedding collected from three brothers two with a CC genotype and one with a NC genotype. Fourteen outbred mice were habituated to a CC stimulus (brother 1). The habituation phase was validated only for eight mice that participated in the generalization phase during which they were presented with a CC (brother 2) and a NC (brother 3) stimuli (see Fig. S2 for the habituation results). Time spent by the eight mice sniffing the two discrimination stimuli did not differ significantly (Wilcoxon test for matched samples, bilateral test, $N=8$, $V=29$, $P=0.148$, power=70%), indicating that the smell of two brothers with the same genotype (here CC) were not perceived as more

similar to each other than odours of two littermates with a different genotype (CC/NC).

Collection and identification of VOC present in the odour stimuli

We analysed the VOC composition of three samples (3 g each) per stimulus used during the behavioural tests (two during the habituation/generalisation and one during the preference test, per health status CC/NC). This approach was aimed to assess variation within and between the stimuli, which were pools made of soiled bedding of several individuals. In parallel, clean bedding, kept under the same conditions as the experimental stimuli, was also sampled and used as a technical control: VOC present both in the control and in the test stimuli were considered as non-informative (Fig. 5).

The VOC content of all samples was collected with a solid phase micro extraction fibre (SPME, 65 μm diameter PDMS-DVB composed of polydimethylsiloxane-divinylbenzene, Sigma-Aldrich, Bellefonte, PA, USA). An SPME fibre was exposed to a stimulus and analysed after desorption of the fibre content in a gas chromatographer coupled to a mass spectrometer (GC-MS). The stimuli and the control kept at -20°C were thawed and maintained on ice for the time of sampling. A 3 g sample was transferred into a 125 ml glass vial, which was then sealed. An SPME fibre was introduced into the vial after piercing its stopper with a needle. The position of the fibre in the vial was controlled so that the distance from the stimulus (~ 2 cm) was similar between samplings. Following an equilibration time (3 min) in an oven maintained at 22°C , the SPME fibre was exposed to the stimulus for 1 h, before being introduced into the GC-MS injector (quadrupole mass spectrometer Shimadzu QP2010-SE (Shimadzu, Kyoto, Japan). The GC was equipped with an Optima 5-MS fused silica capillary column (30 m \times 0.25 mm \times 0.25 μm film thickness, Macherey-Nagel, Düren, Germany). Helium was used as carrier gas (1 ml min^{-1}). The oven temperature was maintained at 40°C for 2 min, after which the temperature increased by 5°C every minute until it reached 175°C , and by $12^{\circ}\text{C min}^{-1}$ until it reached 220°C . Injection of the SPME fibre in the GC for desorption was done while the injector was at 250°C . The

chromatograms were analysed with the resident software (GCMS Solution, Shimadzu, Kyoto, Japan). We used the peak retention times (RT) and mass spectra to identify a compound. RT were then transformed into retention time indices (RI), using as a reference the retention time of a series of *n*-alkanes, that were injected in the same GC-MS (Alkanes standard solution, 04070, Sigma-Aldrich). Final identification of compounds was based on comparison with those in mass spectrum databases (NIST 2007, Wiley Registry Ninth) and RI available compounds databases (e.g. Adams, 2007, Pubchem, <https://pubchem.ncbi.nlm.nih.gov/>). Compounds that were present both in the technical controls (clean bedding) and in our studied samples (stimuli) were considered as potential pollutants and were excluded from the analyses. Compounds present as very small peaks and in a very small number of samples [three compounds: unknown compound 2; (x)-2,4,4-trimethyl-Pent-2-enal; 3,6,6-trimethyl-2-Norpinanone] were also excluded from the analysis. For the other compounds, we calculated their peak area on the total ion current chromatogram (TICC). Compounds present in the form of traces were given an arbitrary area value corresponding to 10% of the area of the smallest peak present in our dataset. Peak area for all compounds were then summed up per individual, and a relative surface area was calculated as the surface area of a given compound divided by the sum of all compounds for a given individual. Hereafter we refer to these values as relative concentrations.

Statistics

All statistical analyses were performed with R version 3.4.4 in Rstudio (RStudio Team 2020 RStudio: Integrated Development for R. RStudio, PBC, Boston, MA, USA. URL <http://www.rstudio.com>). We used the following packages: vegan (Oksanen et al., 2020), lme4 (Bates and Maechler, 2009), ggplot2 (Wickham, 2014) and lmm (Schafer et al., 2020). Significance level was set at $\alpha=0.05$. Normality and heteroscedasticity of the distribution of residuals were checked visually after plotting the model residuals, and data were transformed when it was necessary. G* power version 3.1.9.7 was used for power calculation (Faul et al., 2007).

Experimental condition

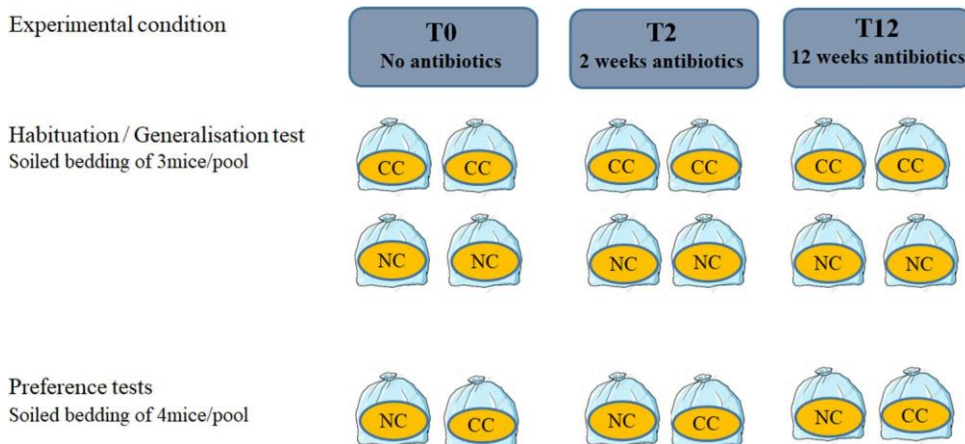
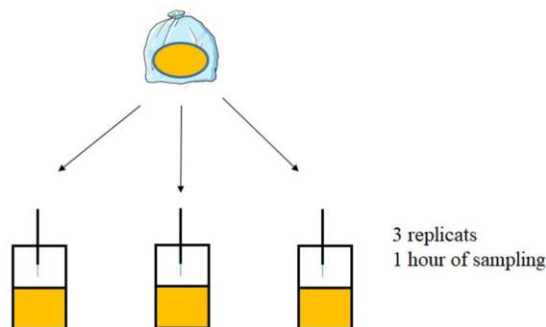


Fig. 5. Protocol for stimuli (soiled bedding) preparation and VOC extraction. Each bag contained a stimulus made of a pool of soiled bedding obtained from four (pref. test) or three (habituation/generalisation) mice. We mixed 40 g soiled bedding/individual to obtain a 120 g bag for the habituation/generalisation tests and 20 g soiled bedding/individual to obtain 80 g of litter for the female tests. For the chemical analysis, we mixed 10 g soiled bedding/mouse.

Chemical analysis



Behavioural study

Wilcoxon signed ranks test (Siegel and Castellan, 1988) were used to compare paired variables: for the preference test, time spent by a given individual sniffing stimulus NC versus CC, and for the habituation/generalisation test, time spent, by a given individual, investigating the two stimuli presented during the generalisation phase. One-way tests were performed when our predictions were directional. Power tests (G*power) were performed to calculate the power of tests that failed to reject the null hypotheses.

LMM were applied to test the total time a mouse spent in contact with the two stimuli presented during the choice tests, with one fixed factor, experimental conditions (T0, T2, T12), and the mice identity as a random factor to control for the repeated use of some of the mice. To test variation of total duration of investigation of the habituation stimulus and latency to approach the habituation stimulus (i.e. did attraction or repulsion guide the mouse behaviour?), we also used the LMM procedure, with two fixed factors, experimental conditions (three modalities) and health status (touch modalities) and mice identity as a random factor.

VOC study

In order to compare VOC composition of CC and NC stimuli at each of the experimental conditions (T0, T2, T12) we used a multivariate approach [redundancy analysis, RDA, followed by a permutation *F* test (Hervé et al., 2018) R package *vegan*]. For this, relative proportions of VOC identified in the stimuli were first transformed using the Centred Log-Ratio transformation. As our data included zeroes, a constant value being one order of magnitude smaller than the smallest non-zero value (i.e. 0.001) was added to the entire data set. Then the data were autoscaled following the method proposed by Hervé et al. (2018). Three different pools of soiled bedding per health status were involved in the behavioural tests and this variable was included in the RDA as a fixed factor (three modalities) together with health status (two modalities).

To test whether heterogeneity in tumour development between CC mice may be reflected in their VOC composition, we compared multivariate dispersion within the odour bouquets among the CC and the NC pools using the function *betadis* of the R package *vegan* (Euclidean distance based on *clr*-transformed and autoscaled VOC relative proportions). The test was performed separately for the two experimental conditions that followed antibiotic ingestion (T2, T12).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: F.G., L.D., F.T., G.G.; Methodology: F.G., B.B., G.G.; Software: M.R.H., F.G., G.G.; Validation: B.B., M.R.H., L.D., F.T., G.G.; Formal analysis: F.G., M.R.H., G.G.; Resources: M.M., A.M., F.T.; Data curation: F.G.; Writing - original draft: F.G., G.G.; Writing - review & editing: B.B., M.R.H., M.M., A.M., L.D., F.T.; Supervision: G.G.; Project administration: L.D., F.T.; Funding acquisition: F.G., F.T.

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Summary: Male mice could discriminate the smell of cancerous congeners even when the tumour was hardly detectable by other means; however, females did not discriminate against the smell of males carrying cancerous tumours. Odorant molecules other than volatile organic compounds analysed here might explain the observed behaviour.

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1	MAVA Foundation	http://dx.doi.org/10.13039/100013324	
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3	MIVEGEC: Maladies Infectieuses et Vecteurs Ecologie Genetique Evolution et Controle		

SUPPLEMENTARY MATERIAL:

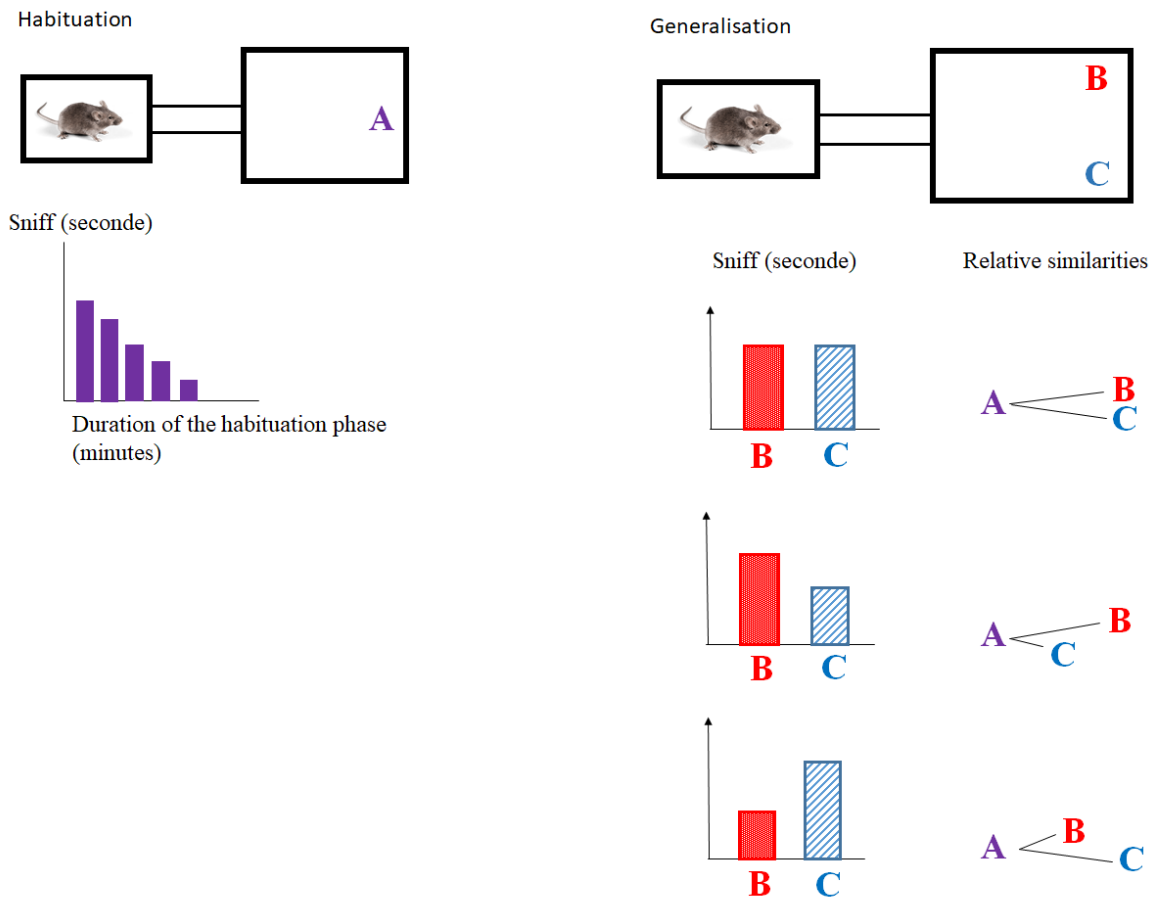


Fig S1: A scheme describing the habituation / generalisation procedure and predictions. The generalisation phase follows the habituation phase only if the habituation phase is validated by a significant reduction of the time spent sniffing the habituation stimulus at the end of that phase. The generalisation phase allows evaluating relative similarities between the habituation stimulus and the two stimuli presented to the mouse during this phase.

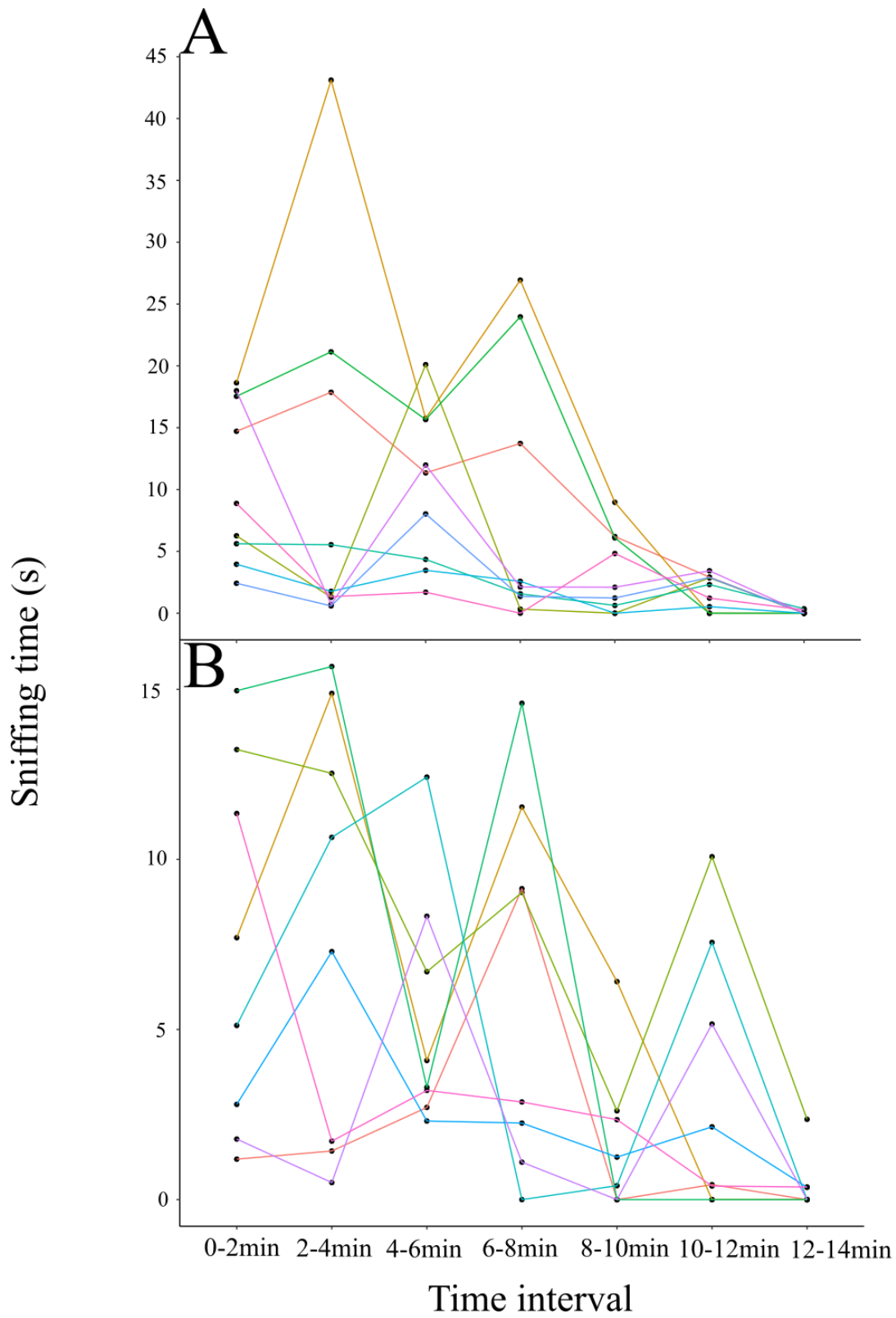
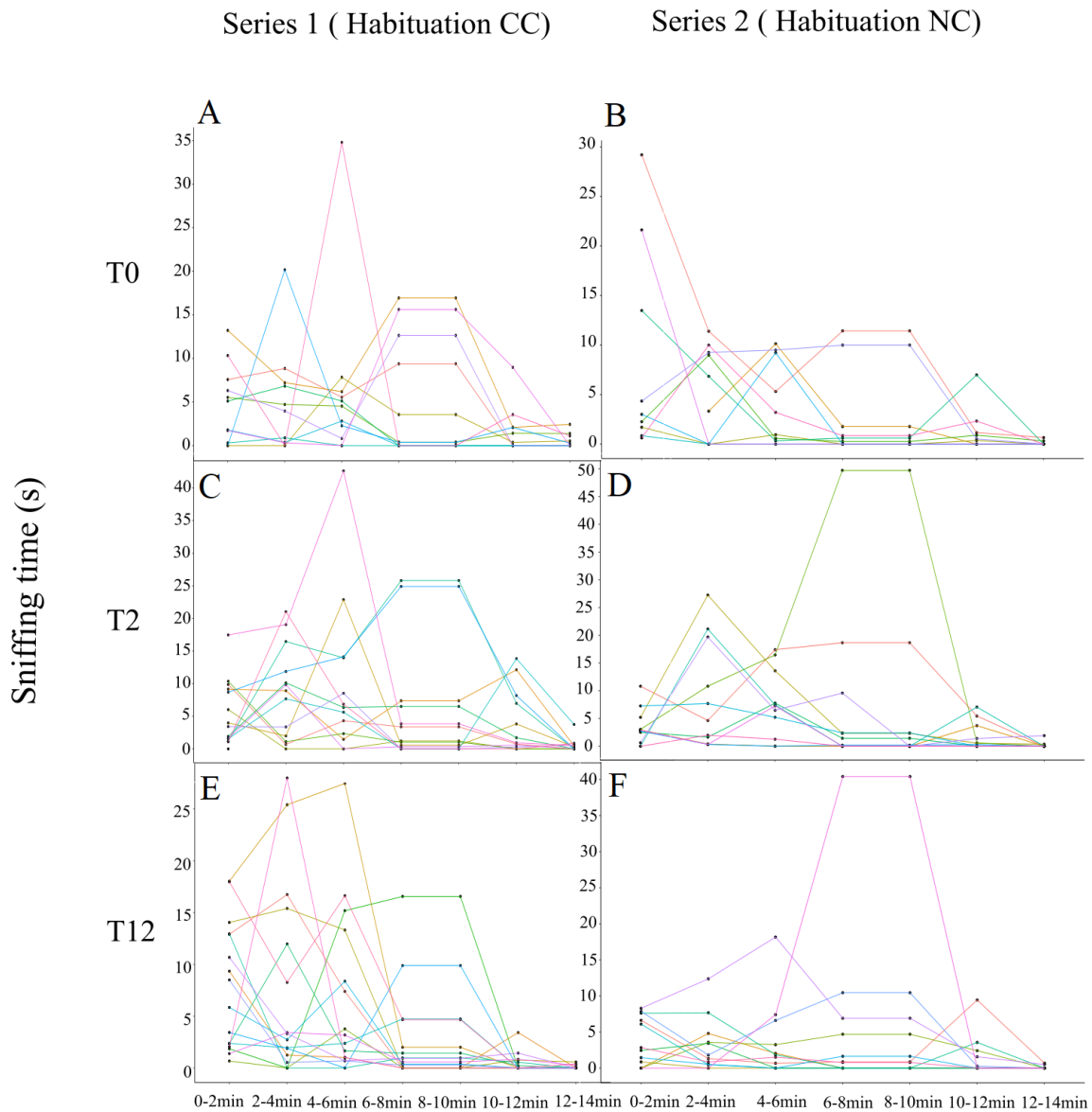


