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Effet des polluants organiques persistants et des pré-adipocytes dans la progression tumorale dans le cancer du sein.

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RESUME

Malgré les avancées dans le diagnostic et le traitement du (CS), plus de 12 000 femmes décéderont de cette maladie en France cette année. Ces décès sont en grande partie dues à la dissémination de la maladie, la survie globale à 5 ans n'étant que de 26% dans les cas de formes métastatiques. Comprendre les facteurs de risque de progression tumorale et les mécanismes sous-jacents est un enjeu majeur en termes de santé publique notamment dans les populations à risque comme les patients obèses.

Le tissu adipeux, composant majeur du microenvironnement tumoral dans le CS, est un réservoir important de polluants organiques persistants (POP) à la fois cancérigènes et perturbateurs endocriniens, et également une source interne chronique de ces molécules qui sont libérées de façon constante dans le sang. Outre leur effets toxiques et pro-invasifs directs, nous avons postulé l'hypothèse que ces POP, dont les dioxines, pouvaient avoir des interactions avec le tissu adipeux et modifier le phénotype des adipocytes péri-tumoraux dont l'effet pro-invasif sur les cellules tumorales est déjà connu.

Les résultats de l'étude clinique METAPOP qui évalue l'association entre les taux sériques et adipeux de POP et la présence d'une atteinte métastatique ganglionnaire chez les patientes prise en charge pour un CS montrent que les concentrations de dioxines et de certains autres polluants donc plusieurs polychlorobiphényles dans le tissu adipeux sont positivement associées 1) au risque de métastase ganglionnaire et à la taille de la tumeur et 2) que cette association était encore plus forte chez les patientes présentant un IMC égal ou supérieur à 25 kg/m^2 (c'est à dire une majorité de la population dans les pays Européens).

Au niveau expérimental, à l'aide d'un système de co-culture en insert de cellules cancéreuses mammaires et de cellules pré-adipocytaires, exposées ou non à la dioxine, nous avons montré que la co-exposition « pré-adipocytes/polluant » entrainait des modifications majeures du phénotype des cellules cancéreuses mammaires avec l'acquisition d'un caractère « cellule souche cancéreuse » associé à la progression tumorale métastatique et à la chimiorésistance. Ce phénotype agressif et pro-métastatique est confirmé *in vivo* sur un modèle de xénogreffe chez l'embryon de poisson zèbre. La génération par la co-exposition de cellules géantes polynucléées est l'une des hypothèses pouvant expliquer l'acquisition de ces propriétés pro-invasives.

Les POP pourraient ainsi contribuer à la progression tumorale et au développement métastatique dans le CS d'une part par leurs effets toxiques directs et d'autre part en ciblant le microenvironnement tumoral. Ces résultats pourraient expliquer le pronostic sombre chez les patientes obèses. Compte tenu du nombre important de cas, cette problématique représente potentiellement un problème majeur de santé publique, qui nécessite des investigations complémentaires pour mieux appréhender les mécanismes d'action mis en jeu.

MOTS-CLES : Cancer du sein, métastases, adipocytes associés au cancer, polluants organiques persistants, dioxine, microenvironnement tumoral, cellules souches cancéreuses.

ABSTRACT

Despite advances in the diagnosis and treatment of breast cancer (BC), more than 12 000 women in France will die this year of this disease. Most of these deaths are due to the spread of cancer cells in the body, the 5-year overall survival is only 26% in metastatic forms. Understanding tumor progression risk factors and the underlying mechanisms is a major challenge in terms of public health, especially in high-risk populations such as obese patients.

Adipose tissue, an important component in BC tumoral microenvironment, is a major reservoir of carcinogenic persistent organic pollutants (POP) and also a chronic internal source of pollutants that are released slowly in the blood. In addition to their direct toxic and pro-invasive effects, we postulated the hypothesis that these POP including dioxins, could interact with adipose tissue and change peritumoral adipocytes phenotype already known for their pro-invasive effects on cancer cells.

The results of the METAPOP clinical trial, which assesses the association between serum and adipose levels of POPs and the presence of metastatic lymph node involvement in BC patients, show that concentrations of dioxins and some other pollutant as polychlorinated biphenyl in adipose tissue are positively associated 1/with the risk of lymph node metastasis and tumor size, and 2/ this association was even stronger in patients with BMI greater than or equal to 25 kg / m2 (more than 50% of the European population).

At the experimental level, through an insert co-culture system of mammary cancer cells and pre-adipocyte cells, exposed or not to dioxin, we have shown that pre-adipocyte / pollutant co-exposure lead to major phenotypical modifications of breast cancer cells with the acquisition of "cancer stem cell" properties associated with metastatic tumor progression and chemoresistance. This aggressive and prometastatic phenotype is confirmed *in vivo* on a zebrafish embryo xenograft model. The generation by co-exposure of polynucleate giant cells is one of the hypotheses to explain the acquisition of these pro-invasive properties.

POP could contribute to tumor progression and metastatic development in BC by their direct effects and by targeting the tumor microenvironment. These data could explain the poor prognosis in obese patients. Given to the high number of cases, this question is potentially a major public health concern and further investigations are needed to understand the mechanisms involved.

KEYWORDS : Breast cancer, metastasis, cancer-associated-adipocytes, persistent organic pollutants, dioxin, tumoral microenvironnement, cancer-stem-cells.

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Annexe 4 : Classification OMS 2012 (4ème édition) des carcinomes mammaires infiltrants
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Liste des abbréviations

AAC: Adipocyte associé au cancer
ASC : Adipose stem cell
ADN: Acide désoxyribonucléique
AhR : Aryl hydrocarbon receptor
ARN: Acide ribonucléique
CAR: Constitutive androstane receptor
CS : Cancer du sein
CSC: Cellule souche cancéreuse
DDE : Dichlorodiphényldichloroéthylène
DDT : Dichlorodiphényltrichloroéthane
HAP : Hydrocarbures aromatiques polycycliques
HER2 : Human epidermal growth factor receptor-2
hMADS : Human multipotent adipose-derived stem cells
HBCD: Hexabromocyclododecane
HpCDF : 1,2,3,4,7,8,9-heptachlorodibenzofuran
HxCDD : Hexachlorodibenzo-p-dioxin
IL : Interleukine
IMC: Indice de masse corporelle
MCF-7 : Michigan cancer foundation – 7 cells
MDA-MB-231 : Triple negative breast cancer cells (isolated at M.D. Anderson)
MEC : Matrice extracellulaire
MMP : Métalloprotéase matricielle
MVP : Major vault protein
OMS : Organisation mondiale de la santé
PBDE : Polybromodiphényléther
PBB : Polybromobiphényl
PCB : Polychlorobiphényle
PCDD : Polychlorodibenzo-p-dioxines
PCDF : Polychlorodibenzo-p-furanes
PFOSA : Perfluorooctane-sulfonamide
POP : Polluant organique persistant
PXR : Pregnane X receptor
RE : Récepteur aux œstrogènes
RP : Récepteur aux progestatifs
TCDD : 2,3,7,8-tétrachlorodibenzo-p-dioxine
TEM : Transition épithélio-mésenchymateuse
TNM: Tumor node metastasis
VEGF : Vascular endothelial growth factor

Docteur en médecine spécialisée en Gynécologie-Obstétrique et plus spécifiquement en chirurgie cancérologique gynécologique et du sein, j'ai découvert le monde de la recherche fondamentale et de la toxicologie au cours de mon année de Master 2 Sciences Chirurgicales, option cancérologie, réalisé en 2015 au sein de l'équipe 1 de l'unité INSERM 1124. L'encadrement et la formation exceptionnels que j'ai reçus pendant cette année ainsi les premiers résultats très encourageants obtenus m'ont convaincue de poursuivre ce projet dans le cadre d'un doctorat d'université. Je remercie le Dr Céline Tomkiewicz et le Pr Xavier Coumoul pour leur accueil et leur soutien au cours de ces dernières années.

Les travaux présentés comportent les résultats d'une étude clinique, METAPOP, réalisée au sein du service de chirurgie gynécologique et du sein de l'hôpital Européen Georges Pompidou dans lequel je suis actuellement Chef de Clinique Assistante, dont le but est d'évaluer l'association entre les taux sériques et adipeux de polluants organiques persistants et la présence d'une atteinte métastatique ganglionnaire chez les patientes prise en charge pour un cancer du sein et une seconde partie expérimentale dont l'objectif est d'étudier les interactions entre les préadipocytes et la dioxine dans le développement de métastases dans le cancer du sein. Pendant ma 2ème année de thèse, j'ai eu la chance d'effectuer une mobilité internationale à l'université de Boston (12 mois) pendant laquelle j'ai pu confirmer mes résultats expérimentaux in vitro sur un modèle in vivo de développement de métastases après xénogreffes chez le poisson zèbre. Je remercie l'Académie Nationale de Médecine, la Fondation ARC, la Fondation de France ainsi que le collège des Gynécologues-Obstétriciens de France pour leur soutien ainsi que le Dr David Sherr pour m'avoir accueillie au sein de son laboratoire à Boston.

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Lauréate de l'Académie Nationale de Médecine 2017, Prix Elisabeth Taub
Bourse Jacques Lansac du Collège National des Gynécologues de France 2017
Bourse Aide à la mobilité internationale de la Fondation de France et Unicancer 2017
Prix Mobilité internationale de la Fondation ARC pour la recherche contre le cancer 2017
3ème prix Kerner de vulgarisation scientifique, Journées Jeunes Chercheurs de l'ARC 2018

INTRODUCTION GENERALE

Le cancer du sein (CS) est une maladie fréquente, atteignant environ une femme sur huit au cours de son existence. Grâce au dépistage précoce et aux avancées de la recherche, le pronostic du cancer du sein s'est beaucoup amélioré mais reste mauvais chez les patientes ayant développé des métastases à distance et dont les cellules tumorales ont acquis des propriétés de résistance à la chimiothérapie. Depuis plusieurs années, de nombreuses études se sont intéressées au rôle des facteurs environnementaux notamment des polluants dans l'initiation du cancer du sein, suggérant une association positive, mais peu d'entre elles se sont questionnées sur le fait que ces polluants pouvaient aussi être des facteurs aggravants du cancer. Les polluants organiques persistants (POPs), appelés comme cela car ils persistent dans l'environnement de très nombreuses années après leur production, se stockent du fait de leur propriétés lipophiles dans le foie, le cerveau et le tissu adipeux qui représente un composant majeur du microenvironnement tumoral dans le CS. Dans des études précédentes, nous avons montré que le tissu adipeux pouvait être une source interne chronique de polluants (dont un grand nombre sans cancérigènes) qui sont libérés à bas bruit dans le sang pour être éliminer (1). Cette libération interne constante et cette recontamination sont par exemple responsables de la toxicité chronique associée à l'exposition aux POP.

L'objectif de ce travail de thèse est d'explorer les effets directs de ces POP sur la progression tumorale ainsi que leurs interactions avec le tissu adipeux qui pourraient contribuer au développement métastatique et expliquer par exemple le pronostic sombre des patientes obèses atteinte de CS.

La première partie de ce travail comprend une synthèse bibliographique sur le cancer du sein, le tissu adipeux, les POP et le rôle connu des adipocytes dans la progression tumorale. Une revue de la littérature s'intéressant au rôle des POP et produits toxiques environnementaux dans la progression tumorale, l'invasion et la formation de métastases dans le CS, ainsi qu'à l'acquisition d'une résistance à la chimiothérapie par les cellules carcinomateuses mammaires sous l'effet de toxiques complète cette partie (article 1).

Dans une seconde partie, nous présenterons les résultats de l'étude clinique METAPOP qui évalue l'association entre les taux sériques et adipeux de POP et la présence d'une atteinte métastatique ganglionnaire chez les patientes prise en charge pour un CS.

Enfin, afin d'améliorer la compréhension des mécanismes impliqués dans les interactions POP/tissu adipeux/cellules cancéreuses, nous avons mis en place un système de co-culture en insert de cellules cancéreuses mammaires et de cellules pré-adipocytaires, exposé ou non à la dioxine, et exploré la réponse des cellules cancéreuses mammaires (MCF7 et MDA-MB-231) *in vitro* et le développement de métastases *in vivo* dans un modèle de xénogreffe chez le poisson zèbre.

PARTIE 1 :
SYNTHESE BIBLIOGRAPHIQUE

I. Le cancer du sein

1. Anatomie et histologie du sein

Le sein est une unité cutanéo-glandulaire composé d'une glande mammaire, de fibres de soutien (ligaments de Cooper) et de tissu adipeux ; le tout est recouvert par la peau (2). La glande mammaire dont la principale fonction est la lactation, est divisée en 15 à 20 sections appelés lobes et eux-mêmes composés de lobules qui se drainent à travers un réseau ramifié de canaux galactophoriques jusqu'au mamelon. Chaque lobule forme avec un canal galactophore terminal qui lui est associé, une unité terminale ductulo-lobulaire. Ces unités sont très sensibles aux variations hormonales et il est admis que la plupart des lésions bénignes et carcinomateuses se développent à partir de l'unité terminale ductulo-lobulaire. Une représentation schématique du sein est présentée Figure 1.

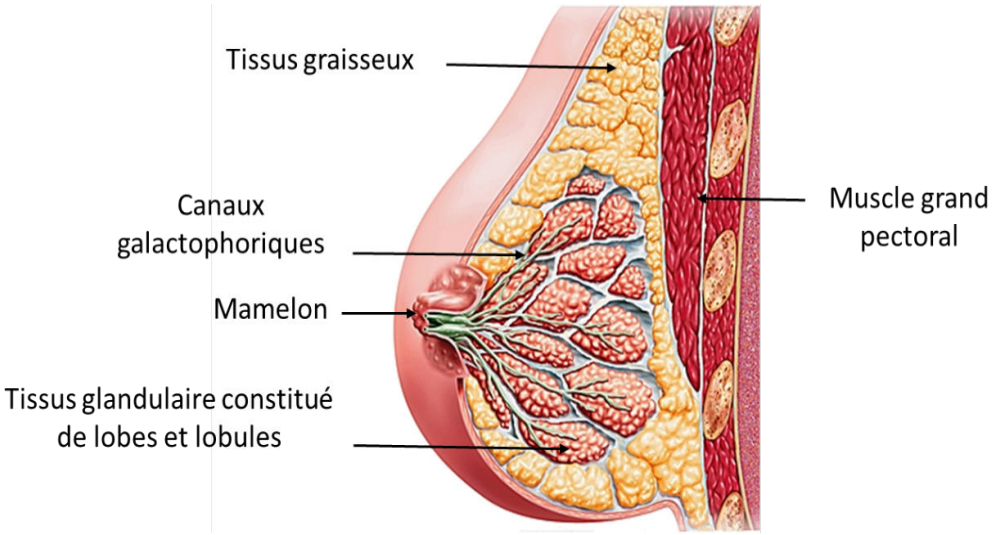

Figure 1: Structure de la glande mammaire : représentation schématique d'une coupe sagittale du sein *(Illustration Médicale d'après Patrick Lynch)*

Sur le plan histologique, la glande mammaire proprement dite présente des formations glandulaires composées d'acini mammaires et de canaux excréteurs et de formations conjonctives intra et interlobulaires. Le tissu conjonctif intralobulaire renferme des fibres de collagènes, des fibroblastes, un riche réseau capillaire sanguin et lymphatique et des fibres nerveuses vasomotrices. Le tissu conjonctif interlobulaire est composé de ces mêmes structures mais aussi de nombreuses cellules adipocytaires organisées en amas (Figure 2).

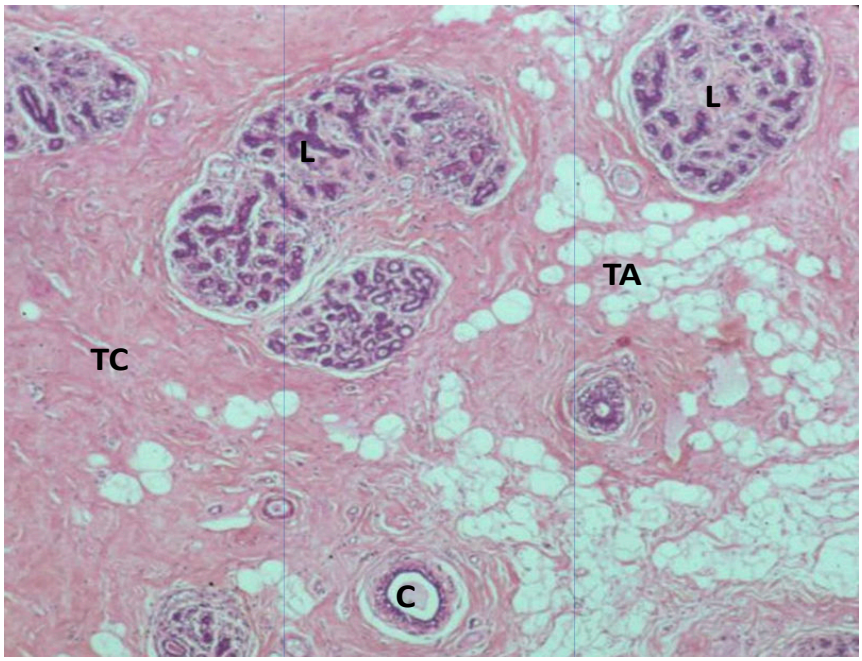

Figure 2 : Coupe histologique de tissu mammaire normal.
TA tissu adipeux, TC tissu conjonctif interlobulaire, L lobules, C canal galactophore.

Le rapport entre le volume de la composante fibro-glandulaire et le volume de la composante graisseuse dépend principalement de l'âge de la patiente. L'involution du sein caractérisée par le remplacement progressif du tissu fibroglandulaire par du tissu adipeux commence à partir de 30 à 35 ans et s'accentue en phase ménopausée. A la mammographie, ce remplacement progressif entraîne la diminution de la densité mammaire et plusieurs classifications radiologiques en fonction du pourcentage graisseux, sont utilisées en routine.

Ainsi, les cellules qui composent le tissu adipeux telles que les cellules progénitrices adipocytaires (pré-adipocytes) et les adipocytes sont des composants majeurs du microenvironnement dans le cancer du sein (CS).

2. Épidémiologie du cancer du sein

Le CS est un problème majeur de santé publique dans le monde avec plus de 2 millions de nouveaux cas estimés en 2018. Il représente 12% des cancers nouvellement diagnostiqués, 25% des cancers de la femme et est responsable de 600,000 décès par an (3). En France, le CS est le premier cancer chez la femme avec plus de 58 000 nouveaux cas estimés en 2018 et représente un tiers de l'ensemble des nouveaux cas de cancer chez la femme (4). Le taux d'incidence standardisé Monde est de 99,9 cas pour 100 000 personnes-années. Entre 1990 et 2018, le nombre annuel de nouveaux cas a presque doublé et son incidence reste en augmentation sur la période récente 2010-2018 (+0,6% par an en moyenne). C'est aussi la première cause de mortalité par cancer chez la femme, avec 12 146 décès estimés en 2018.

L'âge médian au diagnostic est 63 ans mais plus de 20% des patientes ont moins de 50 ans. Le CS est un cancer de bon pronostic avec une survie globale tout stade confondu à 5 ans estimée à 88 % pour les cancers diagnostiqués en France entre 2005 et 2010 (5). Celle-ci est d'autant meilleure que le cancer est diagnostiqué à un stade précoce. En effet, elle est de 99% dans les formes localisées mais chute à 85% en cas d'atteinte ganglionnaire pour atteindre seulement 26% dans les formes métastatiques à distance (6,7).

3. Facteurs de risque de cancer du sein

Le CS est une maladie multifactorielle. Les facteurs de risque reconnus sont :

- ✓ *L'âge :* L'incidence augmente après 30 ans avec un pic entre 50 et 74 ans.
- ✓ *Les facteurs hormonaux endogènes :* une ménarche précoce (avant 12 ans), la nulliparité, une 1ère grossesse tardive (après 30 ans), l'absence d'allaitement, une ménopause tardive (8). La modification des facteurs hormonaux et reproductifs au fil des générations (diminution de l'âge à la puberté, diminution du nombre moyen d'enfants par femme pour les femmes nées après 1930, augmentation de l'âge au premier enfant pour les femmes nées après 1950, diminution des pratiques d'allaitement et ménopause plus tardive) peuvent expliquer par exemple l'évolution de l'incidence du CS (9).
- ✓ *Les facteurs hormonaux exogènes* : de nombreuses études épidémiologiques se sont intéressées à l'effet de l'utilisation de contraceptifs oraux sur le risque de cancer du sein. Globalement, une augmentation faible du risque de cancer du sein chez les utilisatrices d'œstro-progestatifs a été observé, mais ce sur-risque semble disparaître 10 ans après l'arrêt du contraceptif (10). Aujourd'hui la littérature montre qu'il n'y aurait pas d'effet du type d'hormones (estrogènes ou progestatifs), ni de la durée d'utilisation. A contrario, il existe une association positive entre la prescription de traitement hormonal de substitution de la ménopause et le risque de CS. Dans l'étude américaine de la Women's Health Initiative (11), il a été constaté une augmentation significative du risque de CS chez les patientes traitées par une association hormonale à base d'œstrogènes conjugués et d'un analogue synthétique de la progestérone (RR = 1,26, IC95% 1,00 - 1,59). Ces données ont été confirmées par une vaste étude de cohorte, la Million Women Study ayant recruté plus d'un

million de femmes britanniques de 1996 à 2001 dans le cadre du National Health Service Breast Screening Programme (12).

- ✓ *Les antécédents de lésions épithéliales prolifératives non invasives* augmente le risque de développer un CS. C'est le cas des lésions d'hyperplasie canalaire simple (RR = 1,5), d'hyperplasie canalaire atypique (RR = 3 à 5), du carcinome canalaire in situ (RR = 8 à 10) (13) et des lésions épithéliales prolifératives lobulaires (hyperplasie lobulaire atypique et carcinome lobulaire in situ).
- ✓ *Mode de vie :* Les taux d'incidence plus élevés observés en Amérique du Nord, en Europe du Nord et de l'Ouest, et en Australie suggèrent un rôle important des facteurs environnementaux et du mode de vie à l'occidentale dans les pays développés. Cette hypothèse est corroborée par des études sur les migrations géographiques qui ont montré par exemple que les japonais ou les hispaniques ayant émigré d'un pays à bas risque vers les États-Unis, présentent le même profil épidémiologique que le reste de la population américaine après 2 ou 3 générations (14,15), traduisant une exposition liée à l'environnement dès le début de vie (voire *in utero*). L'alimentation riche en graisses animales ainsi que la sédentarité dans les pays développés sont incriminés (16). Le rapport du Centre International de Recherche sur le Cancer publié récemment, attribue 8% des cas de CS en France au surpoids ou à l'obésité et 3% au manque d'activité physique (17). L'obésité est associée à un profil hormonal soupçonné de favoriser le développement du cancer du sein. Elle augmente d'environ 50% le risque de cancer du sein chez les femmes ménopausées, probablement en raison de l'augmentation des concentrations sériques d'œstradiol libre (le tissu adipeux exprimant l'aromatase, enzyme produisant les œstrogènes à partir des androgènes) (13). L'alcool serait lui responsable de 15% des cas de CS (18).

- ✓ *Les facteurs environnementaux* : L'impact de l'environnement sur la survenue de cancers est difficile à évaluer et à étudier mais ne fait plus de doute. Certains agents, chimiques, physiques ou biologiques sont classés comme cancérigènes avérés et certains types de cancers leur sont imputables. Des études *in vitro* ont identifié plus de 250 perturbateurs endocriniens qui imitent ou interfèrent avec les œstrogènes naturels et qui peuvent aussi stimuler la prolifération des cellules mammaires cancéreuses (19). Plus de 200 composés chimiques sont reconnus comme cancérogènes mammaires, chez l'animal (20). Plusieurs études de cohorte réalisées après des expositions accidentelles aux polluants organiques persistants (POP, qui seront définis dans le chapitre suivant) ont montré une augmentation du risque de survenue à long terme d'un CS. L'exemple le plus étudié est l'accident industriel de Seveso en Italie en 1976 (21–23). Une revue de la littérature publiée récemment semble montrer une association entre le risque de CS en cas d'exposition à de nombreux POP dont le dichlorodiphényltrichloroéthane (DDT, un pesticide organochloré), les dioxines, le perfluorooctane-sulfonamide (PFOSA) ainsi que les hydrocarbures aromatiques polycycliques (HAP) (24). Cependant, les études restent insuffisantes avec des résultats discordants sur le plan méthodologique.
- ✓ *Les antécédents d'exposition aux radiations ionisantes* (notamment dans l'enfance).
- ✓ *Les mutations génétiques* : 5 à 10 % des CS sont liés à des mutations génétiques héréditaires. Les gènes les plus connus sont les gènes BRCA1 (Breast Cancer 1) et BRCA2, à transmission autosomique dominante, impliqués dans la réparation des cassures double brin de l'ADN et qui prédisposent au CS et de l'ovaire. Le risque cumulé de développer un cancer du sein est de 47 à 66% en cas de mutation BRCA1, et de 40 à 57% en cas de mutation BRCA2 (25). Les autres syndromes de prédisposition génétique au CS connus sont le syndrome de Li-Fraumeni (mutation P53), le syndrome de Cowden (mutation

PTEN), le syndrome de Peutz-Jeghers (mutation STK11), l'ataxie-télangiectasie (mutation ATM) et la mutation du gène PALB2.

- ✓ *Autres facteurs* : le travail de nuit, les perturbateurs endocriniens ou certaines expositions professionnelles pourraient être responsables de l'augmentation de l'incidence du CS mais ne sont pas encore clairement établis.

4. Diagnostic et classifications

Le diagnostic initial d'un CS repose sur l'examen anatomo-pathologique d'une biopsie ou d'une pièce opératoire. La classification anatomo-pathologique de référence utilisée est la 4ème édition de la classification de l'Organisation Mondiale de la Santé (OMS) parue en 2012 qui contient 21 types histologiques différents de carcinomes invasifs différents (26). Elle est présentée en Annexe 1. Le plus fréquent est l'adénocarcinome de type non spécifique qui représente 40 à 75% des carcinomes invasifs du sein. L'examen anatomopathologique comprend de façon systématique l'évaluation des facteurs pronostics et prédictifs suivants : taille tumorale, grade histo-pronostique de Scarff-Bloom-Richardson de I à III, l'index mitotique ou Ki67, l'évaluation du statut des récepteurs hormonaux aux œstrogènes (RE) et à la progestérone (RP), et la recherche d'une amplification du gène ERBB2/HER2 (récepteur transmembranaire, de type tyrosine kinase, à certains facteurs de croissance). La tumeur est considérée comme récepteurs hormonaux (RH) positive si plus de 10% des cellules tumorales expriment le RE ou le RP (l'expression de RP sans RE étant rare).

En pratique clinique, on utilise la classification TNM qui est un code standard international à travers lequel les équipes de cancérologie décrivent l'extension d'un cancer. La lettre T (Tumor) décrit la taille de la tumeur et son extension à la peau ou à la paroi du thorax sous le sein, la lettre

N (Node pour ganglion) le statut ganglionnaire axillaire et la lettre M (Metastasis) indique s'il existe une extension à distance. Proposé pour le sein en 1957, le système appliqué est celui de l'American Joint Committe on Cancer (27). La classification TNM la plus récente est présentée plus en détail dans l'annexe 2.

Au début des années 2000, l'essor des nouvelles techniques d'analyse du matériel génétique des tumeurs utilisant les puces à ADN ou microarrays (étude à large échelle), a permis d'étudier simultanément l'expression de milliers de gènes (ARN messager) et de proposer une nouvelle classification moléculaire intrinsèque des tumeurs en 5 grandes sous-classes de CS invasifs en fonction de leurs altérations génomiques d'expression (ou signatures) qui sous-tendent des pronostics très différents (28,29) (Figure 3). On distingue ainsi :

- ✓ Les CS dits « luminaux », appelés ainsi car leurs gènes codent les protéines des cellules épithéliales de la lumière des canaux ou des lobules du sein. Ils sont caractérisés par l'expression du RE et de gènes associés à la voie du RE. Ce sont les formes les plus fréquentes de CS (2/3 des cas). Elles comprennent deux sous-groupes A et B selon qu'elles expriment fortement (luminal A) ou faiblement (luminal B) les récepteurs des œstrogènes (RE+). Ces tumeurs expriment les cytokératines (CK) 8/18. Dans plus de 10 % des cas, ces tumeurs sont associées à une mutation du gène de maintien du génome, le TP53.
    - Le sous-type luminal A est caractérisé par une expression des récepteurs hormonaux œstrogènes (RE+) et/ou à la progestérone (PR+), l'absence de surexpression de géne HER2 et un taux de mutations p53 faible. Les tumeurs sont souvent de bas grade histologique.
    - Le sous-type luminal B a les mêmes caractéristiques en ce qui concerne les récepteurs hormonaux (RE+et/ou RP+) mais il y a, plus souvent, une surexpression du gène HER2 +. Les tumeurs sont habituellement de haut grade histologique.

- ✓ Les CS « HER2-like », caractérisés par la surexpression et l'amplification du gène HER2 (qui surexpriment l'oncoprotéine HER2, protéine appartenant à la superfamille des récepteurs à l'epidermal growth factor (EGF)) ; ils peuvent exprimer ou non les récepteurs aux œstrogènes et à la progestérone ;
- ✓ Les CS de phénotype « basal » (basal/myoépithélial se rapporte au nom donné à l'autre type de cellules du tissu mammaire normal), définis par l'absence d'expression des récepteurs hormonaux, et de l'oncoprotéine HER2 ; ils sont plus connus sous le nom de « triple négatifs » (associés à une faible survie). Ils expriment un certain nombre de gènes des cellules basales de l'épithélium ;
- ✓ Les CS dits « normal breast-like », caractérisés par un profil triple négatif et par l'expression de gènes observés dans le tissu mammaire normal et le tissu adipeux.

Cette identification de l'hétérogénéité tumorale a permis d'établir des facteurs pronostiques basés sur des caractéristiques génétiques, de prédire la réponse aux traitements anticancéreux, d'adapter la thérapeutique et d'identifier de nouvelles cibles moléculaires et ainsi développer de nouveaux traitements ciblés.

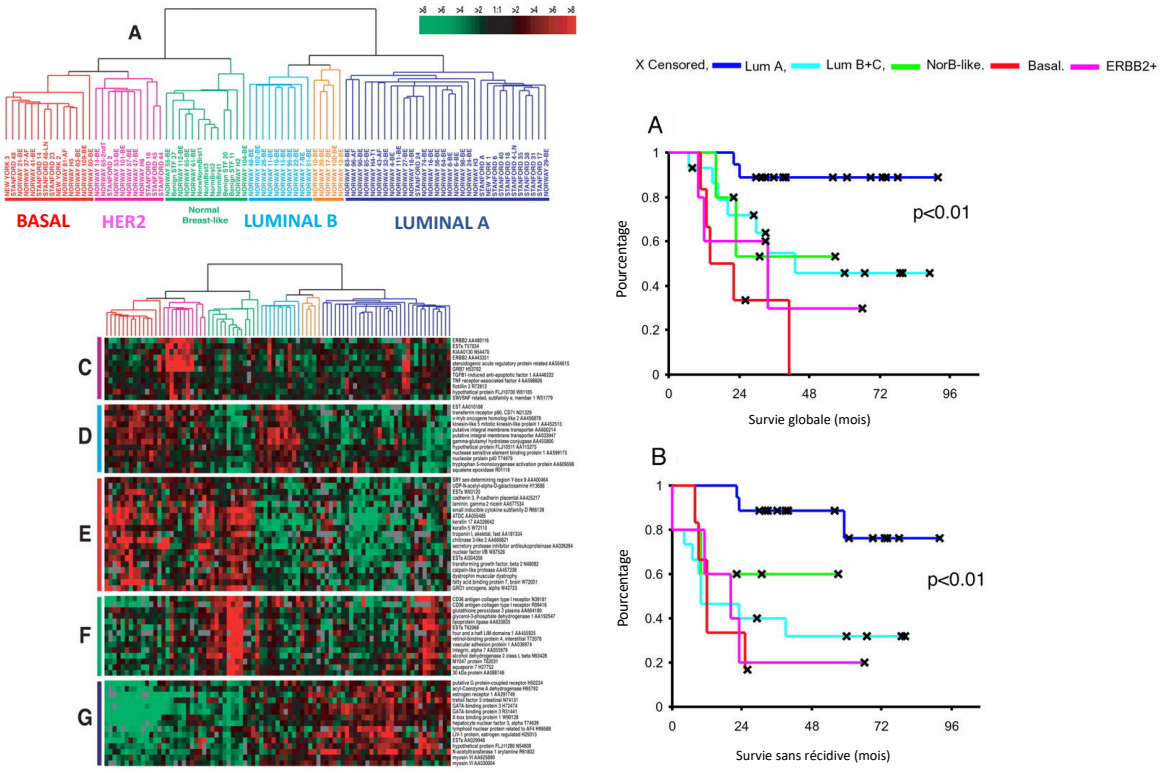

Figure 3 : Classification moléculaire intrinsèques des cancers du sein réalisée à partir d'une analyse transcriptomique par puces à ADN (microarrays). On distingue 5 sous-types présentant des profils d'expression génique différents et des pronostics différents. (*Adapté et traduit à partir des publications de Perou et al, Nature, 2000 et Sorlie et al, PNAS, 2001* (28,29)).

5. Prise en charge

Le traitement d'un cancer du sein repose sur la chirurgie, la radiothérapie, la chimiothérapie (incluant les thérapies ciblées) et l'hormonothérapie en fonction du statut des récepteurs hormonaux. Les traitements proposés dépendent des données cliniques (taille de la lésion, taille du sein, atteinte ganglionnaire ou à distance, stade TNM) et histologiques et moléculaires. L'indication des différentes thérapeutiques ainsi que la séquence de traitement est toujours discutée de façon collégiale en réunion de concertation pluridisciplinaire. Les grands principes de traitement (d'après le référentiel de traitement 2016/2017 coordonné par l'Institut Curie et l'Institut Gustave Roussy) sont présentés ci-dessous (30).

Le traitement chirurgical comprend la résection chirurgicale de la tumeur primitive qui peut être réalisée par mastectomie partielle conservatrice ou mastectomie totale et une évaluation du statut ganglionnaire axillaire par la technique du ganglion sentinelle (GS) en cas d'exploration axillaire préthérapeutique négative (clinique, échographique +/- cytoponction) et de petite tumeur (T1 ou T2) ou par curage axillaire dans les autres cas, ou en cas de non-détection du GS ou de GS macrométastatique. Une radiothérapie adjuvante est indiquée systématiquement en cas de mastectomie partielle et selon des critères cliniques et histologiques en cas de mastectomie totale. La radiothérapie des aires ganglionnaires dépend également de certains critères cliniques et histologiques. Une chimiothérapie peut être administrée de façon adjuvante ou néo-adjuvante. Elle est fréquente pour les tumeurs HER2 amplifiées (HER2-like) ou triple-négatives, chez les patientes jeunes et en cas de critères histo-pronostiques péjoratifs (prolifération importante évaluée par l'index KI67, grade élevé, présences d'emboles vasculaires). Pour les tumeurs RH+, HER2 non amplifiés, la chimiothérapie est discutée en fonction du grade histologique, de la taille, du statut ganglionnaire, de la présence d'emboles vasculaires ou d'une prolifération tumorale élevée et, dans certains cas, de tests génomiques. Une hormonothérapie est indiquée en cas d'expression significative des récepteurs hormonaux par les cellules tumorales (seuil > 10%). Il s'agit d'antagonistes des récepteurs aux œstrogènes comme le tamoxifène ou le fluvestrant ou d'inhibiteurs de l'aromatase chez les patientes ménopausées comme le létrozole ou l'anastrozole. %). Le traitement est administré par voie orale pour une durée recommandée de 5 ans. Enfin, le trastuzumab, un anticorps monoclonal recombinant spécifique des récepteurs HER2 est proposé en cas d'une amplification d'HER2, pour une durée totale de 1 an en situation non métastatique.

Dans les formes métastatiques d'emblée (5% des malades) ou secondairement, le traitement repose sur la chimiothérapie et/ou sur l'hormonothérapie ou des thérapies anti-HER2, selon le profil du

carcinome. Plusieurs lignes de chimiothérapies peuvent être entreprises. Un traitement local par chirurgie et/ou radiothérapie peut également être discuté selon la réponse aux traitements entrepris ou en cas de nécessité d'obtenir un contrôle local (pour les lésions bourgeonnantes ou nécrotiques afin d'éviter les complications infectieuses par exemple).

6. Facteurs pronostiques

Il existe plusieurs facteurs pronostiques cliniques et anatomopathologiques qui permettent d'estimer le risque de récidive et de décès et donc d'adapter les thérapeutiques.

- ✓ L'âge jeune avant 40 ans est associé à des formes souvent plus avancées et plus agressives et présentent des survie sans récidive et survie globale plus faibles (31,32).
- ✓ La taille tumorale élevée qui est directement corrélée au risque d'atteinte ganglionnaire (33,34).
- ✓ Le statut ganglionnaire est un facteur pronostique indépendant et la survie globale et sans récidive diminue avec le nombre de ganglions envahis (35,36). On notera que la présence de cellules tumorales isolées ou de micrométastases (taille <2mm) au sein du ganglion est précisée spécifiquement maintenant sur le compte rendu anatomopathologique car les implications cliniques sont différentes (geste chirurgical axillaire et indications de chimiothérapie).
- ✓ Le type histologique, les plus agressifs étant les carcinomes micropapillaires et les carcinomes métaplasiques.
- ✓ Les caractéristiques anatomopathologiques suivantes : grade élevé, présence d'embols lymphovasculaires, absence d'expression du récepteur aux œstrogènes et surexpression de HER2. Les cancers du sein appelés triple négatifs (ou « basal-like » selon la classification

moléculaire) sont les plus agressifs, le plus souvent de haut grade. Ils atteignent plus souvent des patientes jeunes, parfois associées à des mutations génétiques. Ils sont rarement diagnostiqués par les examens mammographiques de dépistage car ils se développent rapidement (c'est ce que l'on appelle des cancers d'intervalle, entre 2 examens de dépistage normaux). Ils ont une dissémination hématogène rapide qui favorise le risque de survenue de métastases, ou diagnostic ou au cours de suivi. Enfin, même si ces tumeurs sont initialement chimiosensibles, on assiste souvent à un échappement thérapeutique (avec la nécessité de changer de classe thérapeutique) et le taux de récidive est élevé (37,38).