Fig S2 : Time spent by the mice sniffing the stimulus during the habituation phase for A) preliminary test 1 with $n = 9$ and B) preliminary test 2 with $n = 8$



Time interval

Fig S3 : Time spent by the mice sniffing the stimulus during the habituation phase for T0 A) series 1 with n =11 and B) series 2 with n = 10, for T2 C) series 1 with n = 12 and D) series 2 with n = 11 and for T12, for E) series 1 with n = 16 and F) series 2 with n = 12.

Table S1: Identification of the 21 compounds, and their occurrence in the different stimuli used during the behavioural tests for NC (non-cancerous) and CC (cancerous) mice at the three experimental conditions (T0, T2 and T12). Retention indices (RI), retention times (RT).

Compound	RT	RI	NC			CC		
			T0	T2	T12	T0	T2	T12
Unknown compound 1	2.14	n.c	0	7/9	9/9	0	9/9	9/9
propan-2-ol	2.23	n.c	9/9	9/9	9/9	9/9	9/9	9/9
propanoic acid	4.31	707	9/9	9/9	9/9	9/9	9/9	9/9
3-methyl-butan-1-ol	4.89	733	3/9	8/9	9/9	8/9	9/9	9/9
butanoic acid	6.47	802	9/9	9/9	9/9	9/9	9/9	9/9
(x)-2-ethyl-hex-2-enal ^{TI}	6.68	809	0	9/9	9/9	9/9	9/9	9/9
Unknown compound 2 *	7.57	839	6/9	2/9	3/9	6/9	1/9	0/9
3-methylbutanoic acid	7.98	853	9/9	8/9	9/9	9/9	9/9	9/9
2-methylbutanoic acid	8.26	862	9/9	9/9	9/9	9/9	9/9	9/9
hexan-1-ol	8.45	869	9/9	9/9	9/9	9/9	9/9	9/9
(x)-2,4,4-trimethyl-pent-2-enal* ^{TI}	8.59	873	9/9	9/9	0	0	0	0
benzaldehyde	11.19	956	9/9	8/9	9/9	9/9	6/9	9/9
2,3-dehydro-exo-brevicomine	13.80	1039	9/9	9/9	9/9	9/9	9/9	9/9
3,4-dehydro-exo-brevicomine	14.06	1047	9/9	9/9	9/9	9/9	9/9	9/9
fenchone	15.36	1089	9/9	9/9	9/9	9/9	0	9/9
2-sec-butyl-4,5-dihydrothiazole	15.82	1103	9/9	9/9	9/9	9/9	9/9	9/9
nopinone	16.79	1135	9/9	9/9	8/9	9/9	8/9	9/9
camphor	17.06	1144	9/9	9/9	9/9	9/9	9/9	9/9
camphene hydrate	17.25	1150	9/9	9/9	9/9	9/9	9/9	9/9
(E)-3-pinocamphone	17.53	1160	9/9	9/9	9/9	9/9	1/9	9/9
3,6,6-trimethyl-2-norpinanone* ^{TI}	18.89	1205	8/9	7/9	6/9	0	0	9/9

* Compounds were removed from the subsequent statistical analyses because of their very low concentration (less than 1% of the mixture), and of uncertainty about their identification.

n.c : not calculated

TI : attempt of identification; in some cases CC, the configuration could not be determined.

~ Chapitre VI ~

**Impact du cancer sur les modifications d'odeur corporelles :
utilisation des urines de souris.**

Contexte et objectifs

Comme mentionné précédemment, le cancer du poumon est la principale cause de décès par cancer dans le monde. Plus que jamais la détection précoce de ce cancer reste un point essentiel pour maximiser les chances de guérison. Un intérêt potentiel d'étudier les COVs est de trouver des biomarqueurs précoces. Il est alors important de déterminer à quel stade dans le développement du cancer des modifications dans l'émission de COVs apparaissent, et pourraient ainsi servir dans le diagnostic. Dans cette étude nous avons voulu, en parallèle des essais sur les litières, voir si nous pouvions observer des variations de COVs liées au cancer grâce à des analyses chimiques d'urine réalisées sur les souris EGFR. Ayant effectué des prélèvements d'urine toutes les deux semaines à partir du début du traitement, nous avons ainsi pu suivre l'évolution des COVs émis dans les urines de souris au cours de l'évolution du développement d'un adénocarcinome pulmonaire. Pour cela l'urine de 20 souris, 10 CC et 10 NC à 7 temps de prélèvement allant de T0 avant le début du traitement jusqu'au prélèvement T12 qui correspond à 12 semaines après le début du traitement ont été analysées par GC-MS, soit 140 chromatogrammes.

Résumé de l'article

Nos expérimentations ont permis d'identifier une liste de 36 COVs, parmi lesquels 19 ont été trouvés chez chaque souris et chaque prélèvement. Aucune différence qualitative n'a été détectée entre les souris NC et CC. Les deux souris n'ayant pas déclenché de cancer parmi les CC ont été retirées des analyses. Nous avons pu mettre en évidence que des modifications d'odeurs apparaissent entre les souris NC et CC dès le deuxième prélèvement à T2 (voir bilan Figure 18). Quant aux souris CC nous pouvons voir l'évolution du profil d'odeurs qui se différencie dès T2 et continue d'évoluer comme différent jusqu'en T12. Cette différence est due à plusieurs COVs, quatre dont les proportions relatives diminuent, le 3-méthylbutan-2-one, pentan-2-one, hept-4-en-2-one et le 3,4-déhydro-*exo*-Brevicomine et quatre autres qui augmentent le phénylacetone, 2-*sec*-butyl-4,5-dihydro-Thiazole, 4-méthylhept-6-en-3-one et le 6-méthylheptan-3-one. Nous avons pu voir que certains composés étaient impactés soit par l'âge ou la prise de doxycycline aussi bien chez les souris CC que chez les souris NC. Parmi ces composés, seulement deux ont déjà été retrouvés chez l'Homme dans des études liées au cancer, 3-méthylbutan-2-one et le pentan-2-one. Cette étude a permis de déterminer à partir de quel stade des variations de COVs liées au cancer sont détectables ainsi que les composés associés à cette variation. Le stade étant très précoce, cela permet d'envisager une réelle utilité du diagnostic à l'aide des COVs dans le diagnostic précoce du cancer.

Composées biomarqueurs

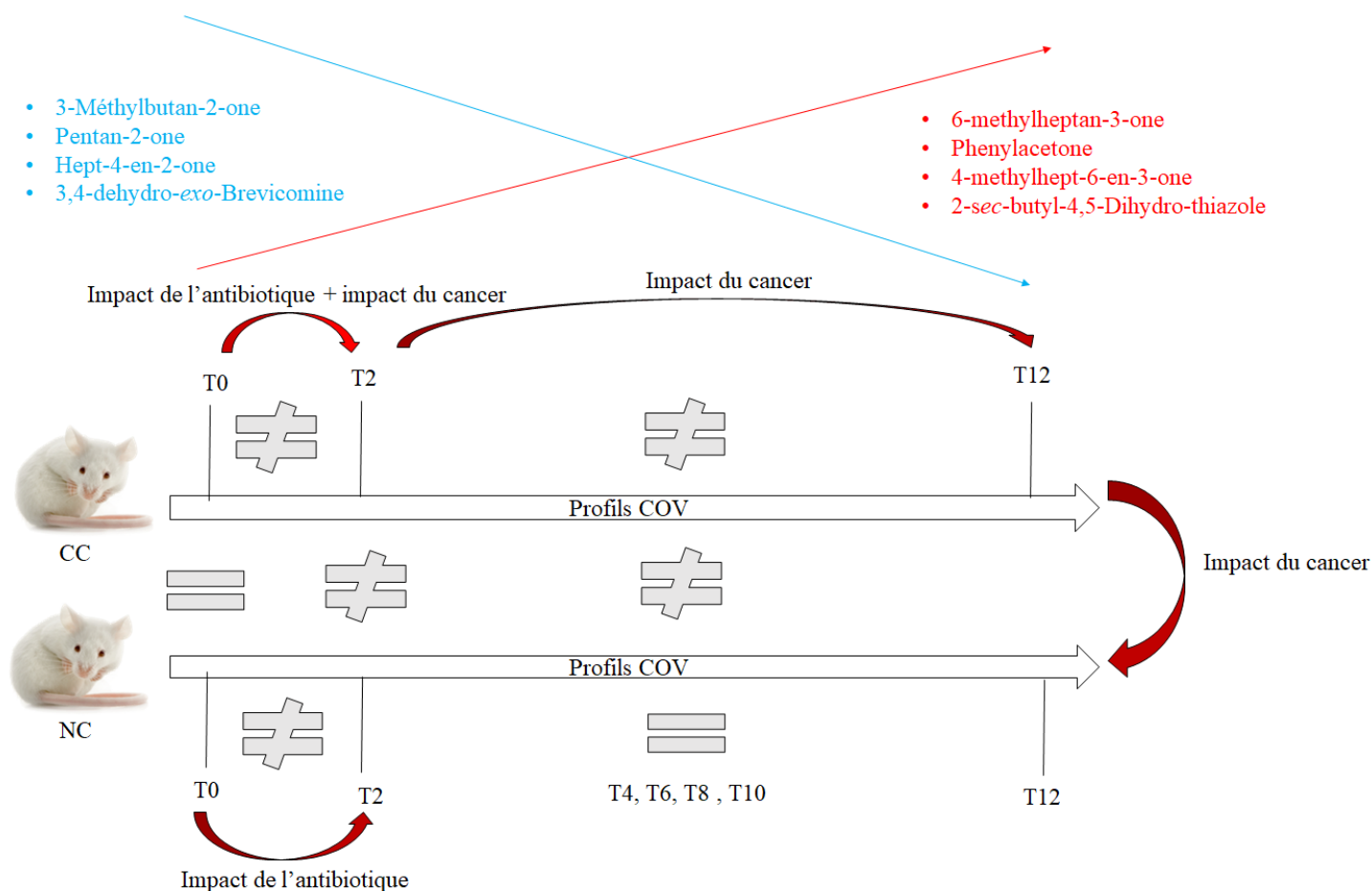


Figure 18: Schéma bilan résumant l'impact du cancer sur les COVs issus des échantillons d'urine de souris. Avec les comparaisons des différences de profils d'odeur des souris NC (non cancéreuses) et CC (cancéreuses) au cours des différents prélèvements analysés, de T0 avant le début du traitement jusqu' à T12 en stade terminal du cancer. Cette comparaison est à la fois faite entre les souris NC et CC et entre chaque groupe de souris. Les flèches rouges expliquent la différence entre les profils d'odeurs induit soit par la prise d'antibiotique soit par le cancer.

Manuscrit associé à ce chapitre :

Manuscrit V :

- Gouzerh, F., Buatois, B., Hervé, M. R., Mancini, M., Maraver, A., Dormont, L., Thomas, F. & Ganem, G. Urinary VOCs as Biomarkers of lung cancer in Mice. (*In prep for Cancers*)

Urinary VOCs as biomarkers of lung cancer in Mice.

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Abstract

Lung cancer is the most important cause of cancer induced death worldwide. Besides prevention and treatment improvement, it is increasingly established that early detection is often critical to cure cancer. The aim of this study was to identify urinary volatile organic compounds (VOCs) that could help diagnose lung cancer at an early stage of development. We analyzed urine VOCs composition obtained from a genetic engineered lung adenocarcinoma mouse model with oncogenic EGFR doxycycline inducible lung specific expression, hereafter CC mice. We compared the VOCs urine profile of CC mice and their littermate negative controls (hereafter NC), before doxycycline induction and at several time points until 12 weeks of doxycycline treatment. We used SPME fibres and GC-MS analysis of urine to detect cancer-related odour variations over time. Our main results showed quantitative differences in the urinary VOCs profiles of cancerous *versus* non-cancerous mice pointing out the influence of tumour development on the VOCs profile of CC mice and pointed out eight VOCs as potential candidates for detection of lung tumours, from noninvasive urine samples, as early as 2 weeks after doxycycline induction, much before small imaging techniques identify them.

Keyword: Lung cancer, biomarkers, urinary volatile organic compounds, EGFR oncogenic mutation, *Mus musculus domesticus*

Introduction

Cancer is one of the deadliest diseases in the world with more than 9 million casualties in 2020 (Sung et al., 2021). Of the many cancers affecting humans, 18% are attributable to lung cancer, and in 90% of the cases deaths are accountable to metastasis rather than primary tumours (Chaffer and Weinberg, 2011; Dillekås et al., 2019). Lung cancers, like many other cancers, are difficult to detect at early stages. Further, besides prevention and treatment's improvements, there is growing evidence that early detection is often critical to curing success: patients whose cancer was diagnosed and treated at an early stage were shown to have better long-term survival than patients whose tumours were not detected before symptoms appearance (e.g. cervical, colorectal, breast cancers (Ludwig and Weinstein, 2005; Saadatmand et al., 2015)). Among the current diagnostic techniques are radiography, magnetic resonance imaging, and biopsy (Al-shamasneh and Obaidellah, 2017; Prabhakar et al., 2018). These methods are effective, but several are invasive, expensive, time-consuming, or necessitate facilities only present in large hospitals (Aruleba et al., 2020; Cui et al., 2020; He et al., 2020). In addition to these diagnostic techniques, the search for specific biomarkers has become very popular with the progress of high-throughput techniques (Gramolini et al., 2008). Among identified diagnostic biomarkers are proteins, tumour antigens, or genes' transcripts found in urine or blood, for example (Ludwig and Weinstein, 2005). However, none of the biomarkers identified so far has sufficient sensitivity, specificity and reproducibility to diagnose lung cancer early enough to prevent metastasis. Therefore, the identification and validation of non-invasive biomarkers that could effectively diagnose lung cancer at an early stage remains critical to tackle the main cause of death by cancer in the world (WHO | World Health Organization).

Over the past 20 years, increased attention has been devoted to exploring the potential use of volatile organic compounds (VOCs) as non-invasive biomarkers of early cancers. Two-thirds of these studies concerned lung cancer (Gouzerh et al., 2021). Although many studies pointed out significant differences in the VOCs profiles of healthy versus cancerous patients in humans (Baldini et al., 2020; Szulejko et al., 2010) or in mice (Matsumura et al., 2010; Sever et al., 2015), we still do not know how early in the process of cancer development variation in VOCs composition occurs and could be detected.

The aim of this study was to identify candidate VOCs that could help diagnose early cancer development. Precisely, we used a genetic engineered mouse model (GEMM) harboring two different transgenes, the lung-specific CCSP-rtTA and a human EGFR with the activating EGFR^{L858R} mutation that upon 8 to 12 weeks of doxycycline treatment induce EGFR-driven lung adenocarcinoma (Li et al., 2007). We compared the VOCs profiles of cancerous mice (CC) mice and their littermate negative controls (hereafter NC), taking into account that doxycycline is an antibiotic and it is reported that changes in gut microbiota-induced by antibiotics can influence the mice urinary VOCs (Lanyon et al., 2007). Hence, we compared VOCs in the urine of CC and NC mice before and after doxycycline

treatment, and compared the VOCs profiles of CC and NC at different stages of tumour development seeking to identify specific VOCs that could help diagnose lung cancer at an early stage of development.

Material and methods

1. Ethical clearance

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

2. Mice

Urine stimuli were obtained from CCSP/EGFRTL transgenic mice, that were generated by crossing the Tet-ON-^{EGFRT790M/L858R} transgenic mouse strain (hereinafter EGFRTL) with the CCSP-rtTA strain (hereinafter CCSP) carrying rtTA, an inverse tetracyclin responsive element, under the control of the lung specific promoter. In CCSP/EGFRTL mice the EGFR gene, contains both L858R mutations (causing EGFR constitutive activation in the absence of ligands as EGF) and T790M gate keeper mutation (conferring resistance to the first generation of EGFR inhibitors, i.e. gefitinib/erlotinib). Crosses were kept in heterozygosity, thus resulting littermate could have any of the four possible genotypes: WT/WT, WT/EGFRTLtg, CCSPtg/WT and CCSPtg/EGFRTLtg. In our experimental setting we used WT/WT mice, lacking both CCSP and EGFRTL transgenes, and hence unable to develop tumours upon doxycycline induction, hereafter non-cancerous (NC) mice, and CCSPtg/EGFRTLtg mice carrying both transgenes and able to develop a lung tumour upon doxycycline induction, hereafter cancerous (CC) mice. This system allows the expression of EGFRTL specifically in the lung but only upon doxycycline exposure, thus leading to the development of peripheral adenocarcinomas with bronchioloalveolar features in alveoli as well as papillary adenocarcinomas (Li et al., 2007; Mur et al., 2020). Mice were 13 weeks old when cancer was induced.

Twenty transgenic male mice were involved in our study: 10 (CC) and 10 (NC). They were bred at the IRCM (Montpellier Cancer Research Institute) at the age of 6 weeks and maintained at the breeding facilities of the IRD (Institute of research and development) of Montpellier. Mice were kept in groups of 2 to 4 mice in transparent cages (26.8cmL*21.5cmL*14.1cmH) before the start of the experiment. The cages contained sawdust, cellulose square, hay, and a cardboard tunnel. From the age of 12 weeks the mice were kept isolated to avoid influence of social interactions on their VOCs profile (Hurst, 2005; Hurst et al., 1997). During all the experimentation period their mass was checked to monitor their health (euthanasia if loss of 10% of mass). Lung adenocarcinoma induction was done by ingestion of food

pellets containing doxycycline (1mg/kg), available ad libitum, starting at 13 weeks of age, for 12 weeks (**Figure 1**).

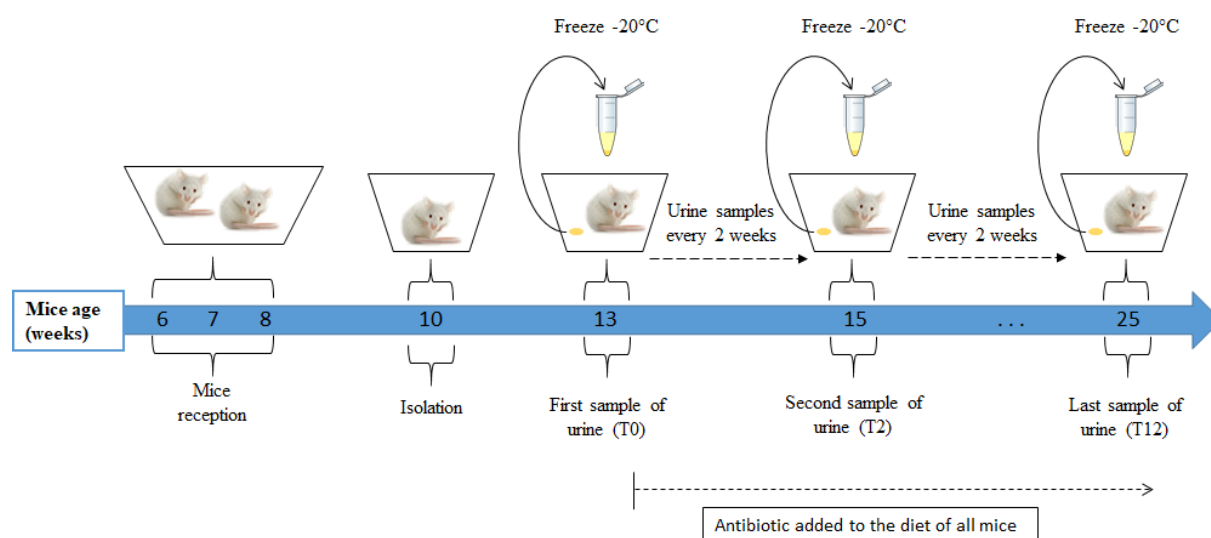


Figure 1: Urine collection protocol. Mice were received at 6-8 weeks and the experiment started when they were 13 weeks old and lasted for 12 weeks. Mice were fed with a diet containing doxycycline from the age of 13 weeks and for 12 weeks. All mice were euthanized at the age of 25 weeks for tumour screening. Samples of urine were collected every two weeks in eppendorf tubes and kept at -20°C .

At the age of 25 weeks, all mice (NC and CC) were euthanized at the IRCM where their lungs were inspected. The presence or not of lung adenocarcinoma was confirmed by analysis of hematoxylin-eosin staining on formalin-fixed paraffin embedded lung sections obtained at the end of the experiment (**Figure 2**). After necropsy two mice of the CC genotype showed no apparent lesions and these two mice were excluded from our data set. We therefore analysed the odour profiles of 10 NC and 8 CC mice.

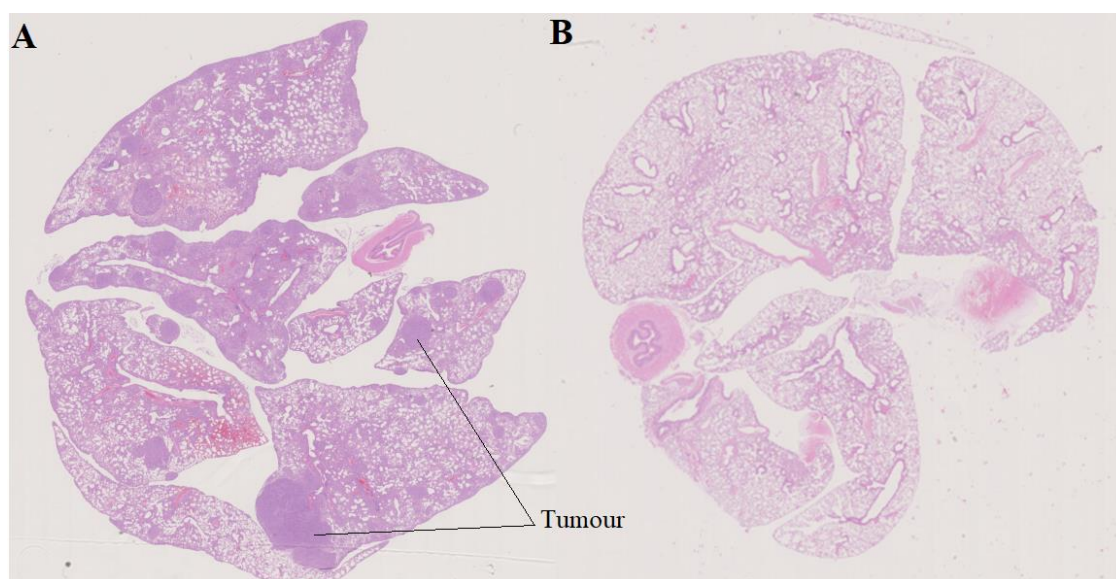


Figure 2: Lung sections, fixed with hematoxylin-eosin staining on formalin-fixed paraffin embedded, following a 12 weeks doxycycline treatment. A) cancerous mice (CC) and B) healthy mice (NC).

3. Urine sampling

The stimuli used to characterize the nature of the chemical variation between cancerous mice and healthy ones were urine samples. Urine samples of 18 male mice were collected every two weeks for 12 weeks. The first one was sampled before the addition of doxycycline (T0) and the last one was sampled 12 weeks following the start of the doxycycline treatment (T12), i.e. a total of 7 samples collected over 12 weeks (**Figure 1**). The urine collection protocol was as follows: each mouse was placed individually in an empty cage previously washed with alcohol and left to dry. Mice were left to urinate in the cage for a maximum of 1 hour. The urine was collected with a micropipette transferred to an eppendorf, and kept frozen at -20° . A mouse was sampled several times over 3 days in the morning / and in the afternoon. Distilled water was deposited on a cage washed with alcohol and collected following the same protocol as for the urine to serve as a control for further chemical analysis.

4. Analysis and identification of VOCs in mice urine

VOCs present in the mice urine were sampled using solid phase micro extraction (SPME, $65\mu\text{m}$ 277 diameter PDMS-DVB fibre composed of polydimethylsiloxane-divinylbenzene, Sigma-Aldrich, Bellefonte 278 PA, USA). Sampling took place in a oven at 22°C . A SPME fibre was introduced into a vial containing $40\mu\text{l}$ urine, then after 3min equilibration time the fibre was exposed to the vial headspace for an hour (**Figure 3**). The position of the fibre in the vial was controlled so that the distance from the stimulus ($\sim 1\text{cm}$) was similar between samplings. After extraction the same fibre was introduced into a Gas Chromatographer coupled to a Mass Spectrometer injector (GC-MS) (quadrupole mass spectrometer Shimadzu QP2010-SE (Shimadzu, Kyoto, Japan)). The GC-MS was equipped with an Optima 5-MS fused silica capillary column ($30\text{ m} \times 0.25\text{ mm} \times 0.25\text{ }\mu\text{m}$ film thickness, Macherey-Nagel, Düren, Germany), with helium as the carrier gas (1 ml/ min). All spectra were analysed with the resident software (GCMS Solution, Shimadzu, 292 Kyoto, Japan).

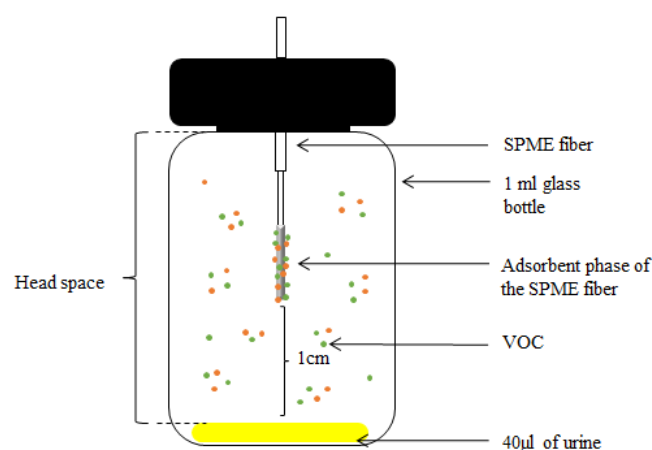


Figure 3: A scheme of the protocol for sampling VOCs present in $40\mu\text{l}$ of mice urine and in water (control) samples using the solid phase microextraction (SPME) method.

As described elsewhere (Gouzerh et al., 2022), VOCs were identified using peak retention time (RT) and mass spectra for each compound visible on the chromatogram. Retention indexes (RI) were calculated with reference to n-alkanes injected in the same GC-MS (Alkanes standard solution, 04070, Sigma-Aldrich, Aldrich, Darmstadt, Germany). Final identification of compounds was based on comparison with mass spectrum databases (NIST 2007, Wiley Registry Ninth) and available

compounds RI databases (e.g. Adams, 2007, Pubchem, <https://pubchem.ncbi.nlm.nih.gov/>). After identification of all compounds and exclusion of those present in the control samples, we calculated the surface of each compound peak area based on the total ion current chromatogram (TICC). The surface area of compounds that were only present as traces could not be calculated, hence, they were given an arbitrary value corresponding to 10% of the value obtained for the smallest peak present in our dataset. The peak areas of all compounds were then summed (total sum) up per individual, and a relative surface area was calculated for each compound by dividing the surface area of each peak by the total sum. Hereafter we refer to these values as relative proportions.

5. Statistics

All statistical analyses were performed with the Rstudio software version 3.4.4 (R Core Team, 2020). We used the following packages: *ade4* (Thioulouse et al., 2018), *vegan* (Oksanen et al., 2020), *mixOmics* (Rohart et al., 2017), *ggplot2* (Wickham, 2014), *RVAideMemoire* (Hervé, 2022), *Hotelling* (Curran, 2021), *nlme* (Pinheiro et al., 2017). Normality and heteroscedasticity of the distribution of residuals were checked visually after plotting the model residuals, and data were transformed when it was necessary.

To compare variation of relative proportions of VOCs emitted by NC and CC mice across experimental conditions (T0, T2, T4, T6, T8, T10, T12) the redundancy analysis (RDA), a multivariate approach, followed by permutation F tests was realised (Hervé et al., 2018). Relative proportions were CLR-transformed prior to RDAs, and as our data included zeroes, a small constant of an order of magnitude smaller than the smallest non-zero value was added to all values prior to the transformation (e.g. 0.01 if the smallest non-zero value was 0.1). Factors included in the RDA depended on the hypothesis tested. We first questioned the impact of the doxycycline treatment, i.e. experimental conditions (7 modalities, T0 to T12), on the urine VOCs composition and to address this question we analysed separately CC and NC mice. These models included experimental conditions as a constrained explanatory variable, and both fibre (2 fibres used) and mice identity as conditional explanatory variables. To assess VOCs differences between NC and CC across experimental conditions we designed a model including as constrained explanatory variables mice identity nested in health status, health status, experimental conditions and interaction between the two last factors, while fibre identity was included as a conditional explanatory variable. VOCs showing an absolute correlation coefficient > 0.8 with the main RDA axes were retained as potential diagnostic molecules, and were involved in univariate analyses. The latter analyses consisted in a series of linear mixed models considering experimental conditions, status and their interaction as fixed explanatory factors, and mice identity and fibre as random factors. Data were log-transformed prior to these analyses to normalize the residuals distribution. Post-hoc pairwise comparisons were performed with the Estimated Marginal Means method (EMMeans). Significance level was set at $\alpha = 0.05$, and adjusted for multiple comparisons with the sequential Bonferroni method (Rice, 1989).

Results

Thirty-six compounds were identified in our biological samples after exclusion of molecules also present in the technical controls (**Table S2**). Nineteen of these compounds were found in all individuals, while the other 27 were found both in CC and NC although not in every individual (**Table S1**). Twenty-six of these compounds were identified, while the other 11 were not (**Table S1**).

Results indicated that the VOCs profiles of both CC and NC were influenced by doxycycline treatment (Model 1 and 2: **Table 1** and all post-hoc tests comparing T0 with T2-T12 $p < 0.05$). However, the influence of experimental conditions was more marked among the CC profiles ($p = 0.005$) than among the NC ones ($p = 0.041$) suggesting that the antibiotic treatment alone may not explain variation in the CC mice VOCs profile (**Figure 4**). Further, the impact of the doxycycline treatment on variations of the VOCs profiles in both NC and CC mice, were not linear (**Fig S1**).

Table 1: Results of the permutation F tests based on redundancy analyses linking VOCs composition to health status (NC, non-cancerous mice, versus CC potentially cancerous mice).

	conditional variables	variance explained	constrained variables	variance explained	F	P
Model 1 : Impact of doxycycline treatment on VOCs profiles of NC mice	Fibre	8.57%	Exp condition(T0,T2...T12)	6.96%	1.34	0.041
	Identity	43.13%				
Model 2 : Impact of doxycycline treatment on VOCs profiles of CC mice	Fibre	9%	Exp condition(T0,T2...T12)	17.45%	3.44	<0.001
	Identity	34.76%				
Model 3 : Impact of doxycycline treatment and health status on the VOCs profile of all mice	Fibre	5.07%	Status(NC /CC)	55.36%	14.38	<0.001
			Exp condition(T0,T2...T12)		2.73	<0.001
			Status : Identity		5.83	<0.001
			Exp condition : Status		1.27	0,09

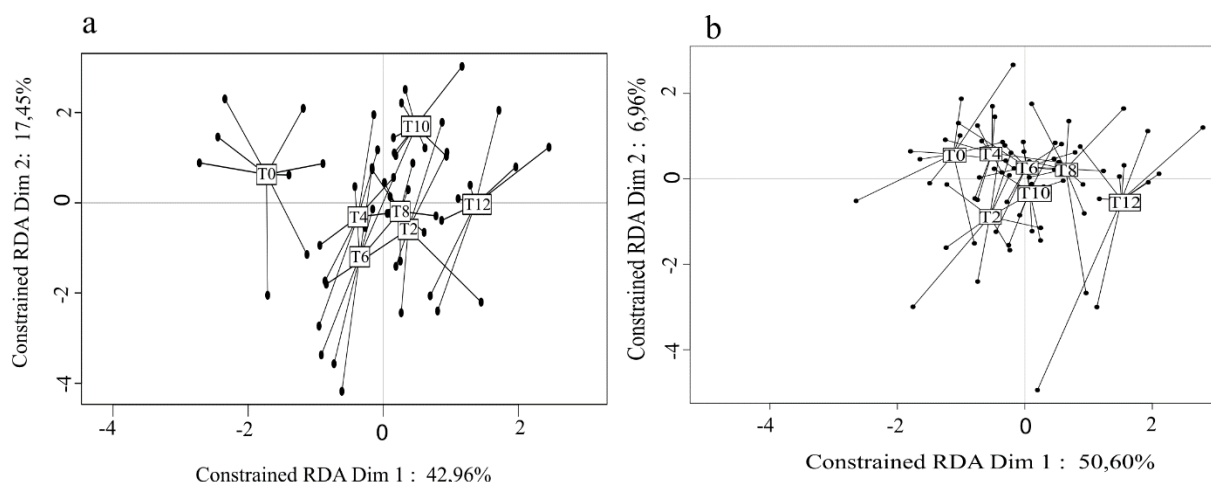


Figure 4: Graphical representation of the RDA results testing the impact of experimental conditions (doxycycline treatment) on the VOCs profiles of: A) Cancerous mice (Table 1, model 2) and B) Non-cancerous mice (Table 1, model 1).

The global RDA model, comparing the profiles of CC and NC at the different experimental conditions pointed out significant main and interactive effects of health status and experimental conditions ($p < 0.005$; model 3: Table 1; **Figure 5**). Posthoc pairwise comparisons did not point out significant differences in the VOCs profiles of CC and NC mice at T0 ($p = 0.897$), however, during the doxycycline treatment (T2-T12), NC and CC mice VOCs profiles were significantly different (for four of the comparisons $p < 0.01$, for the two others $p < 0.05$).