II. Les Polluants Organiques Persistants (POP)

1. Définitions

Les POP sont des substances chimiques qui répondent aux propriétés suivantes :

- ✓ Toxicité prouvée avec un impact sur la santé humaine
- ✓ Résistance aux dégradations biologiques naturelles et persistance dans l'environnement
- ✓ Bioaccumulation dans les tissus vivants
- ✓ Transport longue distance (possibilité de se déposer loin de leur d'émission).

Parmi les plus représentés et les plus étudiés, on peut citer les dioxines, les polychlorobiphényles (PCB), les hydrocarbures aromatiques polycycliques (HAP) et certains pesticides (notamment les organochlorés, désormais interdits au sein de l'union européenne). Ils sont produits et rejetés dans l'environnement par les activités humaines. Certains étaient fabriqués dans un cadre industriel et commercial (ex : PCBs), d'autres involontairement du fait des processus de synthèse (ex : dioxines, voir ci-dessous). Leur accumulation se fait préférentiellement dans certains tissus comme le foie, le système nerveux ou le tissu adipeux (en raison de leur lipophilie) (1).

Je détaillerai plus particulièrement les dioxines car il s'agit du polluant étudié dans la partie expérimentale de ce travail de thèse.

La famille des dioxines regroupe un ensemble de composés structurellement et chimiquement apparentés, appartenant à la famille des HAP chlorés. A ce jour, il existe 210 types de dioxines, dont 17 sont considérées comme ayant une toxicité importante sur l'homme. Parmi celles-ci, la TCDD (2,3,7,8-tétrachlorodibenzo-p-dioxine ou dioxine de Sévéso), un perturbateur endocrinien reconnu, est considérée comme la plus toxique.

Les principales sources de production de dioxines sont l'incinération de déchets et les combustions incomplètes, industrielles ou naturelles. Les dioxines peuvent ainsi provenir de gaz d'échappement

de voitures, de la fumée de cigarette, de la combustion de bois, des éruptions volcaniques, de la production de pesticides organochlorés, du traitement du papier. Les sources primaires d'exposition aux dioxines sont les graisses alimentaires, notamment les produits laitiers, le poisson et la viande (du fait de leur accumulation progressive dans l'échelle alimentaire). Les résidus de dioxine existent à des teneurs très faibles, de l'ordre du picogramme/g, dans tous les produits d'origine animale quel que soit le pays considéré. C'est dans le sol, les sédiments et les animaux que la teneur y est la plus forte. Plus de 90% de la contamination de dioxines se fait par ingestion, absorption puis digestion d'aliments contaminés. Un schéma récapitulant les sources de dioxines et les voies de contaminations pour l'homme sont présentées Figure 4.

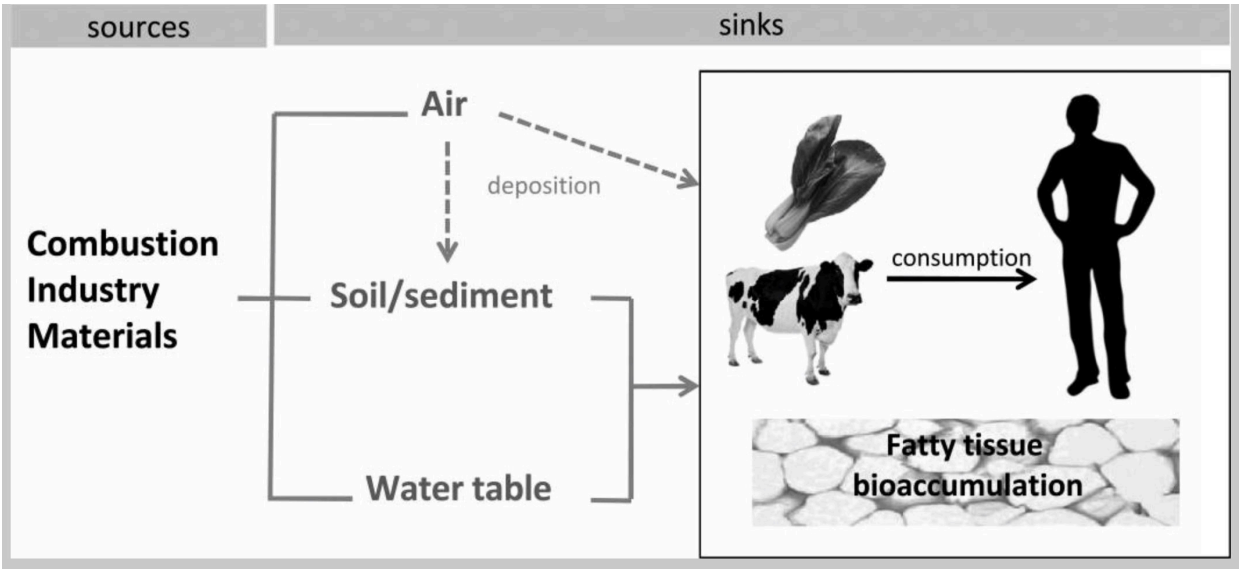

Figure 4: Schéma récapitulant les sources de dioxines et voies de contamination pour l'homme *(D'après Rysavy, J Appl Toxicol, 2013 (39)).*

Les dioxines sont des molécules hydrophobes et chimiquement stables, ce qui explique leur forte persistance dans l'environnement une fois qu'elles y sont « installées » ou stockées. Dans l'organisme, du fait de leur lipophile, leur accumulation après ingestion se fait préférentiellement au niveau des tissus adipeux avec une demi-vie qui varie considérablement en fonction des animaux

considérés (7,1 ans chez l'Homme). On a pu observer ainsi que chez un patient obèse, la quantité de POP est 3 fois plus élevées que chez un patient sain (les concentrations adipocytaires étant proches) (1). Les interactions entre tissu adipeux et POP sont nombreuses et seront détaillées dans un prochain chapitre.

2. Métabolisme des xénobiotiques

Les POP sont des xénobiotiques c'est-à-dire des substances étrangères à l'organismes qui doivent être métabolisés et éliminés par l'organisme, principalement dans le foie. De faible poids moléculaire (<1000Da), ils sont capables de passer la membrane cellulaire, de s'accumuler dans la cellule et d'avoir des effets néfastes. Au cours de leur évolution, les organismes se sont adaptés afin de favoriser l'efflux d'un très grand nombre de ces xénobiotiques indésirables, qui sont en général des molécules hydrophobes. Dans la cellule, ils sont reconnus par des récepteurs nucléaires comme le Constitutive Androstane Receptor (CAR), le Pregnane X Receptor (PXR) ou l'Aryl hydrocarbon Receptor (AhR), qui sont activés et agissent comme des facteurs de transcription pour réguler l'expression d'enzymes du métabolisme des xénobiotiques et les transporteurs des xénobiotiques. Les xénobiotiques sont alors activés par des enzymes de phase I comme par exemple les *cytochromes P450* (CYP), puis conjugués par les enzymes de phase II qui ajoutent un composé hydrophile (comme par exemple les *glutathion S-transférases*) et enfin sont expulsés hors de la cellule à travers des transporteurs actifs (ATP dépendants) de phase III (dont le *multiple drug resistance* MDR) ce qui permet leur élimination dans les fluides biologiques (sueur, urine, selles) (Figure 5).

Les POP sont bel et bien des xénobiotiques activant le métabolisme décrit ci-dessus mais une de leurs particularités est de ne pas être reconnus par le système enzymatique activé. De ce fait, ils conservent leur lipophilie et s'accumulent dans les tissus gras.

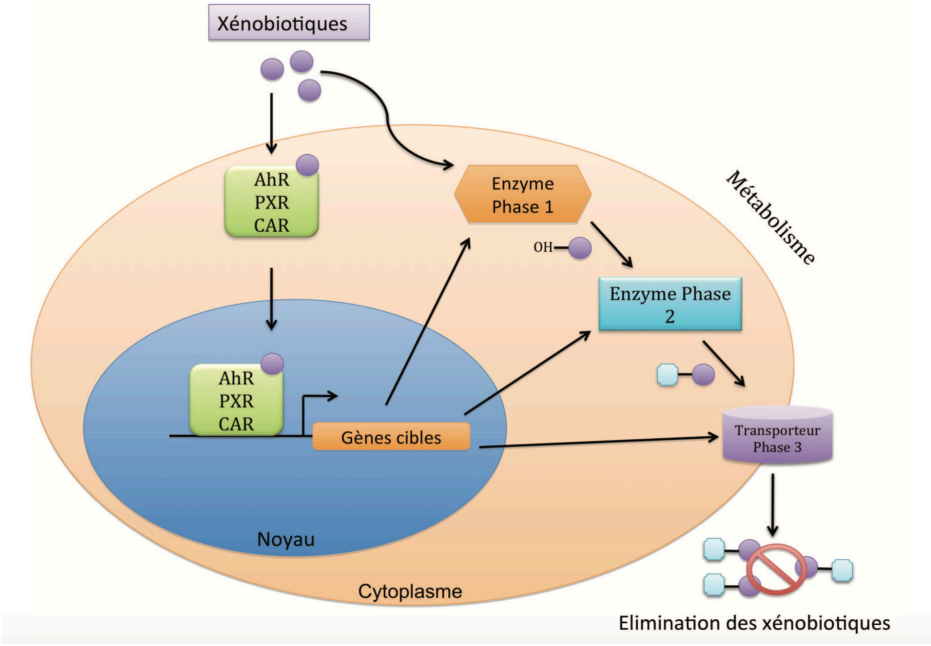

Figure 5 : Schéma simplifié du métabolisme des xénobiotiques (*d'après Aline Chevalier* (40)
AhR : « Aryl hydrocarbon Receptor », PXR : « Pregnane X Receptor », CAR : « Constitutive Androstane Receptor ».

3. L'Aryl hydrocarbon Receptor

L'AhR est un facteur de transcription hautement conservé découvert par Poland en 1976 (41). Il est détecté chez presque tous les vertébrés et est présent dans de nombreux tissus comme la glande mammaire, le foie, le système nerveux central, le système cardiovasculaire, et l'utérus. Communément appelé récepteur aux « dioxines », il possède en fait de nombreux ligands exogènes, dont les polychlorodibenzo-p-dioxines (PCDD), les polychlorodibenzo-p-furanes (PCDF) et certains PCB (dits « dioxin-like » ou DL) mais aussi des ligands endogènes comme les dérivés du tryptophane dont les rôles et les modes d'actions ne sont pas totalement élucidés aujourd'hui.

Le AhR est localisé dans le cytoplasme de la plupart des cellules, complexé à un certain nombre de protéines chaperones (Heat shock protein 90 (Hsp90), p23, et X-associated protein (XAP)), dans une conformation lui conférant une haute affinité pour ses ligands. Après fixation à ses ligands, le complexe se dissocie et le AhR est transloqué dans le noyau où il forme un hétérodimère avec son partenaire AhR Nuclear Translocator (ARNT). Le AhR et ARNT appartiennent à la même famille de protéines « basic-helix-loop-helix-Per ARNT Sim» (bHLH/PAS). Ils ont des structures similaires, incluant un domaine bHLH de liaison à l'ADN et d'hétérodimérisation, ainsi qu'un domaine PAS, qui, seulement dans le cas du AhR, est un domaine de liaison au ligand contribuant à la formation de l'hétérodimère. Cet hétérodimère se lie à des séquences spécifiques d'ADN, appelées XRE (Xenobiotic Responsive Element) présents dans des promoteurs de gènes cibles. Le complexe AhR-ARNT interagit avec des co-activateurs permettant le recrutement de l'ARN polymérase II et la régulation de la transcription de ces gènes cibles. La voie de transduction du AhR est présentée de façon schématique Figure 6.

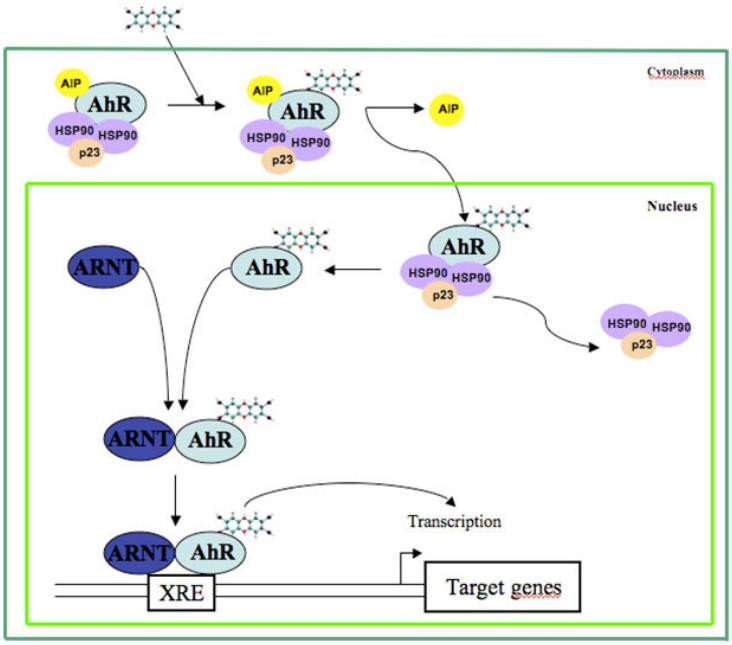

Figure 6 : Voie de transduction du AhR

Outre son rôle connu dans la régulation du métabolisme des xénobiotiques, l'AhR apparait désormais intervenir également dans des processus biologiques et physiopathologiques très variés comme l'inflammation (42) , la régulation métabolique (43), la régulation du système immunitaire (44), les interactions hôte-microbiome ou la progression du cancer (45).

4. Rôle des polluants et toxiques environnementaux dans la progression tumorale dans le cancer du sein

Il existe de plus en plus de preuves épidémiologiques et de données dans la littérature en faveur d'un lien entre l'exposition aux produits chimiques et les polluants environnementaux et la cancérogénèse mammaire (24). Plus récemment, la question du rôle de l'exposition à des perturbateurs environnementaux, non pas dans l'initiation du cancer mais dans la progression tumorale et la formation de métastases a été soulevée. Plusieurs études ont suggéré en effet que les POP et autres produits chimiques auxquels nous sommes exposés de façon chronique, pourraient favoriser l'invasion tumorale et la cascade métastatique (46).

Dans ce travail de thèse, j'ai réalisé une revue de la littérature sur le rôle des contaminants de l'environnement au sens large (POP, perturbateurs endocriniens, alimentation, alcool, tabac, métaux lourds) dans la progression tumorale, le développement de métastases dans le CS ainsi que l'acquisition d'une chimiorésistance par les cellules carcinomateuses mammaires.

Ce travail est présenté dans l'article 1.

ARTICLE 1

<u>Synthèse de l'article 1</u> : "Environmental pollutants /disruptor chemical in cancer progression and drug resistance in breast cancer."

La présence ou survenue de métastases et plus encore l'échappement thérapeutique avec l'acquisition progressive d'une chimiorésistance sont deux phénomènes résultant en un pronostic très sombre chez les patientes atteintes de CS.

Nous avons donc cherché à savoir si les contaminants environnementaux pouvaient avoir un rôle dans ces 2 phénomènes et par quels mécanismes.

Cette revue de la littérature inclut tous les articles expérimentaux et épidémiologiques référenciés sur PubMed, en anglais et sans limite de date, s'intéressant au rôle des POP et produits toxiques environnementaux dans la progression tumorale, l'invasion et la formation de métastases dans le CS, ainsi qu'à l'acquisition d'une résistance à la chimiothérapie par les cellules carcinomateuses mammaires sous l'effet de toxiques.

Dans mes recherches, 2 mécanismes cellulaires impliqués dans la progression tumorale étaient fréquemment cités en tant que cibles des polluants environnementaux : la transition-épithélio-mésenchymateuse (TEM) et l'acquisition par les cellules tumorales d'un caractère « cellule souche » ou « stem-cell » (CSC).

La transition épithélio-mésenchymateuse caractérise un processus dynamique au cours duquel les cellules perdent un nombre important de leurs caractéristiques épithéliales pour gagner des propriétés de cellules mésenchymateuses (Figure 7)(47). Longtemps controversé, le rôle de la TEM dans la progression tumorale et la dissémination métastatique des carcinomes est maintenant admis (48,49). Ce processus nécessite des modifications architecturales et fonctionnelles complexes : une perte des jonctions intercellulaires, une dépolarisation des cellules épithéliales et l'acquisition de

capacités de motilité cellulaire (50,51). Ce phénomène, dynamique et réversible (d'autant plus important pour mettre l'ancrage des cellules migrantes au niveau du site secondaire), est régulé par un ensemble de signaux issus du microenvironnement, comme les cytokines pro-inflammatoires ou le TGFβ, entrainant l'activation de voies de signalisation conduisant à l'expression de multiples facteurs de transcription. Ces facteurs de transcription, incluant les protéines bHLH de la famille Twist (Twist1, Twist2) et des facteurs de transcription à doigts de zinc de la famille Snail (Snail1/Snail, Snail2/Slug) et de la famille Zeb (Zeb1, Zeb2/Sip1), contrôlent l'expression de gènes impliqués dans le contrôle des interactions cellules-cellules, la différenciation et la motilité cellulaires. Or de nombreux polluants environnementaux et produits chimiques, dont la dioxine de Sévéso sont capables de réguler l'expression de ces facteurs de transcription et les voies de signalisation associées.

Par ailleurs, il a été montré que l'initiation d'un processus de TEM était souvent associée à l'acquisition par les cellules transformées de caractéristiques et de propriétés de « cellules souches cancéreuses » (CSC) (52,53).

Les « cellules souches cancéreuses » sont une classe de cellules pluripotentes observées dans la plupart des cancers solides et hématologiques. Les CSC constituent un faible pourcentage (0,05 à 1%) de cellules tumorales dans la masse tumorale. Ils n'en demeurent pas moins essentiels pour la tumeurs : en effet, ils expriment des gènes marqueurs de cellules souches, notamment Oct4 (octamer-binding transcription factor 4), Sox2 ((sex determining region Y)-box 2), Nanog, c-kit (tyrosine-protéine kinase kit), ABCG2 (ATP-binding cassette super-family G member 2) et ALDH (aldéhyde déshydrogénase). Ces cellules possèdent une capacité d'auto-renouvèlement qui les rend immortelles. Elles sont impliquées dans le développement tumoral, la prolifération cellulaire, la dissémination métastatique et la résistance à la chimiothérapie et à la radiothérapie (54–56).

Plusieurs études ont montré que l'exposition chronique à des toxiques pouvaient avoir un rôle dans l'émergence, l'auto-renouvèlement et le maintien des CSC.

Cette revue de la littérature est donc structurée en 3 parties : la première est une brève revue des mécanismes biologiques impliqués dans les processus métastatiques en mettant l'accent sur ces deux mécanismes pré-cités, la seconde est la revue systématique à proprement parlé et la dernière est une discussion sur les voies de signalisations sous-jacentes et le rôle, encore discuté, de l'AhR dans la progression tumorale.

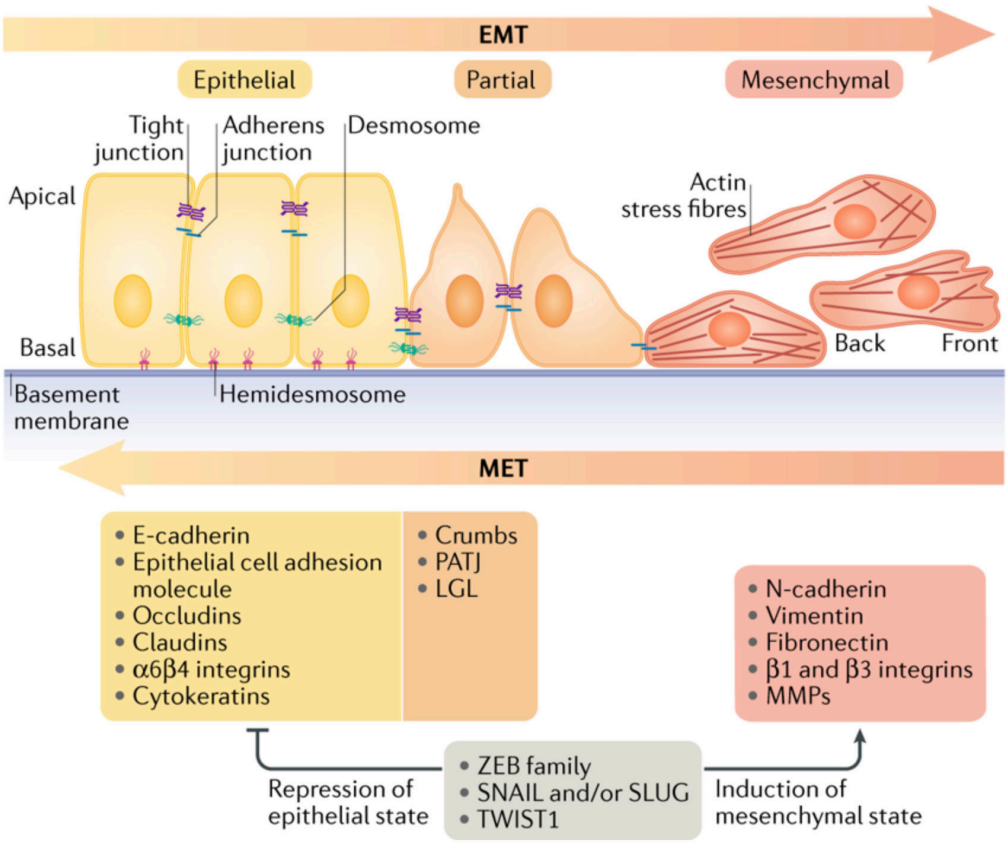

Figure 7 : Processus de transition épithélio-mésenchymateuse.

Les cellules épithéliales présentant une polarité apicale-basale sont maintenues ensemble par des jonctions serrées, des jonctions adhérentes et des desmosomes et sont attachées à la membrane basale sous-jacente par des hémidesmosomes. Ces cellules expriment des marqueurs épithéliaux et des molécules qui participent au maintien de la polarité cellulaire (énumérées dans les cases jaune et orange clair, respectivement). L'induction de l'EMT conduit à l'expression des facteurs de transcription ZEB, SNAIL et TWIST, qui inhibent l'expression des gènes associés à l'état épithélial et activent de manière concomitante l'expression de gènes associés à l'état mésenchymateux (énumérés dans la case orange foncé). L'EMT est un processus réversible. Les cellules migrantes peuvent subirent une transition mésenchymato-épithéliale (MET) ce qui permet leur ancrage au niveau du site secondaire. *(Adapté de Dongre et Weinberg, Nature Reviews Molecular Cell Biology, 2019 (49)).*

Title: Environmental chemicals, breast cancer progression and drug resistance.

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Abstract: 250 words

Breast cancer is one of the most common causes of cancer in the world and the second leading cause of cancer deaths among women. Mortality is mainly associated with the development of metastases. Identifying the mechanisms involved in the appearance of these elements is therefore a major public health issue. Among the proposed risk factors, the role of the chemical environment and pollution is increasingly suggested as having an effect on the signaling pathways involved in the appearance and progression of metastatic tumor cells. The purpose of this article is to summarize current knowledge about the role of environmental chemicals in breast cancer (BC) progression and metastasis formation. First, we will describe two main mechanisms suspected of playing a key role in the development of metastases and recently showed to be triggered by contaminants: epithelial-to-mesenchymal transition and cancer-stemness. Then through a systematic review, we will highlight the effects of a wide variety of environmental toxicants including persistent organics pollutants and endocrine disruptors on invasion mechanisms and metastatic processes in BC and finally discuss the recently described pro-migratory and pro-invasive Ah receptor signaling pathway.

Introduction

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Link between environmental chemicals and chemoresistance

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7,12-Dimethylbenz(A)Anthracene

5. *Polycyclic aromatic carbons*

Cigarette smoke

7,12-Dimethylbenz(A)Anthracene

6. *Alcohol*

7. *Heavy Metals*

III. Role of AhR in cancer progression and metastasis

Conclusion

Introduction

The high and increasing incidence of breast cancer (BC) in women is a major public health concern. Even if the prognosis is excellent when the cancer is located in breast (5-year rate survival: 99%), survival rates decrease rapidly in case of disseminated disease (26%, if distant metastasis [1, 2]. Moreover, drug-resistance to chemotherapy especially in some subtypes of BC, brings great challenge to clinicians in improving survival of BC patients. Cancer progression in the last phase of tumor development (which can occur in case of drug-resistance) defined by the increased growth speed and invasiveness of the tumor cells which leads the acquisition of metastatic potential in cancer cells. Metastasis involves the spread of cancer cells from the primary tumor to surrounding tissues and to distant organs. It is the primary cause of cancer morbidity and mortality, responsible for about 90% of cancer deaths [3]. Although many types of cancers are initially susceptible to chemotherapy, over time they can develop resistance through mechanisms, such as DNA mutations and metabolic changes that promote drug inhibition and degradation [4].

Environmental chemical exposures are ubiquitous. During our lives, we are exposed to many known toxicants as well as a number of potentially hazardous chemicals with less well-characterized risks. Plastic food and beverage containers, cosmetics, sunscreen, cleaning products, and garden products all contain chemicals. Chemical pesticides are used in many commercially grown fruit, vegetable, and grain crops. Moreover, several chemicals are defined as persistent organic pollutants (POPs) for their resistance to degradation, their environmental persistence and their bioaccumulation in the food chain. They have been banned about decades in most countries because of human health concerns but are still accumulated in soils, sediments, air and biota because of long half-lives but humans are still exposed to these chemicals through several routes [5].

Increasing evidence from epidemiological studies, as well as a better understanding of mechanisms linking toxicants with development of cancer, all suggest that exposures to some environmental chemicals found in common products may lead to increased risk of developing cancer. More recently, the role of exposure to low-doses of environmental pollutants, not only in cancer initiation but also in cancer progression, has been raised by several studies suggesting that these chemicals may promote cancer invasion and metastasis [6]. Thus the purpose of this review is to summarize the main findings related to the role of environmental contaminants on promotion of invasion and metastasis in BC and their link with chemoresistance. This review is structured first with a brief review of biological mechanisms involved in metastatic process with a focus on epithelial-mesenchymal transition and cancer-stemness, two mechanisms recently identified to be triggered by contaminants and linked to chemoresistance and, secondly a

systematic review of experimental studies and the evidence for chemicals associated with BC cancer progression and metastasis. Finally, an overview on the pathways triggered by the aryl hydrocarbon receptor (AhR), a major transcription factor and its role on the stages of cancer progression will be discussed.

I. Epithelial-to-mesenchymal transition and cancer stem cells: 2 critical steps in cancer progression and chemoresistance

Cancer metastasis is the spread of cancer cells to distant tissues and organs. The formation of metastases constitutes a complex process of molecular and biochemical events performed by multiple actors and called the "invasion-metastasis cascade". The metastatic cascade requires that tumor cells first detach from the primary tumor, allowed by the loss of epithelial proteins, invade through extracellular matrix, intravasate into the bloodstream and/or the lymphatic circulation, migrate to a target site with specific adhesion properties, attach to target endothelium, extravasate and invade into the target tissue, and establish tumor growth including vascularization [7]. Among the mechanisms involved in cancer metastasis, two critical pathways have been particularly linked to the exposure to low-dose doses of environmental pollutants (toand resistance to anticancer drugs: the epithelial-to-mesenchymal transition (EMT) and the emergence and progression of cancer stem cells (CSCs).

Epithelial-to-mesenchymal transition

EMT is a biological process by which differentiated epithelial cells lose epithelial characteristics and acquire a migratory, mesenchymal phenotype. It occurs during normal embryonic development or tissue regeneration. However, this process is also involved in tumor progression, cancer cells invasion and metastasis; more specifically, the generation of tumor cells is accompanied with the acquirement of stem cell properties that play a major role in resistance to cancer treatment [8–10]. Cancers cells undergoing EMT sustain important morphological changes induced by signal transduction pathways that reduce E-cadherin expression (one of the major cell adhesion proteins), drive the disassembly of intercellular adhesion complexes, and promote actin stress fiber and focal adhesion formation. These types of cellular changes result in the phenotypic transition to an elongated, mesenchymal cell that expresses extracellular matrix-remodeling enzymes and has an increased capacity for migration and invasion. Loss of E-cadherin expression, one of the most frequently used markers for EMT, is associated with a more invasive phenotype in many types of human carcinomas [11, 12]. We show in a previous work that 2,3,7,8-Tetrachlorodibenzodioxin (TCDD), the

most studied and most toxic of all dioxins, decrease E-cadherin expression and favors EMT and cell migration *in vitro* through the activation of the xenobiotic receptor named aryl hydrocarbon receptor (AhR) [13, 14]. Several other studies using different cancer cell lines suggested that exposure (sometimes low-doses ones) of various environmental chemicals, could trigger EMT, including benzo(a)pyrene (BaP), a polycyclic aromatic hydrocarbon (PAH), an AhR agonist, produced by incomplete combustion of organic components [15], nicotine [16–18], heavy metals as cadmium and chromium[19, 20], pesticides [21] or other endocrine-disrupting chemicals [5]. The literature is prolific and shows that environmental and synthetic chemicals have the potential to induce cancer metastasis through regulation of EMT markers and activation of migratory processes. Those toxics are able to regulate many transcription factors, including Snail, Slug, ZEB1, ZEB2, and Twist and several signaling pathways mediated by transforming tumor growth factors ß (TGFß), the Wnt/ß-catenin, Notch, Hedgehog, NFkappaB pathways and tyrosine kinases- receptor. Interestingly, several of them have been shown to be crucial for the regulation of the self-renewal and the maintenance of CSCs[22].

Cancer stem cells emergence and progression

Cancer stem cells are a class of pluripotent cells that have been observed in most of solid and hematologic cancers. CSCs constitute a small percentage (0.05-1%) of tumor cells within the tumor mass. They express stem cell marker genes, including Oct4 (octamer-binding transcription factor 4), Sox2 ((sex determining region Y)-box 2), Nanog, c-kit (tyrosine-protein kinase Kit), ABCG2 (ATP-binding cassette super-family G member 2), and ALDH (aldehyde deshydrogenase). These cells possess a capacity for self-renewal that makes them immortal and it is now clear that they are involved in tumor development, cell proliferation, metastatic dissemination and resistance to chemotherapy and radiotherapy[23–25]. Accumulating evidence has suggested that CSCs are the ones that exhibit invasive properties, support the formation of metastasis and contribute to cancer recurrence. The identification of CSCs and their recognition as a major actor in cancer progression through their invasive properties have constituted a landmark discovery in cancer research and specifically in cancer therapy with promising CSC-targeting drugs preclinical and clinical studies in progress [26].

The involvement of chronic exposure of current background exposure levels carcinogens in the regulation of self-renewal and maintenance of CSCs has been reported by several studies. Such molecules act on several cellular processes including the **emergence** and **proliferation** of CSCs: normal breast tissue is composed of epithelial cells, fibroblasts, adipocytes, blood vessels and stem cells (which are able to renew the tissue through aging). The

homeostasis of this tissue is maintained by the balance of several cytokines including Bone Morphogenetic Proteins, bone morphogenetic proteins (BMP) 2 and BMP4, which respectively act on the proliferation and the differentiation of breast luminal cells. Interestingly, BMPs have been implicated in late stages of tumorigenesis and metastasis. Chapellier et al. showed that bisphenol A (BPA) and bisphenol S are able to shift the balance of secreted BMP molecules in favor of BMP2 which promotes malignant transformation and regulate the emergence of a niche of mammary stem/progenitor cells with similar features to CSCs [27, 28]. This impact of xenobiotics on the emergence of CSC is also observed with cadmium which significantly induces the gene expression of CSC markers in the breast and liver cancer cell lineage and promotes the conversion of non-CSCs to CSC in MCF-7 and HepG2 cell lines [29]. Beyond breast CSCs, arsenic, a class I carcinogen, triggers the proliferative potential of epidermal keratinocytes, decreasing their exit from the germinative compartment under conditions that promote differentiation of untreated cells [30]. Moreover, CD34-positive cells isolated from a human skin keratinocyte line and exposed to arsenic appeared to contain proportionally more putative CSCs than normal stem cells isolated from control cells [31]. Finally, exposure to low doses of arsenic (over 18 weeks) is reported to transform human prostate epithelial stem/progenitor cells, WPE, into CSCs, a process linked to the downregulation of the tumor suppressor, phosphatase and tensin homolog expression (PTEN) [32–35]. This suggests that various EDCs are able to perturb human stem cell self-renewal and influence the acquisition of CSC-like properties by normal stem cells.

EDCs are also able to stimulate CSC proliferation: Benzyl butyl phthalate, a toxic phthalate used as plasticizer in diverse products, has been shown to promote breast CSCs through the activation of the AhR and the stimulation of sphingosine kinase 1 (SPHK1)/sphingosine 1-phosphate (SP1)/sphingosine-1-phosphate receptor 3 (S1PR3) signaling. In a mice xenograft model, BBP increased the incidence of lung metastasis and this effect was reversed with SPHK1 or S1PR3 knockdown tumor cells [36]. In addition, Hu *et al*, have shown that cultured prostaspheres composed of prostate stem/progenitor cells expressing estrogen receptors alpha and ß (ERa, ERb), and GPR30 (GPER1) proliferated in response to low-dose estradiol and BPA) increasing the number of stem-like cells in the side populations [37].

All these data support the fact that exposure to environmental chemicals promotes the cancer cells dedifferentiation to CSCs which have a crucial role in cancer progression. Therefore, the emergence of those sub-types of cancer cells in the primary tumor contributes to tumor heterogeneity, one of the main sources for resistance to chemotherapy.

Link between environmental chemicals and chemoresistance

Resistance to chemotherapy is an important issue in treating the most commonly seen solid tumors. Although many studies have been interested in the past in the role of pollutants in the initiation of cancer, and more recently in the invasion and metastasis process, to our knowledge only one recent paper has investigated the influence of environmental disruptor on the response of cancer patients to chemotherapy. Authors show that BaP reversed the effect of cisplatin, 5-flurouracil, and paclitaxel in the WHCO1 esophageal cancer cell line by reducing drug-induced cell death and apoptosis by 30–40% compared to drug-treated cells [38]. The mechanism(s) by which cancer cells develop chemoresistance is debatable with many reports implicating cancer stem cells, EMT and the tumor microenvironment [4, 39–41]. Considering the effects of environmental chemicals on such mechanisms, we examined the literature linking them to cancer chemotherapy resistance and found several papers suggesting a dispensable role of EMT and even more CSCs in cancer cells drug resistance. In 1992, Sommers et al. described the loss of epithelial markers and the acquisition of vimentin expression in Adriamycin- and Vinblastine-resistant human breast cancer cell lines and hypothesized that EMT cells have advantages in growth capabilities compared to non-EMT cells after drug treatment [42]. Since this first report, chemoresistance to different drugs such as oxaliplatin and paclitaxel was often linked with EMT and has been reported in several cell lines and animal models [43–48] but the subject doesn't reach consensus [8, 49, 50] and more experimental studies are needed to debate. Conversely, the association between stem-cell properties and resistance to chemotherapy is well established. In BC, a lot of signaling pathways in cells indirectly promote CSC-mediated chemoresistance increasing stemness property and self-renewal of CSCs [51–59]. The association between environmental chemicals and CSCs emergence described above call for exploring the potential direct link between pollutants and anti-cancer drug resistance.

II. Effect of chemicals on mechanisms of invasion and metastasis in breast cancer (Figure 1)

We present here a review of the evidence for chemicals associated with invasion and metastasis in BC as well as association with resistance to chemotherapy. We searched PubMed for peer-reviewed articles published before December 2018 that reported on experimental studies of BC, environmental pollutants and cancer spreading. Searches included "breast cancer" in combination with terms for specific chemicals, chemicals groups product classes used in a previous published review [60] and terms linked with cancer progression and aggressivity : "cancer proliferation", "cancer invasion", "cancer invasiveness", "cancer spreading", "metastasis formation". After removing duplicates, articles not focus on BC and articles interested in cancer initiation, we identified 66 relevant articles investigating the role of chemical in BC progression and finally included 53 *in vitro* and *in vivo* experimental articles for full-text review.

The information flow diagram of the study selection is presented in Figure 2. Main findings with suspected targeted signal pathway or mechanisms are summarized in Table 1.

1. Persistent Organics Pollutants

Dioxin

TCDD is one of the most potent carcinogens even tested and the most active congener within the group of AhR agonists. As describe previously, the role of AhR in cancer progression and metastasis is complex and still controversial. Although dioxin is clearly a potent tumor promotor, it has been suggested to have a protective effect for BC progression. It has been shown that the effects of AhR ligands on tumor growth are related to the ability of the receptor to antagonize ERα signaling. In both *in vitro* and *in vivo* models, dioxin was shown to completely reverse the proliferative effects of estrogens [61, 62]. Previous studies have demonstrated that dioxin can disrupt CXCL12/CXCR4 axis which has been shown to limit the metastasis of BC cells to the lung in mice. Dioxin down-regulates both the G-protein-coupled receptor CXCR4 and its unique chemokine ligand, CXCL12 in MCF7 cells and decrease cell migration toward a CXCL12 gradient [63]. Dioxin also suppress proliferation, invasiveness and colony formation *in vitro* through the AhR signaling pathway, regardless of ER status and promote differentiation in a BC stem cell line [64]. *In vivo*, dioxin was found to suppress metastasis by approximately 50%, in a xenograft model with no effect on cancer cells proliferation neither tumor growth [65]. In another study investigating the effects of a single non-toxic dose of dioxin on the development of mammary tumors in female Sprague-Dawley rats treated with an oral dose of 7,12-dimethylbenzanthracene (a tumor initiator), authors demonstrate that tumors volume in the controls were 3.9-fold increased after 21 days. In contrast, a second group of rats with mammary tumors treated with dioxin, the mean tumor volumes decreased from 89.7 ± 53 mm^3 to 24.9 ± 28.5 mm^3 [66]. However, in opposition to these anti-tumorigenic data, other interesting studies show that dioxin induces EMT, increases migration [14, 67] and induces mitochondrial dysfunction, stress signaling, and tumor invasion both *in vivo* and *in vitro* by a mechanism similar to that described for mtDNA-depleted cells, directly targeting mitochondrial transcription and induction of mitochondrial stress signaling [68].Those paradoxical results reported in the literature may reflect differences in tumor cell lines or assays but not only: numerous parameters could influence the behavior of such cells including dose and kinetics of cell culture or composition of the cell culture medium (and for example of competitive tryptophan-derivative AhR ligands).Further mechanistic studies are warranted to understand the pro- or anti-tumor effects of AhR agonists and antagonists and the contextual role of the AhR in those processes.