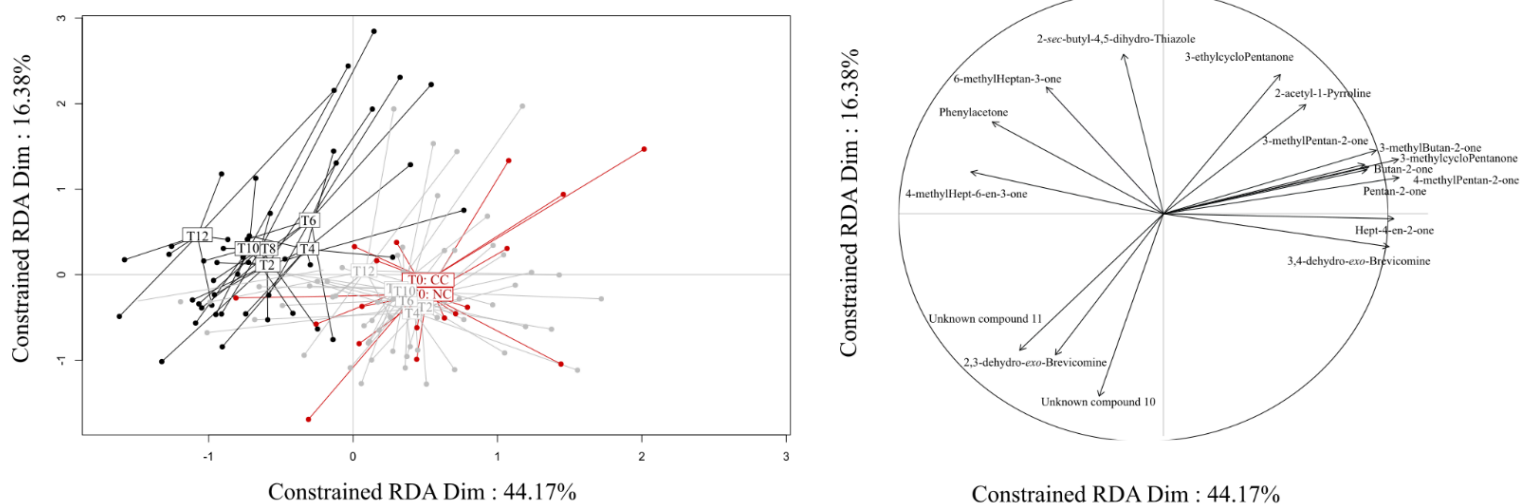


Figure 5: Left panel: Graphical representation of the RDA results testing the influence of health status (CC/NC) and experimental condition (doxycycline treatment) on the VOCs profiles. Represented in red is the first experimental condition (T0, control) for both CC and NC. All the other conditions are represented in grey for NC and in black for CC mice. Right panel: Representation of the VOCs showing an absolute correlation coefficient with the main axes > 0.8 .

We retained 17 VOCs showing an absolute correlation coefficient > 0.8 with the main RDA axes, that we further analysed with linear mixed model (**Table 1**). Among these VOCs three, 3-methylcyclopentanone, 3-ethylcyclopentanone and butan-2-one, varied across treatment but not

between CC and NC (**Table 2**). Eight VOCs were significantly different between CC and NC either at some or at all of the experimental conditions (**Table 1**). Two of the compounds, 3-methylbutan-2-one and pentan-2-one, showed a decrease in their relative proportions both in NC and CC mice from T2 through to T12, however, their relative proportions differed significantly at T10 and T12, decreasing more in CC than in the NC, suggesting that cancer tumour may have a delayed effect on these two compounds (**Figure 6**). The relative proportion of hept-4-en-2-one, decreased more significantly in response to the doxycycline treatment in CC as compared to NC mice, while 2-*sec*-butyl-4,5-dihydrothiazole (thiazoline) relative proportions increased significantly more in the CC profile than in the NC one, also suggesting a tumour effect. Four VOCs showed a significant influence of doxycycline treatment only in the CC profiles: 4-methylhept-6-en-3-one, 6-methylheptan-3-one and phenylacetone showing a marked increase, and 3,4-dehydro-*exo*-brevicomine (brevicomine) a marked decrease in their relative proportions. Absence of variation in the NC profiles suggested no direct effect of the doxycycline, but an indirect effect on CC mice through tumour development.

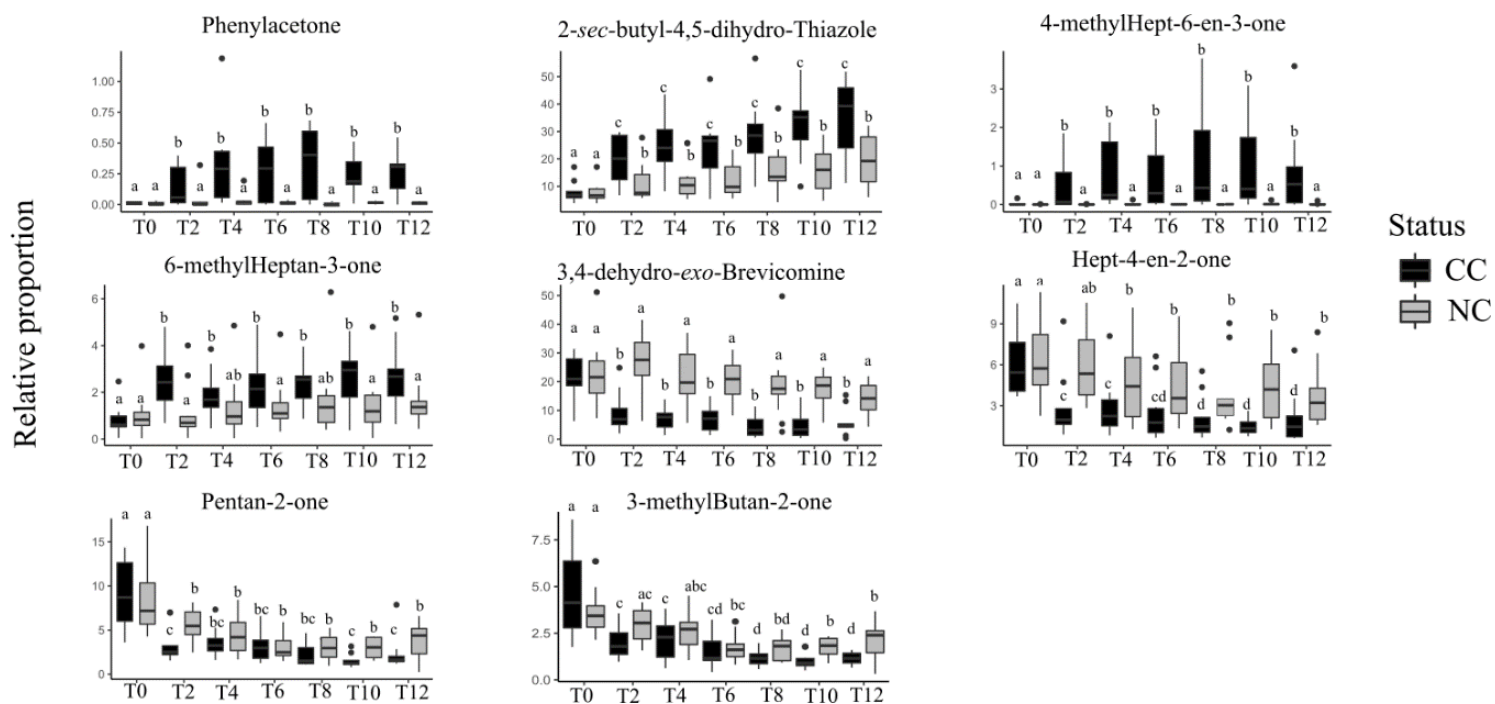


Figure 6 : Patterns of variation of the eight VOCs, retained as candidate to diagnose lung cancer in mice, across treatment (CC, cancerous mice, in black; NC, non-cancerous mice, in grey). Boxplots represented relative proportions of each compounds for each sample time at T0 to T12. Each box plot represents minimum, lower quartile, median, upper quartile, and maximum values. Different letters among boxplots indicate statistically significant differences for the respective trait, at a significance level of $p < 0.05$.

Table 2: Summary of the results of the linear mixed models applied on the relative proportions of 17 VOCs retained from the redundancy analysis (see Fig.5) with relative proportions (%mean \pm se), retention time (RT) and retention indices (RI)

Compound	RT ^a	RI ^b	Status of mice	Experimental condition ^c							Results of linear model					
				T0	T2	T4	T6	T8	T10	T12	experimental condition		status		interaction	
											χ^2	p	χ^2	p	χ^2	p
											df = 6		df = 1		df = 6	
Butan-2-one	2,383	612	NC	11.8 \pm 2.12	7.30 \pm 0.72	6.60 \pm 0.96	7.45 \pm 1.40	7.53 \pm 1.41	7.52 \pm 2.06	7.49 \pm 1.96						
			CC	8.43 \pm 1.29	8.03 \pm 1.85	6.41 \pm 1.23	4.94 \pm 1.12	5.41 \pm 1.22	3.41 \pm 0.43	4.68 \pm 1.10	18.48	0.001	3.10	0.08	3.92	0.74
3-methylButan-2-one	2,987	656	NC	3.62 \pm 0.41	2.95 \pm 0.28	2.54 \pm 0.33	1.73 \pm 0.24	1.69 \pm 0.20	1.75 \pm 0.16	2.16 \pm 0.32						
			CC	4.67 \pm 0.86	2.03 \pm 0.33	2.15 \pm 0.39	1.56 \pm 0.34	1.17 \pm 0.16	0.98 \pm 0.13	1.15 \pm 0.12	144.83	< 0.001	3.3	0.07	15.61	0.02
Pentan-2-one	3,365	684	NC	8.72 \pm 1.36	5.52 \pm 0.59	4.46 \pm 0.76	2.94 \pm 0.44	3.00 \pm 0.46	3.00 \pm 0.37	3.86 \pm 0.64						
			CC	9.25 \pm 1.52	2.96 \pm 0.61	3.70 \pm 0.66	2.92 \pm 0.65	1.72 \pm 0.38	1.42 \pm 0.27	1.58 \pm 0.10	269.43	< 0.001	4.09	0.04	28.51	< 0.001
4-methylPentan-2-one	4,322	733	NC	0.55 \pm 0.11	0.63 \pm 0.06	0.76 \pm 0.15	0.57 \pm 0.11	0.75 \pm 0.14	0.71 \pm 0.16	0.84 \pm 0.17						
			CC	0.47 \pm 0.08	0.49 \pm 0.13	0.46 \pm 0.09	0.42 \pm 0.15	0.38 \pm 0.14	0.32 \pm 0.05	0.26 \pm 0.05	4.37	0.63	4.45	0.04	11.35	0.08
3-methylPentan-2-one	4,6	745	NC	0.92 \pm 0.13	1.35 \pm 0.16	1.35 \pm 0.19	1.61 \pm 0.27	0.75 \pm 0.14	1.30 \pm 0.17	1.37 \pm 0.34						
			CC	1.18 \pm 0.21	0.99 \pm 0.14	1.28 \pm 0.35	0.85 \pm 0.19	0.38 \pm 0.14	1.03 \pm 0.39	0.71 \pm 0.11	7.67	0.29	1.8	0.18	8.88	0.23
3-methylcycloPentanone	6,995	841	NC	1.60 \pm 0.12	2.32 \pm 0.13	2.02 \pm 0.21	2.26 \pm 0.14	2.20 \pm 0.22	2.27 \pm 0.15	2.40 \pm 0.24						
			CC	1.72 \pm 0.32	2.27 \pm 0.19	1.97 \pm 0.14	2.03 \pm 0.14	1.85 \pm 0.11	1.92 \pm 0.12	2.09 \pm 0.15	30.35	< 0.001	0.89	0.35	1.74	0.94
Hept-4-en-2-one	8,467	892	NC	6.26 \pm 0.96	5.90 \pm 0.87	4.85 \pm 0.98	4.26 \pm 0.85	3.67 \pm 0.93	4.32 \pm 0.77	3.78 \pm 0.73						
			CC	6.09 \pm 0.83	2.70 \pm 0.94	2.64 \pm 0.84	2.03 \pm 0.69	1.39 \pm 0.19	1.30 \pm 0.15	1.23 \pm 0.24	102.50	< 0.001	9.58	0.002	25.94	< 0.001
2-acetyl-1-Pyrroline	9,272	919	NC	0.66 \pm 0.22	0.63 \pm 0.16	0.75 \pm 0.20	0.83 \pm 0.16	0.79 \pm 0.26	1.01 \pm 0.35	1.18 \pm 0.28						
			CC	1.12 \pm 0.53	0.73 \pm 0.47	0.56 \pm 0.19	0.70 \pm 0.28	0.48 \pm 0.18	0.49 \pm 0.13	0.57 \pm 0.23	10.22	0.12	0.89	0.35	4.49	0.61
6-methylHeptan-3-one	10,253	950	NC	1.11 \pm 0.34	1.16 \pm 0.39	1.41 \pm 0.43	1.44 \pm 0.37	1.76 \pm 0.60	1.50 \pm 0.42	1.67 \pm 0.44						
			CC	0.86 \pm 0.26	2.86 \pm 0.37	2.16 \pm 0.32	2.71 \pm 0.44	2.64 \pm 0.24	3.03 \pm 0.36	3.05 \pm 0.44	97.56	< 0.001	5.63	0.016	55.04	< 0.001
3-ethylcycloPentanone	10,323	952	NC	1.24 \pm 0.24	1.3 \pm 0.17	1.48 \pm 0.11	1.78 \pm 0.21	2.32 \pm 0.34	1.95 \pm 0.23	2.37 \pm 0.28						
			CC	1.20 \pm 0.17	1.95 \pm 0.22	1.65 \pm 0.25	1.83 \pm 0.14	1.95 \pm 0.15	2.04 \pm 0.22	2.19 \pm 0.26	66.78	< 0.001	0.34	0.56	9.23	0.16
4-methylHept-6-en-3-one	10,793	967	NC	0.001 \pm 0.001	0.002 \pm 0.002	0.02 \pm 0.01	0.01 \pm 0.00	0.01 \pm 0.00	0.02 \pm 0.01	0.01 \pm 0.01						
			CC	0.03 \pm 0.02	0.57 \pm 0.26	0.94 \pm 0.32	0.89 \pm 0.32	1.27 \pm 0.49	1.08 \pm 0.37	1.05 \pm 0.41	110.06	< 0.001	11.52	< 0.001	35.61	< 0.001

2,3-dehydro- <i>exo</i> -Brevicomine*	13,073	1040	NC	2.91 ± 1.07	2.51 ± 0.86	2.77 ± 0.67	2.36 ± 0.61	3.33 ± 0.93	3.24 ± 1.35	3.76 ± 1.48						
			CC	3.55 ± 1.51	5.49 ± 1.55	3.37 ± 1.17	2.87 ± 0.92	4.56 ± 1.11	3.79 ± 0.96	3.89 ± 1.12	4.03	0.67	0.64	0.42	2.81	0.83
3,4-dehydro- <i>exo</i> -Brevicomine	13,327	1048	NC	23.34 ± 3.77	25.8 ± 3.54	21.12 ± 3.29	20.64 ± 2.34	19.38 ± 4.49	17.11 ± 2.15	14.22 ± 1.86						
			CC	22.24 ± 2.96	6.32 ± 0.99	5.68 ± 1.09	5.44 ± 1.20	2.82 ± 0.82	3.38 ± 0.94	3.72 ± 0.66	79.78	< 0.001	37.42	0.001	40.78	< 0.001
Unknown compound _10	13,792	1063	NC	2.85 ± 0.58	2.20 ± 0.47	2.38 ± 0.45	1.89 ± 0.54	2.01 ± 0.54	1.91 ± 0.40	1.82 ± 0.59						
			CC	2.98 ± 0.86	2.78 ± 0.77	1.69 ± 0.67	1.46 ± 0.47	2.24 ± 0.46	2.12 ± 0.62	2.05 ± 0.57	9.68	0.14	0.009	0.92	5.37	0.49
Unknown compound _11	14,345	1081	NC	0.28 ± 0.15	0.24 ± 0.11	0.31 ± 0.12	0.29 ± 0.18	0.35 ± 0.12	0.24 ± 0.08	0.31 ± 0.10						
			CC	0.38 ± 0.21	0.53 ± 0.21	0.34 ± 0.18	0.25 ± 0.09	0.42 ± 0.10	0.46 ± 0.15	0.39 ± 0.13	1.55	0.96	0.65	0.42	3.19	0.78
2- <i>Sec</i> -butyl-4,5-dihydro-Thiazole	15,058	1104	NC	7.79 ± 1.19	11.07 ± 2.29	12.41 ± 2.36	12.4 ± 1.99	16.38 ± 3.45	16.33 ± 2.73	19.73 ± 3.17						
			CC	7.47 ± 1.43	20.89 ± 2.88	24.69 ± 3.64	26.95 ± 4.19	31.25 ± 4.62	33.70 ± 4.26	38.61 ± 4.88	222.66	< 0.001	8.17	0.004	31.84	< 0.001
Phenylacetone	15,702	1125	CC	0.008 ± 0.004	0.04 ± 0.03	0.03 ± 0.02	0.01 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00						
				0.01 ± 0.004	0.18 ± 0.06	0.40 ± 0.12	0.33 ± 0.08	0.42 ± 0.09	0.28 ± 0.06	0.32 ± 0.05	36.1	< 0.001	41.32	0.001	40.54	< 0.001

^a retention time

^b calculated retention indices

^c relative proportion of the compounds (%; mean +/- SE)

Discussion

The use of urine to identify volatile biomarkers to diagnose diseases such as lung cancer is very promising, as it is a minimally invasive and could be a rapid way to obtain a diagnosis. In this study, a lung cancer model was used to investigate the impact of tumour development on mice urine VOCs profiles and identify potential candidate molecules that would help diagnose cancer tumour at an early stage of development. Lung cancer is represented by several clinical types; we chose a mouse model developing non-small cell lung adenocarcinoma (NSCLC) in order to best simulate a human cancer. We detected 36 urinary VOCs present in the mice urine, of which 11 could not be matched with known molecules. Among the 25 identified VOCs, four were never reported in mice VOCs literature, all the other compounds were described as being part of laboratory and wild type mice VOCs profiles (Cavaggioni et al., 2006; Goodrich et al., 1990; Röck et al., 2006) or/and in cancer studies involving mice (Matsumura et al., 2010; Sever et al., 2015).

The present study reports new evidence for candidate molecules that could allow the detection of lung cancer in mice' urine at an early stage of tumour development, and, hence, supports a urine VOCs based diagnostic approach in the context of early detection of lung cancer (Hanai et al., 2012; Matsumura et al., 2010). We detected both an effect of doxycycline (no differences between CC and NC, and T0 different from T2-T12), and the influence of doxycycline induced cancer tumour (CC and NC different at all or some doxycycline treatments) on the mice VOCs profiles. Remarkably, this study points out a VOCs signature specific of early stages of tumour development. Our two groups of mice', NC and CC, only differed in the presence or not of two transgenes. This slight difference in genotype did not seem to influence the mice' urinary VOCs profiles at T0, although a sister study pointed out differences in the VOCs profile obtained from their soiled bedding (Gouzerh et al., 2022), the seven compounds involved in these differences were not found in the mice urine.

The doxycycline treatment altered the odour profiles of both CC and NC mice. This treatment was continuous and lasted for 12 weeks for the two groups of mice. Doxycycline driven alteration concerned three compounds, one (butane-2-one) showing a decrease in relative concentration and the other two (3-methylcyclopentanone and 3-ethylcyclopentanone) showing an increase in their relative concentrations. Butane-2-one, which is derived from fatty acids (Cheng, 2010), decreases in NC and CC mice. It is a compound that can be found in the faeces (Goodrich et al., 1990) and urine of mice (Fujita et al., 2019; Röck et al., 2006). This compound was shown to be a biomarker for epilepsy, and was found to be present in higher relative proportion in epileptic mice (Fujita et al., 2019). The 3-methylcyclopentanone and 3-ethylcyclopentanone, were not reported in other mice' studies. However, they were found in all mice involved in this study, at all experimental conditions.