Polychlorinated Biphenyls (PCBs)

PCBs are a family of aromatic compounds used in industrial applications and electrical equipment until the early 1980s when they were banned in most countries because of human health concerns. However, due to their environmental persistence (as POPs), the general population may be exposed to PCBs from a variety of routes and sources, including diet, ambient air, occupational settings, and consumer products. PCBs (105 and 118) have been suggested to contribute to high-grade BC tumors and overall poor prognosis in BC patients [69]. PCBs enhance migration *in vitro* in MCF7 and MDA-MB-231 cells and tumor growth and bone, lung and liver metastasis development in mice by activating Rho-associated kinase (ROCK) [70]. Furthermore, growing evidence indicates that the direct adhesive interaction between cancer cells and endothelial cells is a critical step in metastasis formation. It has been shown that PCBs can activate vascular endothelial cells resulting in disruption of endothelial barrier function [71, 72]. PCBs induce proinflammatory reactions in human vascular endothelial cells by increasing oxidative stress. It results by the upregulation of the expression of genes encoding for monocyte chemoattractant protein-1 (MCP1), E-Selectin, intracellular adhesion molecule-1 (ICAM-1) and vascular endothelial cell adhesion molecule-1 (VCAM-1) which have been demonstrated to play an important role in brain metastasis formation [73]. In particular in BC, exposure of human microvascular endothelial cell 1 (HMEC-1) to PCB 104 induced endothelial hyperpermeability and markedly increased transendothelial migration of MDA-MB-231 cells [74]. These effects were associated with overexpression of vascular endothelial growth factor (VEGF). PCB 104-mediated elevation of VEGF expression was induced by phosphatidylinositol 3-kinase (PI3K) but not affected by co-treatments with antioxidants or the NF-kappaB inhibitor. In addition, the PI3K-dependent pathway was involved in PCB 104-induced activation of activator protein-1, a transcription factor implicated in the regulation of VEGF gene expression. The VEGF receptor (KDR/Flk-1) antagonist SU1498 and the PI3K inhibitor LY294002 inhibited PCB 104-induced endothelial hyperpermeability. The authors concluded that PCB 104 may contribute to tumor metastasis by inducing VEGF overexpression that stimulates endothelial hyperpermeability and transendothelial migration of cancer cells. The same laboratory used small interfering RNA and pharmacologic inhibitors to show that PCB 104 can activate epidermal growth factor receptor (EGFR) and Janus kinase 3 (JAK3) in a closely coordinated and cross-dependent fashion [75]. In this mechanistic study, activated EGFR and JAK3 stimulated in concert c-Jun NH(2)-terminal kinase and extracellular signal-regulated kinase 1/2 as well as increased DNA-binding activity of transcription factors activator protein-1 and polyomavirus

enhancer activator protein 3 which lead the transcriptional up-regulation of MMP-3 expression. These results indicate that the interplay among EGFR, JAK3, and mitogen-activated protein kinases may play an important role in PCB-induced MMP-3 expression and accelerated transendothelial migration of tumor cells.

2. DDE/DDT and other Organochlorine Pesticides

Organochlorine pesticides (OCPs) are synthetic organic compounds used worldwide as insecticides, herbicides, termiticides and fungicides for agricultural and residential use. While they are banned in the USA and Europe, they are still used in some countries and due to their long persistance in the environment, they are still contaminating food and soils. Epidemiological studies suggest that OCPs including dichlorodiphenyldichloroethane (DDE) and dichlorodiphenyltri-chloroethane (DDT) could influence relevant pathways involved in breast cancer progression and impact BC prognosis and survival [76–80]. For exemple, DDE exposure was associated with a dose-related increased relative risk of exhibiting both lymph-node involvement and a large tumor [81] and a very recent study found that higher DDE and DDT levels in blood were associated with worse overall survival [82]. Experimental data are limited but show that DDT supports hormone-dependent BC cells growth by disrupting the estrogen-androgen balance and opposing the androgen signaling pathway that inhibits growth in hormone-responsive breast cancer cells [83]. *In vitro*, the *o,p'*-DDT isomer (but not the p,p'-DDT), which does not bind to tumor ER, supports estrogen-responsive tumors growth in ovariectomized rats in a dose-response manner [84]. These findings indicate a possible association between OCPs and BC progression maybe through a non-genomic ER signaling pathway.

Hexachlorobenzene (HCB) is a widespread organochlorine pesticide used as a fungicide until the 70' and still released in several industrial processes. HCB was found to induce cell proliferation and insulin-like growth factor I (IGF-I) signaling pathway in MCF7 cells [85] and to activate c-Src/HER1/STAT5b and HER1/ERK1/2 signaling pathways and cell migration, in an AhR-dependent manner in MDA-MB-231 cells [86]. The same team showed that HCB (5µM) enhances MMP2/9 expression, secretion and activity associated to cell invasion, through the non-genomic AhR pathway involving c-Src activation and the HER1/EGFR pathway in MDA-MB-231 cells. *In vivo*, HCB (0.3 and 3mg/kg b.w.) enhances subcutaneous tumor growth in 2 murine models (MDA-MB-231 xenograft and C4-HI syngeneic model) [87]. Coherently to what is observed *in vitro*, the pesticide activated c-Src, HER1, STAT5b, and ERK1/2 signaling pathways and increased MMP2 and MMP9 protein levels. They also observed that HCB stimulated lung metastasis regardless of the tumor hormone-receptor status. Another study investigated the molecular effects of

TGF-β1 signaling and AhR/TGF-β1 crosstalk in MDA-MB-231 cells and found that HCB reduced AhR mRNA expression through TGF-β1 signaling but enhances TGF-β1 mRNA levels through AhR signaling [88]. TGF-β1 is a secreted growth factor which has notably been linked to EMT. In this study, at the protein level, HCB increased TGF-β1 levels and activation, as well as the phosphorylation of downstream effectors such as Smad3 but also JNK and p38. Authors showed that low and high doses of HCB had differential effects on AhR protein levels, localization, and activation, and that a high dose (5μM) induced AhR nuclear translocation and AhR-dependent CYP1A1 expression. Taken together, these findings suggest that c-Src and AhR are involved in HCB-mediated activation of Smad3, a mediator of TGF-β1 signaling, a pathway linked to EMT. Furthermore, HCB enhanced cell migration and invasion through the Smad, JNK, and p38 pathways, while ERK1/2 was only involved in HCB-induced cell migration.

3. Consumer product chemicals

Bisphenol A

The BPA is a xenoestrogen used in the manufacturing of polycarbonate plastics and epoxy resins. The major source of exposure for the general population is food packaging. Because of its widespread presence in the environment and its estrogenic activity both in vivo and in vitro, numerous studies have investigated the potential for adverse effects of BPA exposure on human health and suggest that BPA is significantly correlated with diseases such as diabetes, cardiovascular diseases, increased inflammation and cancer. The role of BPA in BC progression and resistance to chemotherapy has been suggested in several studies.

Bisphenol A was firstly described as a pro-estrogenic compound and it has been shown that exposure of breast cancer cells to BPA presents a gene expression profile of tumor aggressiveness associated with poor clinical outcomes for breast cancer patients [89]. Studies conducted in the MCF-7 cell line estrogen receptor-positive cells (ER+) showed that low levels of BPA significantly increased proliferation [90] and induces MCF-7 breast cancer cell proliferation through upregulation of genes that promote cell cycle and downregulation of anti-proliferative genes [91, 92]. However, BPA is a low-affinity ligand of the estrogen receptors and it has been shown, at environmentally relevant doses, to antagonize the cytotoxicity of multiple chemotherapeutic agents (doxorubicin, cisplatin, and vinblastine) in both ER-alpha-positive and -negative breast cancer cells independent of the classical ERs, by increasing expression of antiapoptotic proteins. The authors showed that both cell types express alternative ERs, including G-protein-coupled receptor 30 (GPR30) and members of the estrogen-related receptor family [93]. The involvement of these alternative

targets of ERs, was confirmed by Castillo Sanchez et al. who showed that BPA promotes migration, invasion and increase the number of focal contacts in MDA-MB-231 breast cancer cells through a GPER-dependent pathway [94]. This proposed pathway is still a matter of controversy as Zhang and al. described that nanomolar concentrations of BPA can increase wound closure and invasion of both BC cell lines MDA-MB-231 and BT-549 cells through increased expression of both protein and mRNA levels of matrix metalloproteinases 2 (MMP2) and MMP9, while it displays no effect on the expression of vimentin and fibronectin in triple negative breast cancer (TNBC) cells. The expression of GPR30, which has been suggested to mediate rapid estrogenic signals, was not modified in BPA-treated MDA-MB-231 and BT-549 cells. Its inhibitor G15 also had no effect on BPA-induced MMPs expression and cell invasion suggesting that GPR30 is not involved in all BPA effects [95]. However, the BPA effects may be mediated through its high-affinity nuclear receptor named estrogen-related receptor γ (ERRγ) (but not ERRα or ERRβ); indeed, the knock-down of ERRγ can markedly attenuate BPA-induced expression of MMP-2 and MMP-9 [96]. Also, BPA treatment can activate both ERK1/2 and Akt in TNBC cells. Both inhibitors of ERK1/2 and Akt can attenuate BPA-induced ERRγ expression and cell invasion of MDA-MB-231 cells. Collectively, BPA appears to increase the expression of MMPs and in vitro motility of TNBC cells via ERRγ. Both activation of ERK1/2 and Akt also participated in this process. To conclude, Castillo-Sanchez et al. also characterized that BPA induces activation of FAK, Src, and ERK2, three mediators which activate cell migration. BPA also induces an increase on AP-1- and NFκB-DNA binding activity through an Src- and ERK2-dependent pathway [94].

Phtalates

Phtalates are one of the more abundant synthetic chemical contaminants. Phthalates including butyl benzyl phthalate (BBP), di(n-butyl) phthalate (DBP), and di(2-ethylhexyl) phthalate (DEHP) are used as plasticizers, and also widely used in food wraps and cosmetic formulations. They are suspected to be endocrine-disrupting chemicals and were shown to increased cell proliferation [54], tumor mobility, and invasiveness of tumor cells [97]. Phthalates are also suspected to cause the proliferation and metastasis of BC cells and tumor progression via up-regulating histone deacetylase 6 (HDAC6) [98]. In this study, phthalate-induced HDAC6 expression was mediated by the ER-alpha/EGFR/PKA/AP-2a pathway and lead to vimentin expression that involved Akt, GSK3β, and β-catenin signaling cascade. The same team has shown that the effects of DBP and BBP on HDAC6 gene expression were mediated through the nongenomic pathway of the AhR in ER-negative breast cancer cells (MDA-MB-231). They indicated that

phthalates stimulated the AhR located at the proximity of the cell membrane and triggered the downstream cyclic AMP (cAMP)-PKA-CREB1 signaling cascade [99]. Phthalates can also activate peroxisome proliferator activated receptors (PPARs) that may eventually lead to high proliferation of MCF7 cells [100]. Furthermore, phtalates were shown to affect the sensibility to tamoxifen, suggesting a role in chemotherapeutic drug resistance. They can inhibit tamoxifen-induced apoptosis in ER-positive MCF-7 cells (but not in ER-negative cells) [101].

Benzophenone / Nonylphenol

Benzophenone-1 (2,4-dihydroxybenzophenone, BP1) and nonylphenol (NP), which are discharged from numerous industrial products (lubricating oil additives, laundry and dish detergents, emulsifiers…) are known EDC defined as environmental compounds that produce human adverse effects by disrupting the endocrine system. One study has studied the effects of BP1 and NP on proliferation and metastasis of MCF-7 human BC cells expressing estrogen receptors (ER) [102] . Treatment with BP1 (10^{-5}-10^{-7} M) and NP (10^{-6}-10^{-7} M) promoted proliferation of MCF-7 cells similar to the positive control 17-beta-estradiol (E2). This response was lost in presence of the ER antagonist ICI-182,780. Moreover, addition of BP1 or NP markedly induced migration of MCF-7 cells similar to E2. Alterations in transcriptional and translational levels of proliferation and metastasis-related markers were investigated and data showed increase in the expression of cyclin D1, a cell cycle progressor, and cathepsin D and decrease in the expression of p21, a negative regulator of cell cycle progression at G1 phase, at both transcriptional and translational levels. BP1- or NP-induced alterations of these genes were blocked by ICI-182,780, suggesting that changes in expression of these genes may be regulated by an ERα-dependent pathway. Authors concludes that BP1 and NP may accelerate growth of MCF-7 breast cancer cells by regulating cell cycle-related genes and promote cancer metastasis through amplification of cathepsin D. Similar data about BP1 effects were found on BG1 human ovarian cancer cells expressing ER with the up-regulation of cyclin D1 [103]. In xenograft mouse models transplanted with BG1 cells, BP1 or E2 treatment significantly increased the tumor mass formation compared to a vehicle within 8 weeks. In histopathological analysis, the tumor sections of E2 or BP1 group displayed extensive cell formations with high density and disordered arrangement, which were supported by the increased number of BrdU-positive nuclei and the over-expression of the cyclin D1 protein. Taken together, these results suggest that BP1 exerts xenoestrogenic effects by stimulating the proliferation of ER-positive cancer cells via the ER signaling pathway.

Per And Polyfluoroalkyl Substances (PFOS/ PFOA)

Perfluoroalkyl acids (PFAAs) are prominent environmental toxicants used in industrial and consumer products because of their stain-resistant and water-repellant characteristics. The two most widely known PFAAs are perfluorooctanoic acid (PFOA), and perfluorooctane sulfate (PFOS) [104]. They are suspected to be endocrine disruptors and potential linked with BC, but the mechanisms underlying their actions are unknown. There is no article studying specifically the role of PFAAs in cancer progression, but some data suggest their role in late stage of cancer development [105, 106]. Tumorigenic activity of PFOA (50-100 μM) and PFOS (1-10 μM) has been shown in MCF10 human breast epithelial cells which demonstrated a higher growth rate compared to controls. PFOA promoted MCF-10A proliferation by accelerating G0/G1 to S phase transition of the cell cycle, due to increased cyclin D1 and CDK4/6 levels, concomitant with a decrease in p27. PFOS had the same effect on the cell cycle due to increased CDK4 and decreased p27, p21, and p53 levels. Authors also showed that PFOA and PFOS are able to stimulate cell migration and invasion, underlying their role in BC progression. Moreover, the ER antagonist ICI 182,780 had no effect on PFOA-induced cell proliferation, whereas the PPARα antagonist GW 6471 was able to prevent the MCF-10A proliferation, indicating that the underlying mechanisms involve PPARα.

4. Food Preparation

Genotoxic carcinogens are potentially present in the human diet; two important examples are BaP and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) which are by-products of food processing and preparation. BaP is a polycyclic aromatic hydrocarbon generated by incomplete combustion of organic substances, thus contaminating numerous foodstuffs and PhIP is a heterocyclic amine formed when meat is cooked. They are both also found in cigarette smoke.

Benzo(a)pyrene

BaP is identified as a mammary carcinogen in rodents and contributes to the development of human breast cancer. It significantly increased invasion in MDA-MB-231 cells. Treatment of MDA-MB-231 cells with Vomitoxin (a selective COX-II inducer) also enhanced invasion; interestingly, co-treatment with NS398 (a selective COX-II inhibitor) attenuated BaP-induced invasion suggesting the involvement of COX-II in the effects of BaP. The number of COX-II immunopositive cells and COX-II protein levels were increased in immunohistochemical staining and western blots

respectively upon exposure to BaP. BaP exposure also induced a profound (46-fold) increase in PGE2 production by MDA-MB-231 cells. The AhR antagonists, resveratrol (RES) and alpha-naphthaflavone (alpha-NF) had no effect on their own, but co-treatment with RES and alpha-NF inhibited BaP-induced invasion suggesting a potential role for the AhR signaling pathway [107]. Another study demonstrated that BaP induces cell migration through a lipoxygenase- and Src-dependent pathway, as well as the activation of focal adhesion kinase, Src, and the extracellular signal-regulated kinase 2 in MDA-MB-231 cells but not in the mammary nontumorigenic epithelial cells MCF12A underlying the role of BaP in BC invasion and metastasis process and not BC initiation [108]. The researchers also showed that BaP promotes an increase of αvβ3 integrin-cell surface levels and an increase of metalloproteinase (MMP)-2 and MMP-9 secretions. In another work, Guo *et al* showed that the pro-oxidant properties of BaP (enhanced ROS production) stimulate the ERK signaling pathway activating the expression and activity of MMP in MCF7 and MDA-MB-231 cells; as a a consequence, their cell migration and invasion were enhanced [109] and cumulative BaP exposure leads to increased tumor growth &, liver and lung metastasis in a mouse model. All these data suggest that BaP can act on several steps of the metastasic cascade and participate to BC progression.

2-Amino-1-Methyl-6-Phenylimidazo[4,5-B]Pyridine

The cooked meat-derived genotoxic 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) induces cancers of the colon, prostate and mammary gland when fed to rats. Carcinogenic effects of cumulative exposures to PhIP at physiologically achievable, pico to nanomolar concentrations were also *demonstrated in vitro* using human breast epithelial MCF10A cells [110]. In this study, progressive carcinogenesis was measured by increasingly-acquired cancer-associated properties of reduced dependence on growth factors, anchorage-independent growth, acinar-conformational disruption, proliferation, migration, invasion, tumorigenicity with metastasis and increased stem-like cell populations. These biological changes were accompanied by biochemical and molecular changes, including upregulated H-Ras gene expression, extracellular signal-regulated kinase (ERK) pathway activation, Nox-1 expression, reactive oxygen species (ROS) elevation, increased HIF-1α, Sp1, tumor necrosis factor-α, matrix metalloproteinase (MMP-2, MMP-9), aldehyde dehydrogenase activity and reduced expression of E-cadherin. Another study shown that sub-nanomolar concentrations PhIP treatment of MCF7 and T47D enhance invasion and migration in a dose-response manner [111]. These results were inhibited by the antiestrogen ICI 182,780. The PhIP-induced

invasive phenotype was associated with enhanced expression of cathepsin D and cyclooxygenase-2 & activity of matrix metalloproteinase (MMP9).

5. Other polycyclic aromatic carbons

Cigarette smoke

Cigarette smoke is an extremely complex chemical composition and contains numerous toxic and carcinogenic substances, including many polycyclic aromatic hydrocarbons (PAHs). Multiple epidemiological studies have established the association between active and involuntary exposure to tobacco and increased risk of BC. Little is known about tobacco carcinogens effects on cancer progression and metastasis, but several epidemiological studies have shown that women who smoke have a higher rate of fatal BC than nonsmoking women [112–115]. Two early works have demonstrated an association between smoking and pulmonary metastasis among women with BC [116, 117]. A prospective randomized animal research showed that cigarette smoke exposure is associated with an increase in the total pulmonary metastatic burden in a murine model of metastatic mammary cell cancer [118]. Experimental animals were exposed to cigarette smoke in specialized exposure chambers at different concentrations chosen to approximate active cigarette smoking. One week after the initiation of exposures, mouse mammary tumor cells were injected into the tail veins of experimental and pulmonary metastases were counted and measured 3 weeks later. The mean metastatic burden in the lungs was consistently greater for smoke-exposed animals. However, molecular mechanisms are poorly investigated. In a chronic exposure model to cigarette smoke (several weeks), both non-tumorigenic (MCF10A, MCF12A) and tumorigenic (MCF7) breast epithelial cells acquire mesenchymal properties such as fibroblastoid morphology, increased anchorage-independent growth, and increased motility and invasiveness [119]. Analysis by flow cytometry showed that treatment with cigarette smoke extract leads to the emergence of a CD44(hi)/CD24(low) population in MCF10A cells and of CD44+/CD49f+ in MCF7 cells, indicating that cigarette smoke causes the emergence of cell populations bearing markers of self-renewing stem-like cells with increased colony formation. *In vivo*, treatment with cigarette smoke extract renders MCF10 cells more capable to survive and colonize the mammary ducts while MCF7 cells are more prone to metastasize from a subcutaneous injection site, independent of cigarette smoke effects on the host and stromal environment. These phenotypical modifications were associated with gene expression changes associated with EMT.

Several studies have tried to identify which compound of cigarette smoke among the more than one hundred potential carcinogens, could lead to enhanced BC metastasis. CS contains ligands of the Aryl hydrocarbon Receptor (including BaP) but also nicotine, the major addictive component of cigarette. Nicotine, while not carcinogenic by itself, has been found to induce cell proliferation and angiogenesis, to induce morphological changes characteristic of a migratory, invasive phenotype and to confer resistance to apoptosis in lung cancer cell lines through the nicotinic acetylcholine receptors (nAChRs) [16]. These pro-invasive effects of nicotine are mediated by alpha7-nAChRs on nonsmall cell lung cancer. RT-PCR analysis showed that the alpha7-nAChRs were also expressed on human BC and nicotine was found to promote proliferation and invasion in 2 human breast cancer cell lines (MCF7, MDA-MB-468) through a nAChR, Src and calcium-dependent signaling pathway. Similarly in this study, nicotine induced changes in gene expression consistent with EMT. Furthermore, a recent study confirmed that nicotine-treated MCF7 cells exhibited changes in cell structure, cellular motility (related to relocation of F-actin) and an enhanced number of MCF7 CD44+CD24- cancer stem cell population [120]. Authors also demonstrated the chemoresistance effects induced by nicotine (towards doxorubicin, a chemotherapy used in routine in BC).

7,12-Dimethylbenz(A)Anthracene

7,12-Dimethylbenz[a]anthracene (DMBA), is a widely studied polycyclic aromatic hydrocarbon that has long been recognized as a very potent carcinogen. It was shown that DMBA can promote a more invasive, mesenchymal phenotype in the Rel-3983 cell line, a cell line established from a mouse mammary tumor virus (MMTV)-c-*rel* transgenic mouse model made to test directly the role of the nuclear c-Rel subfamily of NF-κB protein in breast cancer [121]. Indeed, NF-κB was found to be constitutively activated in BC cells and high levels of nuclear c-Rel were found in a large number of primary human BC. Authors shown that DMBA-treated cells displayed an increased rate of proliferation, displayed growth to a higher cell density, acquired the ability to grow in soft agar and matrigel and showed loss of E-cadherin expression. Compared with control cells, DMBA-treated cells displayed increased NF-kappaB binding and higher levels of the NF-kappaB transactivating subunits c-Rel, RelA, and RelB, which seemed functional as judged by induction of c-Myc and vimentin, two NF-kappaB target genes. In this mechanistic study, ectopic expression of a super repressor mutant of IkappaB-alpha reduced cell growth and invasive morphology in Matrigel, confirming the role of NF-kappaB in epithelial to mesenchymal transition.

6. Alcohol

Accumulating evidence indicates that alcohol exposure may enhance the progression and aggressiveness of existing mammary tumors with multiple potential mechanisms involved [122]. Alcohol may increase the mobility of cancer cells by inducing cytoskeleton reorganization and enhancing the cancer cell invasion by causing degradation and reconstruction of the extracellular matrix [123–126]. It promotes the epithelial-mesenchymal transition (EMT)[127], impairs endothelial integrity, thereby increasing the dissemination of breast cancer cells and facilitating metastasis in animals [128]. Alcohol may also stimulate tumor angiogenesis through the activation of cytokines and chemokines which promotes tumor growth [129, 130]. Additionally, alcohol may increase the cancer stem cell population which affects neoplastic cell behavior, aggressiveness, and the therapeutic response [128]. Alcohol can be metabolized in mammary tissues and breast cancer cells which produce reactive oxygen species (ROS), causing oxidative stress. Several experimental studies suggest that the epidermal growth factor receptor (EGFR) family, particularly ErbB2 (a member of this family), is involved in alcohol-mediated tumor promotion. For example, breast cancer cells or mammary epithelial cells over-expressing ErbB2 are more sensitive to alcohol's tumor promoting effects suggesting that oxidative stress and EGFR/ErbB2 signaling play an important role in this process [122].

7. Heavy Metals

Increased amount of heavy metals (Iron, Copper, Zinc, Lead, Chromium, Nickel) in breast malignant tumors (measured by atomic absorption spectrophotometry and energy-dispersion spectrometer) has been shown to be associated with poor molecular prognostic factors [131]. Tumor growth induced by heavy metals was accompanied with the increase of HER2/neu, p53, Ki-67, 06-methylguanine-DNA methyltranferase expression and decrease of ER-alpha and PR expression. The same authors also showed that the increment of pathological DNA methylation was accompanied with the accumulation of heavy metals in tumor tissue and concluded that heavy metals could stimulate the progression of breast cancer and reduce its sensitivity to treatment. Higher concentrations of Ca, Fe, Cu, and Zn trace elements (TE) were found in neoplastic breast tissues (malignant and benign) when compared with normal tissues [132]. The expressions of all TE were found statistically correlated with well-known prognostic factors for breast cancer. The element copper also showed statistical correlation with overall survival suggesting its potential use as a biomarker. Long-term cadmium exposure has been shown to promote migration and invasion of BC cells through TGIF/MMP2 signaling axis [133]. Tungsten was shown to target the tumor microenvironment to enhance breast cancer metastasis [134]. In a BC- xenograft model, tungsten slightly delayed primary tumor growth, but significantly enhanced lung

metastasis; *in vitro*, tungsten did not enhance BC cells proliferation or invasion. These data suggest that tungsten was not directly acting on BC primary tumor cells to enhance invasion. In contrast, tungsten changed the tumor microenvironment, enhancing parameters known to be important for cell invasion and metastasis (including activated fibroblasts, matrix metalloproteinases (MMP9), and myeloid-derived suppressor cells), and associated with a poor prognosis in humans. Furthermore, heavy metals are able to cause various epigenetic dysregulations, which are believed to play important roles in their carcinogenicity, but the mechanisms are largely unknown. A very recent review about metal carcinogen (Arsenic, cadmium, nickel and hexavalent chromium) suggests that metal could produce CSC-like cells through dysregulated epigenetic mechanisms [135].

III. Role of AhR in cancer progression and metastasis

Many of the pollutants describe above are AhR ligands. The AhR has been linked to the regulation of EMT and the proliferation of CSC; however, historically, it has been described as a xenobiotic-activated transcription factor involved in the detoxication pathways [136]. It is indeed activated by many of environmental pollutants including dioxins, furans, polychlorinated biphenlys and polycyclic aromatic hydrocarbons, allowing the protection of organisms against chemical toxicity. In the last decade, AhR has been shown to play a critical role in many alternative pathways like autoimmunity, metabolic imbalance, inflammatory skin, gastro-intestinal disease among others. Thereby, accumulating evidence indicates that the AhR may be involved in proliferation, cell migration and angiogenesis [13, 14, 136–141] and plays an important role in cancer progression of a variety of cancer types [142–153]. However, studies are controversial, and it is still unclear if the AhR promotes or inhibits cancer aggressiveness in any given tumour type [154–161]. For example, a recent study has shown that AhR inhibition or knockdown/knockout consistently reduced human cell invasion, migration, and metastasis in triple negative and inflammatory breast cancer cell lines (decrease in invasion-associated genes (e.g., Fibronectin, VCAM1, Thrombospondin, matrix metalloproteinase 1) and increase in genes associated with decreased tumour progression (CDH1/E-cadherin) [162]. Authors found that 2.3.7.8-TCDD and 3,3′-diindolylmethane (another AhR agonist), inhibited irregular colony formation in Matrigel (correlated with stem-cellness) and blocked metastasis *in vivo* while accelerating cell migration. This example among many others demonstrate a complex role of the AhR in cancer progression and metastasis and show that 1/ underlying mechanisms are still unclear and could depend on the nature of the ligand, and also on the

cellular context such as the microenvironment and 2/ further investigations using relevant *in vitro* and *in vivo* models are warranted in order to elucidate the potential effect of the AhR in cancer spreading.

CONCLUSION

Despite encouraging advances in both local and systemic treatment over the last years, cancer remains a leading cause of death worldwide and metastasis is the major cause of cancer morbidity and mortality. In this study, we report important links between numerous environmental pollutants and several cell functions implicated in breast cancer progression and metastasis.

Several processes and/or signaling pathways involved in the occurrence of metastasis, deserve a better characterization; for example, as stated in part II, the AhR signaling pathway is complex to describe as numerous ligands can activate the receptor but with a different outcome. We indeed showed that according to the type of ligand, the AhR regulates different types of target genes. Moreover, the presence of endogenous ligands (including tryptophane derivatives) probably interfere with the outcome of the signaling pathway. Overall, opposite outcomes which have been identified for this signaling pathway (or others such as heavy metals) would probably deserve a better contextual characterization to properly understand which conditions are pro- or anti-metastatic. This also means that environmentally-relevant mixtures of pollutants might contain pro- and anti-metastatic compounds suggesting that the effects of pollutants on migratory and invasive pathways would deserve a better characterization not only as a single molecule but in relevant "cocktails". As stated in this manuscript, numerous xenobiotics stimulate the metastatic processes and such properties should be considered also at the epidemiological level and regarding their role in the exposome. This is a major public issue considering the huge number of death due to advanced stages of breast cancer and other types of tumors.

FIGURE 1 : Epithelial-to-mesenchymal transition and appearance of cancer stem cells are two mechanisms which are suspected to lead to the occurrence of metastasis. EMT is associated to the phenotypical acquisition of cellular properties which leads to the migration and invasion of primary tumor cells while cancer stem cells conserve cellular properties of stem cells (important for maintenance and renewal of the breast cellular epithelium) which maintain cancer proliferation, metastatic dissemination and resistance to anticancer treatment. The figure also synthesized the processes which are targeted by environmental pollutants (orange squares). This figure was drawn using the software « Smart Servier Medical Art ».

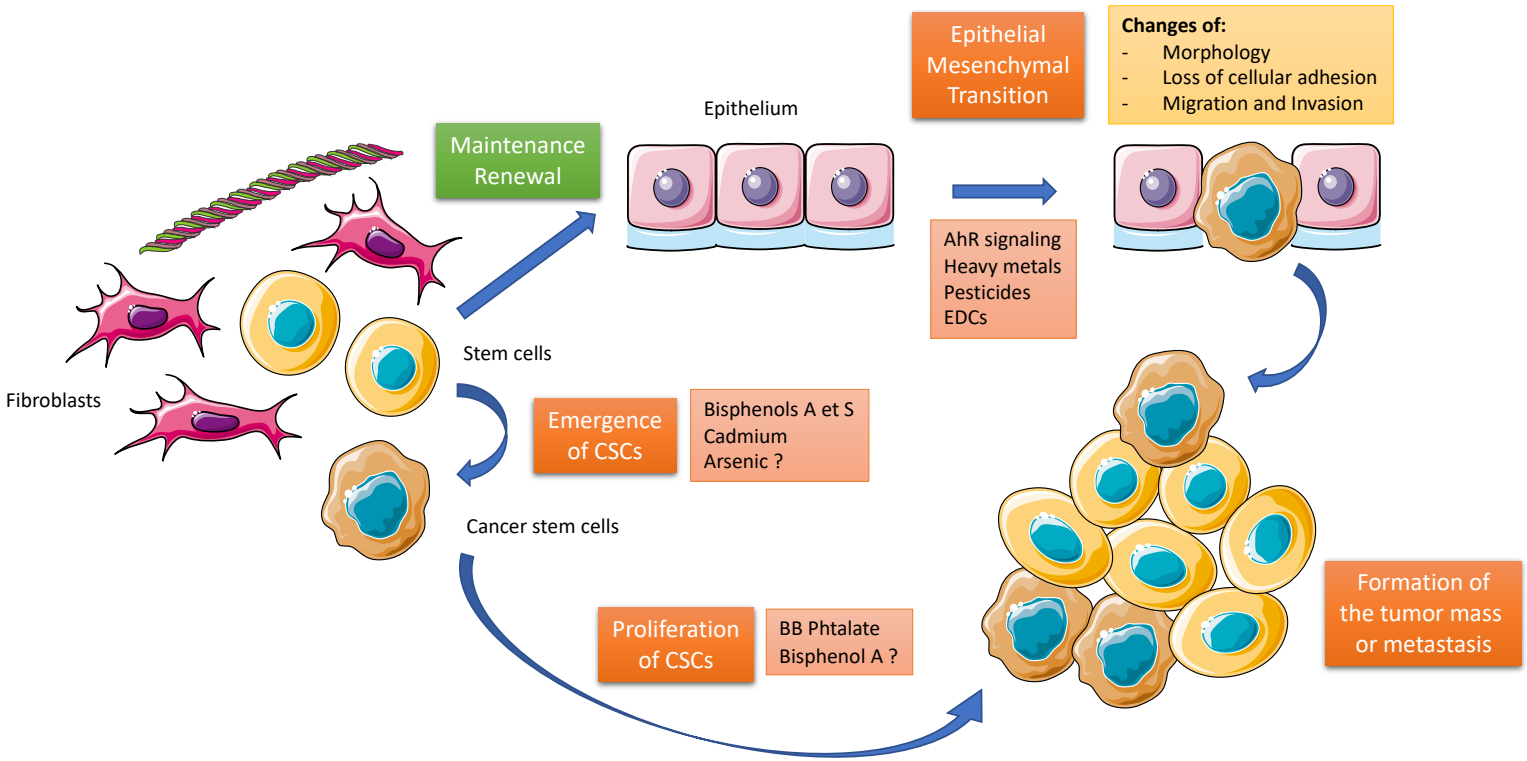

Figure 2: Flow diagram of the study selection

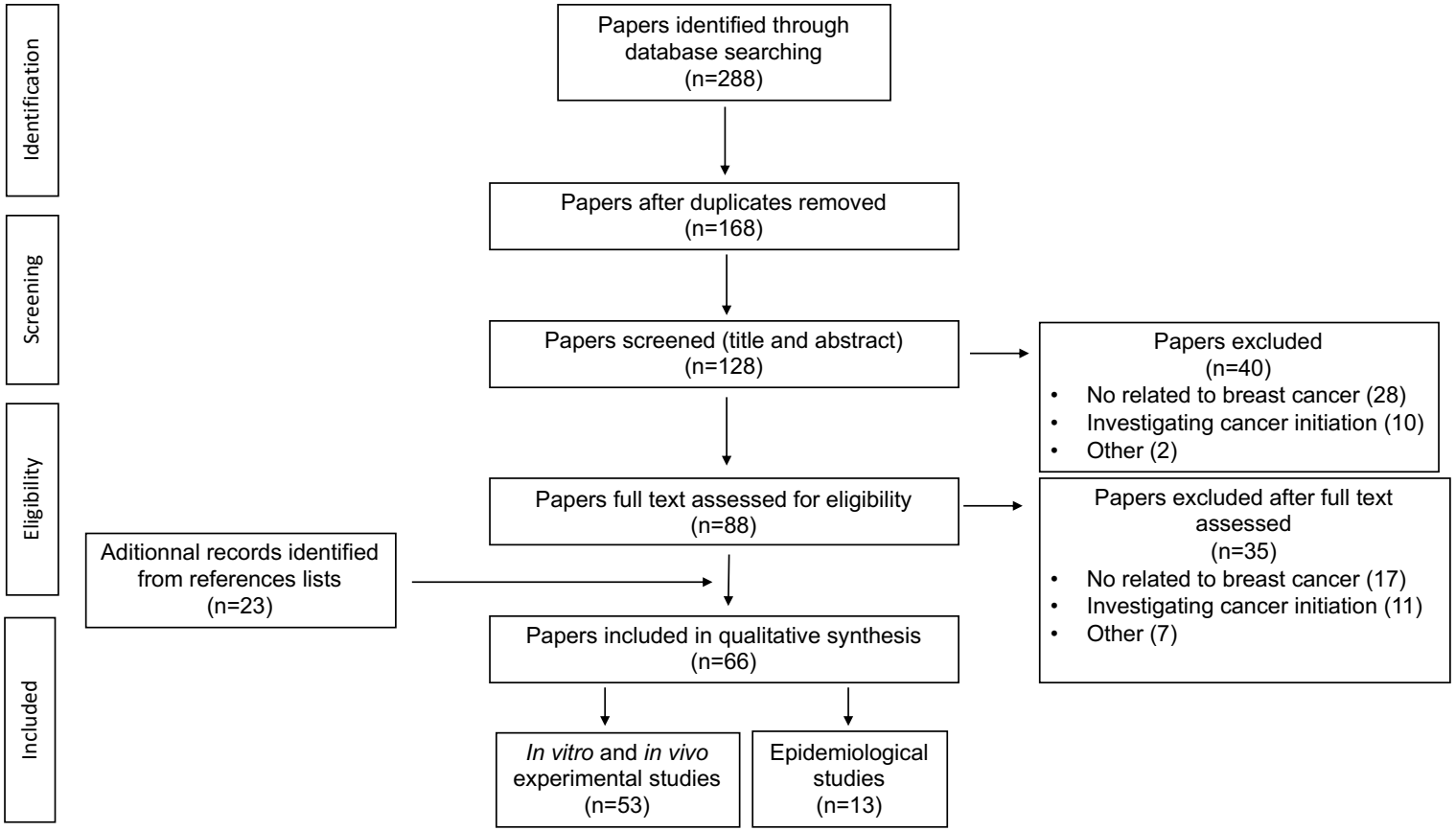

Table 1: Breast cancer findings for environmental chemical disruptors in experimental studies that investigated cancer progression and targeted signal pathway.