Remarkably, our results also indicated significant differences in the urine VOCs profile of NC and CC at an early stage of lung cancer development. Indeed, two weeks after the start of the doxycycline treatment, the relative proportions of six VOCs differed significantly between CC and NC mice profiles, while the first cancer lesions triggered by doxycycline ingestion are visible on MRI only after a 5 to 6 weeks doxycycline treatment (Mur et al., 2020). Two other VOCs allowed detection of cancer tumour at a later stage of development (10 weeks post induction)

Among these eight VOCs allowing to distinguish cancerous from healthy mice, four were present in lower relative proportions in cancerous mice, and the other four were present in higher proportions. Among the compounds that increased in proportions, phenylacetone, which represents a very small proportion of the VOCs emitted by the mice (less than 1%), increased as early as T2 in the cancerous mice. It is a metabolite of amphetamine that is also regularly found in mice urine (Miyashita and Robinson, 1980; Röck et al., 2006; Schwende et al., 1986). In general, phenylacetone increases in mice during periods of day between morning and afternoon and decreases with age (Schaefer et al., 2010a). The increase in phenylacetone in our cancer mice might be an indication of abnormal metabolic activity during cancer development. An increase in phenylacetone has also been reported in Alzheimer's disease (Kimball et al., 2015). This compound was only found in mice, still, an equivalent biomarker of abnormal or important metabolic activity might be worth investigating in humans affected by cancer. Two other compounds were found in higher proportions in CC mice, 4-methylhept-6-en-3-one and 6-methylheptan-3-one. Both these compounds were also described in other studies involving mice (Fujita et al., 2020; Tian et al., 2021). The 6-methyl-heptan-3-one was found in higher relative proportions in presence of melanoma (Kokocinska-kusiak et al., 2020; Sever et al., 2015) and lung cancer (Hanai et al., 2012) at an advanced stage of cancer. However, 4-methyl-6-hepten-3-one was found to increase in the profiles of mice carrying a melanoma and to decrease in those with lung cancer (Hanai et al., 2012). Other studies pointed out an increase in 4-methyl-6-hepten-3-one linked to Alzheimer's disease (Tian et al., 2021). Concerning the 6-methyl-heptan-3-one, its decrease was reported to be linked to anxiety and depression in mice (Fujita et al., 2020; Kwak et al., 2008; Schaefer et al., 2010). Three other discriminating compounds found to decrease in CC compared to NC mice, hept-4-en-2-one, pentan-2-one and 3-methylbutan-2-one, are known mice urine compounds (Fujita et al., 2019; Fujita et al., 2020; Röck et al., 2006). Hept-4-en-2-one is a compound present only in mice (Fujita et al., 2019), but was not described in humans so far. The other two compounds were both found in the VOCs profile of healthy mice (Fujita et al., 2019; Fujita et al., 2020; Röck et al., 2006) but also in humans cancer studies (Rudnicka et al., 2014; Schallschmidt et al., 2016; Shigeyama et al., 2019). Pentan-2-one was found in other mice cancer studies, i.e. melanoma (Kokocinska-kusiak et al., 2020), breast cancer (Woollam et al., 2019a). This compound was shown to increase in response to tumour development in these two studies while in our study its relative proportions decreased. Liu and collaborators (Liu et al., 2019) reported the same pattern of reduction in the relative proportion of this VOC in colorectal cancer cell

culture. In our study, the decrease of pentan-2-one was observed 10 weeks after cancer inoculation. Pentan-2-one decreased in proportions in the profile of cancerous mice in our study, but was shown to increase in most human studies, except in a human lung cancer study (Schallschmidt et al., 2016). 3-methylbutan-2-one was reported to be linked to cancer in a single publication analysing the VOCs profile of the saliva of patients affected by head and neck cancer.

Among the compounds that our study pointed out as discriminating between CC and NC were two testosterone dependent pheromones, thiazoline and brevicomine. These two pheromones play a role in advertising dominance (Novotny et al., 1985) and attracting females (Jemiolo et al., 1986). In our study, the relative proportions of thiazoline increased in CC mice during cancer development, while the relative proportion of brevicomine decreased. Both pheromones were affected very early in the development of cancer, i.e. at least two weeks following the start of the doxycycline treatment. Our results hence confirm earlier studies pointing out the impact of cancer development on the emission of these two pheromones in mice urine (Kokocinska-Kusiak et al., 2020; Matsumura et al., 2010). Brevicomine was shown to increase in a study analysing VOCs associated with melanoma development (Kokocinska-Kusiak et al., 2020) while in our study its relative proportions decrease, further suggesting different effects of different cancer types on the mouse metabolism. Variations in thiazoline pointed out in another study on lung cancer (Matsumura et al., 2010). In our study, variation in proportions of thiazoline consisted in a more limited increase in the course of the doxycycline treatment in NC individuals than in CC mice. Thiazoline variations in NC mice may indicate either or both a doxycycline or an age effect, since the mice were 3 months old at the beginning of the experiments and 6 months old at the end of the study. However, the only significant increase in proportions is observed between T0 and T2, which strongly suggests a treatment rather than an age effect. The variations of these two pheromones in cancerous mice raise questions about the potential impact of cancer in natural populations as variation in these pheromones are expected to influence sexual selection if breeding animals had cancer tumours. Finally, the results of our study, and others, indicate that cancer might affect metabolism paths involving testosterone, further suggesting that targeting testosterone-related compounds in humans might be of interest on the scent of diagnosing cancer at an early stage in humans.

Conclusion

Our study showed that although the doxycycline treatment could have a direct influence on the mice VOCs profiles, it also had an indirect effect, through cancer induction in CC mice. Remarkably, our results pointed out cancer induced VOCs changes at a very early of cancer development (2 weeks), further suggesting that the path taken by research focusing on VOCs as diagnostic tool is promising.

Our study also pointed out that pentan-2-one and 3-methylbutan-2-one could be used to diagnose early cancer development in mice, and might be transferable to diagnose humans cancers.

AUTHOR'S CONTRIBUTION

FG, GG, FT, LD developed the concept, FG and GV realized the experiments, FG and GG performed the analytical measurements and analyzed the data with MH. BB supervised the chemical ecology aspects that GV realized. MM and AM performed euthanasia of EGFR mice and verification of their cancer status. FG and GG prepared and edited the manuscript and all authors have reviewed the manuscript.

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COMPETING INTERESTS

The authors declare no competing financial interests.

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SUPPLEMENTARY MATERIAL:

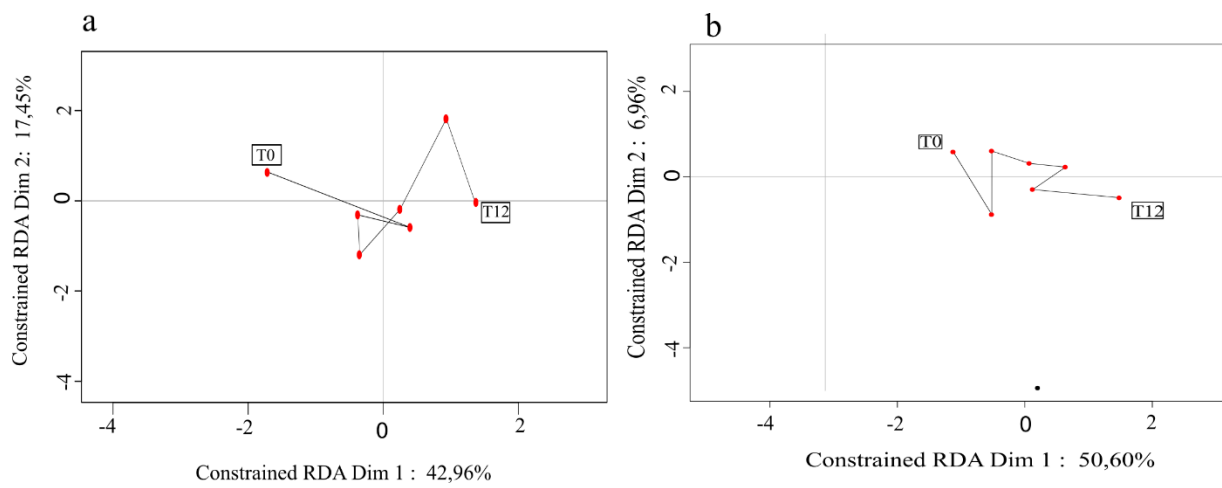


Fig S1: Temporal dynamics of the evolution of VOCs composition in a) CC and b) NC mice at the seven experimental conditions. Variation is larger in CC than NC and not linear in both.

Table S1: Complete list of VOCs found in the urine of NC and CC mice, with their frequency of occurrence (between brackets), retention indices (RI) and retention time (RT), and reference to other studies reporting detection of these molecules in mice or humans.

Compound	RT ^a	RI ^b	T0		T2		T4		T6		T8		T10		T12		References		
			NC (10)	C (8)	NC (10)	C (8)	NC (10)	C (8)	NC (10)	C (8)	NC (9)	C (8)	NC (10)	C (8)	NC (10)	C (8)	Studies involving house mice	Studies targeting cancer in house mice	Studies targeting cancer in human
Butan-2-one	2,383	612	10	8	10	8	10	8	10	8	9	8	10	8	10	8	Fèces : (Goodrich et al., 1990) Urine : (Fujita et al., 2019; Fujita et al., 2020b; Röck et al., 2006)		Breast, colorectal and lung cancer Urine (Porto-Figueira et al., 2018a)↗ Exhaled breath gastric cancer : (Amal et al., 2016)↘ Leukemia Culture cells : (Forleo et al., 2018)↘ Lung cancer Exhaled breath: (Bajtarevic et al., 2009; Buszewski et al., 2012; Fu et al., 2014; Gashimova et al., 2020; Schallschmidt et al., 2015; Schallschmidt et al., 2016; Wang et al., 2014)↗ (Peng et al., 2009) ↘ (Ligor et al., 2009) appaer (O'Neill et al., 1988) Tissue : (Buszewski et al., 2012; Gashimova et al., 2020)↗ Cell culture : (Filipiak et al., 2008)↘ (Hanai et al., 2012; Schallschmidt et al., 2015; Schallschmidt et al., 2016)↗
Unknown compound _1	2,505	621	6	3	4	4	8	4	10	2	6	5	8	5	6	2			
3-methylButan-2-one	2,987	656	10	8	10	8	10	8	10	8	9	8	10	8	10	8	Urine : (Röck et al., 2006)		Head and neck salivary : (Shigeyama et al., 2019) appaer

Pentan-2-one	3,365	684	10	8	10	8	10	8	10	8	9	8	10	8	10	8	Urine : (Fujita et al., 2019; Fujita et al., 2020b; Röck et al., 2006)	Urine Breast cancer : (Woollam et al., 2019b) ↗ Cells culture colorectal cancer : (Liu et al., 2019)↘ Urine Melanoma : (Kokocinska-kusiak et al., 2020)↗	Breast cancer cell culture : (Silva et al., 2017) appear Liver cell culture : (Mochalski et al., 2013)↗ Lung cancer : Exhaled breath (Buszewski et al., 2012; Rudnicka et al., 2014)↗ (Schallschmidt et al., 2016)↘ Cell culture : (Filipiak et al., 2010; Hanai et al., 2012)↗
Unknown compound _2	3,727	706	8	6	8	5	10	6	10	6	9	6	10	8	9	4			
2-methylButanenitrile	3,943	716	10	8	10	8	10	8	10	8	9	8	10	8	10	8			
Unknown compound _3	4,138	725	9	6	8	8	8	7	8	8	7	8	9	8	10	8			
4-methylPentan-2-one	4,322	733	10	8	10	8	10	8	10	8	9	8	10	8	10	8	Urine : (Miyashita and Robinson, 1980; Schaefer et al., 2010)		Colorectal cancer exhaled breath : (Altomare et al., 2013; Altomare et al., 2015)
3-methylPentan-2-one	4,6	745	10	8	10	8	10	8	10	8	9	8	10	8	10	8	Urine : (Miyashita and Robinson, 1980b)	Urine Breast cancer : (Woollam et al., 2019b) ↘	Lung cancer exhaled breath : (Saidi et al., 2020)↗
Hexan-3-one	5,33	778	10	8	10	8	10	8	10	8	9	8	10	8	10	8	Urine : (Fujita et al., 2020; Jemiolo et al., 1987; Miyashita and Robinson, 1980)		Lung cancer urine : (Porto-Figueira et al., 2018a)
Hexan-2-one	5,452	783	10	8	10	8	10	8	10	8	9	8	10	8	10	8	Urine : (Jemiolo et al., 1987; Miyashita and	Urine Melanoma : (Sever et al., 2015) Urine Breast cancer : (Woollam et al., 2019b) ↗	Lung cancer Exhaled breath : (Broza et al., 2013) ↗

																	Robinson, 1980)	Cell culture : (Hanai et al., 2012) ↗ Tissue : (Filipiak et al., 2014)↘ Colorectal cancer feces :(Bond et al., 2019)↗
2-ethyl-Hex-2-enal	6,066	808	10	8	10	8	10	8	10	8	9	8	10	8	10	8		
Unknown compound _4	6,265	815	7	6	8	6	7	5	7	6	3	5	8	6	7	6		
Unknown compound _5	6,327	817	10	8	10	8	10	8	10	8	9	8	10	8	10	8		
3- methylecycloPentanone	6,995	841	10	8	10	8	10	8	10	8	9	8	10	8	10	8		
Unknown compound _6	7,318	852	10	8	10	8	9	8	10	8	8	8	10	8	9	8		
Heptan-4-one	7,777	868	9	8	9	8	10	8	10	8	9	8	10	8	10	8	Urine : (Miyashita and Robinson, 1980b)	Lung cancer exhaled breath : (Rudnicka et al., 2014) appaer Colorectal and breast cancer urine : (Porto-Figueira et al., 2018b)↗
Unknown compound _7	7,933	874	10	8	10	8	10	8	10	8	9	8	10	8	10	8		
Heptan-3-one	8,197	883	3	4	3	7	9	7	8	8	9	8	8	8	6	8	Urine : (Miyashita and Robinson, 1980b)	Urine Breast cancer : (Woollam et al., 2019b) ↗ Cells culture colorectal cancer : (Liu et al., 2019)↗ Breast cancer urine (Silva et al., 2012) ↗ Colorectal leukemia urine : (Silva et al., 2011) Disappear Lymphoma urine (Silva et al., 2011)↗ Liver cell culture : (Mochalski et al., 2013)↗ Lung cancer : Exhaled breath (Broza et al., 2013)↗ Urine : (Santos et al., 2017)↗
Heptan-2-one	8,312	887	10	8	10	8	10	8	10	8	9	8	10	8	10	8	Urine : (Fujita et al., 2019; Fujita et al., 2020; Miyashita and	Urine lung cancer : (Matsumura et al., 2010) Blood Colorectal cancer (Wang et al., 2019)↗ Breast cancer urine (Silva et al., 2012) appaer Liver cell culture : (Mochalski et al., 2013)↗

																	Robinson, 1980; Mucignat-Caretta et al., 2010; Novotny et al., 2007; Osada et al., 2008; Röck et al., 2006; Schaefer et al., 2010; Soini et al., 2009; Zhang et al., 2007)	Cells culture colorectal cancer : (Liu et al., 2019)↗	Lung cancer urine : (Porto-Figueira et al., 2018a)
Hept-4-en-2-one	8,467	892	10	8	10	8	10	8	10	8	9	8	10	8	10	8	Urine : (Fujita et al., 2019)		
Unknown compound _8	8,925	908	10	8	8	8	9	8	10	8	9	8	10	8	10	8			
2-acetyl-1-Pyrroline	9,272	919	10	8	10	8	10	8	10	8	9	8	10	8	10	8	Urine : (Fujita et al., 2019; Fujita et al., 2020; Schaefer et al., 2010; Tian et al., 2021)	Urine lung cancer : (Matsumura et al., 2010) Urine Melanoma : (Kokocinska-kusiak et al., 2020)↗	
('E)-Hept-3-en-2-one	9,752	934	8	6	8	8	8	8	9	8	7	8	9	8	8	8	Urine : (Arnáiz et al., 2014; Osada et al., 2008; Schwende et al., 1986)		
6-methylHeptan-3-one	10,253	950	10	8	10	8	10	8	10	8	9	8	10	8	10	8	Urine : (Arnáiz et al., 2014; Fujita et al., 2020a; Tian et al., 2021)		

3-ethylcyclopentanone	10,323	952	10	8	10	8	10	8	10	8	9	8	10	8	10	8		
4-methylhept-6-en-3-one	10,793	967	1	3	2	6	4	8	5	8	5	8	7	8	3	7	Urine : (Osada et al., 2008; Tian et al., 2021)	Melanoma : (Kokocinska-kusiak et al., 2020) ⁷
4-methyl-Hept-6-en-3-one	11,528	991	10	6	10	8	10	8	10	8	9	8	10	8	10	8		
Unknown compound_9	12,35	1017	9	7	10	8	9	8	10	8	9	7	10	7	10	7		
2,3-dehydro- <i>exo</i> -Brevicomine*	13,073	1040	9	7	10	7	10	8	10	7	9	7	10	8	9	8	Urine : (Mucignat-Caretta et al., 2010; Novotny et al., 2007; Röck et al., 2006a)	Urine lung cancer : (Matsumura et al., 2010)
3,4-dehydro-<i>exo</i>-Brevicomine	13,327	1048	10	8	10	8	10	8	10	8	9	8	10	8	10	8	Urine : (Fujita et al., 2019; Fujita et al., 2020a; Harvey et al., 1989; Jemiolo et al., 1987; Novotny et al., 1984; Osada et al., 2008; Schaefer et al., 2010; Schwende et al., 1986; Zhang et al., 2007)	Melanoma : (Kokocinska-kusiak et al., 2020) ⁷ Urine lung cancer : (Matsumura et al., 2010)

Unknown compound _10	13,792	1063	9	6	10	7	10	8	10	6	8	7	10	7	8	7		
Unknown compound _11	14,345	1081	4	3	4	6	9	5	6	5	7	7	7	7	6	6		
Linalool	14,94	1100	1	2	3	3	5	5	6	5	6	8	5	8	6	7	Urine : (Arnáiz et al., 2014; Schaefer et al., 2010; Schwende et al., 1986)	Urine lung cancer : (Matsumura et al., 2010)
2-sec-butyl-4,5-dihydro-Thiazole	15,058	1104	10	8	10	8	10	8	10	8	9	8	10	8	10	8	Urine : (Arnáiz et al., 2014; Harvey et al., 1989; Kayali-Sayadi et al., 2003; Miyashita and Robinson, 1980; Mucignat-Caretta et al., 2010; Novotny et al., 2007; Osada et al., 2008; Röck et al., 2006; Schaefer et al., 2010b; Schwende et al., 1986; Zhang et al., 2007)	Urine lung cancer : (Matsumura et al., 2010)
Phenylacetone	15,702	1125	4	4	4	7	7	8	7	8	8	8	8	8	7	8	Urine : (Schaefer et al.,	

Table S2: List of VOCs identified both in the biological samples and the controls. In blue compounds found only in urine and retained for our analyses. In pink: the n-alkanes used as standards for the identification of the compounds.