Potential environmental chemical disruptors	Studies (ref)	Type of study *(in vitro/in vivo)*	Effects that chemicals may have	Targeted signal pathway
Persistent EDCs				
Dioxin	[66]	*In vivo*	↓ tumor growth (Sprague-Dawley rat)	Unestablished
	[64]	*In vitro*	↓ invasion, motility and colony formation (MDA-MB-231, MCF7, ZR75, SKBR3) ↑ differenciation in a putative mammary cancer stem cell line	AhR dependant pathway regardless to ER status
	[61]	*In vivo/ In vitro*	↓ proliferation (MCF7) ↓ tumor growth (mice)	Antagonistic effect on ER signaling
	[62]	*In vitro*	↓ proliferation (MCF7, TD47, ZR75)	Antagonistic effect on ER signaling
	[63]	*In vitro*	↓ migration (MCF7)	Downregulation of CXCR4 and CXCL12
	[65]	*In vivo/ In vitro*	↓ metastasis formation (mice) No effect on tumor growth nor cell proliferation	Unestablished
	[67]	*In vitro*	↑ cell migration	NFATc1/ATX-signaling pathway
	[14]	*In vitro*	EMT, ↑ migration	AhR pathway
	[68]	*In vitro*	↑ invasion Resistance to apoptosis Mitochondrial dysfonction (↑ cytosolic [Ca(2+)](c) and RyR1-specific Ca(2+) release, ↑ calcineurin (CnA) levels and activation of its factors.	Mitochondrial transmembrane potential disruption in a time-dependent way Mitochondrial transcription and translation inhibition Activation of CnA-sensitive NF-kappaB/Rel (IkappaBbeta-dependent) factors.
	[162]	*In vivo/ In vitro*	↓ colony formation (BP1, Hs578T, SUM149) ↑ migration ↓ metastasis (zebrafish)	AhR signaling pathway
Polychlorinated biphenyls	[70]	*In vivo/ In vitro*	↑ migration (MCF7, MDA-MB-231 ↑ growth tumor and metastasis (mice)	ROCK signaling pathway

	[74] [75]	*In vitro*	↑ transendothelial migration (MDA-MB-231) ↑ transendothelial migration, ↑MMP3 (human endothelial cells)	Overexpression of VEGF through PI3K pathway signaling Activation EGFR and JAK3 in a coordinated and cross-regulated way AK3 and EGFR stimulate in concert PCB-induced activation of JNK and ERK1/2 followed by increased DNA binding of AP-1 and PEA3 and transcriptional up-regulation of MMP-3 expression.
DDT/DDE and organochlorine pesticides				
DDT/DDE	[84] [83]	*In vivo* *In vitro*	↑ ER + tumor growth (ovariectomized rats) ↑ proliferation	ER signaling pathway Estrogen-androgen balance disruption
Hexachlorobenzene	[85] [86] [87] [88]	*In vitro* *In vitro* *In vivo/ In vitro* *In vitro*	↑ proliferation (MCF7) ↑ migration (MDA-MB-231) ↑ invasion and MMP2/9 (MDA-MB-231) ↑ metastasis in mice (regardless ER status) ↑ migration and invasion	IGF-I) signaling pathway c-Src/HER1/STAT5b and HER1/ERK1/2 signaling pathways AhR, c-Src, HER1, STAT5b, and ERK1/2 signaling pathways Modulation of the crosstalk between AhR and TGFβ signaling
Consumer product chemicals				
Bisphenol A	[96] [95] [90] [91] [92]	*In vitro* *In vitro* *In vitro* *In vitro*	↑ expression MMP2/ MMP9 in TNBC –triple negative breast cancer (MDA-MB-231 and BT-549) ↑ proliferation and invasion in TNBC –triple negative breast cancer (MDA-MB-231 and BT-549) ↑ proliferation (MCF7) Alteration of the expression of cell cycle related genes	Activation ERRγ through ERK1/2 and Akt pathway Unestablished Upregulation of cell cycle genes Downregulation of antiproliferative genes Activation of Estrogen Receptor dependent signaling pathway

	[89]	*In vitro/in vivo*	↑ proliferation Induction of a profile of tumor aggressiveness in high-risk cells from breast cancer patients	Unestablished
	[94, 108]	*In vitro*	↑ migration and invasion (MDA-MB-231)	Activation GPER dependant pathway Activation of FAK, Src, ERK2-dependant pathway
Phtalates	[98]	*In vitro*	Phenotypical and gene expression changes associated with EMT (R2d cells, stem cell derived human breast epithelial cell line)	Activation of EGFR-PKA signaling cascade that increase AP2a transcriptor factor which upregulate histone deacetylase 3
	[99]	*In vitro/In vivo*	↑ proliferation, migration and colony formation (MDA-MB-231) ↑ tumor formation (mice)	AhR/HDAC6/c-Myc signaling pathway
	[100]	*In vitro*	↑ proliferation (MCF7)	Activation of PPARα and γ
	[54]	*In vitro*	↑ proliferation (MCF7)	P13K/AKT signaling pathway
	[101]	*In vitro*	↓ tamoxifen-induced apoptosis in ER+ cells (MCF7) but not ER- cells (MDA-MB-231	Increased Bcl-2 to Bax ratio through an Estrogen Receptor dependent signaling pathway
Benzophénone-1/ Nonylphenol	[102]	*In vitro*	↑ proliferation and migration (MCF7)	Upregulation of cyclin D1 and cathepsin D and downregulation in p21 regulated by an ERα-dependent pathway
Per and polyfluoroalkyl acids PFOA	[105]	*In vitro*	↑ proliferation, migration and invasion (MCF10)	Upregulation of cyclin D1 and CDK4/6 and downregulation and in p27 through PPARα-dependant pathway
PFOS	[106]	*In vitro*	↑ proliferation, migration and invasion (MCF10)	Upregulation of CDK4 and down regulation and downregulation in p27, p21 and p53
Food preparation				
Benzo(A)pyrene	[108]	*In vitro*	↑ migration (MDA-MB-231, MCF7) ↑ αvβ3 integrin-cell surface levels and an increase of metalloproteinase (MMP)-2 and MMP-9	Lipoxygenase- and Src-dependent pathway Activation of FAK, Src and extracellular signal-regulated kinase 2
	[107]	*In vitro*	↑ invasion (MDA-MB-231)	Upregulation of COX II and PGE2 through an AhR signaling pathway

	[109]	*In vivo/In vitro*	↑ migration and invasion, ↑MMP9 (MCF7) ↑ growth tumor and liver and lung metastasis (in an accumulative mouse model mimicking the cumulative effects of chronic BaP exposure)	Upregulation of ROS-induced ERK signaling pathway
2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine	[111]	*In vitro*	↑ migration and invasion, ↑ MMP9 (MCF7, TD47)	Upregulation of Cathepsin D and cyclooxygenase 2 through ER signaling
	[110]	*In vivo/In vitro*	Carcinogenesis effects, ↑ proliferation, migration, invasion, ↑ colony formation and stem-like cell populations (MCF10) ↑tumorigenicity, ↑ lung metastasis	Ras-ERK-Nox-ROS signaling pathway
Other polycyclic aromatic carbons				
Cigarette smoke	[118]	*In vivo*	↑ total pulmonary metastatic burden in smoke-exposed animals (female sexually mature BALB/cAnN mice)	Unestablished
	[119]	*In vitro/ In vivo*	Phenotypical and gene expression changes associated with EMT (MCF7) Emergence of stem-like cells population, colony formation	Unestablished
	[16]	*In vitro*	↑ proliferation and invasion ↑ migration in a dose-dependent manner Phenotypical and gene expression changes associated with EMT (MCF7 and MDA-MB-468)	Activation of nAChRs, Src and calcium-dependent signaling pathway
	[120]	*In vitro*	↑ proliferation ↑ of stem-like cells population Resistance to Doxorubicin	Unestablished
7,12-dimethylbenz(a)anthracene	[121]	*In vitro*	↑ proliferation and invasion (mice) ↑ colony formation EMT (↓ E cadherin)	NFκB pathway
Alcohol	[122] (review)	*In vitro/ in vivo*	↑Angiogenesis, migration, invasion, EMT, MMP ↑ Cancer stem cells ↑ Metastasis formation in mice	Cross-talk between oxidative stress and EGFR/ErbB2 signaling
Heavy metals	[135]	*In vitro*	Cadmium ↑ migration and invasion (MCF7, MDA-MB-231)	TGIF/MMP2 signaling axis
	[134]	*In vivo/ In vitro*	Tungsten ↓ primary tumor growth but ↑metastasis (mice) No change observed in invasiveness of cells in vitro (66Cl4 model of breast cancer metastasis to bone)	Targeting microenvironment: activation CAFs at the metastatic site (↑ MMP9) and ↑ of myeloid-derived suppressor cells

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III. Le tissu adipeux : composant majeur du microenvironnement tumoral dans le cancer du sein

1. Le tissu adipeux

Longtemps considéré comme un tissu de soutien et stockage inerte et négligé par la communauté scientifique, il est actuellement bien établi que le tissu adipeux (TA) constitue un véritable organe qui contribue de façon significative à la régulation de l'homéostasie métabolique. Il joue un rôle primordial dans le stockage et la libération des lipides en gérant les réserves énergétiques de l'organisme selon les besoins et les approvisionnements mais agit également comme un véritable organe endocrinien, capable de synthétiser et sécréter des adipokines, qui agissent au niveau local (par voie autocrine ou paracrine) ou systémique et influencent tous les autres organes impliqués dans la physiologie mais aussi en physiopathologie comme dans l'obésité, le diabète et plus récemment le cancer.

a. Anatomie du tissu adipeux

Le TA représente 15 à 25% du poids corporel total chez un individu de poids normal, et jusqu'à 50% dans les cas d'obésité morbide. On distingue plusieurs types de tissus adipeux selon leur localisation et leur fonction. Le tissu adipeux est un tissu hétérogène, en effet il possède des spécificités anatomiques et fonctionnelles importantes selon sa localisation et l'importance de son extension. On distingue le TA blanc, et le TA brun qui présentent une anatomie, une composition cellulaire et des rôles métaboliques distincts. Le TA brun est principalement présent chez le fœtus et le nouveau-né et régresse ensuite rapidement. Sa persistance et son importance chez les humains adultes sont activement étudiées et ses fonctions restent à déterminer. Le TA blanc a pour principale fonction de stocker l'énergie en excès dans l'organisme sous forme de lipides pour ensuite la libérer

sous forme d'acides gras libres par lipolyse. Au sein du TA blanc, on retrouve deux types de TA qui présentent d'importantes différences : le TA viscéral et le TA sous-cutané. Le TA sous-cutané est, comme son nom l'indique, situé sous la peau, plus précisemment dans l'hypoderme, et réparti dans tout l'organisme. Il stocke environ 80% de la graisse corporelle et les principaux dépôts sont situés au niveau de l'abdomen, de la région fessière et au niveau mammaire chez la femme (57). Le TA viscéral comprend des dépôts plus profonds intra et rétro-péritonéaux : au niveau de l'omentum, des viscères et du mésentère, et en moins grande quantité au niveau péri-rénal, périprostatique ou encore péri-vasculaire et péricardique C'est ce TA qui est responsable des complications cardio-vasculaires et métaboliques de l'obésité et plus généralement du syndrome métabolique (58).

b. Composition du tissu adipeux

Le TA est classé histologiquement dans les tissus conjonctifs. Il est composé de nombreux types cellulaires et d'une matrice extracellulaire (MEC). Au niveau cellulaire, on retrouve les adipocytes, composés pour l'essentiel d'une grosse gouttelette lipidique occupant 90% du volume de la cellule, le noyau étant refoulé à la périphérie et le cytoplasme limité à une mince couronne autour des lipides intracellulaires, de cellules progénitrices adipocytaires et de préadipocytes, de cellules endothéliales et de cellules immunitaires : macrophages, polynucléaires et lymphocytes. Il est intéressant de noter que si les adipocytes représentent environ 83% du volume du tissu adipeux, cela ne représente que 20 à 40% du contenu cellulaire en terme de type cellulaire (Figure 8) (59).

La MEC est composée de protéines structurales, les collagènes et de protéines d'adhésion dont la fibronectine, la laminine, les élastines et les protéoglycanes (60). La MEC, constamment remodelée par l'action de métalloprotéases, joue un rôle important dans le processus de différenciation

adipocytaire et un rôle de réservoir de facteurs de croissance. Par exemple, le facteur de croissance proangiogénique VEGF (Vascular Endothelial Growth Factor) est séquestré dans la MEC sous forme liée à l'héparane sulfate et peut être libérer sous le contrôle des protéases (61).

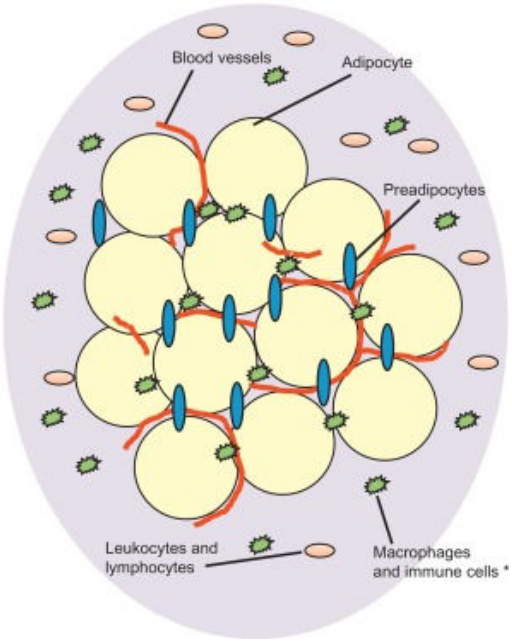

Figure 8: La grande majorité du tissu adipeux est constitué d'adipocytes mais il existe plusieurs autres types cellulaires dont des cellules immunitaires comme les macrophages, les lymphocytes B et T régulateurs, les leucocytes et les lymphocytes. L'ensemble est richement vascularisé *(D'après Tsiloulis, PMBTS, 2015 (62)).*

c. Origine et développement du tissu adipeux

La formation du TA adipeux débute avant la naissance. L'expansion du TA se déroule rapidement après la naissance par une augmentation du nombre et de la taille des cellules adipeuses, phénomènes appelés respectivement hyperplasie et hypertrophie. Les adipocytes sont des cellules matures différenciées incapables de proliférer. L'augmentation du nombre d'adipocytes dans le TA est expliquée par la différenciation de cellules progénitrices présentes dans la fraction stroma-vasculaire (63). De nombreuses études ont montré que ces cellules progénitrices pouvaient se

différencier *in vitro* en cellules adipogénique, chondrogénique, myogénique et ostéogénique en présence de facteurs d'induction spécifiques, confirmant leur caractère multipotent. Si les mécanismes d'orientation d'une cellule précurseur vers la lignée adipocytaire (appelée détermination) sont encore peu connus, la différenciation adipocytaire proprement dite (passage de précurseur déterminé vers la lignée adipocytaire à l'adipocyte mature) a été très étudiée (64,65). Après une phase de croissance exponentielle, l'adipoblaste marque un arrêt de croissance à confluence sous l'influence de deux facteurs de transcription CCAAT/Enhancer binding protein-α (C/EBP-α) et peroxisome proliferator-activated receptor γ (PPARγ), dont l'expression ne cesse d'augmenter au cours de la différenciation et devient un préadipocyte. Les préadipocytes arrêtés en phase G0/G1 du cycle cellulaire expriment des marqueurs précoces tels que la chaîne α2 du collagène de type VI (A2COL6) ou la lipoprotéine lipase et perdent l'expression de pref-1 (preadipocyte factor 1), protéine soluble ayant un effet inhibiteur sur la différenciation adipocytaire. Les préadipocytes subissent ensuite une phase d'expansion clonale liée à une série de réplications de l'ADN ou mitoses post-confluentes et sont alors engagés dans l'adipogénèse sous l'influence d'importants changements transcriptionnels chronologiques. La dernière étape de la différenciation ou maturation terminale permet enfin l'acquisition des propriétés métaboliques et sécrétoires des adipocytes matures. Les principales étapes de la détermination et de la différenciation adipocytaires sont résumées dans la Figure 9.

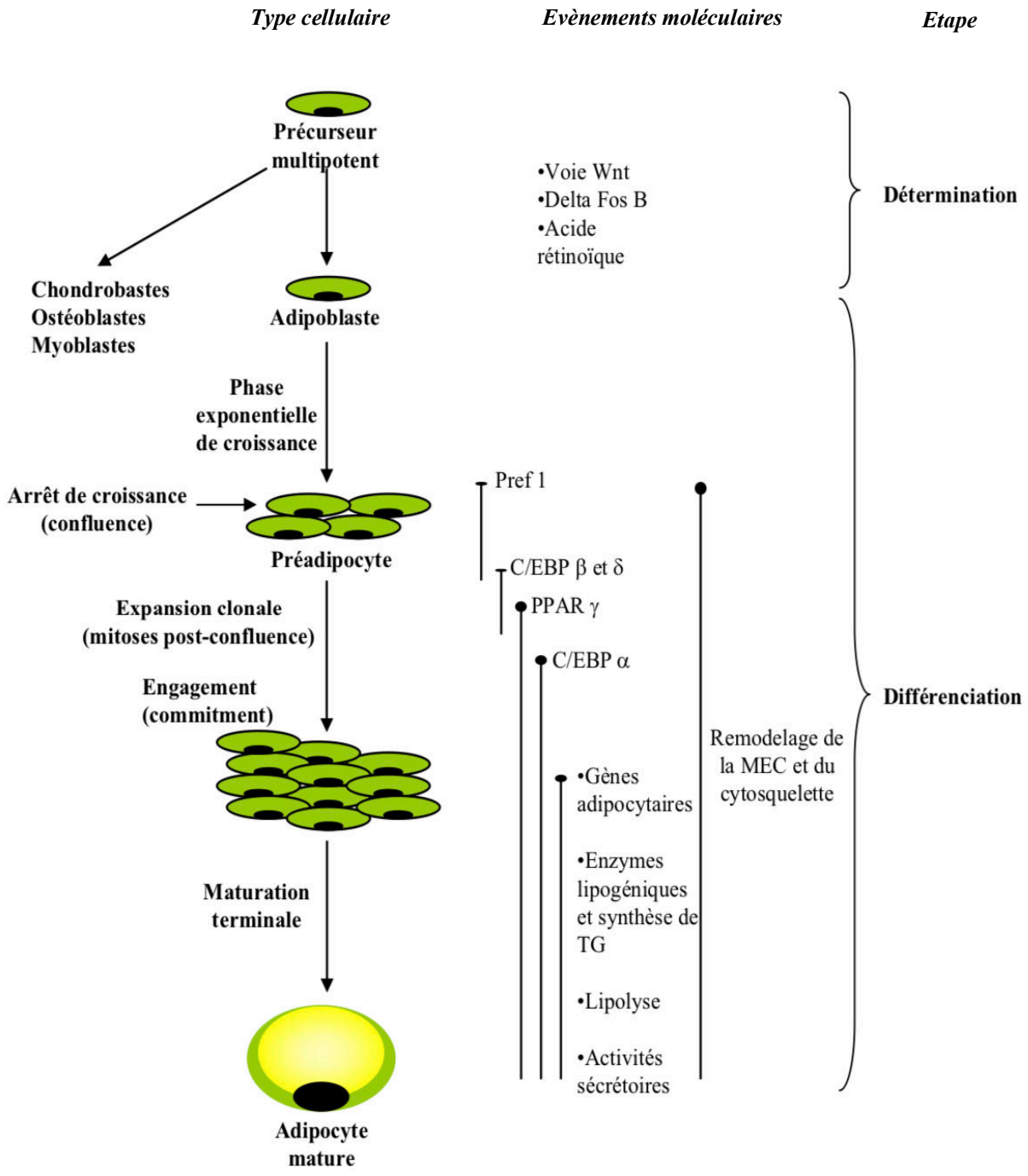

Figure 9 : Détermination et différenciation adipocytaire *(D'après Gregoire F. M., Physiol. Rev., 1998(64)).*

d. Rôles du tissu adipeux

Le rôle principal du tissu adipeux est de réguler les réserves énergétiques de l'organisme en stockant les acides gras libres en excès sous forme de triglycérides, et de les libérer par la suite à la demande par la lipolyse. Par souci de synthèse, ces mécanismes ne seront pas détaillés dans ce travail. Le tissu adipeux est également considéré comme un organe endocrine, c'est-à-dire qu'il est capable de sécréter, sous l'influence de signaux hormonaux divers, de nombreuses molécules appelées adipokines. Quelques exemples de ces facteurs sécrétés par le TA sont présentés dans le tableau 1. Ces médiateurs ont un impact sur de nombreux tissus cibles, comme le foie ou les muscles squelettiques, et jouent un rôle primordial dans la régulation de la balance énergétique ainsi que dans la médiation des complications métaboliques et cardiovasculaires associées à l'obésité. Par exemple, l'angiotensinogène et l'angiotensine II, l'inhibiteur de l'activateur du plasminogène 1, l'adiponectine et la résistine semblent participer à la pathogenèse de l'hypertension, des accidents thrombotiques et de la résistance à l'insuline associée à l'excès de graisse viscérale. Une autre grande catégorie des molécules sécrétés par le tissu adipeux est celle des chimiokines et cytokines pro-inflammatoires (TNFα, IL1, IL6) ou anti-inflammatoire (TGFβ, IL1Rα, IL10, MCP1, IL8, RANTES, IP10, PAI1). Ce profil de sécrétion est modifié par exemple en condition d'obésité (66) et contribue, avec l'infiltration du TA par des cellules immunitaires, à un état sub-inflammatoire chronique responsable des complications métaboliques de l'obésité. Des données de la littérature, de plus en plus nombreuses, soulignent également le rôle de ces cytokines endogènes sécrétés dans le microenvironnement tumoral sur la croissance et la progression tumorale et sur l'immunosuppression de l'hôte, en particulier chez les patients obèses (67,68).

Tableau 1 : Quelques exemples de facteurs sécrétés par le TA (*Adapté de Anghel and Wahli, Cell Research, 2007(69))*

NOM	EXPRESSION	PRINCIPALES FONCTIONS
Leptine	Adipocytes	Hormone, reflet de la masse adipeuse Signal de satiété par effet direct sur l'hypothalamus Stimule la lipolyse, inhibe la lipogénèse, stimule l'oxydation des AG
Adiponectine	Adipocytes	Hormone insulino-sensitive Stimule l'oxydation des AG Action anti-inflammatoire
Adipsine	Adipocytes	Composante du complément Stimule le stockage de TG, inhibe la lipolyse
Pref-1	Pré-adipocytes	Inhibe l'adipogénèse Sa surexpression dans le TA affecte la tolérance au glucose et la sensibilité à l'insuline
TNFα	Pré-adipocytes	Cytokine pro-inflammatoire Induit l'insulino-résistance Augmente la lipolyse dans les adipocytes
IL6	Adipocytes	Cytokine pro-inflammatoire Diminue les voies de signalisation de la leptine et de l'insuline
TGFβ	Adipocytes/macrophages	Facteur de croissance, adipokine anti-inflammatoire Effet sur la prolifération, la différenciation et l'apoptose Expression augmentée au cours de l'obésité
MCP1	Adipocytes/macrophages	Chémokine anti-inflammatoire Recrute les macrophages sur les sites inflammatoires Augmente la lipolyse et la sécrétion de leptine
PAI 1	Adipocytes	Protéines vasculaires, puissant inhibiteur du système fibrinolytique Lié au développement de l'insulino-résistance Niveaux circulants augmentés en cas d'obésité

2. Obésité et cancer du sein

L'obésité, définie par un indice de masse corporelle (IMC) supérieur ou égal à 30 kg/m^2, est un facteur de risque reconnu de cancer et de mortalité par cancer (70,71). En ce qui concerne le CS, l'obésité a un impact sur le risque de développer un CS, sur le pronostic et mais également l'efficacité des traitements oncologiques (72).

L'obésité est un facteur de risque reconnu de CS, particulièrement après la ménopause (73). Il a de plus été démontré au sein d'une grande méta-analyse qu'après la ménopause, le risque de CS augmente de 9% pour 5 unités d'IMC (dans les études européennes et nord-américaines). Les patientes en surpoids (IMC entre 25 et 30 kg/m^2) ont donc un sur-risque de 9% par rapport à la population générale et ce chiffre atteint plus de 30% chez les patientes obèses morbides (IMC>40kg/m^2) (73).

De plus, les patientes obèses présentent habituellement des cancers plus agressifs et plus avancés avec une atteinte métastatique ganglionnaire plus fréquente et un risque de métastases à distance plus important (74,75).

La réponse aux traitements est également moindre chez ces patientes, que ce soit la chimiothérapie, l'hormonothérapie ou la radiothérapie. Dans l'étude ADEBAR comparant une chimiothérapie adjuvante à base d'anthracycline et une à base de taxanes chez les patientes avec un CS métastatique en ganglionnaire, il a été montré que quel que soit le type de chimiothérapie administrée, il existe une diminution significative de la survie globale et de la survie sans récidive des patientes avec un IMC supérieur à 30 kg/m^2 comparées aux autres patientes (76). Concernant l'hormonothérapie, l'étude ATAC (qui a démontré une supériorité des inhibiteurs de l'aromatase (IA) face au tamoxifène chez les patientes ménopausées) a montré que les patientes avec un IMC supérieur à 35 kg/m^2 avaient un bénéfice moindre des IA et un risque augmenté de rechute (77). Il

en est de même chez les patientes non ménopausées en surcharge pondérale ou obèses dans l'étude ABCSG-12 (78). Ces données traduisent une activité des aromatases plus élevée chez les patientes ayant une masse grasse et donc moins bien inhibée par les IA administrés aux doses habituelles. Il n'y a en effet dans ces études, aucune adaptation des doses des traitements hormonaux en fonction du poids.

De façon plus générale, l'obésité est toujours dans les études associée à une diminution de la survie globale et une augmentation du risque de récidive, indépendamment du statut ménopausique (79) et une prise en charge nutritionnelle adaptée associée à une perte pondérale pourrait avoir un impact oncologique avec une diminution du taux de survie sans récidive (2437 patientes de 48 à 79 ans, perte de poids moyenne de 2,7 kg, survie sans récidive de 12,4% dans le groupe contrôle versus 9,8% dans le groupe régime (HR : 0,76 ; p = 0,034)) (80).

Il existe des raisons cliniques pour expliquer ces données épidémiologiques : le diagnostic est souvent retardé et fait à un stade plus avancé car l'examen clinique est moins contributif et les examens radiologiques moins approfondis (81,82); les doses de chimiothérapie et hormonothérapies ne sont pas adaptées à la surface corporelle et au volume de distribution des patientes obèses, par crainte de toxicité (83). En outre, la physiopathologie de l'obésité et également du cancer étant de mieux en mieux comprises, de nombreuses hypothèses biologiques émergent : augmentation de la synthèse d'œstrogènes chez les patientes ménopausées obèses (tissu adipeux riche en aromatase) ; insulinorésistance donc augmentation de l'insuline et de l'insuline-like growth factor (IGF1), ce dernier ayant été identifié comme un facteur favorisant la prolifération et la survie cellulaire tumorales par stimulation des voies MAPK et PI3K (84); augmentation du rapport leptine/adiponectine, certains auteurs suggérant que l'adiponectine aurait un rôle inhibiteur sur les voies PI3K, MAPK et mTOR impliquées dans la prolifération tumorale (85), inflammation chronique (64,65) ; et enfin, le rôle paracrine du tissu adipeux péritumoral et en particulier des

adipocytes, composants majeurs du microenvironnement tumoral dans le CS qui est détaillé au chapitre suivant. Les hypothèses biologiques pouvant expliquer la survenue/récidive/mauvais pronostic du CS chez les obèses sont détaillées dans la Figure 10.

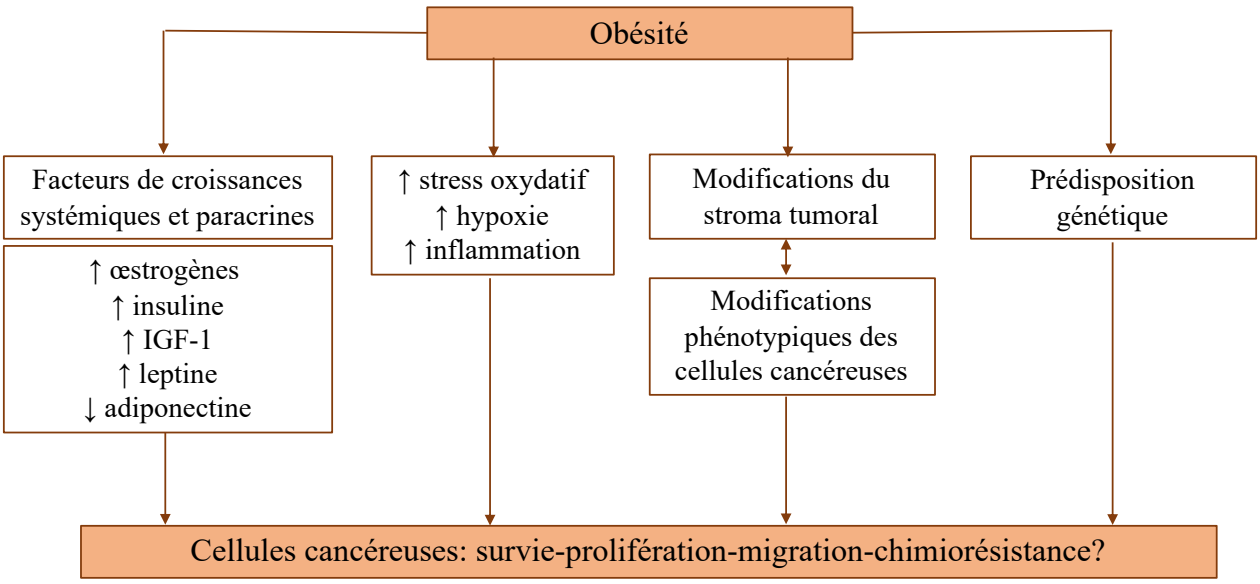

Figure 10 : Hypothèses biologiques pour expliquer la survenue/récidive/mauvais pronostic du cancer du sein chez les obèses *(Adapté de Netgen, Revue Médicale Suisse, 2012(86)).*

3. Influence du microenvironnement tumoral et des adipocytes dans le cancer du sein

Les cellules épithéliales mammaires font partie d'un microenvironnement complexe qui comprend la matrice extracellulaire, des facteurs diffusibles tels que les cytokines ou les facteurs de croissance et une grande variété de cellules non épithéliales comme des cellules adipocytaires, des fibroblastes, des cellules endothéliales, des lymphocytes ou encore des macrophages. Il est maintenant admis que le cancer est une maladie tissulaire au cours de laquelle les cellules tumorales interagissent de façon dynamique avec leur microenvironnement (87). Au cours de la progression tumorale, les cellules tumorales vont profondément modifier leur microenvironnement, qui en

retour va favoriser la croissance et la dissémination de la tumeur. Le rôle des fibroblastes associés au cancer (CAF pour cancer-associated fibroblast) et des macrophages (TAM pour tumor-associated macrophage) dans ce processus a clairement a été décrit il y a de nombreuses années (88,89). Les adipocytes ont longtemps été négligés alors qu'ils sont des composants majeurs du microenvironnement dans le CS. Le rôle du tissu adipeux dans la croissance tumorale et la formation de métastases est aujourd'hui mieux connu tout comme celui des molécules impliquées dans le dialogue entre adipocytes et cellules cancéreuses. Les sécrétions tumorales sont en effet capables de modifier le phénotype des adipocytes en les « activant ». Les adipocytes au contact des cellules tumorales perdent leur différenciation et acquièrent un phénotype pré-adipocytaire ou fibroblastique dont les propriétés sécrétoires sont accrues avec notamment une augmentation de la sécrétion de cytokines pro-inflammatoires. Ces adipocytes activés, nommés CAA pour cancer-associated-adipocytes sécrètent alors des protéases dont MMP-11 et des cytokines pro-inflammatoires dont IL6 qui va jouer un rôle clé dans l'effet pro-invasif des cellules carcinomateuses mammaires (72,90). Cela entraine des modifications majeures des caractéristiques et du phénotype des cellules tumorales et leurs propriétés d'invasion et d'agressivité (Figure 11).

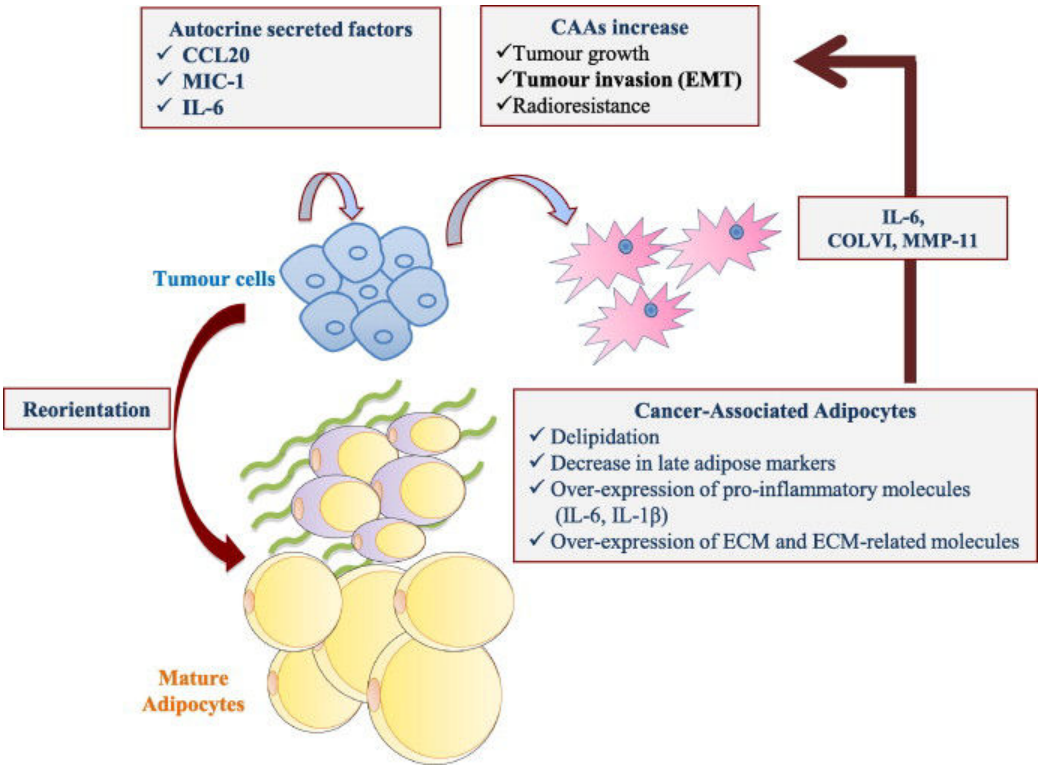

Figure 11: Dialogue bidirectionnel entre les adipocytes et les cellules cancéreuses mammaires et concept d'adipocyte associé au cancer (CAA) *(Wang, Cancer Letters, 2012 (72)).*

De plus, ces CAA, sous l'effet des sécrétions tumorales perdent leur contenu lipidique par lipolyse. Les acides gras libres libérés sont transférés et stockés dans des cellules tumorales sous forme de triglycérides en gouttelettes lipidiques, modulant ainsi leur métabolisme et favorisant leur capacités de survie (91).

Plusieurs études récentes suggèrent plus spécifiquement que les cellules progénitrices adipocytaires ou adipose stem cells (ASC) présentes dans le TA pourraient également jouer un rôle important dans la progression tumorale via l'activation de plusieurs signaux intracellulaires (92,93). Les ASC favoriseraient le remodelage de la matrice extracellulaire et la production des métalloprotéases par les cellules cancéreuses, produiraient des exosomes et des miRNAs impliqués

dans la croissance tumorale et le développement de métastases et agiraient sur plusieurs voies de signalisation impliquées dans le métabolisme des cellules tumorales, la production de leptine ou une fois encore d'IL6.

Enfin, les tissu adipeux et les adipocytes pourraient avoir un rôle dans l'acquisition d'une chimiorésistance. Il a été démontré que la coculture de cellules cancéreuses mammaires avec des adipocytes favorisaient la résistance à la doxorubicine, un agent anticancéreux majeur utilisé dans le traitement du CS, via la surexpression d'un transporteur membranaire, le transport-associated major vault protein (MVP) qui favorise l'efflux de l'agent chimiothérapeutique hors de la cellule (94). Le rôle des pré-adipocytes et des adipocytes a également été suggéré dans l'acquisition d'une résistance au trastuzumab, anticorps monoclonal ayant montré son efficacité pour le traitement des tumeurs HER2 amplifiées.

4. Interactions entre le tissu adipeux et les xénobiotiques

De plus en plus d'études s'intéressent aux relations entre le tissu adipeux et les polluants de l'environnement (95–97). En effet, 70% de la masse du TA est formée par des lipides notamment des triglycérides, faisant ainsi de ce tissu le plus grand réservoir de contaminants et polluants lipophiles de l'organisme.

L'obésité est la maladie la plus étudiée en santé environnementale et il a été montré par exemple que l'exposition prénatale à des perturbateurs endocriniens semble favoriser une augmentation de la masse adipeuse à l'âge adulte et les complications métaboliques associées (98,99). Par ailleurs, des travaux réalisés dans le laboratoire ont montré que plusieurs POP pouvaient avoir un effet direct sur le tissu adipeux et augmenter l'inflammation de ce tissu (42). Dans cette étude, l'exposition de cellules pré-adipocytaires et d'adipocytes à la dioxine mais aussi les PCB 153 et le PCB 126 entrainaient la régulation de plusieurs gènes impliqués dans les voies de signalisation de la réponse inflammatoire (et à un moindre niveau de la réponse immunitaire, métabolique et du cancer) d'une manière AhR-dépendante.

De plus, si le tissu adipeux joue un rôle protecteur de séquestration lors d'expositions aigües, on sait qu'il constitue une source interne chronique de polluants (dont un grand nombre sont cancérigènes) qui sont libérés à bas bruit dans le sang pour être éliminés, peut-être d'autant mieux lors des épisodes lipolytiques (100). Dans certaines situations, la quantité de POP libérée dans la circulation par le tissu adipeux peut augmenter de façon importante avec des potentiels effets sur les organes cibles. Par exemple, une augmentation progressive de la concentration sanguine en dioxines, furanes et PCB a été observée lors d'amaigrissements drastiques obtenus par la combinaison d'une chirurgie bariatrique et d'un régime adéquat (1). Chez les personnes présentant

les concentrations de polluants les plus importantes, l'amélioration des fonctions hépatiques et du profil lipidique étaient plus lentes et moins franches que chez les autres sujets.

A la lumière de ces données et du rôle supposé des xénobiotiques dans la progression tumorale retrouvé dans les études expérimentales, nous avons émis l'hypothèse que les polluants organiques persistants pouvaient influencer le tissu adipeux, composant majeur du microenvironnement tumoral du CS, et favoriser par ce biais la progression tumorale et la formation de métastases.

PARTIE 2 :
ETUDE CLINIQUE METAPOP

ARTICLE 2

Synthèse article 2 : ***"Associations between persistent organic pollutants and risk of breast cancer metastasis"***

Plusieurs études récentes suggèrent que les POP, qui s'accumulent dans le tissu adipeux, peuvent influencer le phénotype de la tumeur et stimuler les processus cellulaires impliqués dans la cascade métastatique tels que l'invasion (voir article 1). Dans le CS, il a été montré par exemple que le dichlorodiphényldichloroéthylène (DDE), un métabolite du dichlorodiphényltrichloroéthane plus connu sous le nom de DDT, est associé au risque d'envahissement ganglionnaire, à la taille tumorale et à une diminution de la survie globale (101) mais il n'existe aucune étude observationnelle disponible dans la littérature.

Nous avons donc mené une étude clinique prospective exploratoire afin d'évaluer l'association entre les taux sériques et adipeux de POP et la présence d'une atteinte métastatique ganglionnaire chez les patientes prise en charge pour un CS.

Dans cette étude cas-témoins, les concentrations de 49 POP dont les dioxines, les polychlorobiphényls (PCB), les polybromobiphénylethers (PBDE), les polybromobiphényls (PBB) et les hexabromocyclododecanes (HBCD) ont été mesurées par spectrométrie de masse dans des échantillons de sérum et de tissu adipeux péritumoral provenant de patientes atteintes de CS et opérée dans le service de chirurgie cancérologique gynécologique et du sein de l'hôpital européen Georges Pompidou. Des modèles de régression logistique ont été utilisés pour étudier les associations entre les concentrations de POP et le risque de métastases et d'autres caractéristiques de l'agressivité du CS comme la taille tumorale. Nous avons également analysé spécifiquement les effets du surpoids et de l'obésité, ces facteurs étant associés comme nous l'avons vu à un mauvais pronostic.

Résultats :

Nous avons montré que les concentrations de 2.3.7.8-TCDD et de deux PCB coplanaires (c'est-à-dire dioxin-like et ligands du AhR) (PCB 77 et 169) dans le tissu adipeux étaient positivement associées au risque de métastase ganglionnaire et à la taille de la tumeur. D'autres produits chimiques tels que le PBDE 153 (2,2',4,4',5,5'-Hexabromodiphenyl ether, un retardateur de flamme), le 1.2.3.4.7.8 HxCDD (une autre dioxine) ou le PCB114 présentaient également des odds ratio (OR) supérieurs à 2 avec des valeurs de p inférieures à 0,1. De façon intéressante, cette association était encore plus forte chez les 43 patientes présentant un IMC égal ou supérieur à 25 kg/m^2 avec un OR à 4,48 (Intervalle de confiance de 95% 1,32–20,71). De plus, chez les 10 patientes atteintes d'un cancer triple négatif connu pour son agressivité et son risque élevé de dissémination métastatique, nous avons observé des concentrations de plusieurs POP ou catégories de POP significativement plus élevées dans le groupe métastatique (1.2.3.4.6.7.8-HpCDF ($p < 0,01$) ; PCB 101, 138, 153, 167, somme des non dioxin-like PCB et somme de 8 PBDE ($p < 0,05$)).

Ces résultats doivent être pris avec précaution car il s'agit d'une étude préliminaire sur un échantillon limité (91 patientes) mais ils permettent pour la première fois de suggérer que l'exposition interne à la 2.3.7.8-TCDD et à certains PCB pourrait être associée à des phénotypes de CS agressifs, en particulier chez les patientes en surpoids et les patientes atteintes d'un CS triple négatif. Des études longitudinales plus larges sont nécessaires pour confirmer ces associations.

De façon plus générale, la compréhension des mécanismes impliqués dans les effets des POP dans la progression tumorale et sur les interactions POP/tissu adipeux/cellules cancéreuses est essentielle.

Ce sont ces aspects que nous avons cherchés à explorer dans la partie expérimentale de la thèse et nos résultats sont présentés dans l'article 3.

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Associations between persistent organic pollutants and risk of breast cancer metastasis

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ABSTRACT

Background: Breast cancer (BC) is a major public health concern with over 2 million new cases diagnosed and over 600,000 deaths in 2018 in women worldwide. When distant metastases are present at diagnosis, the 5-year survival rate is only 26%. Recent studies have suggested that persistent organic pollutants (POPs) that accumulate in adipose tissue (AT) can influence tumor phenotype and stimulate cellular processes important for metastasis such as invasion. We, therefore, tested the hypothesis that POP exposure is associated with BC metastasis.

Methods: We conducted an exploratory case-control study in which the concentrations of 49 POPs were measured in both AT and serum samples from BC patients, with or without lymph node metastasis, who underwent partial or total mastectomies, lymph node biopsies and sampling of the adipocytic tumor microenvironment. Adjusted, unconditional logistic models were used to study the associations between the POP concentrations and the risk of metastasis and other hallmarks of cancer aggressiveness.

Results: 2.3.7.8-TCDD concentrations in AT are positively associated with the risk of metastasis in 43 patients who have BMIs equal or higher than 25 kg/m2 (odds ratio: 4.48 (1.32–20.71)). Furthermore, the concentrations of 2.3.7.8-TCDD and two coplanar PCBs (77&169) in AT also were positively associated with the risk of lymph node metastasis and the tumor size.

Conclusion: Our study suggests that 2.3.7.8-TCDD and some PCBs contribute to the development of tumor metastasis and other hallmarks of cancer aggressiveness. While these results should be considered with caution, this is the first study to identify such potential risk factors. Larger longitudinal studies are necessary to confirm our results.

Clinical Trial Protocol Record: 2013-A00663-42.

1. Introduction

Breast cancer (BC) is a major health concern with over 2 million new cases diagnosed in 2018 in women worldwide (Bray et al., 2018). If the cancer is located only in the breast, the 5-year survival rate is 99% but this rate decreases if there is spread to lymph nodes (85%). However, if distant metastases are present upon diagnosis the survival rate drops dramatically (26%) (Howlader et al., 1975; Siegel et al., 2017).

The exposure to environmental pollutants has been proposed, recently, as a risk factor for BC (Lauby-Secretan et al., 2013; Leng et al., 2016; Rodgers et al., 2018; Zhang et al., 2015). Polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs) have been identified as endocrine-disrupting-chemicals (EDC) and/or as carcinogens. Some of these compounds are persistent organic pollutants (POPs) since

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their hydrophobicity favors their bioaccumulation throughout the food chain. Overall, the scientific evidence concerning the associations between EDC and BC risk in humans has been inconclusive (Ochieng et al., 2015).

To date, researchers have been interested mainly in the link between BC initiation and EDC. Only a few recent studies have explored the associations with late stages of cancer, such as metastasis, which is the main cause of BC mortality. We have shown that TCDD (2,3,7,8-Tetrachlorodibenzo-p-dioxin) triggers invasive processes using human tumor cell lines as model systems (Bui et al., 2009; Diry et al., 2006; Pierre et al., 2014; Tomkiewicz et al., 2013). TCDD is best known for its induction of the AhR signalling pathway. AhR is responsive to many POPs (including PCBs) and aromatic hydrocarbons. Two studies have demonstrated an association between smoking and pulmonary metastasis among women with BC (Murin and Inciardi, 2001; Scanlon et al., 1995). Among POPs, TCDD is suspected to act as an endocrine disruptor similarly to organochlorine compounds (OCs). Several studies which focused on the impact of POPs and non-POPs on the initiation of breast cancer did not demonstrate a relationship between xenobiotics exposure and the incidence of BC (Høyer et al., 2000; Muscat et al., 2003; Parada et al., 2016a, 2016b; Roswall et al., 2018). However, p,p′-DDE was found to be associated with a dose-related increased relative risk of both lymph-node involvement and large tumors which suggests a potential relationship with tumor aggressiveness (Dairkee et al., 2008; Demers et al., 2000; Parada et al., 2019). Further, a very recent study found that higher levels of p,p′-DDE and DDT in the blood were associated with worse overall survival (Parada et al., 2019). Also, it has been shown that breast cancer cells exposed to Bisphenol A, another endocrine disruptor (non-POP), exhibit a gene expression profile of tumor aggressiveness which is associated with poor clinical outcomes for breast cancer patients (Dairkee et al., 2008). Finally, several POPs trigger metastatis formation in mice (Castillo-Sanchez et al., 2013; Guo et al., 2015; Liu et al., 2010; Ochieng et al., 2015; Pierozan et al., 2018; Pontillo et al., 2013; Seelbach et al., 2010; Zhang et al., 2016).

Since no observational studies have been conducted to evaluate the link between EDC and BC metastasis, we conducted an exploratory, hospital-based case-control study to analyze the concentrations of 49 POPs in samples of adipose tissue (AT) and serum from breast cancer patients with and without lymph node metastasis. We also analyzed the effect of obesity since this factor is associated with larger tumors, positive lymph node status and overall survival (Picon-Ruiz et al., 2017).

2. Material and methods

2.1. Study design

A monocentric cohort study (Clinical Trial Protocol Record 2013-A00663-42) was conducted in the Department of gynecological-oncological surgery of the Georges-Pompidou European Hospital (Paris, France) from 12/2013 to 11/2017. Participation in the study was proposed to each female newly diagnosed for BC and operated in the department, aged 18 years or more, who possessed a uni- or multifocal lesion with the principal lesion being > 1 cm in size or palpable. All patients (10% of total BC population) gave informed consent to participate in the study, which was approved by the 'Comité de Protection des Personnes' in 2013 [French equivalent of an Institutional Review Board (IRB)]. Patients unable to give informed consent were not included. The reasons for not participating in the study were: 1) Non-palpable lesions. The lesion had to be palpable so that peritumoral adipose tissue could be easily collected; 2) Patients who have an excessive number of solicitations from many studies in the department with a large number of questionnaires; 3) Men were removed from the study for reasons of homogeneity. At enrollment, the participants completed an assisted lifestyle questionnaire and provided a blood sample to assess POP concentrations before any treatment was initiated. Patients underwent partial or total mastectomy. Lymph node biopsy and axillary lymph node removal (depending upon the lymph node biopsy results) were performed according to the American Society of Clinical Oncology (ASCO) guidelines. During the surgery, an adipose tissue sample (1–3 g) was obtained at a distance of 1 cm from the palpable tumor for the measurement of POPs. The cancer tissue removed during the surgery was sent to the pathology laboratory for clinical analyses. Nodal status was determined for each patient. Patients were classified into 2 groups and participants with lymph node involvement were placed in the metastatic group (classification was performed without any information regarding the POP measurement). All the patients' data were anonymized and recorded on a computerized database (Fig. 1).

2.2. Chemical analysis

The methodologies for the isolation, detection and quantification of the targeted POPs, including dioxins (PCDD/F), polychlorobiphenyls (PCB), polybromodiphenylethers (PBDE), polybromobiphenyls (PBB) and hexabromocyclododecane (HBCD), have been described (Antignac et al., 2006, 2009; Bichon et al., 2015; Cariou et al., 2005; Costera et al., 2006; Ploteau et al., 2016). The complete list of chemicals (IUPAC) can be found in Table S1. Briefly, samples were first submitted to a high pressure and temperature extraction (ASE Dionex, Sunnyvale, CA, USA) (fat) or a liquid/liquid extraction with pentane (serum). The resulting extracts were weighed to measure fat content (gravimetric method for fat, enzymatic determination for serum) and reconstituted in hexane for further sample clean up. PCDD/F, PCB, PBDE and PBB measurements were performed by gas chromatography (Agilent 7890A) coupled to high-resolution mass spectrometry (GC-HRMS) on double sector instruments (JEOL MS 700D and 800D) after electron impact ionization (70 eV; 10% valley) and in the single ion monitoring (SIM) acquisition mode. The three HBCD isomers (alpha, beta and gamma) were quantified using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) on a triple quadrupole instrument (Agilent 6410) using electrospray ionization and selective reaction monitoring as the acquisition technique. All the analyses were conducted in an ISO 17025:2005 accredited laboratory. The lipid content for serum samples was determined with enzymatic kits (Biolabo, Maizy, France) which allowed determination of the concentrations of phospholipids (PHO), triacylglycerides (TAG), total cholesterol (t.CHO) and free cholesterol (f.CHO). Total serum lipids (TSL) were estimated using the following formula (Akins et al., 1989): $TSL = 1.677 * (t.CHO - f.CHO) + (f.CHO + TAG + PHO)$.

2.3. Statistical analysis

Demographic characteristics were summarized using the median and interquartile range, for continuous variables, and the frequency and percentage, for categorical variables. The statistical comparison of demographic characteristics between groups (i.e. metastatic versus non-metastatic) was performed using the Mann-Whitney-Wilcoxon Test and Fisher's exact test, for the numerical and the categorical variables, respectively. The associations calculated with Spearman's correlation coefficients were plotted in heatmaps. The distributions of POPs between groups were summarized by medians and interquartile ranges and compared statistically using Mann-Whitney-Wilcoxon Tests. Those chemicals with detection rates below 75% were excluded from the statistical analysis and for the analytes whose detection rates were between 75 and 100%, the non-detected values were replaced by the limits of detection (Antweiler 2015).

Crude odds ratios (ORs), adjusted ORs (aORs) and corresponding 95% confidence intervals (CI) were calculated for each chemical (transformed to the natural logarithm) using unconditional logistic regression considering the non-metastatic cancer group as referent. Three adjusted models were considered to account for predefined variables known as factors which affect the internal levels of POPs and the risk of

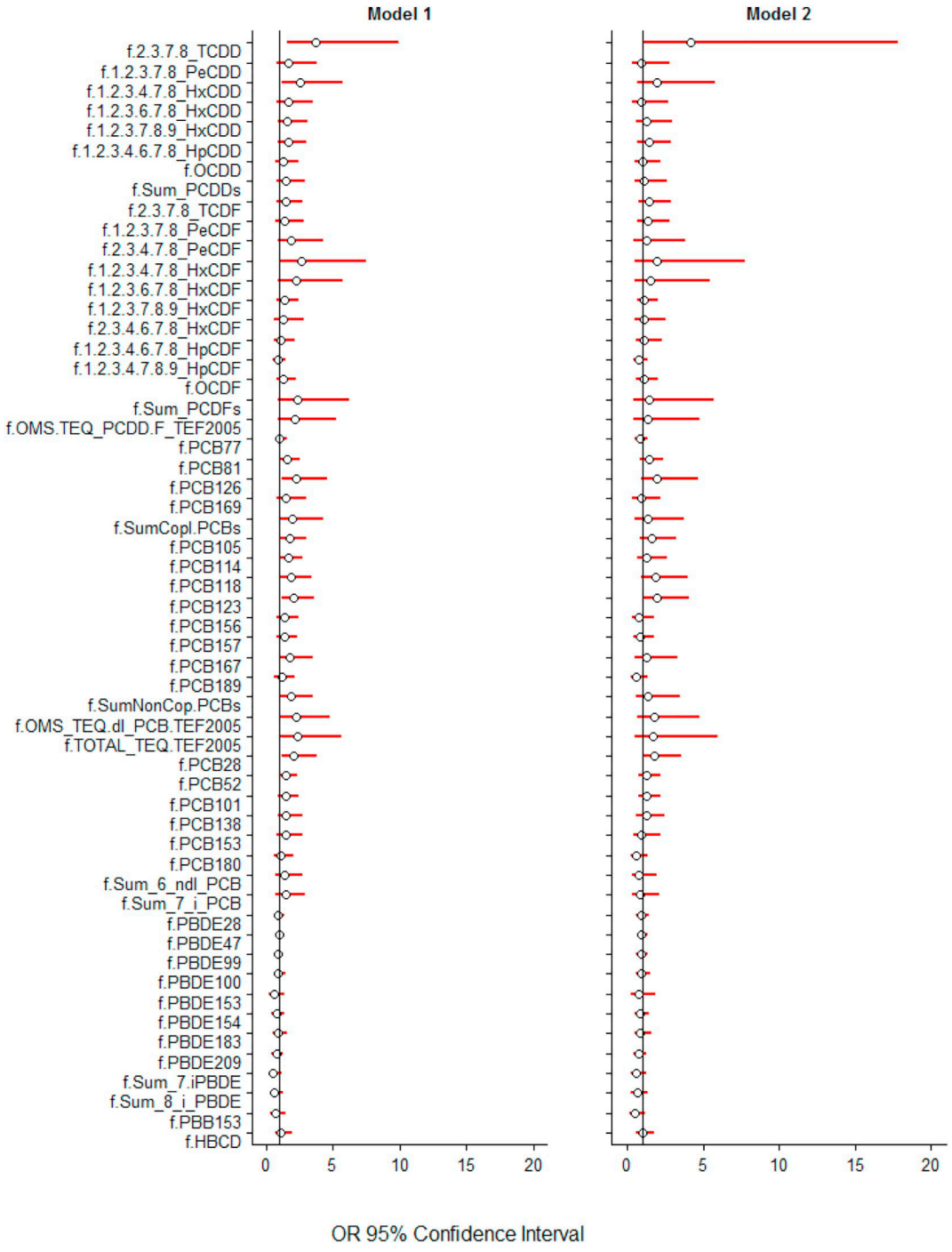

Fig. 1. Associations between the concentrations of persistent organic pollutants in adipose tissue and the presence of large tumor size (≥ 2 cm vs < 2 cm). The forest plots represent the odds ratios and 95% confidence intervals estimated through unconditional logistic regression. Model 1 - Crude model; Model 2 – adjusted for age, body mass index and familial history of breast cancer.

cancer. The confounding variables in the first adjusted model were the age, smoking and menopause. In the second adjusted model, we further included the body mass index (BMI) to evaluate the effect of adiposity as a potential confounder. In the third adjusted model, the familial history of BC also was included in the final model. Due to the small influence of confounding variables in the risk estimates, we only reported the results of the crude and fully adjusted models in the main manuscript.

We further explored whether the concentrations of POPs were associated with the number of lymph nodes affected, classified according to the international TNM classification of breast cancer (N1 $\leq$ 3 nodes, N2 = 4–9 nodes, N3 $\geq$ 10 positive nodes). The groups N2 and N3 were combined for the statistical analysis due to the low number of individuals in each group. This classification aims to better anticipate the aggressiveness of the tumor and its spreading potential. Tumor size was evaluated as an independent outcome of cancer aggressiveness by dichotomizing the cases, according to the median of the distribution of tumor sizes, into a group of small tumor size and another with large size (cut off = 2 cm). The concentrations of POPs were compared between the two groups with adjustments to POPs concentrations for BMI, age and familial breast cancer history in unconditional logistic models. The breast cancer expression of estrogen and progesterone receptors was

further considered in the stratification analysis but was limited to the positive phenotypes due to the low number of receptor-negative tumors.

Serum biomarkers were considered as surrogates of peritumoral AT biomarkers and were considered only in the global models to evaluate the coherence of estimates with AT models. For that reason, these proxy circulating markers were not considered in the stratification analysis and were relegated to secondary outcomes in the results section. Since lipid normalized concentrations of POPs in serum may lead to biased estimates when serum lipids may fall in the causal pathway between the exposures and the outcomes, a systematic comparison of different approaches was conducted (Cano-Sancho et al., 2018; O'Brien et al., 2016). Hence, we considered the Model WW, that uses serum concentrations expressed on a wet basis (pg or ng/mL); the Model WWA, that uses serum concentrations expressed on a wet basis (pg or ng/mL) and the TSA included as a covariate in the regression model; Model LW, that uses serum concentrations on a lipid basis (pg or ng/g lipids); Model LWA, that uses serum concentrations on a lipid basis (pg or ng/g lipids) and the TSA also included as a covariate in the regression model. We further tested the associations between metastasis and the ratio of concentrations between both matrices, serum and adipose tissue ($R_{ser:AT}$), as a multi-compartment biomarker (Ploteau et al., 2016). In equilibrated states, the concentrations of both compartments expressed in lipid weight should be equivalent, and the $R_{ser:AT}$ should be close to 1, conversely, altered states would be reflected by estimates > 1.

Mixtures of parent chemicals were generated by sum of concentrations and/or sum of 2005 World Health Organization dioxin-like toxic equivalents (WHO-TEQ). A threshold for the p-value < 0.05 was used to define associations as statistically significant. Considering the hypothesis-driven design of this study, the relatively small number of multiple tests conducted and the high correlation among variables tested, we opted not to correct for the multiple comparisons which minimizes the risk of inflating the type 2 errors (Rothman, 1990). All the statistical analyses were performed using R (v.3.3.1.) software.

3. Results

3.1. Demographic characteristics

A summary of the study population's characteristics is presented in Table 1 and Table S2 (participants with and without metastases). Both datasets reflected the homogeneity of the groups. The median age of participants ranged from 58 to 67 years. A majority of women had undergone menopause (71–85%). The most frequent histological subtype in both groups was infiltrating ductal carcinoma. There was no difference in the expression of ER, PR and HER2. As expected, the number of lymph nodes, the frequency of larger tumors and the Ki-67 proliferation index > 20%, were higher among patients with metastatic cancer. For several participants, the volume of serum or the amount of adipose tissue was insufficient to complete the chemical analyses and vice versa. Therefore, the serum dataset contained the results derived from 40 participants with metastatic cancer and 47 with non-metastatic cancer, different than the 38 participants with metastatic cancer and 53 with non-metastatic cancer recorded in the AT dataset.

3.2. Distribution of persistent organic pollutants

The distributions of POPs in AT and serum are summarized in Table 2 and in Tables S3 and S4. Chemicals with detection rates lower than 75% (i.e. PBB52, PBB101, β-HBCD, γ-HBCD in AT and serum and α-HBCD in addition in serum) were excluded from the statistical analysis. The POPs distribution was not statistically different between both groups.

After normalization by lipid content, the concentrations in AT and serum were highly correlated for most chemicals (Fig. S1A–B). However, most flame retardants in AT exhibited lower correlations with their circulating levels, although there were several exceptions (Fig. S1C). The correlation structures between chemicals appeared to be almost identical between metastatic and non-metastatic cancers in both AT and serum (Figs. S2–3).

Table 1
Population characteristics (Median and interquartile range (IQR) or counts and frequency percentages) from the adipose tissue dataset for individuals with breast cancer with metastasis or without metastasis. The distributions between groups have been compared with Mann–Whitney *U* test for continuous data and Fisher's exact test for categorical data.

	Metastatic N = 38	Non-metastatic N = 53	p-Value
Age (years)	59.8 (52.9–74.3)	64.0 (51.0–73.0)	0.99
BMI (kg/m^2)	24.0 (21.4–27.8)	24.0 (21.0–27.0)	0.63
Parity (number of infants)			0.94
0	8 (21%)	13 (25%)	
1	8 (21%)	13 (25%)	
2	12 (32%)	15 (28%)	
≥3	10 (26%)	12 (22%)	
Breastfeeding	18 (47%)	25 (47%)	1.00
Missing	1	3	
Menopause	27 (71%)	38 (72%)	1.00
Missing	1	0	
Hormonal contraception	9 (24%)	11 (21%)	0.80
Missing	2	1	
Professional category			0.27
White collar	17 (45%)	30 (57%)	
Blue collar	2 (5%)	2 (4%)	
Pink collar	17 (45%)	15 (28%)	
Un-employed/students	1 (3%)	5 (10%)	
Living area			0.17
Rural	5 (13%)	2 (4%)	
Urban	33 (87%)	50 (94%)	
Mixt	0 (0%)	1 (2%)	
First-hand smoking	8 (21%)	9 (17%)	0.60
Missing	1	0	
Second-hand smoking	4 (11%)	10 (19%)	0.38
Missing	1	0	
Personal history of cancer	2 (5%)	5 (9%)	0.69
Family history of breast cancer	8 (21%)	14 (26%)	0.62
Missing	0	1	
Histopathological examinations			
Triple negative	4 (11%)	6 (11%)	1.00
Estrogen receptor positive	33 (87%)	44 (83%)	0.77
Progesterone receptor positive	27 (71%)	36 (68%)	0.82
Tumor size			0.001
0.1–2 cm	12 (32%)	31 (58%)	
3.1–5 cm	20 (53%)	22 (42%)	
> 5 cm	6 (16%)	0 (0%)	
Affected lymph nodes			< 0.0001
≤ 3 nodes	26 (68%)	0 (0%)	
4–9 nodes	9 (24%)	0 (0%)	
≥10 nodes	3 (8%)	0 (0%)	
No applicable	0 (0%)	53 (100%)	
Grade histology			0.68
1	5 (13%)	10 (19%)	
2	21 (55%)	25 (47%)	
3	12 (32%)	18 (34%)	
Histological classification			0.89
Infiltrating ductal carcinoma	31 (82%)	40 (75%)	
Infiltrating lobular carcinoma	6 (16%)	9 (17%)	
Papillary carcinoma	0 (0%)	2 (4%)	
Mucinous (colloid) carcinoma	1 (3%)	1 (2%)	
Apocrin carcinoma	0 (0%)	1 (2%)	
HER2 Positive	2 (5%)	6 (11%)	0.46
Ki-67			0.03
< 20%	12 (32%)	29 (55%)	
> 20%	26 (68%)	24 (45%)	

Table 2
Summary of the (median (interquartile range)) of persistent organic pollutants measured in adipose tissue from individuals with metastatic and non-metastatic breast cancer. The groups were compared with the Mann-Whitney *U* test.

Chemicals[a]	Units	Metastatic (n = 38)	Non-metastatic (n = 53)	p-Value
2.3.7.8-TCDD	pg/g l.w.	1.4 (1.1–1.8)	1.3 (0.9–2.0)	0.70
1.2.3.7.8-PeCDD	pg/g l.w.	4.6 (3.7–5.9)	4.5 (3.6–6.6)	0.86
1.2.3.4.7.8-HxCDD	pg/g l.w.	2.3 (1.8–2.8)	2.2 (1.4–3.7)	0.72
1.2.3.6.7.8-HxCDD	pg/g l.w.	14.7 (11.9–19.8)	15.4 (9.2–24.4)	0.62
1.2.3.7.8.9-HxCDD	pg/g l.w.	1.6 (1.1–2.3)	1.5 (1.1–2.8)	0.73
1.2.3.4.6.7.8-HpCDD	pg/g l.w.	12.6 (8.1–19.8)	12.9 (6.7–23.8)	0.61
OCDD	pg/g l.w.	91.3 (60.4–123.3)	102.7 (71.8–162.1)	0.18
Sum PCDDs	pg/g l.w.	126.7 (92.4–180.5)	139.8 (96.9–235.1)	0.32
2.3.7.8-TCDF	pg/g l.w.	0.4 (0.3–0.5)	0.4 (0.3–0.6)	0.86
1.2.3.7.8-PeCDF	pg/g l.w.	0.2 (0.1–0.3)	0.2 (0.1–0.3)	0.64
2.3.4.7.8-PeCDF	pg/g l.w.	12.0 (8.7–14.9)	11.2 (8.6–19.3)	0.67
1.2.3.4.7.8-HxCDF	pg/g l.w.	2.0 (1.6–2.6)	2.2 (1.7–3.4)	0.41
1.2.3.6.7.8-HxCDF	pg/g l.w.	2.4 (1.8–2.9)	2.5 (1.9–3.7)	0.46
1.2.3.7.8.9-HxCDF	pg/g l.w.	0.1 (0.1–0.1)	0.1 (0.1–0.1)	0.61
2.3.4.6.7.8- HxCDF	pg/g l.w.	0.8 (0.6–1.0)	0.8 (0.5–1.1)	0.96
1.2.3.4.6.7.8-HpCDF	pg/g l.w.	1.2 (0.8–1.6)	1.3 (1.0–1.8)	0.24
1.2.3.4.7.8.9-HpCDF	pg/g l.w.	0.1 (0.1–0.2)	0.1 (0.1–0.1)	0.83
OCDF	pg/g l.w.	0.3 (0.2–0.6)	0.3 (0.2–0.4)	0.46
Sum PCDFs	pg/g l.w.	19.3 (16.4–26.2)	19.6 (14.9–29.8)	0.62
WHO-TEQ_{2005} PCDD/F	TEF_{2005}/g l.w.	11.8 (10.3–15.1)	11.6 (9.4–17.8)	0.81
PCB 77	pg/g l.w.	4.3 (2.8–7.8)	6.7 (3.4–11.9)	0.16
PCB 81	pg/g l.w.	2.1 (1.1–3.0)	2.4 (1.3–4.7)	0.14
PCB 126	pg/g l.w.	61.0 (42.8–81.5)	62.5 (40.7–103.6)	0.77
PCB 169	pg/g l.w.	79.0 (56.0–117.3)	84.0 (53.4–134.7)	0.94
Sum Copl. PCBs	pg/g l.w.	147.9 (106.9–217.9)	180.8 (103.3–241.4)	0.58
PCB 105	pg/g l.w.	4379.0 (2641.4–6129.3)	5247.4 (2473.8–11,189.3)	0.24
PCB 114	pg/g l.w.	1941.0 (1223.7–3227.5)	2329.2 (857.6–3397.8)	0.75
PCB 118	pg/g l.w.	20,010.1 (13,746.8–28,123.0)	24,288.5 (14,501.8–47,869.5)	0.37
PCB 123	pg/g l.w.	183.2 (132.3–373.8)	268.2 (140.5–573.7)	0.40
PCB 156	pg/g l.w.	21,177.7 (12,023.5–31,398.0)	20,850.0 (11,068.6–37,322.1)	0.88
PCB 157	pg/g l.w.	4496.6 (2325.4–6408.0)	4030.0 (1927.7–7644.8)	0.77
PCB 167	pg/g l.w.	4466.9 (2787.1–7229.4)	4314.9 (2698.6–7531.7)	0.91
PCB 189	pg/g l.w.	2801.2 (1811.9–4222.3)	2871.9 (1639.3–4313.4)	0.81
Sum Non Cop. PCBs	pg/g l.w.	59,011.5 (39,991.2–93,646.3)	62,660.2 (36,089.7–115,909.2)	0.59
WHO-TEQ_{2005} PCB s	TEF_{2005}/g l.w.	10.7 (7.2–15.7)	11.5 (7.8–18.0)	0.57
TOTAL WHO-TEQ_{2005}	TEF_{2005}/g l.w.	22.5 (16.9–30.8)	24.1 (16.1–35.2)	0.71
PCB 28	ng/g l.w.	1.7 (0.9–2.4)	1.9 (1.0–3.0)	0.62
PCB 52	ng/g l.w.	0.3 (0.2–0.6)	0.4 (0.2–0.7)	0.26
PCB 101	ng/g l.w.	0.7 (0.5–1.4)	1.0 (0.5–1.9)	0.17
PCB 138	ng/g l.w.	71.9 (52.9–100.9)	72.3 (40.5–124.7)	0.89
PCB 153	ng/g l.w.	193.0 (131.1–253.3)	166.2 (117.6–283.4)	0.82
PCB 180	ng/g l.w.	168.4 (120.5–270.1)	156.6 (106.1–277.4)	0.63
Sum 6 non dioxin-like PCB	ng/g l.w.	438.6 (291.2–621.3)	391.5 (261.6–710.6)	0.78
PBDE 28	ng/g l.w.	0.021 (0.010–0.036)	0.025 (0.015–0.045)	0.18
PBDE 47	ng/g l.w.	0.238 (0.138–0.491)	0.361 (0.162–0.646)	0.23
PBDE 99	ng/g l.w.	0.067 (0.037–0.112)	0.079 (0.039–0.166)	0.42
PBDE 100	ng/g l.w.	0.123 (0.056–0.207)	0.119 (0.071–0.241)	0.61
PBDE 153	ng/g l.w.	1.440 (1.054–1.899)	1.436 (1.013–1.915)	0.98
PBDE 154	ng/g l.w.	0.025 (0.015–0.034)	0.024 (0.017–0.048)	0.66
PBDE 183	ng/g l.w.	0.122 (0.083–0.201)	0.142 (0.092–0.188)	0.68
PBDE 209	ng/g l.w.	1.761 (1.275–2.199)	1.454 (1.088–1.941)	0.27
Sum penta and hepta BDE	ng/g l.w.	2.296 (1.447–3.039)	2.288 (1.697–3.175)	0.66
Sum penta, hepta and deca BDE	ng/g l.w.	4.381 (3.484–5.537)	3.981 (3.016–5.299)	0.45
PBB 153	ng/g l.w.	0.609 (0.440–0.842)	0.640 (0.488–0.883)	0.47
α-HBCD	ng/g l.w.	1.020 (0.600–1.769)	1.615 (0.965–1.952)	0.18

[a] Note: PBB 52, PBB 101, β-HBCD and γ-HBCD presented < 75% of detected samples and excluded from the statistical analysis.

3.3. Associations between POPs and risk of breast cancer metastasis

The summary of adjusted ORs (95% CI) for the major chemical groups is presented in Table 3 whereas the detailed graphical summary of ORs and 95% CIs for the different models and chemicals is plotted in Fig. S4. Overall, the results from the different models using AT biomarkers did not show any significant association between POPs and the risk of metastasis. The ORs and respective 95% CI were uniformly distributed around the null. The Seveso dioxin 2.3.7.8-TCDD exhibited substantially higher adjusted ORs (1.72 (0.56–6.03)) than the rest of PCDDs or their sum (0.67 (0.28–1.54)) (model 4, Fig. S4, Table 3). Moreover, when these analyses were restricted to a sub-group with a high BMI ($\geq 25\,kg/m^2$), the ORs were larger and the risk of BC metastasis was significantly larger for 2.3.7.8-TCDD (aOR 4.48 (1.32–20.71)). Other chemicals such as PBDE 153 (aOR 2.27 (1.06–6.00)), 1.2.3.4.7.8 HxCDD or PCB114 also displayed higher ORs (> 2) with p values below 0.1 (Table 4). The results for the subgroup with a normal BMI ($< 25\,kg/m^2$) displayed null associations for all chemicals with ORs shifting below one for a large list of chemicals including 2.3.7.8-TCDD, the WHO-TEQ Sum of PCDD/F or the non-coplanar PCBs, which supports the concept that BMI modifies effects.

The stratification analyses taking into account only the sub-groups of individuals with breast cancer and the expression of estrogen and progesterone receptors are summarized in the Table S5. The results from both sub-groups showed very similar association patterns, with a global modification of the ORs towards the null or below. Remarkably,

Table 3
Summary estimates for the associations between the sums of concentrations of PCDDs, PCDFs, PCBs, PBDEs and PBB153 in adipose tissue and the risk of breast cancer metastasis represented with the odds ratios and 95% confidence intervals estimated from unconditional logistic regression (N = 91, 38 cases with metastatic cancer and 53 with non-metastatic cancer). The model was adjusted for age, smoking, body mass index, menopause and familial history of breast cancer. Abbreviations: WHO-TEQ, World Health Organization Dioxin-like Toxic Equivalents. NA: not applicable because the low detection rates. Ndl, non-dioxin like.

Chemical groups	Adipose tissue adjusted OR (95% CI)	p value	Serum adjusted OR (95% CI)	p value
Sum PCDDs	0.67 (0.28–1.54)	0.36	0.81 (0.41–1.59)	0.55
Sum PCDFs	0.70 (0.20–2.45)	0.57	0.93 (0.26–3.31)	0.91
Sum PCDD/Fs WHO-TEQ	0.90 (0.28–3.02)	0.86	0.96 (0.34–2.70)	0.93
Sum Coplanar PCBs	0.85 (0.33–2.19)	0.74	1.15 (0.46–2.96)	0.77
Sum Non-Coplanar PCBs	0.93 (0.38–2.25)	0.86	1.11 (0.57–2.20)	0.75
Sum PCBs WHO-TEQ	0.83 (0.33–2.08)	0.70	1.15 (0.52–2.59)	0.73
Sum Ndl-PCB	1.18 (0.48–2.99)	0.72	1.23 (0.58–2.67)	0.60
Sum PBDEs	1.32 (0.61–3.02)	0.49	1.73 (0.65–4.78)	0.27
PBB 153	0.80 (0.33–1.92)	0.62	1.23 (0.44–3.70)	0.70
α-HBCD	1.02 (0.57–1.80)	0.93	NA	NA

the negative associations with PCB 101, PBDE28, PBDE 47 and PBDE99 became statistically significant for both groups ($p < 0.01$). The number of participants with a negative expression of the estrogen receptor (n = 14) and triple negative breast cancer (n = 10) were too small to allow the computation of regression models, but the metastatic group exhibited, in general, larger concentrations of all POPs with statistically significant differences (Mann-Whitney U test, summary in Table S6) for 1.2.3.4.6.7.8-HpCDF ($p < 0.01$) and PCBs 101, 138, 153, 167 and the sum Ndl-PCBs and the sum of 8 PBDEs ($p < 0.05$).

With respect to serum biomarkers, the ORs and 95% CIs also were distributed around the null for most chemicals with minor effects for confounding variables (Fig. S5). Also, the systematic comparisons of serum models did not reveal a major effect of TSL on the risk estimates for any of the models for most chemicals (See details in Fig. S6). These results also suggested a lack of consistency among the risk models using AT biomarkers with the serum biomarkers for some chemicals where the associations shifted in the opposite direction (e.g. 2.3.7.8-TCDD or OCDF). To further explore the potential implications of the chemical dynamics among both compartments on the risk estimates, we included the ratio among compartments $R_{Ser:AT}$ as an independent variable in the risk models. The results from the logistic regression using $R_{Ser:AT}$ are summarized in the Fig. S7. In general, the ORs for the ratios were close to the null, exhibiting similar trends to those results reported for AT and serum biomarkers; that is, the $R_{Ser:AT}$ of Seveso dioxin, 2.3.7.8-TCDD consistently associated with the risk of metastasis (1.72 (1.02–3.09)) (Table 5).

4. Discussion

Given the role of the cellular microenvironment on BC metastasis, we have explored, for the first time, the associations between the internal dose of a large panel of POPs and the risk of BC metastasis. Our study shows the widespread presence of low amounts of POPs in human tissues despite the fact that most of the chemicals were banned many years ago. The concentrations of these chemicals are similar to those previously reported in France which supports a background exposure in our study individuals comparable with that of the general population (Ploteau et al., 2017).

To date, only a few studies have explored the associations between a large panel of POPs and BC metastasis. For example, positive associations have been found between the internal concentrations of OCP and tumor size (Demers et al., 2000; Høyer et al., 2001). Women with higher concentrations of PCBs (particularly PCB118) were found to have a higher risk of BC recurrence, (Muscat et al., 2003). PCB 118 was one of the PCBs that we found to be associated with tumor size. Our results suggest that 2.3.7.8-TCDD may be a risk factor for BC aggressiveness and a predictor of poor prognosis, especially among overweight women. Obesity is recognized as a factor for poor prognosis in BC due to aggressive features of the tumor and resistance to treatment (Barba et al., 2016; Jiralerspong and Goodwin, 2016). In previous work, we showed that AT releases POPs chronically and slowly (Kim et al., 2011). This internal release may be responsible for the chronic toxicity associated with POPs exposure (Joffin et al., 2018). Moreover, we have shown that POPs promote an epithelial-to-mesenchymal transition which is suspected to promote the formation of metastatic cells (Bui et al., 2009; Pierre et al., 2014). Therefore, in this study, we evaluated the potential involvement of adiposity through stratification of a sub-group of the study population with high BMI. The subgroup analysis revealed a different pattern of associations which enlarged the ORs (95% CI) for 2.3.7.8-TCDD, the most toxic of dioxins.