Compounds	RT ^a	RI ^b
n-Hexane	2,219	600
Pollution	2,373	611
Butan-2-one	2,383	612
Pollution	2,493	620
Unknown compound 1	2,505	621
3-Methylbutan-2-one	2,987	656
Pentan-2-one	3,365	684
NI	3,548	697
n-Heptane	3,630	700
Unknown compound 2	3,727	706
2,4-Dimethylfuran	3,798	709
Pollution	3,83	711
2,4-Dimethylfuran	3,855	712
NI	3,925	715
2-Methylbutanenitrile	3,943	716
2-Methylbutanenitrile	4,052	721
NI	4,072	722
Unknown compound 3	4,138	725
4-Methylpentan-2-one	4,322	733
3-Methylpentan-2-one	4,6	745
Hexan-3-one	5,33	778
Hexan-2-one	5,452	783
NI	5,677	793
NI	5,737	796
n-Octane	5,829	800
2-Ethyl-hex-2-enal	6,066	808
Unknown compound 4	6,265	815
Unknown compound 5	6,327	817
NI	6,445	822
NI	6,700	830
NI	6,868	836
3-Methylcyclopentanone	6,995	841
Unknown compound 6	7,318	852
NI	7,565	861
Heptan-4-one	7,777	868
Unknown compound 7	7,933	874
Bis(methylthio)methane	8,192	883
Heptan-3-one	8,197	883
Heptan-2-one	8,312	887
Hept-4-en-2-one	8,467	892
NI	8,558	895

NI	8,568	896
NI	8,598	897
NI	8,665	899
n-Nonane	8,688	900
NI	8,845	905
NI	8,917	907
Unknown compound 8	8,925	908
(Z)-Pent-2-enyl acetate	9,028	911
NI	9,037	911
NI	9,122	914
NI	9,128	914
2,6-Dimethylpyrazine	9,133	914
NI	9,172	915
2-Acetyl-1-pyrroline	9,272	919
NI	9,558	928
(E)-Hept-3-en-2-one	9,752	934
NI	10,185	948
NI	10,198	948
6-Methylheptan-3-one	10,253	950
3-Ethylcyclopentanone	10,323	952
NI	10,473	957
NI	10,562	960
4-Methylhept-6-en-3-one	10,793	967
Pollution	11,082	976
Dihydro-3,5-dimethyl-2(3H)-furanone	11,140	978
NI	11,273	982
NI	11,323	984
NI	11,325	984
NI	11,332	984
4-Methyl-Hept-6-en-3-one	11,528	991
Octanal	11,815	1000
n-Decane	11,824	1000
Pollution	12,017	1006
NI	12,252	1014
Unknown compound 9	12,35	1017
2-Ethylhexan-1-ol	12,747	1030
2-Ethylhexan-1-ol	12,77	1030
Limonene	12,723	1029
1,8-Cineole	12,787	1031
NI	12,800	1031
2,3-dehydro- <i>exo</i> -Brevicomine	13,073	1040
NI	13,165	1043
3,4-dehydro- <i>exo</i> -Brevicomine	13,327	1048
NI	13,633	1058
NI	13,637	1058

Unknown compound 10	13,792	1063
Acetophenone	13,820	1064
NI	13,898	1066
NI	13,982	1069
NI	13,998	1070
Unknown compound 11	14,345	1081
NI	14,468	1085
NI	14,493	1085
NI	14,618	1089
Linalool	14,94	1100
n-Undecane	14,947	1100
Nonanal	14,982	1101
2-sec-butyl-4,5-dihydro-Thiazole	15,058	1104
NI	15,343	1113
Methyl(methylthio)methylsulfide	15,647	1123
Phenylacetone	15,702	1125
NI	16,302	1145
alpha-Terpineol	17,707	1192
n-Dodecane	17,942	1200
Decanal	18,028	1203
2-AminoBenzaldehyde	18,348	1214
n-Tridecane	20,777	1300
Undecanal	20,917	1305
NI	21,542	1329
NI	21,825	1339
n-Tetradecane	23,448	1400
(E)-beta-Farnesene	24,930	1459
NI	25,147	1467
n-Pentadecane	25,971	1500
(E,E)-alpha-Farnesene	26,198	1510

^a retention time

^b calculated retention indices

NI : non identified

~ Discussion générale et perspectives ~

Le sujet des modifications d'odeurs liées au cancer est en pleine expansion comme j'ai pu le montrer avec les deux synthèses **Manuscrit I et II**. La première a permis de montrer l'étendue des connaissances sur le sujet de la détection du cancer grâce aux COVs chez l'homme, et la seconde a permis d'établir la liste actuelle des animaux capables de détecter des individus cancéreux. Ces synthèses ont permis de voir où en était la recherche dans ce domaine et les grandes questions et challenges méthodologiques qui restent à explorer. Au cours de cette thèse à la fois fondamentale et appliquée, j'ai voulu étudier le cancer sous le prisme de l'écologie chimique et de l'écologie comportementale. Cette thèse s'est articulée autour de trois grands objectifs. Le 1er objectif a consisté à étudier si nous pouvions utiliser des souris comme nez pour discriminer des individus cancéreux sans méthode d'apprentissage ou de conditionnement au préalable, et si ces animaux étaient capables de faire cette discrimination très tôt dans le développement du cancer. Le 2^{ème} objectif était de savoir comment ces modifications d'odeur pouvaient influencer les préférences sexuelles des femelles *Mus musculus domesticus*, ainsi que le comportement de mâles *Mus musculus domesticus*. Et enfin, dans un cadre plus médical, nous avons voulu savoir à partir de quel stade de développement du cancer nous pouvions, avec des outils d'analyses chimiques, détecter des modifications de COVs liées au cancer et identifier des possibles biomarqueurs utilisables chez l'homme.

I) Synthèses des résultats

- Capacité des souris à discriminer des congénères cancéreux

L'utilisation des animaux comme nez pour détecter des maladies et notamment le cancer n'est pas nouvelle comme j'ai pu le montrer dans le **Manuscrit II**. Cependant, peu d'études se sont intéressées aux capacités sans apprentissage des animaux, en dehors des études sur certains invertébrés. L'objectif de cette thèse, était à la fois fondamental et appliqué. Je souhaitais dans un premier temps savoir si dans la nature les animaux, et plus précisément les souris, étaient capables de discriminer des congénères cancéreux. De plus, dans une optique appliquée, il me semblait intéressant de déterminer le moment à partir duquel elles pouvaient faire cette discrimination, et de préciser si avec des tests d'habituation/généralisation, nous pouvions discriminer des individus cancéreux sans avoir à réaliser sur les souris une phase de conditionnement. Ce dernier point est important car il pourrait permettre dans de futures recherches d'utiliser les souris comme nez pour détecter le cancer sans avoir besoin de réaliser un apprentissage au préalable. Mon étude, a permis tout d'abord de montrer que les variations d'odeurs corporelles en relation avec la tumorigenèse pouvaient être détectées très tôt par les souris. En effet, elles sont capables de discriminer les souris CC et NC à partir de seulement deux semaines de développement du cancer, ce qui correspond dans le cas du cancer étudié à l'apparition de lésions précancéreuses visibles uniquement par un examen anatomopathologique et non à l'IRM (Li et al., 2007; Mur et al., 2020). De plus, nous avons pu montrer que cette capacité de discrimination n'était pas

forcement simple à mettre en évidence. En effet, si les souris ont parfaitement réussi à faire la différence entre 2 groupes de souris NC et un groupe de souris CC, en revanche elles n'ont pas pu faire la différence entre 2 groupes de souris CC et un groupe de souris NC. Ce point a soulevé des questions sur l'hétérogénéité des groupes de souris CC et la possibilité que les souris ne soient pas capables de faire correctement la discrimination. Dans ce cas, la non-discrimination des souris pourrait venir du fait qu'il existe une trop grande hétérogénéité des profils d'odeurs entre plusieurs individus atteints d'un même cancer du poumon.

- Impact du cancer sur les relations intra-spécifiques

Le deuxième objectif de cette thèse était d'améliorer notre compréhension des conséquences écologiques du cancer sur l'écologie de leurs hôtes, en particulier les relations intra-spécifiques. Bien que le cancer touche l'ensemble de l'arbre du vivant, il a pour l'instant été étudié majoritairement dans un cadre médical strict et beaucoup moins en écologie et évolution. Ainsi, l'influence du cancer sur l'écologie et l'évolution des espèces et le fonctionnement des écosystèmes reste à l'heure actuel mal compris, comparativement à ce qui a été réalisé sur les interactions hôtes parasites ces dernières décennies. Bien que plusieurs travaux aient mis en évidence un rôle significatif des parasites dans les interactions interindividuelles chez la souris (sélection sexuelle notamment), aucune étude à ma connaissance n'a exploré l'influence du cancer, alors que cette pathologie est commune chez ce rongeur. Pour se rapprocher le plus possible d'un contexte naturel, nos tests de préférences ont volontairement considéré des souris *Mus musculus domesticus* issues du milieu sauvage et maintenues en élevage. Des tests de préférences ont été réalisés sur des souris femelles à qui des stimuli (litières souillées) de souris CC et NC leur ont été présentés. Nos résultats montrent que les souris femelles n'ont pas de préférence ni d'aversion vers l'un ou l'autre des stimuli. Concernant les mâles, en plus d'avoir mis en évidence leur capacité de discrimination, nous avons pu voir qu'ils mettaient plus de temps à renifler le stimulus des souris NC que celui des souris CC et qu'ils passaient moins de temps en présence du stimulus NC lors de la phase d'habituation lorsqu'ils étaient seulement en présence d'un seul stimulus. Cela suggère que les mâles sont plus attirés par le stimulus CC que NC, et qu'il n'y a pas d'évitement, contrairement à ce qui est en général observé vis-à-vis de congénères parasités. D'autre part, nous avons pu voir que les souris étaient plus attirées par les prélèvements T0, lorsque les souris avaient trois mois, soit le même âge que les souris nez, comparé aux prélèvements T12, où elles étaient alors âgées de 6 mois.

- Identification précoce du cancer

Le principal objectif à visée appliquée de cette thèse était de déterminer le stade de développement du cancer à partir duquel il est possible de détecter des variations d'odeurs significatives. Sachant que le dépistage précoce des cancers, notamment celui du poumon, est primordial pour augmenter le taux de survie, l'identification de signatures olfactives précoces de la tumorigenèse reste

une direction de recherche majeure en oncologie. Si l'utilisation des COVs dans ce cadre apparaît comme une méthode peu onéreuse et non invasive, il est toutefois essentiel de vérifier si ces approches permettent un diagnostic plus précoce du cancer comparé aux techniques actuelles comme l'imagerie médicale ou les biopsies par exemple. Pour tenter de répondre à ces interrogations, différentes approches expérimentales ont été réalisées. La première, à l'aide de souris nez, a montré que les souris étaient capables de discriminer des congénères cancéreux très tôt dans le développement du cancer. Une modification détectable par les odeurs émises semble donc apparaître très tôt au cours du développement des tumeurs. Dans une perspective médicale, ces résultats sont aussi pertinents que prometteurs, car ils suggèrent la possibilité, via le nez des souris, d'un diagnostic du cancer du poumon, plus précocement que par d'autres techniques, l'IRM notamment. Cette détection murine semble pouvoir se faire dès l'apparition des lésions précancéreuses. Qu'en est-il des analyses chimiques, basées sur les techniques de GC-MS notamment, sont-elles aussi performantes que le nez des souris ? Au niveau des litières, nous n'avons pas pu mettre en évidence de différences précoces au niveau des COVs, alors que les souris en sont capables dès le prélèvement T2. En revanche, des différences en fin de traitement étaient bien visibles. Parmi les composés discriminants au sein des litières, certains sont des composés propres aux souris et sont des phéromones produites uniquement par les souris mâles: le 3,4 dehydro *exo* brevicomine et le 2-*sec* butyl-4,5 dihydrothiazole. Des composés communs à l'Homme et la souris ont aussi été trouvés : le benzaldéhyde et le hexan-1-ol. Bien que ces résultats appuient l'idée qu'il soit possible de détecter une signature olfactive des cancers par des analyses chimiques, l'intérêt semble ici limité du fait que cette détection est tardive d'après nos résultats. Cependant il ne faut pas oublier que nous n'avons pu analyser que des prélèvements issus des échantillons T2 et T12, soit à 2 semaines de traitement et à 12 semaines de traitement à un stade avancé du cancer. L'analyse des échantillons intermédiaires pourrait nous donner une réponse sur la possibilité que ces deux composés soient des biomarqueurs précoces du cancer. En effet, si nous pouvons observer des variations à T4, ce qui représente 4 semaines de développement du cancer, stade à laquelle l'IRM n'est pas encore fiable pour la détection de ce cancer, cela montrerait que ces composés peuvent servir de biomarqueurs à un stade qui reste précoce dans le développement du cancer.

Concernant les analyses d'urines, nous avons aussi pu mettre en évidence des différences de profils d'odeurs avec huit composés différents entre les souris CC et NC. Parmi ces composés, six permettent de différencier les souris saines et cancéreuses dès le prélèvement T2, i.e. comme le nez des souris. Il s'agit de la phénylacetone, le 4-méthyl-hept-6-en 3-one, le 6-méthyl-heptan-3-one, le hept-4-en-2-one ainsi que les deux phéromones émises par les souris mâles déjà retrouvées dans les litières, c'est-à-dire le 3,4 dehydro-*exo*-Brevicomine et le 2-*sec* butyl-4,5-dihydroThiazole. Les deux autres composés participant à la discrimination plus tardivement dans le développement du cancer, à partir du prélèvement T10 soit 10 semaines après le début du développement du cancer, sont le pentan-2-one et le 3-méthylbutan-2-one.

II) Impacts écologiques et évolutifs des modifications d'odeurs induit par le cancer.

Malgré un intérêt grandissant pour l'exploration du rôle des processus oncogéniques sur l'écologie et l'évolution des organismes multicellulaires, encore très peu de choses sont connues (Vittecoq et al., 2013; Madsen et al., 2017; Thomas et al., 2017). Cette situation est attribuable à plusieurs phénomènes. De la même manière que les parasites étaient autrefois étudiés uniquement par les parasitologues, les processus tumoraux ont jusqu'à une période très récente été exclusivement étudiés par la médecine humaine ou vétérinaire, pour qui l'écologie évolutive n'est pas une préoccupation centrale. Egalement, les écologues et biologistes de l'évolution, en considérant que le cancer était avant tout une pathologie survenant dans la période post reproductive, ont systématiquement occulté la possibilité que les processus cancéreux dans leur ensemble puissent jouer un rôle sur la valeur sélective des individus, et donc influencer en retour leurs traits d'histoire de vie, comportements etc... Toutefois, il est à présent établi que les processus tumoraux sont extrêmement fréquents chez les organismes multicellulaires (Madsen et al., 2017; Vincze et al., 2021). De ce fait, il semble probable que les organismes multicellulaires dans la nature sont souvent confrontés au problème du cancer, et/ou au fait d'avoir à interagir avec des individus (de la même espèce ou non) porteurs de tumeurs. Par ailleurs, si les cancers métastatiques sont certes le plus souvent observés dans la seconde partie de vie, ce n'est pas pour autant le cas des stades précédents de la tumorigenèse. Ces stades intermédiaires, du fait de leur présence et activité, ou indirectement au travers du coût des défenses qu'ils entraînent, ne sont probablement pas neutres sur plusieurs variables reliées à la valeur sélective. Ce champ de recherches est pour le moment quasi vierge (Thomas et al., 2018 ; Boutry et al., 2020).

Nos résultats indiquent que les souris ont la capacité de discriminer des odeurs d'individus cancéreux mais que cela, contrairement à ce qui observé avec les maladies parasitaires, ne semble pas pour autant avoir d'influence sur les préférences des femelles. Cette absence d'effet peut s'expliquer pour plusieurs raisons. L'observation de tumeurs chez les souris de laboratoire (Ward, 1983; Frith, Ward, & Chandra, 1993; Greenacre, 2004), ou chez les souris sauvages élevées en laboratoire (Andervont & Dunn, 1962), est le plus généralement associée au vieillissement. Une première explication à nos résultats serait alors que même si la tumorigenèse influence les performances des individus dès les premiers stades, la balance des coûts et bénéfices à choisir/éviter un partenaire hébergeant des tumeurs aboutit au fait qu'il sera la plupart du temps désavantageux de rejeter les congénères avec des tumeurs. Par exemple, si la majorité des souris hébergent des processus cancéreux, le coût à éviter des partenaires porteurs de tumeurs peut rapidement devenir élevé, couplé au coût de la recherche des rares partenaires sains. Il est aussi possible que même si les processus tumoraux impactent dès les premiers stades les performances de leurs porteurs, c'est surtout en fin de tumorigenèse que l'effet est le plus prononcé. Le bénéfice sélectif réel à éviter des partenaires porteurs de tumeurs est alors

questionnable, même chez des espèces où les mâles délivrent des soins parentaux. De plus, la plupart des cancers n'étant pas dus à des mutations congénitales (i.e. transmissibles à la descendance), mais à des mutations survenant de façon aléatoire au cours de la vie, le risque de doter sa progéniture de « mauvais gènes » en acceptant un partenaire porteur de tumeurs semble limité. Rappelons aussi que même si dans la nature une souris peut développer un cancer, il est sans doute plus probable que la principale cause de mortalité ne soit pas le cancer, mais la prédation, une maladie parasitaire ou un évènement écologique (e.g. sécheresse ou au contraire inondation). Dans ce contexte, il n'est pas attendu que la sélection naturelle ait favorisé, tout au moins fortement, les comportements d'évitement de partenaires sexuels tumoraux. Chez certaines espèces, comme les Diables de Tasmanie (*Sarcophilus harrisi*), les tumeurs sont directement transmissibles (Pearse & Swift, 2006; Pye et al., 2016). Bien que ces tumeurs soient mortelles à court terme (6-9 mois), il n'existe pas actuellement d'évidence d'évitement des individus malades comme partenaire sexuel. La même conclusion semble s'appliquer avec les espèces ayant des cancers d'origine infectieuse. Ces observations suggèrent que la sélection naturelle favorise avant tout la reproduction, même si cela résulte en risque accru de cancers par la suite. La fait que cette réponse ne soit pas là même avec les parasites peut s'expliquer par le fait que les conséquences d'une infection sur la valeur sélective s'observent en général à très court terme, contrairement au cancer.