TCDD (and several PCBs, such as PCB 77 and 169) activate the Aryl hydrocarbon Receptor (AhR) signalling pathway. The AhR regulates the expression of xenobiotic metabolism enzymes (such as cytochromes P450) upon exposure to different types of hydrocarbons including dioxins or various PCBs. Recently, other gene targets have been identified which include transcriptional regulators of the EMT such as Slug and Snail (Pierre et al., 2014). As a consequence, the induction of Slug or Snail by TCDD through the activation of the AhR signalling pathway down-regulates the epithelial marker, E-Cadherin. This facilitates the migration of tumor cells (Diry et al., 2006). The AhR also regulates non-genomic pathways (Src and Focal Adhesion Kinase) which are important for the stability of focal adhesion sites (Tomkiewicz et al., 2013). Thus, the activation of the AhR signalling pathway by xenobiotics has been suspected to play a potential role in tumor progression. This suspicion is supported by the identification of TCDD as a potential biomarker of metastasis. In addition to TCDD, we also have found that two coplanar PCBs (77&169) in AT are positively associated with the risk of lymph node metastasis and tumor size. These two PCBs are two DLCs. The toxic equivalent factor (TEF) of PCB 169 is 0.01, one of the highest among all coplanar PCBs. The identification of these two activators of the AhR signalling pathway as potential biomarkers of tumor size and risk of lymph node metastasis also is consistent with the previous suspicion.

Several hypotheses can be formulated to explain the contribution of the adipocytic microenvironment to tumor aggressiveness which include the secretion of pro-migratory cytokines and chemokines or pro-inflammatory molecules the expression of which could be potentiated by the presence of AhR ligands (Diedrich et al., 2015). Emerging evidence also has shown that POPs may contribute to a dysfunctional adipocyte phenotype. This phenotype is characterized by an increased production of pro-inflammatory cytokines, oxidative stress and lipolytic rates which favor the local over-release of stored chemicals and pro-tumoral molecules by the peritumoral adipocytes (Artacho-Cordón et al., 2016; Howell and Mangum, 2011; Pestana et al., 2017; Ruzzin et al., 2010). These findings suggest that some chemicals may synergistically contribute to the metastatic risk through peritumoral adipocyte dysfunction in addition to the direct effects on breast cancer cells. Such a hypothesis would help to elucidate the gaps between

Table 4
Associations between concentrations of persistent organic pollutants in adipose tissue and metastatic breast cancer for the sub-population group with BMI ≥ 25 kg/m^2 (n = 44) and BMI < 25 kg/m^2 (n = 47). The models were adjusted for age, smoking, body mass index, menopause and familial history of breast cancer.

Chemical	BMI ≥ 25 kg/m^2			BMI < 25 kg/m^2		
	OR	95% CI	p-Value	OR	95% CI	p-Value
2.3.7.8_TCDD	4.48	(1.32–20.71)	0.03	0.90	(0.38–2.5)	0.83
1.2.3.7.8_PeCDD	2.07	(0.78–6.45)	0.17	0.66	(0.28–1.57)	0.33
1.2.3.4.7.8_HxCDD	2.92	(0.94–11.31)	0.09	1.07	(0.45–2.58)	0.88
1.2.3.6.7.8_HxCDD	1.70	(0.67–5.13)	0.29	0.73	(0.31–1.72)	0.45
1.2.3.7.8.9_HxCDD	0.97	(0.40–2.29)	0.95	0.87	(0.4–1.86)	0.71
1.2.3.4.6.7.8_HpCDD	0.89	(0.39–2.00)	0.77	0.71	(0.3–1.62)	0.42
OCDD	0.75	(0.33–1.56)	0.45	0.70	(0.33–1.42)	0.33
Sum_PCDDs	0.79	(0.35–1.73)	0.56	0.74	(0.34–1.53)	0.42
2.3.7.8_TCDF	0.83	(0.40–1.64)	0.59	0.89	(0.46–1.66)	0.71
1.2.3.7.8_PeCDF	0.92	(0.45–1.83)	0.80	0.94	(0.5–1.75)	0.85
2.3.4.7.8_PeCDF	2.09	(0.72–6.92)	0.19	0.60	(0.26–1.36)	0.22
1.2.3.4.7.8_HxCDF	1.01	(0.37–2.81)	0.99	0.72	(0.34–1.52)	0.39
1.2.3.6.7.8_HxCDF	1.36	(0.50–3.91)	0.55	0.65	(0.3–1.37)	0.26
1.2.3.7.8.9_HxCDF	1.33	(0.61–2.91)	0.45	1.15	(0.6–2.31)	0.68
2.3.4.6.7.8_HxCDF	1.09	(0.50–2.47)	0.83	1.15	(0.6–2.22)	0.68
1.2.3.4.6.7.8_HpCDF	0.90	(0.39–1.86)	0.79	0.96	(0.49–1.89)	0.90
1.2.3.4.7.8.9_HpCDF	0.83	(0.35–1.81)	0.65	1.29	(0.68–2.66)	0.45
OCDF	1.66	(0.80–3.92)	0.19	1.28	(0.65–2.76)	0.49
Sum PCDFs	1.48	(0.52–4.46)	0.46	0.71	(0.32–1.57)	0.39
WHO-TEQ Sum PCDD/F	2.22	(0.75–7.69)	0.17	0.70	(0.29–1.7)	0.40
PCB77	0.85	(0.36–1.88)	0.69	0.76	(0.37–1.49)	0.43
PCB81	0.87	(0.40–1.77)	0.70	0.62	(0.28–1.25)	0.20
PCB126	1.17	(0.51–2.74)	0.70	0.87	(0.39–1.9)	0.72
PCB169	2.84	(0.85–12.62)	0.12	0.82	(0.36–1.95)	0.64
Sum Coplanar PCBs	1.44	(0.53–4.24)	0.48	0.80	(0.35–1.83)	0.59
PCB105	1.06	(0.45–2.48)	0.90	0.70	(0.32–1.45)	0.35
PCB114	2.70	(0.94–9.31)	0.08	0.67	(0.29–1.53)	0.33
PCB118	1.39	(0.55–3.64)	0.49	0.67	(0.3–1.45)	0.31
PCB123	1.33	(0.55–3.29)	0.52	0.67	(0.3–1.46)	0.32
PCB156	2.95	(0.89–12.76)	0.10	0.84	(0.37–2)	0.68
PCB157	2.09	(0.80–6.46)	0.16	0.93	(0.4–2.45)	0.88
PCB167	3.61	(0.98–17.00)	0.07	0.70	(0.3–1.62)	0.40
PCB189	1.86	(0.69–5.96)	0.25	1.11	(0.48–2.74)	0.80
Sum Non-Coplanar PCBs	1.94	(0.67–6.33)	0.23	0.71	(0.31–1.64)	0.42
WHO-TEQ PCB	1.33	(0.51–3.59)	0.55	0.79	(0.35–1.78)	0.56
TOTAL WHO-TEQ	1.80	(0.61–5.95)	0.30	0.74	(0.31–1.8)	0.49
PCB28	1.13	(0.56–2.31)	0.72	0.86	(0.41–1.73)	0.68
PCB52	0.69	(0.29–1.41)	0.34	0.66	(0.31–1.33)	0.26
PCB101	0.67	(0.28–1.43)	0.33	0.74	(0.36–1.46)	0.38
PCB138	1.86	(0.73–5.52)	0.22	0.89	(0.44–1.76)	0.72
PCB153	2.01	(0.74–6.41)	0.20	0.96	(0.46–2.05)	0.92
PCB180	1.65	(0.66–4.73)	0.31	1.22	(0.55–2.94)	0.64
Sum 6 non dioxin-like PCB	1.92	(0.70–6.06)	0.22	0.99	(0.47–2.24)	0.99
Sum 7 non dioxin-like PCB	1.98	(0.71–6.34)	0.21	0.97	(0.46–2.19)	0.95
PBDE28	0.68	(0.33–1.33)	0.26	0.70	(0.35–1.29)	0.27
PBDE47	0.64	(0.29–1.31)	0.24	0.88	(0.46–1.64)	0.67
PBDE99	0.71	(0.33–1.47)	0.37	0.87	(0.44–1.66)	0.67
PBDE100	0.90	(0.43–1.89)	0.77	0.89	(0.46–1.68)	0.71
PBDE153	2.27	(1.06–6.00)	0.06	0.63	(0.27–1.26)	0.22
PBDE154	0.68	(0.31–1.39)	0.29	1.02	(0.54–1.94)	0.96
PBDE183	0.78	(0.38–1.54)	0.48	0.96	(0.49–1.88)	0.90
PBDE209	1.17	(0.57–2.43)	0.67	1.07	(0.57–2.08)	0.82
Sum 7 PBDE	1.19	(0.59–2.48)	0.63	0.73	(0.35–1.39)	0.35
Sum 8 PBDE	1.14	(0.56–2.37)	0.72	1.14	(0.6–2.23)	0.68
PBB153	1.40	(0.64–3.50)	0.42	0.58	(0.28–1.13)	0.12
α-HBCD	1.55	(0.60–4.67)	0.39	0.88	(0.37–1.99)	0.76

exposure to POPs, adiposity and breast cancer (Blücher and Stadler, 2017).

Furthermore, the OR (95% CI) was also enlarged in the sub-group of patients with > 3 metastatic lymph nodes which suggests an association between the metastatic stage and TCDD concentration. Finally, larger tumor size was associated with the presence of higher concentrations of TCDD and some PCBs for which statistically significant associations were observed after confounding adjustment. The statistically significant negative associations found for the ER+ and PR+ subgroups suggest receptor status can modify effects. They also highlight the need for studies with larger numbers of individuals with breast cancers with negative expression which are already acknowledged to be the most aggressive forms with the worst prognosis.

Although AT is accepted as the gold standard matrix for the evaluation of the internal dose of POPs, little is known about the dynamics of lipophilic pollutants in the breast microenvironment. In the current study, we made a substantial analytical effort to decipher the complex relationships between the concentrations of chemicals in two major compartments, AT and serum. Although the results showed a strong correlation of chemicals between both compartments, the application of such biomarkers in the regression models resulted in divergent risk estimates. Overall, these results suggest that the use of serum

Table 5
Summary of the levels (median (interquartile range)) of persistent organic pollutants measured in adipose tissue from the population dichotomized on the basis of the median of tumor size distribution. The groups were compared with the Mann-Whitney test.

	Units	Small size < 2 cm N = 43	Large size ≥ 2 cm N = 48	p-Value
2.3.7.8-TCDD	pg/g l.w.	1.2 (0.9–1.5)	1.6 (1.1–2.3)	0.003
1.2.3.7.8-PeCDD	pg/g l.w.	4.4 (3.4–5.7)	5.0 (3.7–6.7)	0.25
1.2.3.4.7.8-HxCDD	pg/g l.w.	1.9 (1.3–2.9)	2.6 (1.9–4.0)	0.017
1.2.3.6.7.8-HxCDD	pg/g l.w.	14.3 (9.1–19.7)	15.8 (11.9–25.6)	0.24
1.2.3.7.8.9-HxCDD	pg/g l.w.	1.4 (1.0–2.0)	1.7 (1.1–2.7)	0.15
1.2.3.4.6.7.8-HpCDD	pg/g l.w.	11.1 (6.2–16.9)	14.5 (8.6–24.0)	0.098
OCDD	pg/g l.w.	89.4 (62.6–132.8)	98.6 (72.1–159.3)	0.37
Sum PCDDs	pg/g l.w.	126.2 (92.8–186.8)	142.7 (102.5–217.4)	0.21
2.3.7.8-TCDF	pg/g l.w.	0.3 (0.2–0.5)	0.4 (0.3–0.6)	0.17
1.2.3.7.8-PeCDF	pg/g l.w.	0.2 (0.1–0.2)	0.2 (0.1–0.3)	0.41
2.3.4.7.8-PeCDF	pg/g l.w.	10.9 (7.9–15.7)	12.2 (9.3–19.3)	0.15
1.2.3.4.7.8-HxCDF	pg/g l.w.	2.0 (1.5–2.6)	2.3 (1.8–3.4)	0.063
1.2.3.6.7.8-HxCDF	pg/g l.w.	2.1 (1.7–3.0)	2.6 (1.9–3.9)	0.096
1.2.3.7.8.9-HxCDF	pg/g l.w.	0.1 (0.1–0.1)	0.1 (0.1–0.1)	0.26
2.3.4.6.7.8- HxCDF	pg/g l.w.	0.7 (0.5–0.9)	0.8 (0.6–1.1)	0.28
1.2.3.4.6.7.8-HpCDF	pg/g l.w.	1.3 (0.8–1.6)	1.2 (0.9–1.6)	0.94
1.2.3.4.7.8.9-HpCDF	pg/g l.w.	0.1 (0.1–0.2)	0.1 (0.1–0.1)	0.52
OCDF	pg/g l.w.	0.2 (0.2–0.4)	0.3 (0.2–0.6)	0.16
Sum PCDFs	pg/g l.w.	18.1 (14.3–29.2)	21.7 (16.8–29.8)	0.11
Sum WHO-TEQ PCDD/F	TEF_{2005}/g l.w.	11.3 (8.6–15.8)	12.6 (10.3–18.4)	0.15
PCB 77	pg/g l.w.	5.9 (3.2–11.9)	5.3 (3.3–10.9)	1.00
PCB 81	pg/g l.w.	1.9 (0.9–3.8)	2.5 (1.5–3.9)	0.11
PCB 126	pg/g l.w.	52.0 (34.3–80.9)	68.2 (49.9–112.5)	0.020
PCB 169	pg/g l.w.	79.9 (47.3–125.0)	85.9 (59.4–140.1)	0.22
Sum Coplanar PCBs	pg/g l.w.	137.1 (95.6–202.9)	180.6 (115.6–279.9)	0.064
PCB 105	pg/g l.w.	3397.2 (2315.0–6149.0)	5020.7 (3321.5–10,981.6)	0.044
PCB 114	pg/g l.w.	1633.8 (811.1–2883.6)	2565.9 (1468.1–4056.5)	0.051
PCB 118	pg/g l.w.	18,165.3 (11,329.7–29,421.4)	25,612.6 (15,978.2–47,962.2)	0.027
PCB 123	pg/g l.w.	179.6 (94.5–288.0)	301.3 (150.7–576.7)	0.009
PCB 156	pg/g l.w.	19,468.7 (9259.6–30,824.5)	23,091.2 (13,586.1–37,411.0)	0.27
PCB 157	pg/g l.w.	3602.2 (1868.1–6655.3)	4584.9 (2438.8–7011.3)	0.34
PCB 167	pg/g l.w.	3814.3 (2330.8–5996.2)	5220.7 (3174.9–7808.8)	0.056
PCB 189	pg/g l.w.	2726.2 (1616.2–4279.3)	3020.1 (1723.9–4290.8)	0.62
Sum Non Coplanar PCBs	pg/g l.w.	56,735.1 (33,999.6–85,933.3)	72,402.4 (46,204.6–115,654.6)	0.081
WHO-TEQ dl-PCB	TEF_{2005}/g l.w.	10.2 (6.2–13.6)	12.4 (8.1–20.6)	0.039
TOTAL WHO-TEQ	TEF_{2005}/g l.w.	21.7 (15.8–30.0)	25.7 (18.7–38.1)	0.048
PCB 28	ng/g l.w.	1.1 (0.8–2.3)	2.2 (1.2–3.5)	0.007
PCB 52	ng/g l.w.	0.3 (0.2–0.6)	0.4 (0.3–0.7)	0.056
PCB 101	ng/g l.w.	0.7 (0.5–1.5)	1.0 (0.6–2.0)	0.13
PCB 138	ng/g l.w.	67.1 (39.9–101.5)	81.0 (56.1–125.5)	0.15
PCB 153	ng/g l.w.	163.6 (119.8–280.4)	201.9 (126.9–289.1)	0.27
PCB 180	ng/g l.w.	160.2 (104.0–277.1)	164.9 (114.0–246.8)	0.74
Sum 6 ndl-PCB	ng/g l.w.	388.5 (275.9–683.3)	474.2 (280.4–717.4)	0.32
PBDE 28	ng/g l.w.	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.86
PBDE 47	ng/g l.w.	0.3 (0.1–0.7)	0.4 (0.2–0.5)	0.72
PBDE 99	ng/g l.w.	0.1 (0.0–0.2)	0.1 (0.0–0.1)	0.53
PBDE 100	ng/g l.w.	0.1 (0.1–0.3)	0.1 (0.1–0.2)	0.57
PBDE 153	ng/g l.w.	1.6 (1.0–2.1)	1.4 (1.0–1.8)	0.28
PBDE 154	ng/g l.w.	0.0 (0.0–0.0)	0.0 (0.0–0.0)	0.22
PBDE 183	ng/g l.w.	0.2 (0.1–0.2)	0.1 (0.1–0.2)	0.44
PBDE 209	ng/g l.w.	1.5 (1.3–2.2)	1.7 (0.9–2.1)	0.42
Sum 7 i PBDE	ng/g l.w.	2.6 (1.7–3.6)	2.2 (1.5–2.8)	0.12
Sum 8 i PBDE	ng/g l.w.	4.5 (3.7–5.7)	3.6 (3.0–5.3)	0.098
PBB 153	ng/g l.w.	0.6 (0.5–0.9)	0.6 (0.4–0.9)	0.44
α-HBCD	ng/g l.w.	1.1 (0.6–2.0)	1.3 (0.9–2.0)	0.34

biomarkers may result in inconsistent findings if used as surrogate biomarkers in peritumoral AT. These results are consistent not only with those of our previous studies in the field of endometriosis where serum biomarkers exhibited substantial disagreement with the estimates using AT biomarkers (Cano-Sancho et al., 2018) but also with growing evidence about the complexity of use and interpretation of circulating biomarkers (O'Brien et al., 2016). Future studies need take into account the use longitudinal markers of exposure that would approximate lifetime trajectories either by optimally using multiple sample time-points or by reconstruction of exposure trajectories through PBPK modeling (Verner et al., 2011).

The results from this study should be considered with caution due to some methodological limitations. For instance, this exploratory study has a limited sample size and was conducted within a clinical setting to deliver definitive inferential conclusions at a population level. For that reason, large population-based studies with greater statistical power and a lower risk of selection bias are necessary to confirm the findings. Furthermore, we purposely opted not to adjust for the multiple tests performed in this exploratory analysis because the hypothesis-based approach, of the high correlation between variables and to avoid the consequential inflation of type 2 errors (Rothman, 1990). For that reason, the results should be interpreted with caution in the light of potential type 1 errors which result in some associations due to chance. Another limitation is the measurement error derived from using proxy biomarkers instead of target tissue before the onset of the metastasis. The design of the present study may not rule out some potential reverse

causality as the main cause of the associations. In addition, in this first analysis we have evaluated the associations for each chemical individually and only using sums of concentrations or bioactivities to evaluate the effect of mixtures. Hence, we emphasize the need for further research to evaluate the simultaneous effects of these chemicals through more efficient statistical models. However, to date, there are few statistical approaches that are available to efficiently identify the combined associations of highly correlated chemicals reaching an optimal balance between bias and variance (Agier et al., 2016).

5. Conclusion

This exploratory study has investigated, for the first time, the association between lymph node metastasis, the first step of BC dissemination, and the internal exposure to POPs. Although the study is preliminary and consists of a limited sample size that precludes drawing definitive conclusions, the results suggest that the internal exposure to 2.3.7.8-TCDD and some PCBs could be associated with the risk of aggressive BC, in particular in obese patients and in patients with receptor negative cancers, conditions which merit further research. Thus, larger population-based longitudinal studies are necessary to confirm these associations. In addition, fundamental research must develop to improve our understanding at the mechanistic level of the effect of POPs on metastatic cellular processes and potential cross-talk with peritumoral adipose tissue.

Declaration of Competing Interest

The authors declare they have no actual or potential competing financial interests.

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Appendix A. Supplementary data

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PARTIE 3 :
ETUDE EXPERIMENTALE

ARTICLE 3

Synthèse article 3: "*Interaction between preadipocytes and the environmental pollutant dioxin leads to increased aggressiveness of breast cancer cells"*

Afin de modéliser au mieux la tumeur et son microenvironnement, nous avons utilisé un système de co-culture en insert de cellules cancéreuses mammaires (MCF7 et MDA-MB-231, lignée triple négative) et de cellules pré-adipocytaires. Le système est exposé ou non à la dioxine pendant 48h puis la réponse des cellules cancéreuses mammaires est étudiée. Dans le protocole, les milieux de culture conditionnés sont congelés et utilisés ultérieurement, par exemple pour traiter les cellules cancéreuses mammaires avant xénogreffe.

Précisions :

* Nous avons volontairement décidé de traiter l'ensemble du système de co-culture par le polluant et non uniquement les adipocytes afin de refléter au mieux l'exposition des individus *in vivo*. En effet, la tumeur et son microenvironnement sont exposées simultanément aux POP lors de contaminations aigües ou chroniques.

* La dose de dioxine utilisée de 25 nM a été choisie après des études préliminaires et a montré l'absence de toxicités cellulaires directes. C'est une dose compatible avec des « taux physiologiques » et qui est utilisée de façon courante dans le laboratoire dans les études expérimentales *in vitro* et *in vivo*.

* Nous avons utilisé dans nos expérimentations, une lignée humaine de préadipocytes appelée human Multipotent Adipose-Derived Stem adipose (hMADS). Les cellules hMADS sont des précurseurs adipocytaires isolés à partir de tissus adipeux de jeunes enfants (102). Elles possèdent des caractéristiques de cellules souches mésenchymateuses et ont une capacité d'auto-

renouvèlement. Notre choix s'est tourné vers cette lignée cellulaire du fait des fortes capacités sécrétoires de ces cellules immatures par rapport aux adipocytes différenciées en réponse à l'exposition aux polluants (42). Par ailleurs, des études réalisées par cytométrie de flux ont montré que la proportion des préadipocytes dans le tissu adipeux pouvait représenter jusqu'à 15% à 35% des cellules adipocytaires (97,98) et que ce pourcentage n'est pas constant et est considérablement augmenté lors de pertes importantes de poids, par exemple après chirurgie bariatrique (Figure 12) (105).

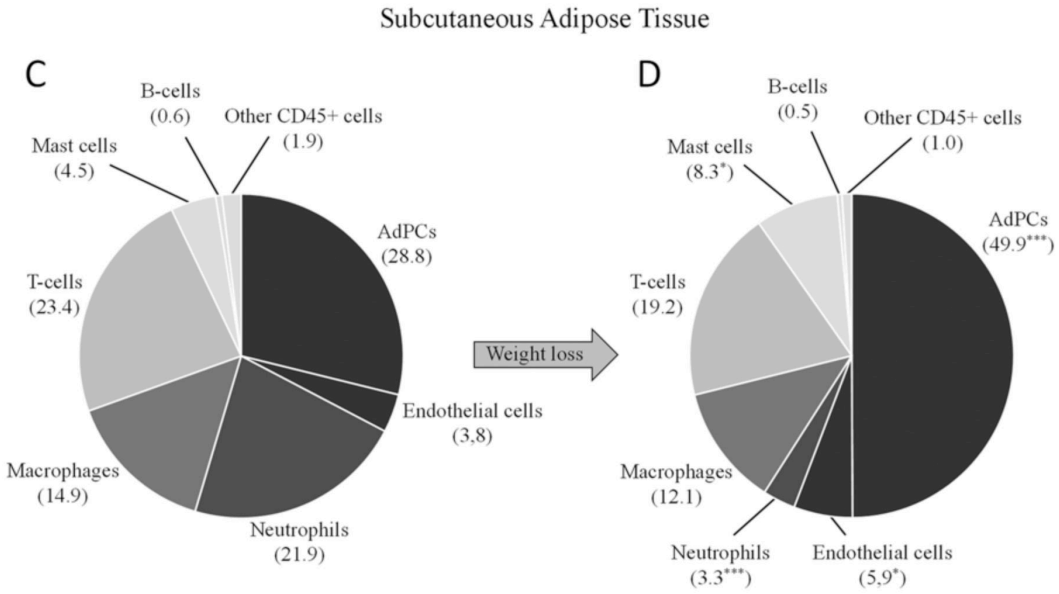

Figure 12: Graphique montrant la composition de la fraction vasculaire stromale dans le tissu adipeux sous-cutané dans une cohorte de patients obèses morbides (IMC moyen=48,5 kg/m2 ±1,5) avant (C) et après une perte importante de poids induite par une chirurgie bariatrique (IMC moyen=31,8 kg/m2±1,2) (D). (AdPCs : Adipose precursors cells) *(D'après García-Rubio, Scientific reports, 2018* (105)*).*

Seuls les cellules hMADS ont été utilisées dans notre étude, mais il serait intéressant de reproduire nos résultats d'une part avec des cellules pré-adipocytaires humaines (et non une lignée immortalisée) et également avec des adipocytes différenciés (les cellules hMADS peuvent être

différenciés en adipocytes en 10 jours). Ces 2 pistes de recherche feront l'objet de travaux futurs au sein du laboratoire.

Résultats :

Nous avons montré que la co-exposition des cellules mammaires à la dioxine et aux pré-adipocytes entraine des modifications phénotypiques majeures responsables d'une augmentation du potentiel agressif et métastatique *in vitro* et *in vivo*.

La co-exposition (et uniquement cette condition) favorise l'acquisition par les cellules cancéreuses mammaires de caractéristiques de « cellules souches cancéreuses » (CSC), dont l'un des marqueurs est l'expression de l'aldéhyde déshydrogénase 1 isoforme 3 (ALDH1A3) (analyses protéomiques). Nous avons confirmé que la protéine surexprimée était bien active en cytométrie de flux (protocole ALDEFLUOR). Cette enzyme intervient dans le métabolisme de l'acide rétinoïque et joue un rôle dans le contrôle de la différenciation cellulaire. L'expression de l'ALDH1A3 est prédictive du risque métastatique en particulier dans le CS (56,106,107). L'augmentation de la capacité de formation de mammosphères dans la co-exposition confirme également l'acquisition de ces propriétés de CSC par les cellules cancéreuses mammaires.

De plus, les cellules co-exposées montrent une capacité accrue à métastaser *in vivo* dans un modèle de xénogreffe chez le poisson zèbre. Nous avons montré une augmentation de la proportion d'embryons développant des métastases, du nombre de métastases par embryon et de façon intéressante, le développement de métastases au niveau du pôle céphalique de l'embryon.

Ces changements s'accompagnent de modifications morphologiques dont certaines sont observées dans la TEM : diminution du nombre de points focaux d'adhérence, lamellipodes. Nous retrouvons

également des modifications du niveau des transcrits de gènes impliqués dans la TEM (E-Cadhérine, SNAIL, SLUG).

Fait le plus marquant de notre travail, la co-exposition entraine la formation de cellules polynucléaires géantes (ou « polynuclear giant cancer cells » pour PGCC). Les PGCC sont une sous-population particulière de cellules cancéreuses qui contribuent à l'hétérogénéité tumorale des tumeurs solides. Elles sont généralement observés dans des conditions extrêmes (telles que l'hypoxie ou la radiothérapie) et sont fortement associées dans la littérature à l'agressivité du cancer et à la chimiorésistance (108). Les PGCC, auparavant considérées comme des cellules sénescentes, sont capables de générer des cellules cancéreuses filles par bourgeonnement (109). Le nombre de PGCC, associé à l'expression de protéines de la TEM, est corrélé aux capacités d'invasion des cellules cancéreuses et à la formation de métastases à distance dans le CS (110). Ces cellules expriment des marqueurs de CSC (ALDH1A3, CD44 et CD133) et sont résistantes à la chimiothérapie et la radiothérapie (111). Nous postulons l'hypothèse que les PGCC et les cellules exprimant un phénotype CSC sont la même entité, qui émerge dans la condition co-exposition et est responsable de l'invasion tumorale et la dissémination métastatique et probablement l'acquisition d'une chimiorésistance.

Ces résultats suggèrent que les POP peuvent, en plus de leur effets pro-métastatiques directs supposés, agir sur le tissu adipeux péritumoral du CS et augmenter ainsi l'agressivité des cellules cancéreuses mammaires en ciblant le microenvironnement tumoral.

<u>Perspectives dans les suites de ce papier :</u>

***La piste de la chimiorésistance** nous intéresse particulièrement et les expérimentations sont en cours pour valider la baisse de la sensibilité des cellules co-exposées aux différentes drogues

habituellement utilisées dans le traitement du CS. De plus, nous avons prouvé que la coculture et la co-exposition avait pour conséquence la perte de l'expression du récepteur aux œstrogènes ce qui a un impact thérapeutique négatif direct (pas de traitement ciblant le système œstrogénique, cellules plus agressives) (Figure 13).

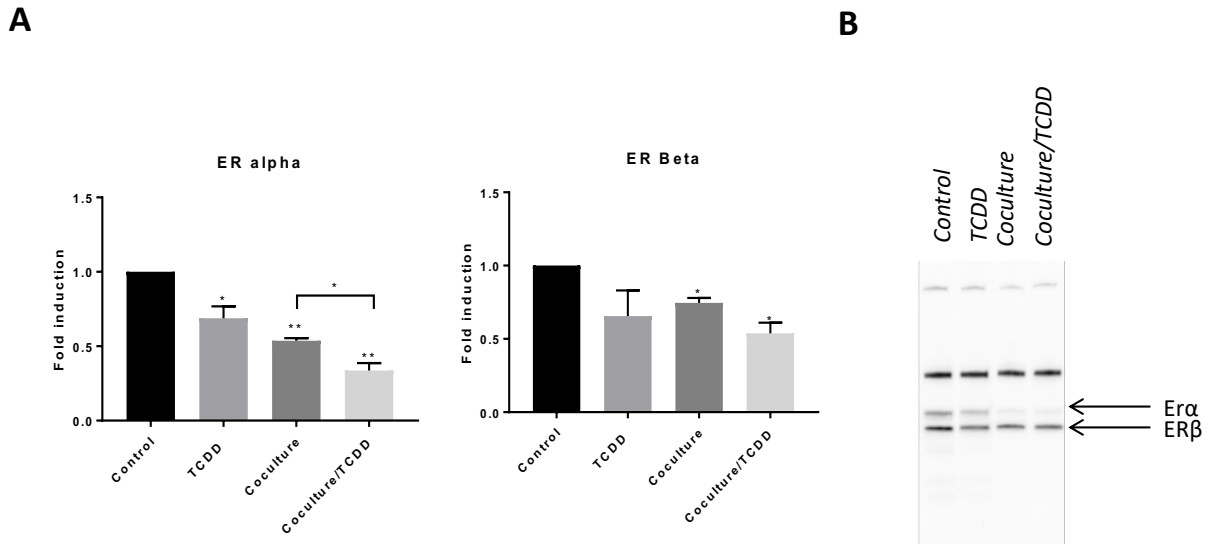

Figure 13: Diminution de l'expression de l'ER α au niveau ARNm (A) et protéine (B). La diminution est d'autant plus marquée dans la condition « co-exposition ».

*L'une de nos hypothèses pour expliquer la formation des PGCC est **l'entose**. L'entose est un mécanisme de cannibalisme cellulaire dont le rôle dans le cancer est débattu (112). Il se caractérise par l'internalisation d'une cellule **vivante** au sein d'une cellule hôte. Il est déclenché par la perte de contact avec la matrice extracellulaire et met en jeu des forces contractiles générées par les jonctions adhérentes de type cadhérine connectées au réseau du cytosquelette d'actine intracellulaire. Dans la plupart des cas, la cellule internalisée est digérée et éliminée mais il a été montré que celle-ci peut aussi se diviser au sein de l'hôte, voire même s'en échapper. Ces observations soulignent bien que la cellule internalisée est vivante. Ce phénomène peut limiter la croissance tumorale en éliminant la cellule internalisée mais il existe également un risque non

négligeable de perturbations du bon déroulement de la division cellulaire de la cellule hôte, induisant l'apparition de cellules tumorales aneuploïdes et l'instabilité génomique de la tumeur. (113). Plus récemment, il a été montré que l'entose était favorisée par la privation de glucose et que ce mécanisme pourrait être un mécanisme d'adaptation cellulaire pour la survie de certaines populations de cellules cancéreuses, les cellules mortes étant des sources de nutrition. Cela pourrait participer à la sélection et la prolifération de cellules cancéreuses sélectionnées et éventuellement expliquer la progression tumorale.

Nous sommes en contact avec une équipe spécialisée dans l'entose pour réaliser une collaboration et affirmer ou infirmer cette hypothèse qui nous parait séduisante.

Title: « Interaction between preadipocytes and the environmental pollutant dioxin leads to increased aggressiveness of breast cancer cells".

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Keywords: AhR, TCDD, breast cancer, chemoresistance, metastasis, stem cells, polyploid giant cells.

Short title: Role of co-exposure to preadipocytes and dioxin on the metastatic potential of breast cancer cells.

ABSTRACT

Breast cancer is a major public health concern and its prognosis is very poor once metastasis occurs. The tumor microenvironment and chemical pollution have recently been suggested to contribute to the development of aggressive tumors. We hypothesized that those two factors may interact to increase tumor aggressiveness. The microenvironment consists, in part, of adipocytes and preadipocytes in which persistent organic pollutants (POP) can be stored. We developed a co-culture model which can reveal the contribution of the microenvironment and POP to the phenotypic variation of breast cancer cells. We found that co-exposure to TCDD, the prototypical dioxin, and pre-adipocytes favors the acquisition by cancer cells of stem cell-like features and aggressiveness along with a dramatically enhanced capability to metastasize *in vivo* in a Zebrafish model. These changes are accompanied by the generation of giant polynuclear cells. Thus, breast cancer cells aggressive phenotype may depend on the interaction of tumor microenvironment and environmental pollutants.

MAIN TEXT

Introduction

Breast cancer (BC) has both a high incidence and mortality among women and critical socio-economic impacts. Over the past 50 years, it has become a major health problem for women worldwide with over 2 million new cases diagnosed in 2018. This represents about 12% of all new cancer cases, 25% of all cancers in women and more than 600,000 deaths [1]. If the cancer is located only in the breast, the 5-year survival rate of people with BC is 99% but this rate decreases to 85% if it has spread to lymph nodes and, more dramatically, to only 26% if distant metastases are present at diagnosis [2,3]. The metastatic process, or the spread of tumor cells throughout the body, is responsible for about 90% of cancer patient deaths [4] and represents the central clinical challenge of solid tumor oncology.

The development and progression of BC are complex processes which involve hormonal factors as well as numerous genetic and epigenetic alterations. During the past 10 years, many studies have focused on the role of the tumor microenvironment and the peritumoral stromal fraction, which is composed of adipose tissue, cancer-associated fibroblasts, endothelial cells and immune cells such as macrophages and leukocytes. During tumor progression, cancer cells will modify their microenvironment extensively which, in turn, will promote the growth and dissemination of the tumor [5,6]. Adipose tissue, which consists mainly of mature adipocytes and progenitors (preadipocytes and adipose-derived stem cells (ADSCs), is the most abundant component surrounding BC cells. Adipose tissue exerts a major endocrine and secretory role and, thus, represents an essential actor in the inflammatory, angiogenic and remodeling responses of the extracellular matrix which influence tumor behavior. Several studies have shown that pre-adipocytes / adipocytes, which are influenced by factors produced by tumor cells in a constant and dynamic intercellular communication, may promote migration and invasion of BC cells participating in local inflammation via the production of interleukins and chemokines [7,8].

Adipocytes also store persistent organic pollutants (POPs) such as dioxins, furans, polychlorinated biphenyls, flame retardants for long periods of time as do pre-adipocytes which, in addition, have higher secretory capacities. POPs are a major public health concern due to their toxic effects and their persistence in the environment and organisms. Due to their lipophilicity, POP accumulation occurs preferentially in the liver, brain and adipose tissues. In previous studies, we have shown that adipose tissue releases POPs slowly and chronically [9]. This constant internal release and re-contamination also is responsible for the chronic toxicity associated with POPs exposure. We have recently shown, from a

mechanistic standpoint, that POPs promote an epithelial-to-mesenchymal transition. This is a cellular developmental phenomenon which is observed during gastrulation in triploblastic organisms, but which is involved also in pathological processes such as fibrosis or the formation of metastatic cells [10,11]. The POP 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, Seveso dioxin) is the most toxic congener of the dioxin family and is also one of the most potent activators of the aryl hydrocarbon receptor (AhR), a transcription factor which regulates xenobiotic metabolism and the epithelial-mesenchymal transition (EMT) [12].

We hypothesized that TCDD targets the tumor microenvironment to enhance BC metastasis. Here, we studied the paracrine role of pre-adipocytes during metastasis in mammary cancer epithelial cells and the aggravating role of exposure to TCDD. To study the interactions between pre-adipocytes and BC cells, we developed an original *in vitro* co-culture model which consists of human mammary tumor cells (MCF-7 or MDA-MB-231) and human pre-adipocytes (hMADS, human multipotent adipose-derived stem cells). We hypothesized that exposure to TCCD modifies the phenotype of adipose cells and the subsequent responses of the BC cells to co-culture. Therefore, we first investigated the effect of the co-culture and TCDD alone or the effect of co-exposure (adipose cells + TCDD) on the phenotype of MCF-7 cells and found that both co-culture and co- exposure markedly affected MCF-7 characteristics as measured by real-time experiments. Next, a proteomics analysis revealed specifically in the co-exposure condition, an increased expression of a stem-cell biomarker, the Aldehyde dehydrogenase 1 family member A3 (ALDH1A3). We then observed that the cancer stem-cell (CSC)-like properties of MCF-7 cells (such as the formation of tumorspheres) also were increased by co-exposure. To ensure that these features were observed *in vivo*, we demonstrated the ability of co-exposed cancer cells to metastasize in a Zebrafish model. Finally, we found that these cells display specific cellular characteristics and that co-exposure leads to the generation of giant polynuclear cells (PGCCs), a cancer sub-population strongly associated with aggressiveness and chemoresistance in cancer.