L'absence d'influence du cancer sur les préférences sexuelles dans nos expériences peut aussi provenir du fait que seules des informations olfactives ont été utilisées comme possible critère de choix. Cela ne signifie pas que, dans la nature, les femelles ne feraient pas un choix préférentiel en faveur d'un partenaire sain pour se reproduire. Pour tester cette hypothèse, des études en open field devraient être réalisées pour savoir quel partenaire sexuel les femelles choisiraient si elles étaient réellement en présence de souris et non pas juste de stimuli odorants. Si les femelles dans ces conditions évitent les mâles tumoraux, cela suggérerait aussi que la communication chimique n'est pas le principal critère utilisé par les souris pour la détection du cancer chez les congénères.

D'autre part, nous avons pu montrer que les souris mâles vont plus rapidement vers des odeurs de souris cancéreuses que vers les odeurs de souris saines. Ce résultat suggère qu'au contraire des femelles, les souris mâles peuvent être impactés par la présence d'un cancer chez un congénère de même sexe. Le fait que les souris mâles passent plus de temps à renifler l'odeur de souris CC et qu'ils soient moins méfiants pour aller renifler ces odeurs, peut venir d'une plus grande curiosité vis-à-vis de cette nouvelle odeur, ou bien il peut s'agir d'un effet lié à une marque de dominance ou bien de subordination. Des études pourraient être réalisées pour mieux comprendre le comportement des mâles vis-à-vis des stimuli NC et CC, pour savoir si les souris se sentent dominantes ou bien subordonnées vis-à-vis d'un individu cancéreux. Cela pourrait se faire notamment en regardant si les souris urinent ou non, en présence des stimuli d'un individu cancéreux. En effet, uriner chez les souris peut être un comportement de marquage, et plus précisément de contremarquage (Arakawa et al., 2008). Les mâles dominants

sentant l'odeur d'un autre mâle marquent souvent au même endroit pour montrer leur dominance. A l'inverse, les mâles subordonnés évitent en général les comportements de marquages pour éviter les agressions (Desjardins, Maruniak, & Bronson, 1973; Rich & Hurst, 1999). En perspective de recherche, je propose d'explorer cette piste en étudiant le comportement de marquage d'individus sains vis-à-vis de stimuli de congénères cancéreuses et vice versa. Ces travaux permettraient par exemple de préciser si le cancer peut, au cours de sa progression, inverser le statut de dominance des souris. De plus, nous avons pu voir que les souris passent moins de temps à renifler les odeurs des souris à T12 (fin de traitement antibiotique) comparé à T0 (sans traitement d'antibiotique). Cette diminution du temps passé peut provenir soit de l'effet prolongé des antibiotiques qui dérangerait les souris ou soit lié à l'âge des échantillons présentés. Les souris en T12 ont plus de 6 mois alors qu'en T0 elles ont 3 mois, soit le même âge que les souris nez. Les souris nez ont peut-être perçu l'odeur des souris plus âgées comme un stimulus dangereux et elles ont donc préféré ne pas rester trop longtemps à renifler la litière.

En dehors des observations comportementales, nous avons pu voir que le cancer semble impacter certaines phéromones émises par les mâles, la brevicomine ainsi que la thiazoline. Ces deux composés jouent un rôle dans la communication sociale, et notamment sexuelle, via la dominance du mâle (Dulac & Torello, 2003). Les variations de ces composés, sans pour autant avoir d'impact sur les préférences des femelles, soulèvent d'autres questions : il y a-t-il par exemple d'autres phéromones qui compensent les variations de ces composés en jouant un rôle dans l'attraction des femelles ? Des questions d'ordre métabolique se posent, notamment sur le lien éventuel entre le développement d'un cancer du poumon et l'évolution de composés ayant un rôle dans la reproduction. Comment expliquer qu'un cancer localisé au niveau du poumon influence l'émission de composés émis dans les urines de souris mâles ? Quelles voies métaboliques, quels processus physiologiques sont impactés par le cancer ? Est-ce que tous les cancers affectent les COVs de la souris de la même manière ? D'autre part, nous avons étudié l'impact du cancer sur les COVs émis par des souris mâles, mais qu'en est-il des souris femelles, ont-elles les mêmes variations de composés ? Concernant les préférences, nous avons réalisé ces tests sur les femelles, car chez les souris, ce sont elles qui choisissent le plus souvent leurs partenaires. Mais qu'en serait-il dans l'autre sens, un mâle se reproduirait-il avec une femelle cancéreuse qui risque de mourir et/ou de ne pas subvenir correctement aux soins parentaux ?

III) Débouché médical de la détection du cancer grâce aux COVs

- Détection précoce du cancer

Que ce soit à l'aide d'analyses comportementales en utilisant la souris comme nez ou grâce à l'analyse chimique sur les urines, la détection précoce du cancer du poumon, « carcinome pulmonaire non à petites cellules », ou NSCLC, semble prometteuse. Dès l'apparition des lésions précancéreuses l'organisme est suffisamment impacté pour que des variations soient visibles dans la composition en

COVs émis par l'organisme vivant. Ces variations de proportion de composés les uns par rapport aux autres dans le bouquet odorant émis par les individus est le fait de seulement quelques composés qui diminuent ou qui augmentent dans le profil d'odeurs. Notre modèle de souris cancéreuse semble prometteur pour l'étude de la détection du cancer du poumon NSCLC qui est le cancer le plus représenté chez l'Homme. Au vu des résultats sur notre modèle de souris, il est fort probable que de telles modifications de COVs dès l'apparition des lésions précancéreuses soient aussi présentes chez l'Homme. Cela semble donc indiquer que l'utilisation des COVs pourrait effectivement être utilisée pour le diagnostic précoce du cancer du poumon et certainement pour d'autres types de cancer. Notons toutefois qu'à moins d'identifier des signatures olfactives précises à chaque cancer, la détection de la présence de tumeurs par l'étude des COVs, telle que réalisée ici, demanderait des investigations plus poussées pour préciser le type de cancer et la localisation de la tumeur. Egalement, il est à présent établi que l'accumulation de processus cancéreux avec l'âge est la règle chez les organismes multicellulaires, mais que seuls certains d'entre eux conduiront à des cancers métastatiques. Dans ce cadre, le véritable enjeu n'est pas de détecter des amas de cellules cancéreuses mais de prédire lesquels sont de nature à évoluer vers des formes graves, pour idéalement ne traiter que ceux-là, afin d'éviter les coûts financiers et/ou psychologiques (Maley et al., 2017). Cela rajoute un défi dans l'utilisation des COVs qui devront pouvoir être suffisamment pertinents pour identifier seulement les amas de cellules voués à se développer en cancer.

- Composés biomarqueurs du cancer du poumon

L'analyse des études réalisées sur l'Homme (**Manuscrit I**) a permis de mettre en évidence que de nombreux COVs avaient déjà été identifiés comme potentiel biomarqueurs précoces du cancer. Cependant il n'existe pas de réel consensus sur le sujet. Une liste de 28 composés a été identifiée comme présente dans une majorité d'études. Nous avons pu montrer que parmi ces 28 composés, 12 n'étaient pas des composés biogéniques, donc non produits par l'Homme et même parfois d'origine industrielle comme par exemple le styrène qui est utilisé pour fabriquer des plastiques comme le polystyrène. La présence d'un grand nombre de composés exogènes dans les échantillons soulève la question de leur provenance. Il peut s'agir soit d'une erreur d'identification, soit d'un problème de contamination dans les échantillons, mais au vu du nombre d'études ayant retrouvé ces composés exogènes, cela semble peu probable. Il se pourrait qu'il s'agisse de composés classiquement présents dans notre environnement et qui pourraient contaminer plusieurs de nos organes, passeraient dans notre système respiratoire, sanguin, et pourraient ensuite être libérés de nouveau dans l'environnement via nos odeurs? Il est aussi possible d'imaginer que la présence de cellules cancéreuses dans notre organisme puisse induire un plus grand rejet de composés volatils ou au contraire garderait en plus grande quantité ces composés exogènes. Il semble primordial de pouvoir répondre aux questions portant sur l'apparition ou la disparition de certains de ces composés pour pouvoir mieux comprendre comment le cancer modifie les odeurs, et comment nous pouvons les utiliser à une fin médicale dans un cadre de détection précoce.

Dans le cadre de ma thèse, j'ai pu mettre en évidence plusieurs composés issus des prélèvements d'odeurs de litières et d'urines, comme étant discriminants à différents stades de développement du cancer. Les analyses de litières ayant été réalisées uniquement à un stade précoce (T2) et avancé (T12), nous ne pouvons pas connaître exactement à quel moment les composés deviennent différents (**Manuscrit IV**). Les proportions relatives du benzaldéhyde et du hexan-1-ol sont modifiées en fin de traitement. Le benzaldéhyde est un aldéhyde, sa présence dans les odeurs augmente chez les souris cancéreuses ce qui est aussi le cas dans certaines études réalisées sur l'Homme. Ce composé a été retrouvé dans plusieurs publications sur le cancer du poumon mais aussi dans d'autres cancers, le cancer du sein, colorectal, la leucémie mais aussi le foie (**Manuscrit I**). Le benzaldéhyde n'a pas été retrouvé dans nos échantillons d'urine ce qui peut signifier qu'il est émis par un autre compartiment que les urines, telles que les fèces ou l'haleine. Ce composé a été retrouvé chez l'Homme uniquement dans des échantillons issus de culture cellulaires et d'air expiré. Il se peut donc qu'il ne soit pas retrouvé dans les urines car émis uniquement par les cellules cancéreuses pulmonaires et qu'on peut par conséquent les retrouver uniquement dans l'air expiré. L'autre composé identifié est le hexan-1-ol qui fait partie de la famille des alcools primaires, il est quant à lui en diminution chez les souris cancéreuses. Ce composé a déjà été identifié dans des études réalisées sur l'Homme. Cependant il n'a été trouvé que dans deux publications, l'une sur le mélanome, en utilisant des échantillons d'odeurs de cultures cellulaires et l'autre au sein de cancers de la tête et du cou à partir d'échantillons de salive. Dans ces deux études, comme dans la nôtre, ce composé a été retrouvé comme diminuant chez les individus cancéreux. Notre étude ayant été réalisée sur des litières de souris, il se peut que ce composé soit émis par la salive des souris étant donné qu'il a été retrouvé dans la salive humaine. Pour savoir si ces composés pourraient être utilisés comme biomarqueurs, il faudrait réaliser des analyses complémentaires pour savoir à quel moment il augmente dans les échantillons de litières et trouver dans quel compartiment ce composé est émis par la souris (e.g. les fèces, la respiration, la salive ou encore les glandes). Cependant, il semble compliqué d'utiliser ces deux composés comme potentiels biomarqueurs du fait qu'ils soient très communs dans de nombreux parfums ou déodorants, mais aussi dans notre environnement, comme dans certains fruits par exemple (Opgrande et al., 2000; Nogueira, Fernandes, & Nascimento, 2003; Verma et al., 2017; Li, Di, & Bai, 2019). De ce fait, il semble indispensable de prendre en compte les composés émis dans l'environnement des individus échantillonnés pour éviter de confondre les composés présents dans l'environnement de l'individu et ceux réellement émis par l'Homme.

Concernant les composés retrouvés dans les urines, nous avons pu mettre en évidence que huit d'entre eux participent fortement à la discrimination entre les souris saines et cancéreuses (**Manuscrit V**). Parmi ces composés, en dehors des deux phéromones, émis par les souris mâles, nous pouvons voir qu'il s'agit de 6 composés de la famille des cétones (Figure 16). Nous avons pu voir dans l'introduction et la figure 2 que la famille la plus représentée dans les urines était les cétones. Il n'est par conséquent pas très surprenant d'observer des différences uniquement sur des composés cétonés. Seulement deux

composés sont communs entre la souris et l'Homme, le 3-méthylbutan-2-one et le pentan-2-one qui sont des isomères, c'est-à-dire qu'ils possèdent la même formule brute mais pas la même formule développée (voir. Figure 19). Malheureusement ces deux composés sont impactés par le cancer tardivement au cours de son développement, seulement au bout de 10 semaines de traitement lorsque les tumeurs sont déjà présentes. Cependant, cela n'est pas dénuée d'utilité dans un cadre médical. En effet, il peut être intéressant de savoir si le cancer détecté est déjà trop avancé ou non pour ensuite pouvoir au mieux adapter le traitement.

Les quatre autres composés n'ayant pas été retrouvés dans des publications sur l'Homme et le cancer sont aussi des cétones. Trois composés sont en augmentation, la phenylactone, le 6-méthylheptan-3-one et le 4-méthylhept-6-en-3-one. La phenylactone n'a jamais été identifiée comme biomarqueur dans d'autres publications cependant ce composé est un métabolite de l'amphétamine et de la méthamphétamine. Il a été retrouvé augmentant chez les souris atteintes d'autres maladies telle que Alzheimer (Kimball, Wilson, & Wesson, 2015). L'étude de la phénylactone comme biomarqueur pourrait être intéressante au vu de son augmentation croissante au cours du développement du cancer dès l'apparition des cellules précancéreuses. Pour les deux autres composés, le 6-méthylheptan-3-one a été retrouvé en augmentation chez les souris stressées et déprimées (Kwak et al., 2008; Schaefer et al., 2010; Fujita et al., 2020) et le 4-méthylhept-6-en-3-one a été identifié dans une étude sur Alzheimer (Tian et al., 2021). Le dernier composé, le hept-4-en-2-one diminue chez les souris cancéreuses, c'est un composé uniquement retrouvé chez les souris. Il serait intéressant d'étudier les voies de biosynthèse de l'ensemble de ces composés identifiés comme potentiels biomarqueurs pour mieux comprendre comment certains peuvent être influencés par l'apparition du cancer. De plus, la connaissance des voies de biosynthèse de ces composés chez les souris pourrait permettre de savoir si on trouve les mêmes voies de biosynthèse chez l'Homme, et d'identifier des potentiels composés qui pourraient être impactés par le cancer.

Parmi les composés retrouvés dans la litière et les urines, nous avons les deux phéromones émises par les souris mâles. Cependant, dans les urines, la thiazoline est en augmentation alors qu'elle était en diminution dans les litières. Cette différence de variation peut être due au fait que pour les échantillons d'urine nous avons étudié l'émission des composés émis par la même quantité d'urine (40µl) pour chaque échantillon de souris, alors que dans les litières les concentrations d'urines représentaient la quantité émise par les souris. Il se peut que les souris cancéreuses urinent moins que les saines ce qui induirait une diminution des phéromones présentes dans la litière. Ces deux phéromones ne peuvent faire l'objet de biomarqueurs chez l'Homme, puisqu'elles sont produites uniquement par les souris mâles. Cependant, cela peut toutefois apporter une piste de recherche. En effet, cela nous a permis de mettre en évidence que le cancer avait un impact sur des composés dérivés de la testostérone. Il pourrait donc être intéressant d'étudier l'évolution des composés stéroïdiens chez l'Homme qui sont dérivés de la testostérone.

Composés discriminants

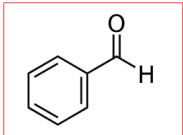
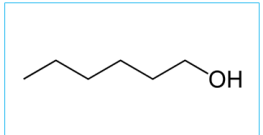
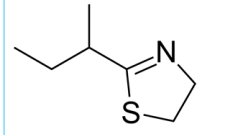
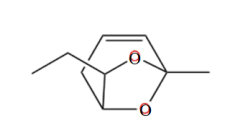
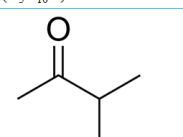
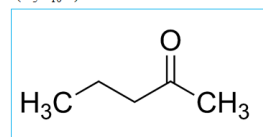
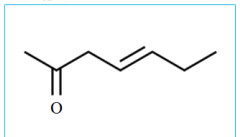
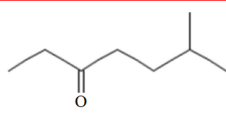
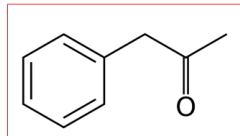
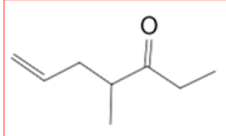
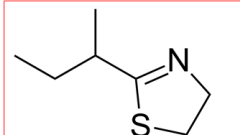
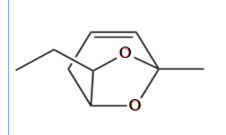
	Composés commun entre la souris et l'homme		Composés retrouvé uniquement chez la souris	
Litières	Benzaldéhyde (C_7H_6O)  Aldéhyde	Hexan-1-ol ($C_6H_{14}O$)  Alcool primaire	2-sec-butyl-4,5-Dihydro-thiazole ($C_7H_{13}NS$)  Phéromones	3,4-dehydro- <i>exo</i> -Brevicomine ($C_9H_{14}O_2$)  Phéromones
	3-Méthylbutan-2-one ($C_5H_{10}O$)  Cétone	Pentan-2-one ($C_5H_{10}O$)  Cétone	Hept-4-en-2-one ($C_7H_{12}O$)  Cétone	6-méthylheptan-3-one ($C_8H_{16}O$)  Cétone
Urines			Phenylacetone ($C_9H_{10}O$)  Cétone	4-méthylhept-6-en-3-one ($C_8H_{14}O$)  Cétone
			2-sec-butyl-4,5-Dihydro-thiazole ($C_7H_{13}NS$)  Phéromones	3,4-dehydro- <i>exo</i> -Brevicomine ($C_9H_{14}O_2$)  Phéromones

Figure 19: Bilan des composés discriminants retrouvés dans les litières et les urines de souris cancéreuses. Avec en rouge les composés augmentant chez les souris cancéreuses et en bleu les composés diminuant.