Results

Co-culture and co-exposure modify MCF-7 cell properties.

We designed an easy-to-implement co-culture model to produce conditioned media and to study the communication between adipose cells and human tumor cells. We used this model to investigate the influence of POPs (TCDD) on the phenotype of MCF-7 tumor cells (Figure 1A). Here, conditioned media are the media derived from cultures of MCF-7 cells alone (control) or exposed to TCDD (TCDD) or co-cultured with hMADS cells (co-culture) and exposed to TCDD (co-exposed). We first used an xCELLigence system to monitor MCF-7 properties (adhesion, spreading, and proliferation of the cells) under control conditions or in co-culture with hMADS cells (co-culture) and following treatment with TCDD alone (TCDD) or in co-culture with hMADS cells (co-exposure). We found that MCF-7 cells co-cultured with hMADS cells exhibited a decreased cell index value (CI, see Materials and Methods) (Fig. 1B). TCDD alone had no significant effect but the combination co-culture/TCDD (co-exposure) decreased the CI as compared to co-culture alone. Similar results were observed with the xCELLigence system when MCF-7 cells were exposed simply to conditioned media from co-exposure condition (Supplemental figure 1)

Co-exposure leads the acquisition of a cancer stem-cell like phenotype.

In light of the preceding results, we performed high-throughput proteomic analysis of extracts of cells grown under the different conditions. We quantified 2238 proteins (Supplementary table). The differences among the different conditions were very low except for ALDHA3, CYPA1 and CYPB1 that are overexpressed specifically in some of the tested conditions. For example, CYP1A1 and CYP1B1 are clearly induced in the presence of TCDD in both TCDD and co-exposure conditions. Interestingly, the analysis has shown very high levels of Aldehyde dehydrogenase 1 family, member A3 (ALDH1A3) only in the co-exposure condition (Fig. 2A). ALDH1A3 is a cancer stem cell (CSC) marker and its expression is predictive of metastasis especially in breast cancer [13–15]. ALDH activity was then evaluated in MCF-7 cells by FACS analysis. ALDH1A3-positive MCF-7 cells were increased significantly in the presence of TCDD but particularly in the co-exposure condition (Fig. 2B).

We also explored the ability of tumor cells to form tumorspheres and showed that both the co-culture and co-exposure conditions decreased the number and increased the diameter of these spheres which is directly correlated with the CSC properties (Supplemental figure 2).

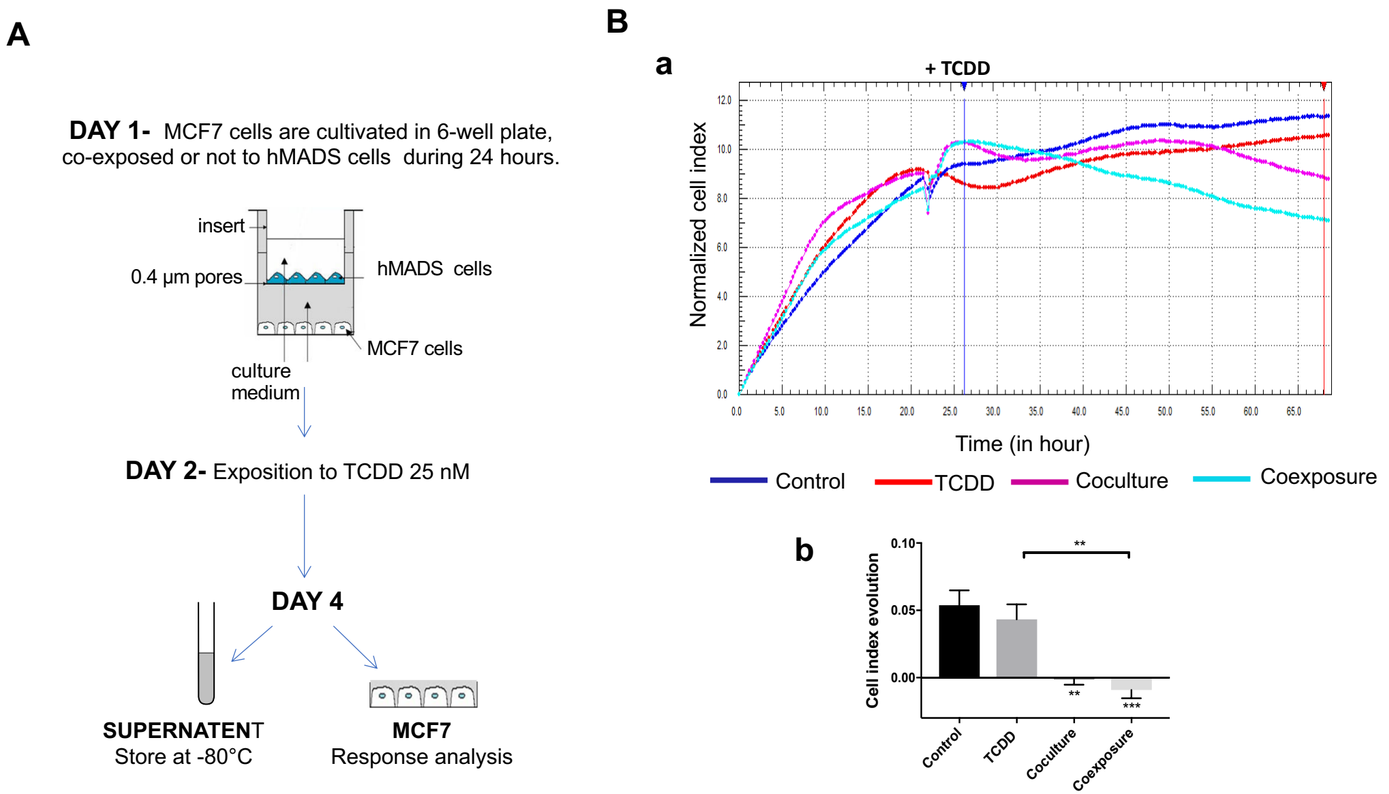
Co-exposure promotes metastasis formation in vivo in a Zebrafish model (Fig 3)

Since cancer stem cells have a high capability of invasion and metastasis, we next explored the ability of cancer cells to metastasize *in vivo*. A well-characterized zebrafish xenograft model of human cancer cell metastasis was used. MCF-7 cells labeled with CM-Dil and RFP-MDA-MB-231 cells were treated with conditioned medium for 48 hours and then injected into the perivitelline space of two-day-old zebrafish larvae. Fish were imaged 24 h later. The number of fishes with metastasis in the tail (vascular plexus) or in the head region and the number of metastasis per fish were counted for each condition. MCF-7 metastasis formation was observed in 53% of fish from the control condition (N=32), 43% from the TCDD condition (N=21), 66 % from the co-culture condition (N= 36), and 76% from the co-exposure condition (N=34). The number of metastases per fish was significantly higher in the co-exposure condition compared to control (p=0.001). Metastases in the head region were observed only from the co-culture and co-exposure conditions with, respectively, 8% and 21% of fish affected. Similar results were found with MDA-MB-231 cells. Metastasis formation was observed in 50% of fish from the control condition (N=46), 51% from the TCDD condition (N=41), 58% from the co-culture condition (N= 24), and 79% from the co-exposure condition (N=42) (Fig. 3). The number of metastases per fish was significantly higher from the co-exposure condition (p=0.006) and metastases in the head region were observed only in the co-exposure condition (40% of fish with head metastases). These *in vivo* results support our hypothesis that the co-exposure of breast cancer cells to preadipocytes and TCDD increases breast cancer cell aggressiveness and metastasis formation.

Co-exposure leads to the generation of polynuclear giant cancer cells (Fig 4)

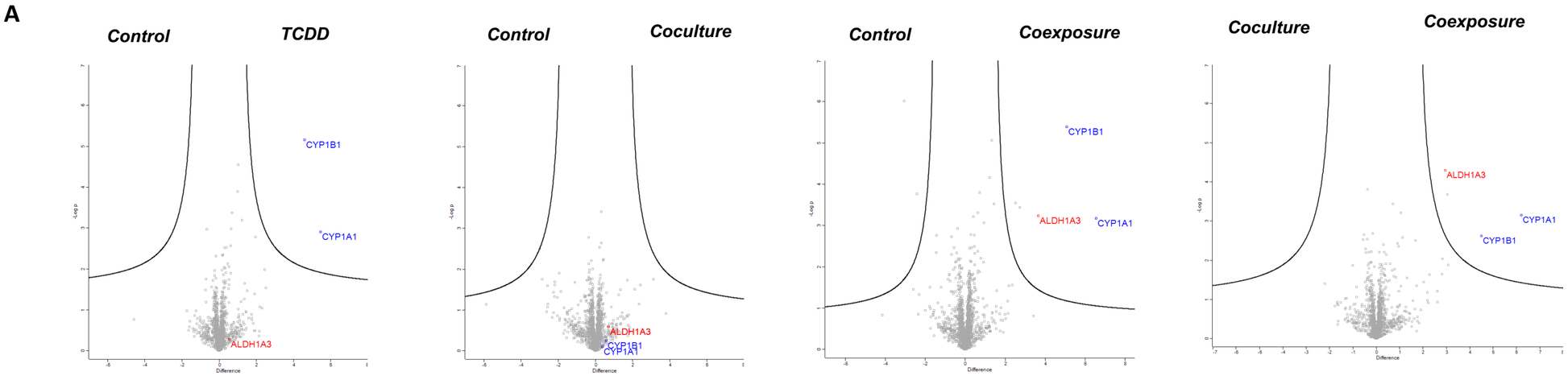
The *in vivo* results suggest that our co-exposed cells acquire specific features which allow the establishment of invasive properties. Consequently, we performed immunofluorescence staining to investigate cellular morphological changes (Figure 4). Antibodies to actin and paxillin were used to examine the cytoskeleton and focal adhesion sites, respectively. Both co-culture and co-exposure conditions led to dramatically altered cellular morphologies. In the presence of TCDD, the signals for paxillin became punctuate and focal adhesions and stress fibers were induced as compared to the control condition. These morphological changes are observed during epithelial-to-mesenchymal transition (EMT). We, therefore, investigated the expression of EMT-regulated genes and observed a down-regulation of E-cadherin together with an upregulation of Slug (SNAI2) and Snail (SNAI1) which are master EMT-regulatory transcription factors (Supplemental figure 3). Under co-culture conditions, cells appeared elongated with tapering cytoplasmic poles having one or more lamellipodia and relatively short filopodia and actin filaments oriented along the long axis of the cell (Fig 4A). However, the co-exposure condition induced polynucleation and the formation of giant cells as the number of cells and the number of nuclei per field decreased. The number of cells and the number of nuclei by field decreased significantly in the co-exposure condition whereas the number of polynuclear giant cancer cells (PGCCs) per field increased (Fig 4B). In parallel, we found that these morphological changes are not due to cell death, senescence, proliferation, or autophagy. No difference was observed among the different conditions for those processes (Supplemental figure 4).

<u>Figure 1</u>: Modifications of MCF-7 cells properties.
(A) Presentation of the 2D co-culture system and the protocol. **(B)** xCELLigence and dynamic monitoring of MCF-7 cells. Co-culture and co-exposure effects on adhesion, spreading, and proliferation of the cells. Results are expressed as a CI value. **(a)** Representative graph from xCELLigence system comparing the growth curve in each condition. **(b)** Rate of proliferation at the different condition as determined by analysing the slope of the line between the 68 and 26 h interval.



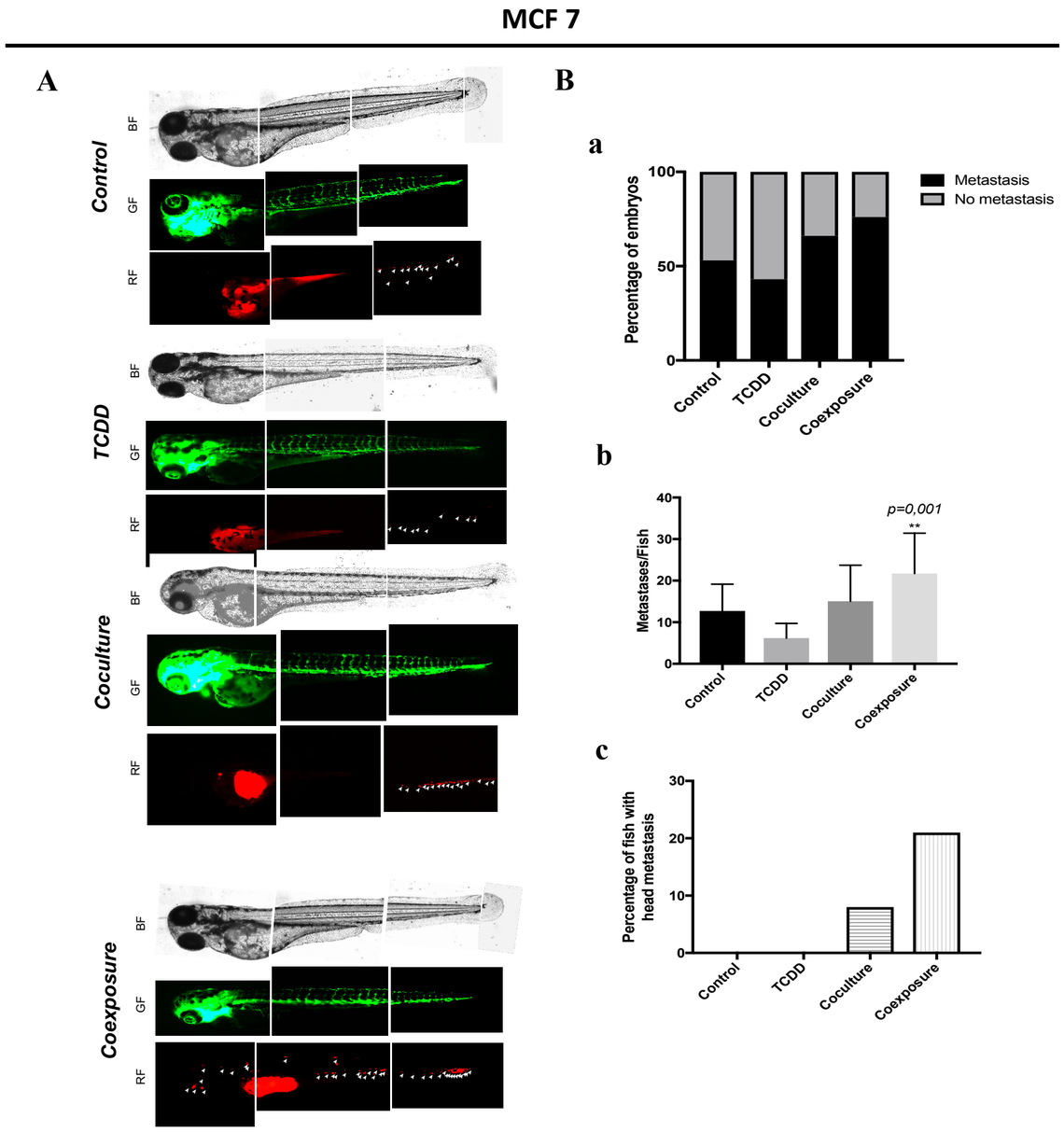
<u>Figure 2</u>: MCF-7 co-cultivated and exposed to TCDD exhibit a cancer stem-cell like phenotype.

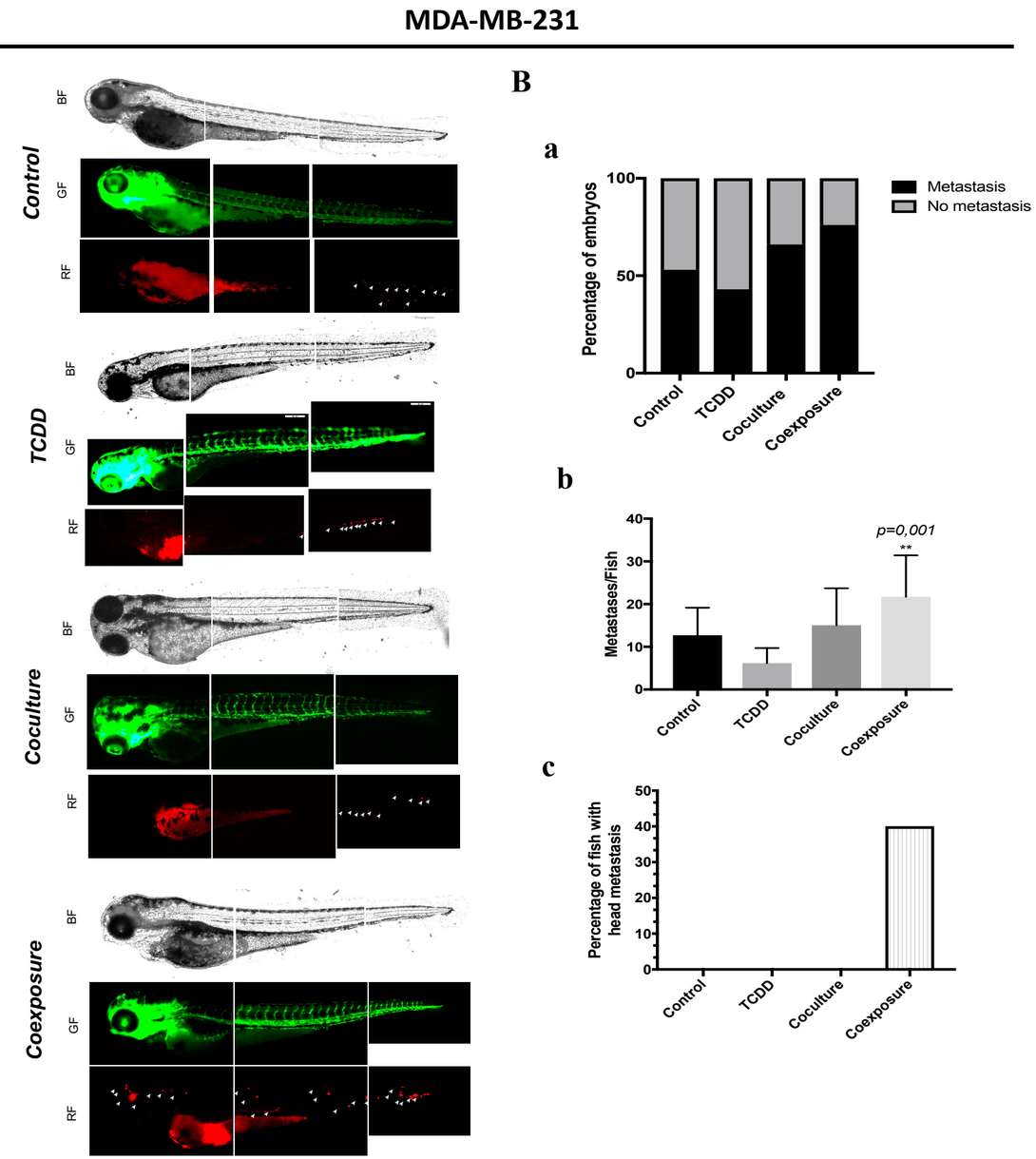
(A) High-throughput proteomic analysis (conditioned medium) showing ALDH1A3 overexpression in co-culture. **(B)** ALDH1 enzymatic activity was detected using the ALDEFLUOR assay. DEAB was used to inhibit the reaction of ALDH with the ALDEFLUOR reagent, providing a negative control. ALDH+ MCF-7 cells were significantly increased in TCDD and in co-exposure. .



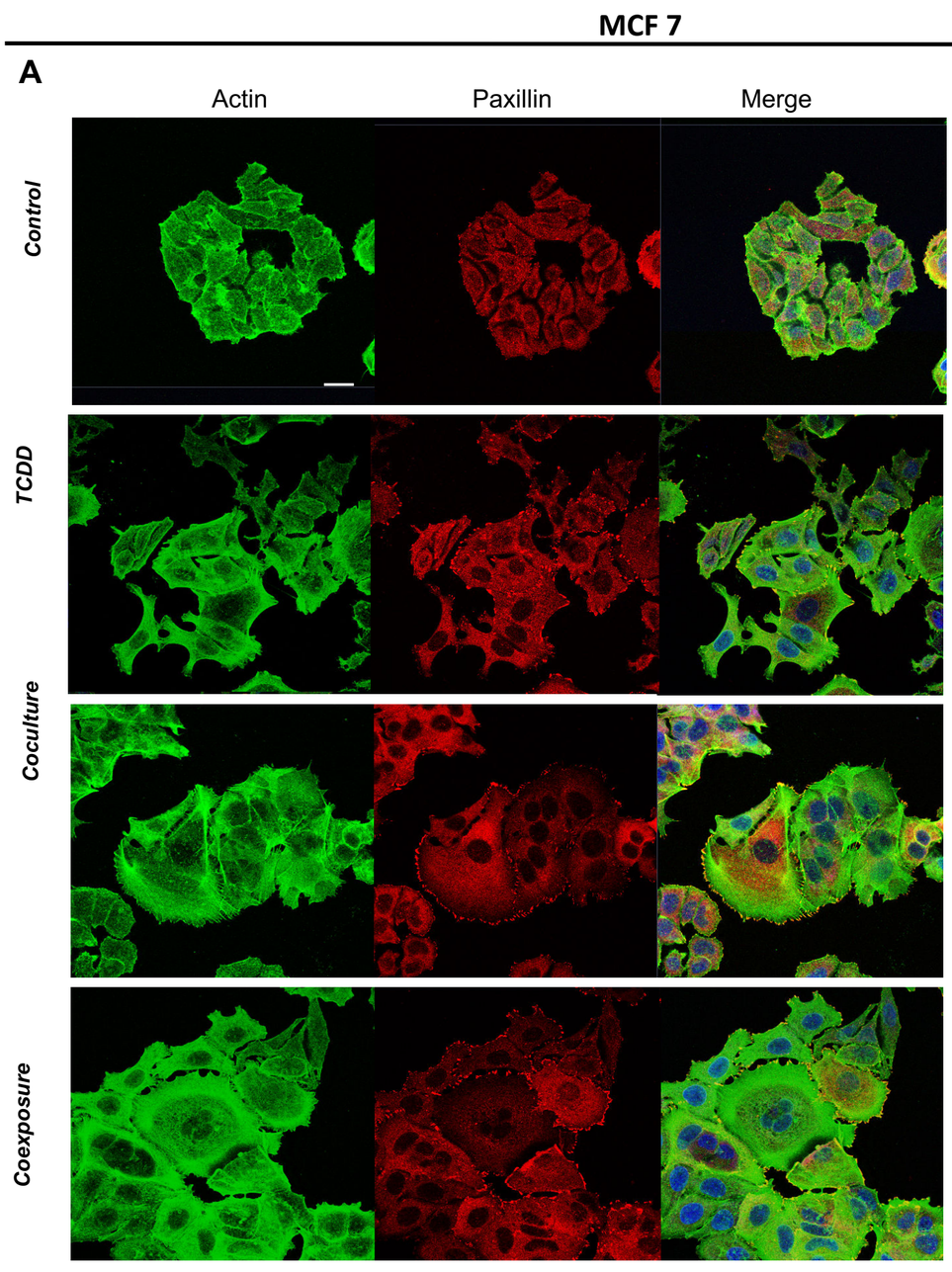
<u>Figure 3</u>: Metastasis formation in vivo in zebrafish larvae

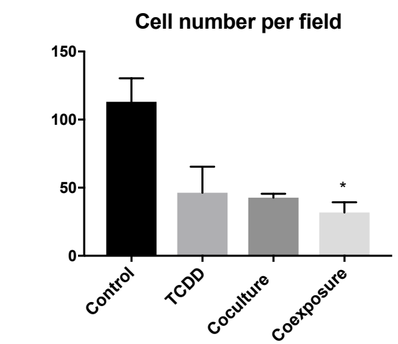
Coexposure enhance human cancer cell metastasis in zebrafish xenograft models. Human RFP-MDA-MB-231 (TNBC) or MCF7 cells were injected into the perivitiline space of two-day-old zebrafish larvae and larvae. Fish were imaged 24 h later at 8.5× magnification by fluorescence microscopy. (**A**) Representative images from 21-46 fish/group. (**B**) Quantitations. **(a)** The number of fish with one or more metastases. (**b**) Data are presented as the average number of metastases/fish + SE. **(c)** Percentage of fish with metastasis in the head region.

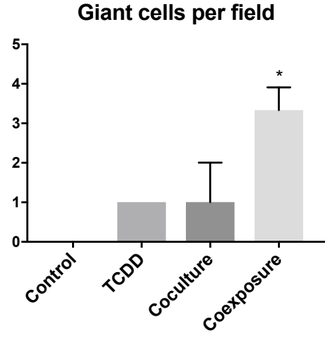


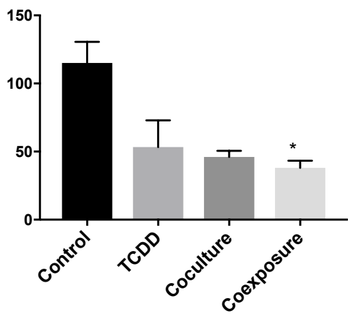


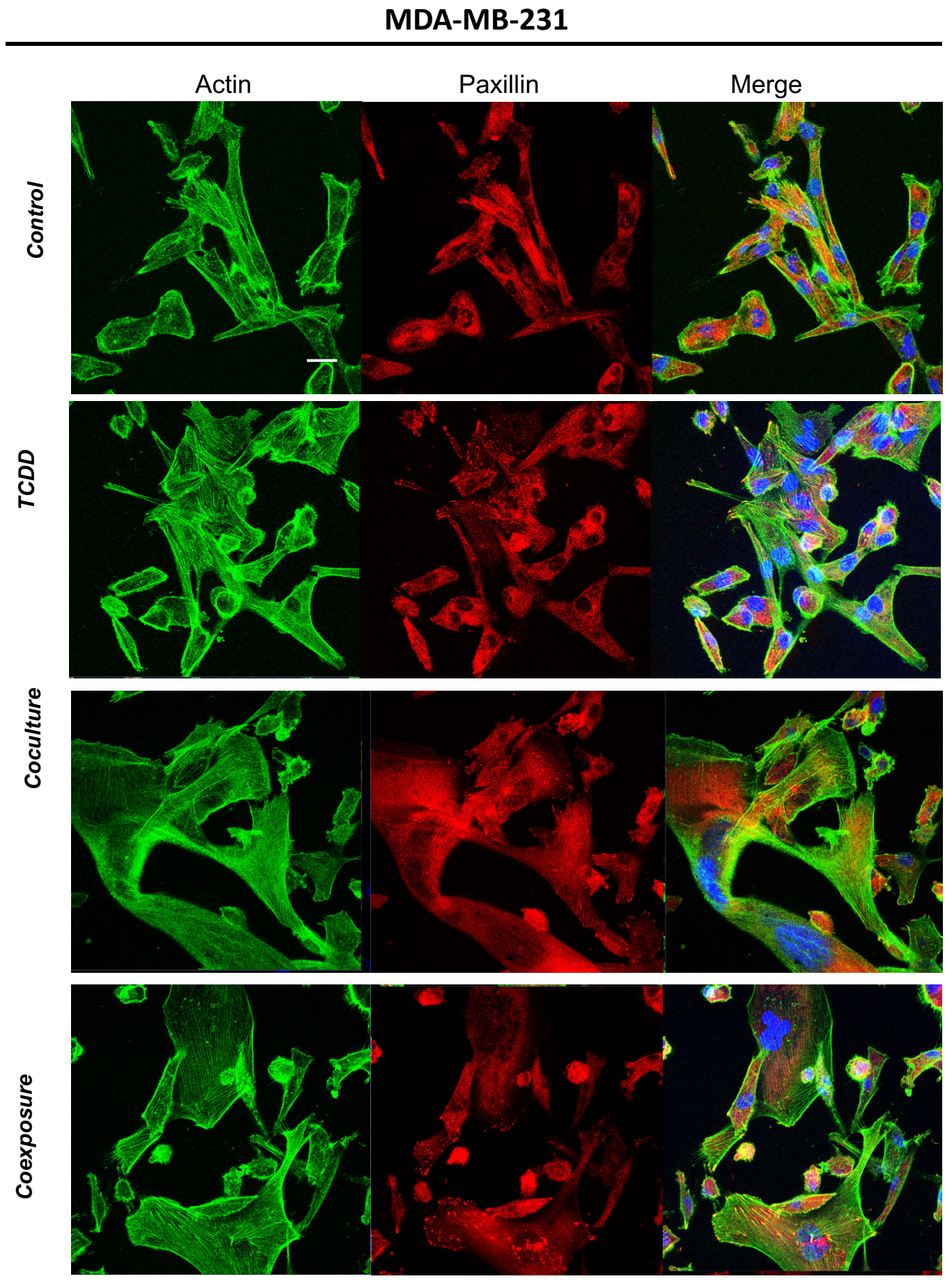
<u>Figure 4:</u> Morphological changes in coexposure: Generation of viable non apoptotic giant multinucleated cells
(A) MCF7 cells were stained for paxillin (red), actin (phalloidin, green) and nucleus (topo-3, blue). Co-exposure induce polynucleation and the formation of giant cells (photographs were taken using a 63× objective, scale bar 20µm **(B)** Quantitation of cell number, nuclei number and giant cells number per field for MCF7 cells.



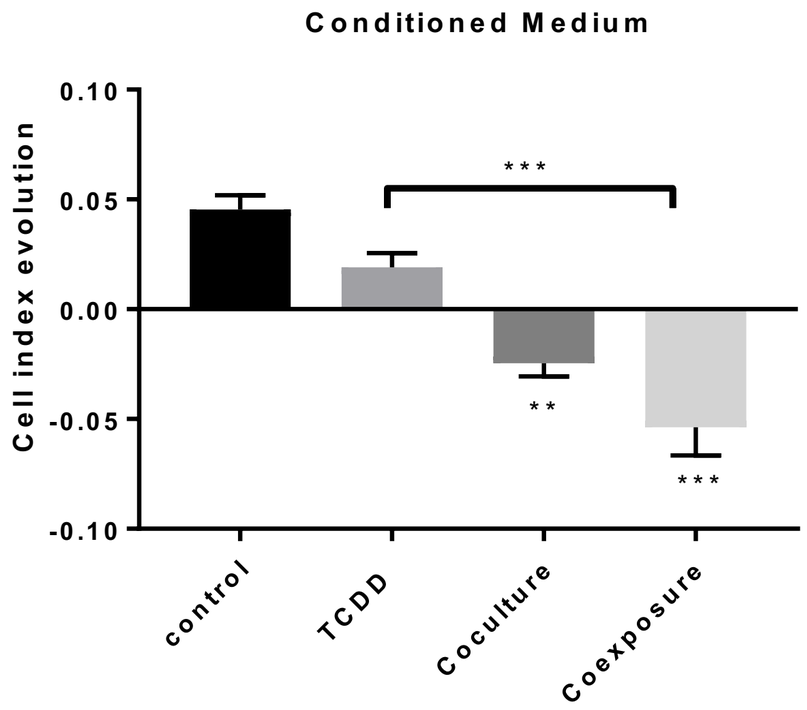




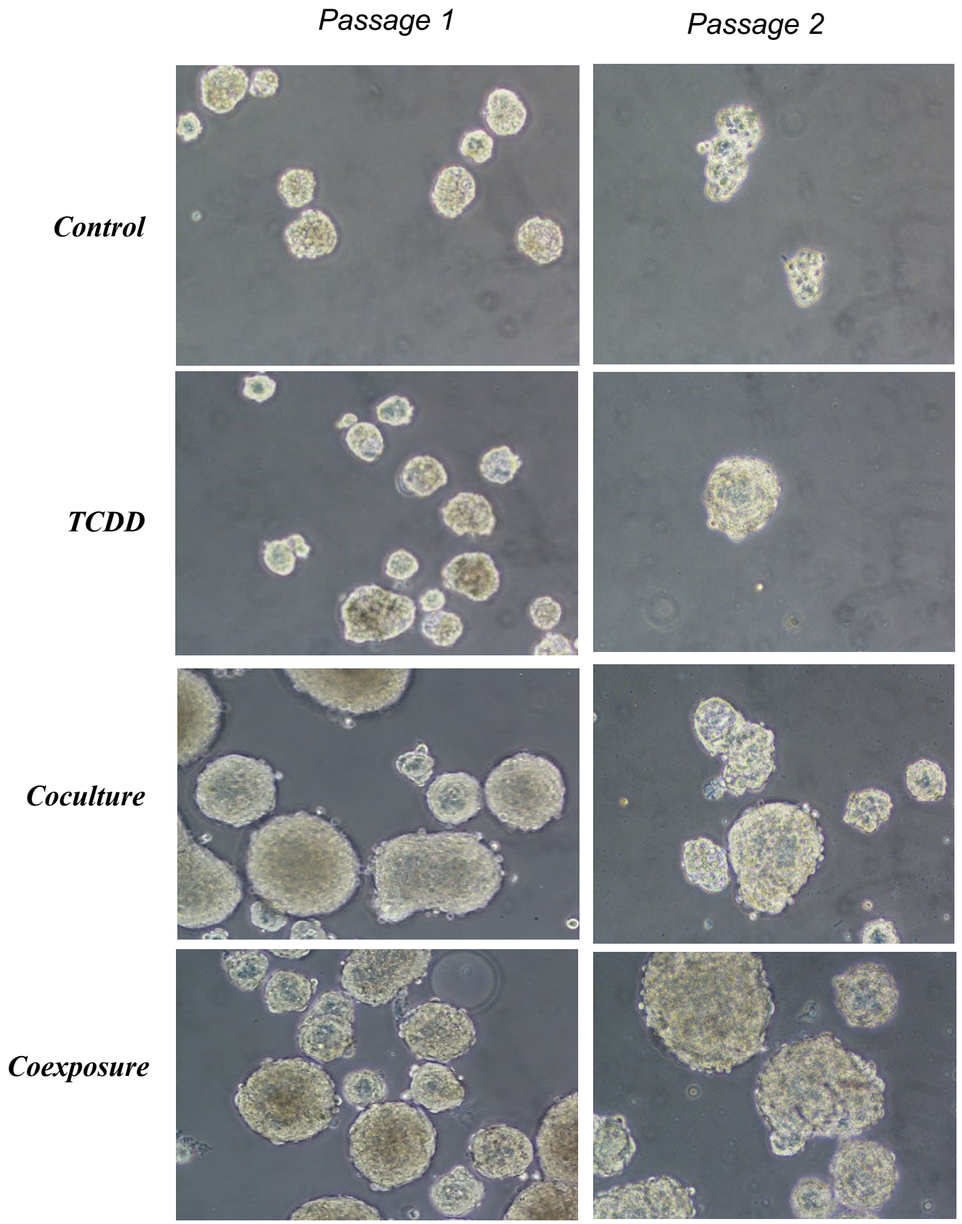


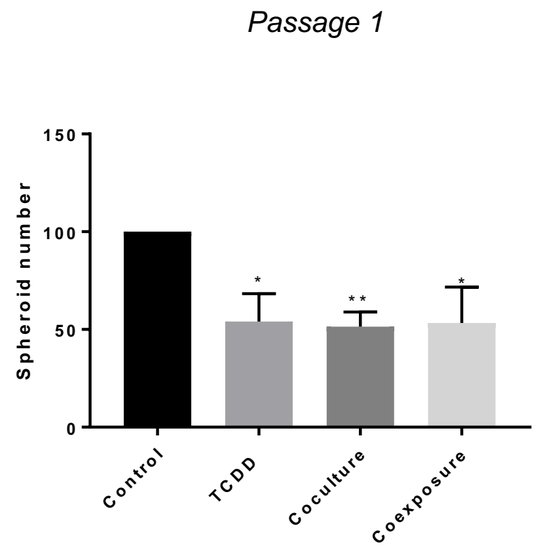


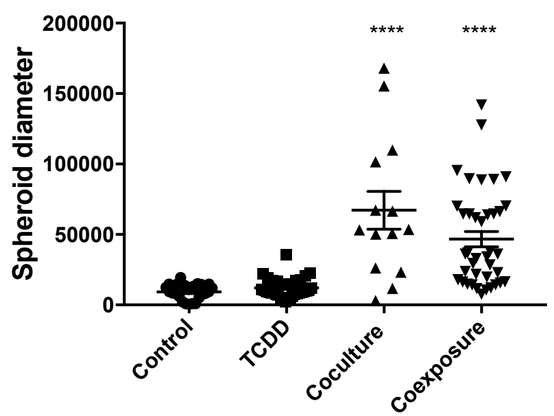
<u>Supplemental Figure 1</u>: Modifications of MCF-7 cell properties after treatment with conditioned medium.
xCELLigence and dynamic monitoring of MCF-7 cells. The results are expressed as CI values. The rate of proliferation (cell index evolution) under the different conditions were determined by analyzing the slope of the cell index over the interval between 26 and 68 h .



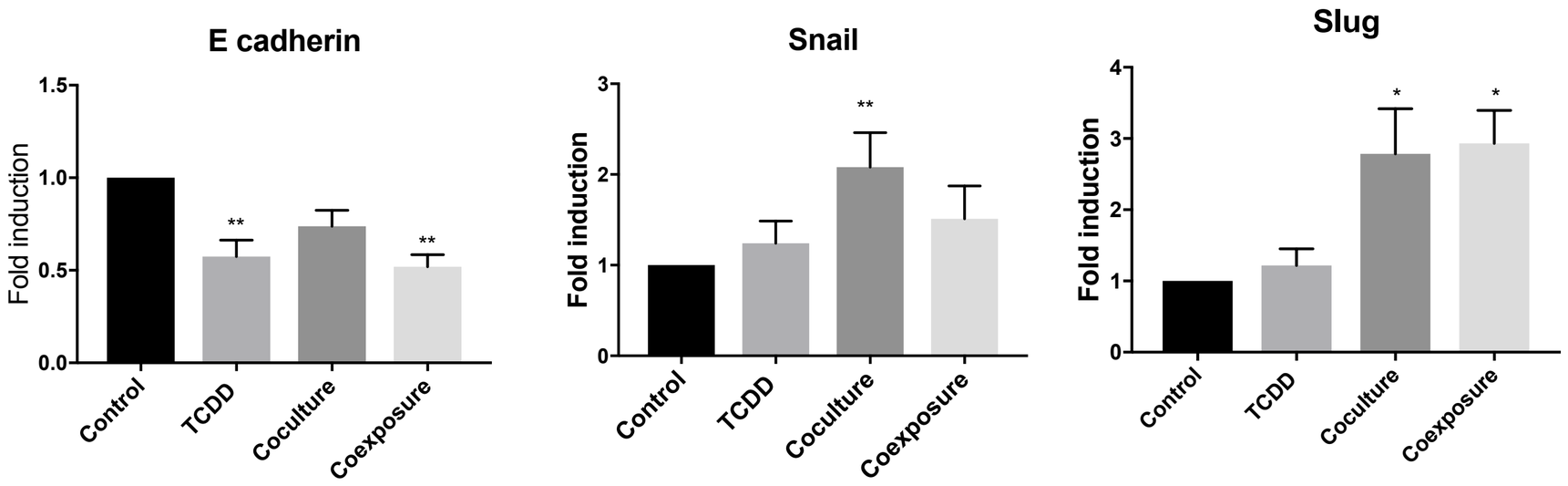
<u>Supplemental Figure 2</u>: Sphere formation assay.
Representative images of sphere formation taken on day 7 on two consecutive generations. Only breast stem/progenitor cells can self renew and grow into a spheroid structure.