- Utilisation des souris comme nez et comparaison avec les autres animaux

L'utilisation de souris comme nez pour la détection du cancer semble être prometteuse d'un point de vue médical. Dans notre étude **Manuscrit III**, nous avons pu montrer que les souris étaient capables de discriminer des congénères cancéreux à un stade précoce de développement du cancer sans avoir eu un apprentissage de l'odeur au préalable. Cela a été réalisé à partir d'échantillons de litières souillées par les souris, contenant ainsi toutes les odeurs émises par les souris durant une période donnée. Cette étude a permis de montrer que nous n'étions pas forcément obligés de réaliser un long entraînement pour réussir à leur faire détecter des odeurs d'individus cancéreux. Le protocole de détection pourrait faire l'objet d'études plus approfondies pour savoir si cette discrimination peut être élargie à d'autres cancers, à d'autres stimuli notamment issus d'odeurs humaines. Le fait que les souris discriminent plus facilement un individu cancéreux parmi des individus sains que l'inverse, semble indiquer, comme dit précédemment, une plus grande hétérogénéité entre individus cancéreux. Nous avons pu voir visuellement à l'aide des coupes histologiques que malgré le traitement identique entre les souris, les tumeurs n'étaient pas forcément réparties de la même manière dans le poumon. Certaines avaient des petites tumeurs éparpillées alors que d'autres avaient peu de tumeurs mais plus grosses (voir Figure 20). Une étude histologique plus approfondie pourrait être réalisée pour savoir s'il existe une corrélation entre les structures des tumeurs et les modifications d'odeurs.

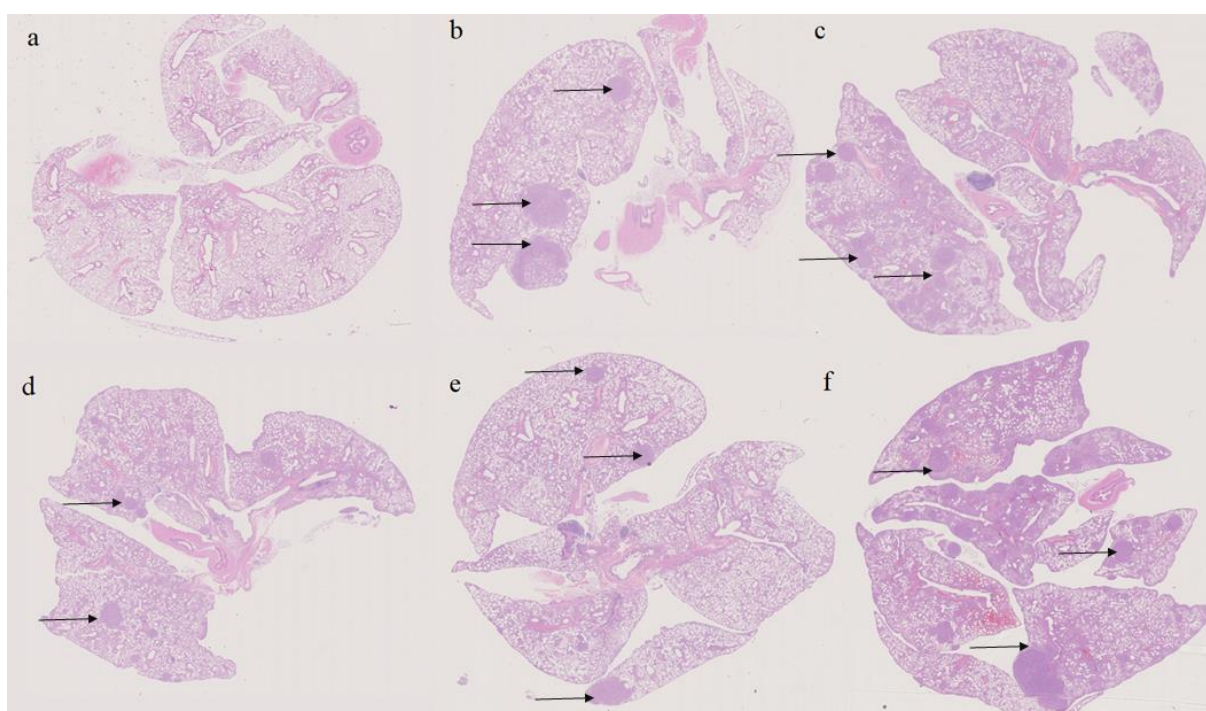


Figure 20: Coupe histologique de poumon de souris EGFR. Les coupes sont fixées avec une coloration d'hématoxyline-éosine sur formalin-fixed paraffin embedded. La coupe a) représente une souris saine NC et les coupes de b) à f) sont des coupes de souris cancéreuses CC avec les flèches représentant des tumeurs.

Nous savons que chez l'Homme il existe une forte variation inter-individuelle même entre individus sains. De plus d'autres pathologies pourraient venir interférer avec les COVs émis, ce qui pourrait rendre difficile la discrimination par les souris. Des études complémentaires semblent nécessaires et obligatoires avant de réussir à mettre en place un protocole valide pouvant utiliser les souris sans réaliser d'apprentissage au préalable, en leur présentant simplement des stimuli différents, qu'elles seraient capables de discriminer. D'autre part, cette capacité de détection nous a permis de mettre en évidence le fait qu'elles possèdent un odorat suffisamment développé pour détecter très précocement d'infimes variations induites par la présence de lésions précancéreuses. L'utilisation des souris comme nez présente des avantages mais aussi des inconvénients par rapport à l'analyse en GC-MS et par rapport à l'utilisation d'autres animaux. En utilisant des souris comme nez, l'identification de composés n'est pas nécessaire ce qui peut être un gros avantage par rapport aux analyses chimiques. Leur capacité de détection étant souvent plus efficace que les méthodes d'analyse chimique, leur utilisation peut nous permettre de s'affranchir des limites technologiques actuelles. Cependant l'utilisation d'animaux sous-entend qu'il faut les maintenir en captivité. Les souris de laboratoire, du fait de leur durée de vie d'environ 1-2 ans, elles ne pourront être utilisées qu'un temps limité, ce qui augmenterait le nombre d'individus à élever et à utiliser, contrairement aux chiens qui peuvent vivre plus de 10 ans, et peuvent ainsi réaliser un nombre élevé de tests. Cependant les chiens, comparés aux souris, demandent plus de temps et d'argent pour ce qui est du dressage, de l'entretien et du maintien en bonne santé. Les chiens ont besoin d'un dressage long, de 2 mois à un an pour certains, avant de réussir à discriminer des individus cancéreux, contre des tests de 30min pour des souris. Nous avons pu voir dans le **Manuscrit II** que l'utilisation d'invertébrés pouvait être une alternative moins chère, qui permet aussi de réaliser des tests sur une plus grande quantité d'individus. Ce point est important à considérer, car il faut un grand nombre d'individus lorsqu'on utilise des invertébrés pour obtenir une réponse correcte : entre 25 à 30 fourmis ou 50 à 100 nématodes par exemple sont nécessaires pour obtenir une réponse fiable. Cependant l'élevage d'insectes ou de nématodes est plus facile à mettre en place d'un point de vue financier, car pas de frais vétérinaires et un coût d'entretien faible. D'autre part, à l'heure actuelle, aucune réglementation n'interdit l'utilisation des invertébrés en expérimentation, ce qui facilite leur exploitation et permet l'utilisation d'un nombre illimité d'individus.

IV) Perspectives et conclusion

Que ce soit dans un cadre médical ou dans un cadre d'écologie évolutive, l'étude du cancer a encore énormément de choses à nous apprendre. Cette thèse a permis d'un point de vue médical de confirmer l'idée selon laquelle l'utilisation des COVs pour diagnostiquer le cancer est une voie prometteuse de recherche. En effet, cela pourrait permettre de diagnostiquer le cancer bien avant nos moyens actuels de détection, dès l'apparition des lésions cancéreuses. Cela permettrait ensuite au

traitement d'être plus efficace et d'obtenir de meilleurs résultats de guérison. Cependant nous avons pu montrer dans le **Manuscrit I** qu'il n'était pas toujours aisé d'identifier des composés prometteurs. De plus, il existe une grande hétérogénéité interindividuelle au niveau des odeurs comme illustré dans ce travail, réalisé sur des souris issues de la même lignée génétique, nourries avec la même alimentation et maintenues exactement dans les mêmes conditions. Cela sous-entend qu'en fonction de l'organe touché, de la taille de la tumeur et certainement d'autres paramètres liés au micro-environnement de chaque tumeur, les modifications des COVs ne seront pas exactement les mêmes. La généralisation et la standardisation des COVs comme outil pour le diagnostic des cancers chez l'Homme semble pour le moment prématuré. Nous avons chacun notre propre profil d'odeurs qui varie en fonction de nombreux facteurs, e.g. comme la nourriture, l'état de stress, l'âge, les maladies (Shirasu & Touhara, 2011) etc ... Des études de cohorte à long terme sur l'Homme permettraient d'étudier une population sur plusieurs années tout en réalisant des analyses d'odeurs à différents pas de temps, ce qui pourrait rétrospectivement permettre de détecter des perturbations spécifiques des COVs associés au développement d'un cancer. Ainsi, l'obtention de biomarqueurs de chaque type de cancer et le suivi de leur évolution au cours du temps à l'aide des techniques d'analyses chimiques semble envisageable mais risque de prendre encore des années avant d'atteindre des niveaux de fiabilité et de spécificité acceptables. L'utilisation de nez animaux semble une option plus rapide de développement. Nous avons pu voir qu'il n'y avait pas que les souris qui pouvaient être de bons bio-détecteurs de maladies cancéreuses. Des organismes tels que les insectes ou les nématodes, présentés dans le **Manuscrit II**, présentent des compétences et des caractéristiques qui pourraient faire d'eux de très bon nez. D'autres tests tels que l'électroantennographie (EAG) ou l'imagerie calcique peuvent être plus facilement réalisables sur ces organismes que sur des vertébrés tels que les chiens ou les souris. Ces tests peuvent permettre d'obtenir des informations sur les composés que ces organismes sont capables de détecter. Cependant ces techniques sont très peu développées, peu de laboratoires en France et dans le monde possèdent ces technologies, qui peuvent être difficiles à mettre en place et pour lesquelles peu de personnes sont formées. Dans tous les cas, comme dit précédemment, si l'utilisation des COVs pour détecter la présence d'un cancer semble prometteuse, il restera compliqué de savoir la localisation exacte du cancer dans l'organisme. D'autres techniques pourraient ensuite être couplées pour détecter l'emplacement exact de la tumeur.

En dehors du cadre médical, la capacité de certains organismes à détecter et discriminer les individus cancéreux soulève de nombreuses questions d'ordre écologique et évolutif. Des questions telles que : Pourquoi et comment arrivent-ils à reconnaître des organismes cancéreux ? Quelles sont les conséquences de ces détections sur les interactions intra et interspécifiques dans la nature et *in fine* sur le fonctionnement des écosystèmes ? Ces questionnements sont d'autant plus pertinents que les écosystèmes de la planète sont de plus en plus pollués par des substances mutagènes. Dans cette thèse, nous avons entrouvert la porte qui mène à toutes ces réponses mais il reste une multitude de questions

et beaucoup de travail en perspective. Nous avons seulement pu étudier les préférences sexuelles des souris, mais qu'en est-il sur d'autres espèces comme celles qui vivent de manière sociale ? Les individus cancéreux peuvent-ils être détectés dans un groupe social parmi les individus sains, et si oui sont-ils exclus de ce groupe comme dans le cas des individus parasités ou au contraire sont-ils traités d'une manière différente sachant que leur pathologie n'est pas transmissible. En plus des relations intraspécifiques, le cancer peut-il influencer les relations interspécifiques ? Par exemple, les prédateurs utilisent-ils les modifications d'odeurs des proies comme un signal de maladies, et donc de plus grande vulnérabilité en cas d'attaques ? L'impact du cancer dans les relations proie-prédateur est inconnu contrairement aux maladies parasitaires pour lesquelles nous avons davantage d'informations. Peu de choses sont également connues quant au rôle des pathologies cancéreuses sur la dynamique des maladies parasitaires, notamment celles véhiculées par des vecteurs souvent sensibles aux odeurs. Nous avons par exemple cité le cas des drosophiles capables de détecter des odeurs d'individus humains cancéreux. Il serait intéressant dans ce contexte d'étudier la réponse des moustiques vis-à-vis de COVs émis par des personnes saines ou cancéreuses. Ces questionnements sont très actuels. On dénombre par exemple de plus en plus de cancers dans les pays du Sud, du fait de l'allongement de la durée de vie. En parallèle, ces pays abritent de nombreux moustiques à l'origine de diverses maladies infectieuses. Si les moustiques piquent différemment les gens qui ont un cancer et ceux qui n'en ont pas, et qu'il y a de plus en plus de cancers dans les pays du Sud, cela pourrait avoir un impact à grande échelle sur la dynamique des maladies qu'ils transmettent. Par ailleurs, comme de nombreux cancers évoluent pendant des années sans générer de symptômes majeurs, il serait aussi intéressant de déterminer à partir de quel moment, les moustiques sont sensibles à un changement du profil d'odeurs.

Une des hypothèses pour expliquer que les souris femelles étudiées dans cette thèse n'ont pas eu de répulsion vis-à-vis de souris mâles cancéreuses est le fait que contrairement aux infections parasitaires, le cancer n'est pas transmissible. Même s'il n'existe pas à ce jour d'évidence de comportement d'évitement chez les espèces ayant des cancers transmissibles (notamment les diables de Tasmanie évoqués précédemment), il serait souhaitable de pousser les investigations. Par exemple, il est possible que la détection des tumeurs, sur la base de COVs ou d'autres variables, existe chez les diables, mais que les comportements d'évitement ne s'expriment que dans des situations qui ne sont pas directement liées à la reproduction (compétition pour l'accès à de la nourriture). Bien que très spéculatif, on ne peut exclure dans le cas des cancers transmissibles, la possibilité que les cellules cancéreuses, au cours du temps, acquièrent la capacité à manipuler les COVs émis par leur hôte dans un sens qui favorise les interactions interindividuelles, et donc la transmission.

Les cancers, comme d'autres pathologies, sont associés à des modifications d'odeurs corporelles chez les patients. Bien que les scientifiques aient fait beaucoup de progrès ces dernières décennies dans l'exploration de ce phénomène, la valeur des COVs comme signatures chimiques des cancers reste un champ d'investigation encore en pleine exploration. Les questionnements sont multiples, allant de la

recherche de biomarqueurs précoces de la maladie à l'étude des conséquences écologiques et évolutives de ces changements d'odeurs, ou encore à l'exploration de leurs conséquences épidémiologiques, dans le cas par exemple d'interférence avec le comportement d'insectes vecteurs. Cette thèse s'inscrit dans un champ de recherches s'annonçant prometteur, passionnant et pertinent pour mieux soigner certaines et nos pathologies et mieux comprendre les interactions entre individus et espèces dans le monde changeant qui nous entoure.

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Résumé :

Conséquences écologiques, évolutives et appliquées des modifications d'odeurs corporelles associées au cancer.

Chez de nombreuses espèces animales, la communication chimique joue un rôle majeur dans les interactions entre individus, tant au niveau intraspécifique qu'interspécifique (sélection sexuelle, compétition, prédation, etc.). S'il est établi que bon nombre de pathologies sont associées à des modifications d'odeurs corporelles, peu de travaux ont été consacrés aux conséquences écologiques et évolutives de ces phénomènes. De plus, la plupart des recherches sur ce sujet concernent les maladies infectieuses, suggérant par exemple un évitement de la contagion entre individus, mais beaucoup plus rarement les cancers. Cette thèse vise à mieux comprendre comment les processus cancéreux (des lésions précancéreuses aux cancers métastatiques) influencent les interactions entre individus au travers des modifications d'odeurs corporelles qu'ils entraînent. Pour mener nos recherches, nous avons utilisé un modèle biologique hôte/cancer : la souris/cancer du poumon. En plus de clarifier le rôle des odeurs associées au cancer dans les interactions entre organismes, ce projet de recherche visait également à identifier le stade de développement du cancer à partir duquel les changements olfactifs sont observables, avec l'objectif appliqué de mettre en évidence de possibles biomarqueurs précoces du cancer présentant un intérêt médical. Concernant l'aspect appliqué, nous avons pu observer des modifications d'odeurs très tôt dans le développement de la maladie, dès l'apparition des lésions précancéreuses, et plusieurs composés biomarqueurs potentiels ont été identifiés. Nos résultats indiquent ainsi que le cancer du poumon chez la souris modifie de manière précoce les odeurs corporelles, et que celles-ci peuvent être détectées aussi bien par des analyses chimiques que par l'utilisation de souris saines comme nez. Ces conclusions ouvrent des perspectives de recherche dans le domaine médical. Concernant l'impact du cancer sur le comportement des congénères sains, nos résultats suggèrent que la maladie ne semble pas jouer de rôle sur les préférences des souris femelles, contrairement à ce qui est généralement observé dans le cadre du parasitisme. Ce nouveau domaine d'étude, qui réunit l'écologie chimique et le cancer, ne fait que démarrer et de nombreuses questions restent à élucider, notamment vis-à-vis d'autres interactions telles que proie-prédateur, ou encore dans le cas des cancers transmissibles comme chez le diable de Tasmanie. Cette thèse ouvre de nombreuses perspectives de recherche, tant sur le plan médical que sur le plan de la biologie fondamentale.

Mots clés : adénocarcinome pulmonaire, *Mus musculus domesticus*, écologie comportementale, écologie chimique

Abstract:**Ecological, evolutionary and applied consequences of body odour changes associated with cancer.**

In many animal species, chemical communication plays a major role in interactions between individuals, both at the intraspecific and interspecific levels (sexual selection, competition, predation, etc.). Although it is known that many pathologies are associated with changes in body odour, little work has been done on the ecological and evolutionary consequences of these phenomena. Moreover, most research on this subject concerns infectious diseases, suggesting for example an avoidance of contagion between individuals, but much more rarely cancers. This thesis aims to better understand how cancer processes (from pre-cancerous lesions to metastatic cancers) influence interactions between individuals through the changes in body odour that they cause. To conduct our research, we used a biological host/cancer model: the mouse/lung cancer. In addition to clarifying the role of cancer-associated odours in inter-organism interactions, this research project also aimed to identify the stage of cancer development at which olfactory changes are observable, with the applied objective of identifying possible early cancer biomarkers of medical relevance. On the applied side, we were able to observe odour changes very early in the development of the disease, from the onset of precancerous lesions, and several potential biomarker compounds were identified. Our results thus indicate that lung cancer in mice alters body odours early on, and that these can be detected both by chemical analysis and by using healthy mice as noses. These findings open up new research perspectives in the medical field. Regarding the impact of cancer on the behaviour of healthy conspecifics, our results suggest that the disease does not seem to play a role in the preferences of female mice, contrary to what is generally observed in the context of parasitism. This new field of study, which brings together chemical ecology and cancer, is only just beginning and many questions remain to be answered, particularly with regard to other interactions such as prey-predator, or in the case of transmissible cancers such as in the Tasmanian devil. This thesis opens up numerous research perspectives, both in the medical field and in fundamental biology.

Key words: Lung adnocaarcinome, *Mus musculus domesticus*, behaviour ecology, chemical ecology.