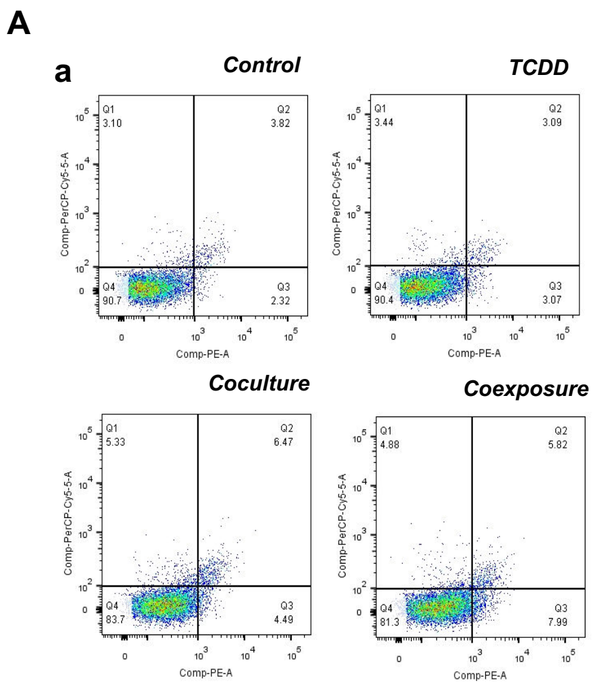


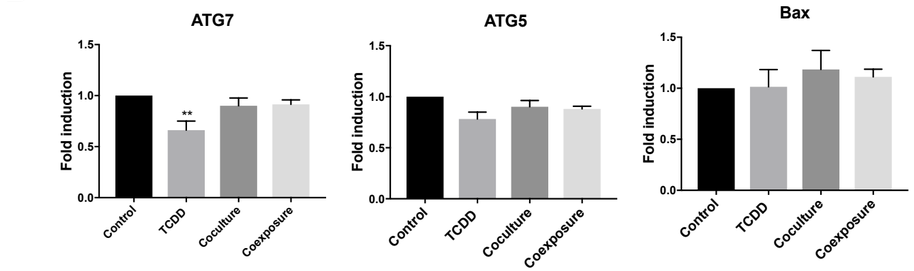


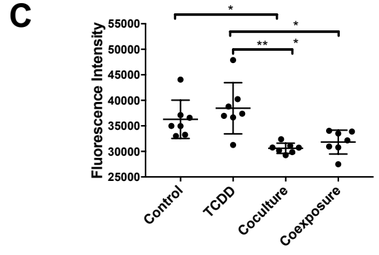
<u>Supplemental figure 3</u>: Transcriptional changes of genes involved in EMT

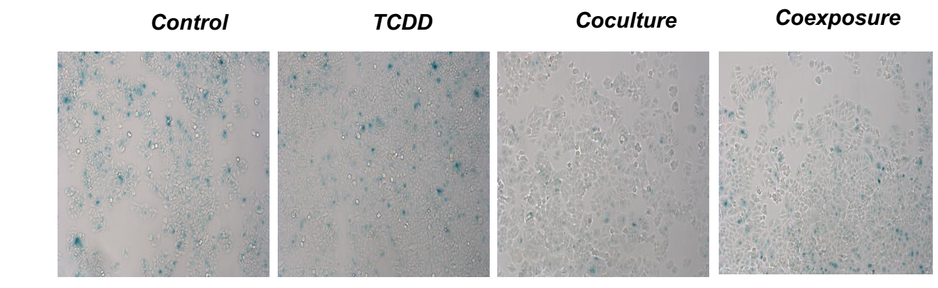


<u>Supplemental figure 4</u>: Cell death experiments.
No difference was observed between each condition in cell death, senescence, proliferation, or autophagy after 48h of treatment. (A) *Evaluation of cell death by apoptosis using annexin V-FITC/PI staining.* (a) Flow cytometer results. Cells were classified as healthy cells (Annexin V−, PI−), early apoptotic cells (Annexin V+, PI−), late apoptotic cells (Annexin V+, PI+), and damaged cells (Annexin V−, PI+). (b) The ratio of apoptosis among different experimental groups. (B) *Evaluation of apoptosis and autophagy*. Relative mRNA expression levels of ATG5, ATG7 and BAX by real-time qPCR. (C) *MCF-7 proliferation exploration using alamarBlue assay*. Mean fluorescence is indicated for each condition as a point and statistically significant differences were determined by ANOVA and indicated as *P* values above the comparison, bars, SD. The fluorescence is decreased in co-culture while the cell death is identical. This is explained by the decrease of the number of cells in co-culture. (D) *Senescence-associated β-galactosidase activity.*









Discussion

In this study, we show that human BC cells acquire pro-metastatic features, *in vitro* and *in vivo,* when cultured with both pre-adipocytes and a persistent organic pollutant (TCDD). Indeed, we demonstrated, using zebrafish larvae, that a large increase in brain metastases was observed only with co-exposed MDA-MB-231 cells. This result is entirely consistent with another model, the non-invasive human MCF-7 cell line. We further showed that co-exposure leads to the generation of polynuclear giant cancer cells (PGCCs) defined by irregular nuclei the sizes of which were at least three to five times larger than those of regular diploid cancer cells [16].

PGCCs, a particular subpopulation of cancer cells which contribute to solid tumor heterogeneity, are usually observed under extreme conditions (like hypoxia or radiotherapy) and are strongly associated with cancer aggressiveness and chemoresistance [17]. Nuclear morphometric parameters can identify an aggressive tumor phenotype and provide significant prognostic information for survival and risk of tumor progression [18,19]. PGCCs, previously, were considered as senescent cells but recent data has revealed that these large cancer cells generate daughter cancer cells via budding [20]. Integrated formation of PGCCs with EMT is associated with invasion and metastasis in human breast cancer [21]. These cells also are positive for cancer stem cells (CSCs) markers (ALDH1A3 or aldehyde deshydrogenase 1A3, CD44 and CD133) and become more resistant to chemotherapy [22]. CSCs were identified for the first time in blood mononuclear cells in human acute myeloid leukemia [23].They possess characteristics that promote tumor metastasis and chemoresistance [13,24]. In the current work, we found that the co-exposure condition dramatically increases ALDH1A3 level and activity. In BC, the ALDH activity of CSCs has been shown to be due to the isoform ALDH1A3 which is a marker for cells with an increased propensity to metastasize. It is recognized also as a marker for metastasis and ALDH1A3 expression could be used as a prognostic factor [14]. However, the role, in this process, of retinoic acid, an ALDH1A3 enzyme metabolite, is still unknown [15,25].

Our work leads to the hypothesis that PGCCs and CSCs are the same entity which emerges, specifically, under the co-exposure condition. In the literature, both are associated with resistance to chemotherapy in several cancers and lead to a less favorable prognosis. A model of the CSC-stimulating crosstalk circuit involves the STAT3, CCL2 and NOTCH pathways. In the tumor microenvironment, paracrine signaling initiated by BC cells induces MCP1 (CCL2) production by stromal fibroblasts through STAT3 activation. The fibroblast-derived MCP1, in turn, promotes cancer progression by regulating CSCs

through activation of the NOTCH pathway [26,27]. In our work, the microenvironment is simulated by the hMADS cells (preadipocytes) cells. Previous work in our laboratory has shown that this cell line is a valuable human adipocyte model to evaluate pollutant toxicity and its response to dioxin, especially for the regulation of the inflammatory pathway [28]. Adipose tissue, the major cell type in the breast, is an abundant source of mesenchymal stem cells which validates the use of the hMADS cell line.

TCDD, which is lipophilic and resistant to xenobiotic metabolism, is stored in adipose tissue which also stores other persistent organic pollutants. TCDD is the most potent dioxin and we have used it in our study to mimic long-term bioaccumulating molecules which also stimulate the AhR signaling pathway [29]. In previous studies, we showed that TCDD leads to EMT in cellular models and to liver fibrosis in animal models [10]. Liver fibrosis is suspected to involve EMT processes also observed during cancer metastasis. It would be interesting to test the effects of mixtures of POPs in our model in order to determine whether they can stimulate tumor progression. Also, mixtures of non-persistent organic pollutants such as phtalates and bisphenols or heavy metals would be interesting to test since although these xenobiotics are non-persistent, they are considered as semi-persistent due to continuous exposure. While they do not stimulate similar signaling pathways, the detoxication processes involving such molecules are quite analogous. Our study provides evidence, for the first time, that environmental pollutants may favor the occurrence of metastases by targeting the tumor microenvironment and stimulating previously unsuspected molecular pathways. This knowledge may be relevant, generally, in terms of public health for the management of tumors.

Materials and Methods

Cell culture. The human breast cancer cell line MCF-7 (ATCC® HTB-22™) cells were cultured at 37°C in Dulbecco's modified essential medium (DMEM) complemented with nonessential amino acids, supplemented with 10 µg/mL human recombinant insulin, 10% fetal bovine serum, 200 U/ml penicillin, 50µg/ml streptomycin (Invitrogen, Cergy-Pontoise, France) and 0.5 mg/ml fungizon in a humidified atmosphere in 5% CO_2. The human breast cancer cell line MDA-MB-231 (ATCC® HTB-26™) cells were cultured at 37% in DMEM complemented with nonessential amino acids, 10% fetal bovine serum, 200 U/ml penicillin, 50µg/ml streptomycin (Invitrogen, Cergy-Pontoise, France). The establishment and characterization of the hMADS cell line have been described previously [30]. Proliferating hMADS cells were seeded into proliferating medium consisting of Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum, 2.5 ng/mL hFGF2, 10 mM HEPES buffer, 50 U/mL penicillin, and 50 µg/mL streptomycin and incubated at 37°C in 5% CO_2. hFGF2 was removed when the cells reached confluence.

The co-culture model. MCF-7 cells or MDA-MB-231 cells, and hMADS preadipocytes were co-cultured in Transwell culture plates in proliferating medium without FGF (Fibroblast Growth Factor). Briefly, 400,000 breast cancer cells were seeded into the wells of a 6-well companion and 400 000 preadipocytes hMADS cells were seeded onto polyester membrane inserts (0.4-µm pore size) in 6-well culture dishes. MCF-7 and MDA-MB-231 cells also were grown alone as controls. After 24 h of incubation at 37°C, the medium was replaced and the cells were treated, or not, with 25nM TCDD for 48h. The MCF-7 or MDA-MB-231 and hMADS co-culture was called "co-culture" and this co-culture system treated with TCDD was called "co-exposure" (co-culture + TCCD). The conditioned media of the lower and upper chambers of the co-culture system were collected after 2 days of treatment and were used to stimulate breast cancer cells for 48 hours.

Chemicals

TCDD (#ED-901, CAS: 1746-01-6) was purchased from LGC Standards (France).

Monitoring of cell growth using real time cell analysis (RTCA). MCF-7 cells were seeded in duplicate for each condition into 16-well xCELLigence E-plates at 40,000 cells/well in a final volume of 100 µL and, immediately, an E-plate insert containing hMADS cells (40 000 cells/well) was added. One day

after seeding, the medium was removed and the cells were treated or not with 25nM TCDD. The impedance value of each well was automatically monitored by the xCELLigence system and expressed as a cell index value (CI). The rate of attachment (CI/h) was calculated using the xCELLigence RTCA software (ACEA Biosciences, Inc., Roche Diagnostics). Each experiment was performed in triplicate.

***Sample preparation for Mass spectrometry analysis*.** The FASP (Filter-aided sample preparation) procedure for protein digestion was performed, as described previously [31] using 30kDa MWCO centrifugal filter units (Microcon, Millipore, Cat No MRCF0R030), on 50µg of each lysate in RIPA buffer. Proteins were reduced with 0.1M dithiotreitol (DTT) for 30min at 60°C, then applied to the filters, mixed with 200µL of 8M urea, 100mM Tris-HCl pH 8.8 (UA buffer) and finally centrifuged for 15min at 15,000g. In order to remove detergents and DTT, the filters were washed twice with 200µl of UA buffer. Alkylation was carried out by incubation for 20min in the dark with 50mM iodoacetamide. Filters were then washed twice with 100µl of UA buffer (15 000 x g for 15min) followed by two washes with 100µl of ABC buffer (15 000 x g for 10min) to remove urea. All centrifugation steps were performed at room temperature. Finally, trypsin was added in a 1:30 ratio and digestion was achieved by incubation at 37°C overnight.

nanoLC-MS/MS protein identification and quantification. Samples were vacuum dried and resuspended in 50 µL of 10% acetonitrile, 0.1% trifluoroacetic acid, for LC-MS/MS. For each run, 1 µL was injected into a nanoRSLC-Q Exactive PLUS (RSLC Ultimate 3000, Thermo Scientific, Waltham, MA, USA). Peptides were separated on a 50cm reversed-phase liquid chromatographic column (Pepmap C18, Thermo Scienfitic). Chromatography solvents were (A) 0.1% formic acid in water, and (B) 80% acetonitrile, 0.08% formic acid. Peptides were eluted from the column with the following gradient 5% to 40% B (120 min), 40% to 80% B (10 minutes). At 131 min, the gradient returned to 5% B to re-equilibrate the column for 30 minutes before the next injection. Two blanks were run between triplicates to prevent sample carryover. Peptides eluting from the column were analyzed by data dependent MS/MS using the top-10 acquisition method. Briefly, the instrument settings were as follows: resolution was set to 70,000 for MS scans and 17,500 for the data dependent MS/MS scans in order to increase speed. The MS AGC target was set to 3.106 counts, while MS/MS AGC target was set to 1.105. The MS scan range was from 400 to 2000 m/z. MS and MS/MS scans were recorded in the profile mode.

Dynamic exclusion was set to 30 sec duration. Three replicates of each sample were analyzed by nanoLC-MS/MS.

Data Processing Following nanoLC-MS/MS acquisition. The MS files were processed with the MaxQuant software version 1.5.8.30 and searched with an Andromeda search engine against the Uniprot Human database. To search parent mass and fragment ions, we set a mass deviation of 3 ppm and 20 ppm respectively. The minimum peptide length was set to 7 amino acids and strict specificity for trypsin cleavage was required, allowing up to two missed cleavage sites. Carbamidomethylation (Cys) was set as fixed modification, whereas oxidation (Met) and N-term acetylation were set as variable modifications. The false discovery rates (FDRs) at the protein and peptide level were set to 1%. Scores were calculated in MaxQuant as described previously [32] The reverse and common contaminants hits were removed from MaxQuant output. Proteins were quantified according to the MaxQuant label-free algorithm using LFQ intensities [33]. Statistical and bioinformatic analyses, including heatmaps, profile plots and clustering, were performed with Perseus software (version 1.5.5.3) freely available at http://www.perseus-framework.org [34]. For statistical comparisons, we defined four groups, each containing biological quadruplicates. Each sample was run in technical triplicates as well. We then filtered the data to keep only proteins with at least 4 valid values out of 4 in at least one group. Next, the data were imputed to fill missing data points by creating a Gaussian distribution of random numbers with a standard deviation of 33% relative to the standard deviation of the measured values and 2.5 standard deviation downshift of the mean to simulate the distribution of low signal values. We performed t-tests using FDR=0.05, S0=1, for all pertinent pairs and represented the results using volcano plots.
The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [35] partner repository with the dataset identifier PXD014328.

Aldefluor assay. The Aldefluor kit (Stem Cell Technologies) was utilized to profile and isolate cells with high and low ALDH activity as previously described [36]. Cells were incubated in Aldefluor assay buffer containing the ALDH substrate (1 μM), BODIPY-aminoacetaldehyde (BAAA), at 37°C for 30 min. Cells that were able to catalyze BAAA to its fluorescent product, BODIPY-aminoacetate (BAA), were considered $ALDH^+$ cells. The enzymatic activity of ALDH was blocked by a specific inhibitor, DEAB. Sorting gates for fluorescence-activated cell sorting (FACS) were drawn relative to baseline

fluorescence, which was determined by DEAB-treated samples (negative control). After incubation, cells were resuspended in fresh assay buffer. $ALDH^+$ and $ALDH^-$ cells were sorted by a BD Canto II flow cytometer.

Spheroid formation*.* MCF-7 cells (input of 3,000 cells per spheroid) were transferred to conditioned medium. Cell suspensions (150 µl) were transferred to each well of a 96-well plate coated with 1% agarose to allow spontaneous spheroid formation within 7 days. After 7 days, the number of mammospheres per microscopic field over five fields was determined. The diameter of the mammospheres were measured. Technical triplicates were used for statistical analyses ($p < 0,05$, t test). After 7 days, cells were trypsinized and re-plated in growing medium (second passage).

Zebrafish larva metastasis assays. Zebrafish husbandry was performed as described [37] in the zebrafish facility at the Boston University School of Medicine and in accordance with IACUC-approved protocols. AB x Fli-GFP fish breeders were crossed and overexpressing-GFP embryos (in the vasculature) were obtained. RFP-labelled MDA-MB-231 cells and MCF-7 cells were pre-seeded into a 6-well plate and treated with the different conditioned medium for 48 hours. For CM-DiI labelling, MCF-7 cells were trypsinized and centrifuged to obtain a pellet and resuspended in 5µl/ml CM-DIL dilution (Invitrogen), incubated in the dye at 37° at 5% CO_2 for 10 min and then rinsed once for PBS. For micro-injections, the RFP-labelled MDA-MB-231 cells and CM-DiI-labelled MCF-7 to be transplanted were resuspended at a concentration of 50×10^6 cells/mL in DMEM with 10% FBS. One day post-fertilization (1 dpf) larvae were enzymatically dechorionated using Pronase (Roche Diagnostics, Westborough, MA, USA) and allowed to recover overnight at 28 °C, in the dark, and in sterilized egg water. The 2-dpf zebrafish larvae were anesthetized with tricaine and subsequently immobilized before injections. Borosilicate glass capillaries (1.0 mm outside diameter × 0.78 mm) (World Precision Instruments, Sarasota, FL, USA) used for micro-injection were pulled using 500 Volts (pull = 100, velocity = 250) in a capillary machine (Sutter Instrument, Novato, CA, USA). Human tumor cell transplantations were performed by injection of ~1 nL directly into the perivitelline space of the larvae using a needle holder and a micro-injection station (World Precision Instruments) as described [38]. Xenografted zebrafish larvae were then rescued in sterile egg water and transferred to a standard 6-well plate and incubated in the dark at 33°C Twenty-four hours later, zebrafish larvae were individually mounted in a low melting 1.5%

agarose gel (Fisher) for side view immobilization using a thin eyelash. Metastases were quantified using an inverted fluorescence microscope (Leica DMIL LED). Each experiment was repeated at least in triplicate (MCF-7) or quadruplicate (MDA-MB-231).

Immunocytochemistry and confocal microscopy. MCF-7 cells were seeded onto coverslips, cultured with or without preadipocyte hMADS cells and treated or not with 25nM TCDD. After 2 days of co-culture, the cells were fixed with 4 % paraformaldehyde for 20 min and permeabilized with 0.2 % Triton X-100 for 3 min at room temperature, then washed with PBS. The cells were incubated in blocking solution (PBS-bovine serum albumin 1%) for 1 h and then incubated with the primary antibody in PBS for 1 h 30 min at room temperature. After washing with PBS-T (PBS containing 0.1 % Tween-20), the cells were incubated with a second antibody conjugated with a fluorescent dye for 1 h at room temperature. For the staining of actin and nuclei, FITC-conjugated phalloidin and TO-PRO-3 (Invitrogen) were included during the incubation with the secondary antibody. The coverslips were sealed with Dako Faramount Aqueous Mounting Medium Ready-to-use (Invitrogen) and images were recorded using a Zeiss LSM 510 confocal microscope (Carl Zeiss Meditec France SAS, Le Pecq, France) using a 40 Plan-Neofluar 1.3 NA oil objective and LSM Image Browser (Zeiss).

Preparation of RNA and reverse transcription quantitative PCR. RNA was prepared using the *GenElute* Mammalian Total RNA Miniprep *Kit* (Sigma Aldrich). Reverse transcription was performed using the high capacity cDNA reverse transcription Kit (Life technology, France). Quantitative real-time polymerase chain reaction (qRT-PCR) was carried out using the CFX384 System (Biorad) and the *SensiFAST* SYBR Master Mix (Bioline). The relative amounts of mRNA were estimated using the delta-delta Ct method with RPL13A as the reference.

Annexin V- PE/7-AAD dual staining assay. The Annexin V-PE/7-AAD dual staining assay was carried out using FITC Annexin V Apoptosis Detection Kit (Biolegend). After 48h of co-culture and treatment, MCF-7 cells were collected by trypsinisation. The collected cells were washed two times with ice-cold PBS and then incubated in binding buffer containing Annexin V-PE and 7-AAD for 5 min at room temperature in the dark. After 15 min of incubation, cells were analyzed for apoptosis with a BD Canto II flow cytometer.

AlamarBlue cell proliferation assay. Cells were plated at 5000 cells per well in a 96-well plate, grown for 24 h and treated with conditioned media. All conditions were assessed in triplicate. Plates were incubated for 48h at 37°C. AlamarBlue was added to each well according to the manufacturer's protocol. Cells were incubated for 2 h at 37 °C and the absorbance at 540 nm was recorded. Data were expressed as the means ± standard error of the mean (SEM).

SA-β-Galactosidase Activity*.* SA-β-galactosidase enzymatic activity was assayed *in vitro* using a β-galactosidase staining kit (Senescence Cell Staining Kit; Sigma-Aldrich). Briefly, 10,000 cells were plated in 12-well plates, treated for the indicated times, incubated at 37 °C overnight and then washed with PBS (pH 6.0), fixed and stained with 5-bromo-4- chloro-3-indolyl-h-D-galactopyrano- side (X-Gal) overnight at 37 °C. Only senescent cells stain at pH 6.0.

Statistics. Each experiment was performed at least in triplicate. The results of 3 or more independent experiments are expressed as the mean +/- SD (standard deviation). Statistical analysis was performed using H Kruskal–Wallis's test (nonparametric comparison of k independent series) followed by a 1-factor ANOVA test (parametric comparison of k independent series). A value of $P<0.05$ was considered statistically significant.

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Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al, 2019) partner repository with the dataset identifier PXD014328.

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Author contributions

MK: design & perform experiments, funds rising, wrote the article

CT: design & perform experiments, wrote parts of the M&M section

ICC: perform experiments, wrote parts of the M&M section

DS: design experiments

RB: design experiments, wrote the article

XC: design experiments, funds rising, wrote the article

Competing financial interests

The authors declare no competing interests.

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Supplementary information

Supplemental Figure 1: Modifications of MCF-7 cell properties after treatment with conditioned medium.

Supplemental Figure 2: Sphere formation assay.

Supplemental Figure 3: Transcriptional changes of genes involved in EMT.

Supplemental Figure 4: Cell death experiments.

Supplemental Table: Proteomics data

CONCLUSION

Le tissu adipeux constitue une réserve de POP et donc une source chronique interne de polluants. Dans ces travaux de thèse, nous avons montré, entre autres, pour la première fois que les concentrations d'un POP dans le tissu adipeux, la dioxine, était un facteur de risque d'agressivité, en particulier chez les patientes en surpoids. Nos résultats expérimentaux qui démontrent l'acquisition par les cellules cancéreuses mammaires d'un phénotype agressif, enrichi en CSC, favorisant la formation de métastases lors de la co-exposition pré-adipocytes/dioxine sont cohérents et soulignent le rôle des interactions entre le microenvironnement tumoral et des polluants environnementaux.

Plusieurs hypothèses peuvent être formulées pour expliquer la contribution du microenvironnement adipocytaire à l'agressivité tumorale, notamment la sécrétion de cytokines pro-inflammatoires et de chimiokines dont l'expression pourrait être potentialisée par la présence de ligands AhR comme la dioxine (114). Plusieurs études ont également montré que les POP pouvaient modifier le phénotype adipocytaire et moduler leur fonctions entrainant une production accrue de cytokines pro-inflammatoires, des perturbations du métabolisme cellulaire associées à une augmentation du stress oxydatif ou des perturbations de la lipolyse (affectant la libération de POP) (115–118). Ces dysfonctions adipocytaires pourraient contribuer à la libération locale excessive de produits chimiques et de molécules pro-tumorales par les adipocytes péritumoraux et potentialiser les effets pro-invasifs déjà décrits des adipocytes associés au cancer.

Au-delà des effets directs des POP sur les cellules cancéreuses mammaires, l'ensemble de nos résultats mettent en exergue le rôle synergique que certains polluants pourraient avoir sur le risque métastatique par le biais d'un dysfonctionnement des adipocytes péritumoraux.

Ces travaux sont également une contribution en termes de santé publique et peuvent permettre d'alerter une fois de plus les services publics sur la dangerosité des POPs et la nécessité de les contrôler au mieux.

PERSPECTIVES

Les résultats de l'étude METAPOP particulièrement intéressants nécessitent d'être confirmés dans une cohorte plus importante. Nous avons à notre disposition les échantillons tumoraux des patientes. Une analyse des profils transcriptomiques des tumeurs et leurs mises en relation avec les concentrations de POP apporteraient des informations importantes sur les voies de signalisation ciblées par les différents polluants. Nous nous intéressons aussi au laboratoire aux effets de « mélanges » de polluants qui mériteraient d'être étudiés dans ce domaine de la progression tumorale. Nous sommes en train de travailler sur des modèles statistiques de modélisation de ces effets sur la cohorte de patientes inclues dans METAPOP.

La poursuite des travaux expérimentaux sont nécessaires afin de confirmer nos résultats sur des préadipocytes humains mais aussi des adipocytes différenciés. Nous envisageons également de charger des adipocytes avec de la TCDD avant la mise en culture pour voir si les effets observés dans notre étude résultent de la seule modification du phénotype adipocytaire ou également de modifications phénotypiques des cellules cancéreuses. Ici encore, tester des mélanges pertinents de polluants serait intéressant. Nous avons débuté par ailleurs une étude financée par l'INCA sur les effets de la fumée de cigarette sur l'environnement péritumoral du CS et le risque métastatique, utilisant le même modèle *in vitro*. Nous participons également à un dépôt de projet européen pour étudier l'effet des additifs alimentaires (aliments ultra-transformés) sur le modèle de co-culture.
D'un point de vu mécanistique, de nombreuses pistes doivent encore être explorées. On peut citer le rôle du AhR dans les effets observés à travers l'utilisation d'antagonistes. Il existe par ailleurs de nombreux ligands endogènes identifiés de l'AhR (comme les dérivés du tryptophane qui jouent

un rôle important dans la régulation du système immunitaire) et il serait intéressant d'étudier les interrelations entre ses ligands endogènes et exogènes.

De plus, l'origine des PGCC ainsi que leur rôle dans la progression tumorale sont encore débattus. Confirmer que les PGCC observées dans nos expérimentations sont générées par le phénomène d'entose apporterait des éléments importants de compréhension de ce mécanisme dont le rôle est encore mal connu.

Enfin, de façon plus large, en collaboration avec le laboratoire LABERCA à Nantes (Laboratoire d'étude des résidus et contaminants dans les aliments), nous nous intéressons aux rôles des POP dans l'endométriose qui est une maladie fréquente et handicapante et dont les étiologies ne sont actuellement pas connues. Au cours de l'endométriose, les cellules endométriales acquièrent des propriétés migratoires et des propriétés d'invasion dans le cas de l'endométriose profonde (infiltration des tissus adjacents). Les hypothèses concernant l'implication des POPs dans le développement et la progression de la maladie sont nombreuses : activation du récepteur AhR (Aryl hydrocarbon Receptor) induisant la formation des œstrogènes (119,120), production excessive d'espèces réactives de l'oxygène (ERO) pouvant induire des lésions cellulaires et une augmentation de l'expression génique pro-inflammatoire par l'activation de la voie NFkappaB, sécrétion accrue de prostaglandines péritonéales (121). Cependant, les études, nombreuses, sont peu conclusives et parfois contradictoires. Une revue de la littérature est en cours de rédaction dont le protocole a été publié (Article 4 présenté en annexe 1). Un projet expérimental explorant le rôle des POP et du microenvironnement (constitué de cellules mésothéliales péritonéales, de cellules monocytaires mais aussi de cellules adipeuses du grand omentum) dans cette maladie est en attente de financement.

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Annexe 1: Article 4 :

"Associations between exposure to organochlorine chemicals and endometriosis in experimental studies: A systematic review protocol ."

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Review article

Associations between exposure to organochlorine chemicals and endometriosis in experimental studies: A systematic review protocol

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ABSTRACT

Background: Endometriosis is a hormone-dependent gynaecological disease characterised by the presence and growth of endometrial tissues outside of the uterus. There is growing experimental evidence that suggests environmental endocrine disrupting chemicals, specifically organochlorine chemicals (OCCs), may play a role in the pathogenesis of endometriosis, but to date, there are no studies attempting to gather and synthesise the published literature systematically.

Objectives: The main objective of this SR is to evaluate the associations between the exposure to OCCs and endometriosis in experimental models (*in vivo* and *in vitro*).

Methods: The SR framework has been developed following the guidelines established in National Toxicology Program/ Office of Health Assessment and Translation (NTP/OHAT) Handbook for Conducting a Literature-Based Health Assessment, which provides a standardised methodology to implement the Grading of Recommendations Assessment, Development and Evaluation (GRADE) approach to environmental health assessments. The review process will be managed and documented through HAWC, an open-source content management system, to guarantee transparency.

Eligibility criteria: Only experimental studies, *in vivo*, *ex vivo* or *in vitro*, exploring associations between controlled exposures to OCCs and endometriosis and related outcomes will be included. Eligible studies will include peer reviewed articles of any publication date which are sources of primary data. Only studies published in English will be considered.

Information sources: We will apply the search strings to the scientific literature databases NCBI PubMed, Web of Science and SCOPUS. Manual searches will be performed through the list of references of included articles.

Data extraction and synthesis or results: Data will be extracted according to a pre-defined set of forms and synthesised in a narrative report. Given sufficient commensurate data, a meta-analysis may also be performed.

Risk of bias: A quality assessment will be performed for *in vivo* and *in vitro* studies using the NTP/OHAT Risk of Bias Rating Tool for Human and Animal Studies.

Level of evidence rating: Following a comprehensive assessment of the quality of evidence for both *in vivo* and *in vitro* studies, a confidence rating will be assigned to the body of literature and subsequently translated into a rating on the level of evidence (high, moderate, low, or inadequate) regarding the research question.

Systematic review registration: PROSPERO CRD42018102618.

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1. Introduction

1.1. Background and rationale

Endometriosis is a hormone-dependent gynaecological disease characterised by the presence and growth of endometrial tissues outside of the uterus. Estimates of prevalence range from 10 to 45% of people who menstruate although the true burden of this disease is likely higher due to the unknown prevalence among those who are asymptomatic (Buck Louis et al., 2011; Rawson, 1991). The precise aetiology is also unknown, and, while several hypotheses have been suggested, it is most likely to be multifactorial, involving genetic and environmental factors (Sourial et al., 2014; Van der Linden, 1996).

There is presently a growing body of literature analysing the association between endometriosis and the exposure to synthetic organochlorine chemicals (OCCs), a group of organic compounds with chlorinated aromatic molecules which are recognised to have an adverse impact on human health, such as polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), biphenyls (PCBs), and pesticides (OCPs) (Anger and Foster, 2008; Arisawa et al., 2005; Bruner-Tran et al., 1999; Guo et al., 2009). OCCs are characterised to be highly lipophilic, persistent and bioaccumulative in trophic chains. Because of this, despite the installation of laws banning or regulating these substances in the 1970s and 1980s, OCCs are still found in human blood and fatty tissues worldwide (Pumarega et al., 2016; UNEP, 2017).

Some epidemiological studies have reported positive associations between PCDD/Fs, PCBs and other OCCs and endometriosis (Buck Louis et al., 2012; Martinez-Zamora et al., 2015; Ploteau et al., 2017; Porpora et al., 2009), but the overall body of evidence remains inconclusive, often due to acknowledged methodological limitations (Smarr et al., 2016). Human epidemiological evidence, however, provides only an incomplete picture of the total existing evidence linking such pollutants and endometriosis, missing the potential causal and mechanistic relationships. Controlled experimental studies *in vivo* and *in vitro* can help fill the gap left by epidemiological studies. Endometriosis occurs spontaneously in only humans and several other non-human primates, including rhesus and cynomolgus monkeys and baboons, which are considered referent animal models due to their similarities to humans in reproductive anatomy and physiology (D'Hooghe et al., 2009; Nishimoto-Kakiuchi et al., 2018). In fact, the question was raised regarding the role of environmental chemical exposures on endometriosis following a study that demonstrated an increase in incidence and severity of endometriosis in an experimental population of rhesus monkeys exposed to dioxin (Rier et al., 1993).

Non-human primate and murine models can act as human analogues to elucidate the processes involved between environmental exposures and the onset or progression of endometriosis, and *in vitro* testing allows for the exploration of potential molecular mechanisms of action and more complex cellular interactions. Due to low frequency of spontaneous endometriosis in non-human primates, endometriosis is sometimes surgically induced by transplantation in experiments to provide insight into the pathophysiology of disease progression. This approach has been also shown to be successful in rodents, either by transplantation of endometrial tissue from the same or syngeneic animal (autologous models), or by transplantation of human endometrial tissue (humanised models) in immunocompromised mice (Bruner-Tran et al., 2018; Grümmer, 2006).

For *in vitro* models of endometriosis, there exist several immortalised human endometriotic cell lines (*e.g.* epithelial 12Z; stromal 22B), but it is often preferable to obtain primary cultures of endometrial stromal or epithelial cells isolated from human biopsies (Arosh et al., 2015; Banu et al., 2009). Cell co-cultures and 3D models have shown the existence and complexity of cross-talk between epithelial and stromal cells, endocrine and paracrine responses, and the extracellular matrix (Arnold et al., 2001; Wild et al., 1994). Overall, *in vitro* studies allow for more in depth analysis of the mechanisms of action and signalling cascades involved following exposure to certain environmental pollutants.

To date, the underlying pathophysiology of endometriosis remains tenuous, as does the mechanistic understanding linking specifically OCCs and endometriosis. Several theories (*i.e.* induction, transplantation) have been proposed to explain the plethora of processes underlying such heterogeneous disease (further discussed by Signorile and Baldi, 2010; Sourial et al., 2014). At the cellular and molecular level, it has been acknowledged that progesterone resistance, estrogen dependence, oxidative stress and pro-inflammatory conditions, and/or immunosuppression with genetic epigenetic predisposition are hallmarks favouring the migration, adhesion and progression of endometriotic tissues (Bulun et al., 2002). Endocrine disrupting chemicals such as OCCs are likely to interact with nuclear receptors and impact molecular pathways of direct relevance for endometriosis. For instance, dioxin and dioxin-like PCBs have been shown to interact with the estrogen pathway through the estrogen receptor or by binding aryl hydrocarbon receptor (AhR), leading to downstream effects on gene and protein regulation involved in cell adhesion, matrix remodelling, proliferation and apoptosis of endometrial cells (Bruner-Tran et al., 2010). The action of OCCs on the AhR pathway independent of estrogen-mediated actions may also alter endometrial cell stress responses, metabolism and cell migration (Willing et al., 2011). Furthermore, the potent 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) may alter the progesterone action by downregulation of progesterone receptors, a pattern of progesterone resistance leading to the dysregulation of matrix metalloproteinases (MMPs), promoting the growth and invasion of endometriotic tissue (Bruner et al., 1997; Bruner-Tran et al., 2010). Other physiological processes tightly linked to endometriosis have been associated with OCCs, including immunosuppression, inflammation or oxidative stress, however the directed acyclic relationships and causal ordering of these underlying molecular events remains still unclear (Birnbaum and Cummings, 2002).

Systematic reviews (SR) are a powerful tool that allow for an unbiased synthesis of all existing evidence in a specific field, which may assist in the development and justification of regulatory and healthcare decisions through evidence summaries and quality assessments (Higgins and Green, 2011; Hooijmans et al., 2018; Rooney et al., 2014). During the last decade, a substantial effort has been made to develop and adapt SR workflows to environmental health domains, including tailored protocol guidelines and risk of bias tools, such as the Navigations Guide or the National Toxicology Program/ Office of Health Assessment and Translation (NTP/OHAT) Handbook (Higgins and Green, 2011; Hooijmans et al., 2018; Rooney et al., 2014). As a result, we have seen a substantial increase of SR-based publications focusing on environmental chemicals, especially for epidemiological studies with a few emerging reviews on experimental studies. The lack of validated tools developed to assess *in vitro* studies constrains the use of mechanistic data to supporting decisions within chemical health risk settings (Al Saadi et al., 2016; Rooney et al., 2016; Samuel et al., 2016). To date, only narrative reviews have been conducted to synthesise the available evidence from experimental studies on the associations between environmental chemicals and endometriosis (Birnbaum and Cummings, 2002; Bruner-Tran et al., 2010; Bruner-Tran and Osteen, 2010; Guo et al., 2009). Recently, we have conducted a systematic-review focusing on the associations between OCCs and endometriosis in human epidemiological studies using a rigorous and comprehensive GRADE-based protocol following the NTP/OHAT guidelines (Cano-Sancho et al., 2018). There is thus a need to conduct a SR covering the experimental body of evidence (*in vivo* and *in vitro*) to complement our existing knowledge from epidemiological studies and to provide a complete systematic summary of the overall body evidence on the associations between OCCs and endometriosis.

1.2. Objectives

The main objective of this SR is to evaluate the associations between the exposure to organochlorine chemicals (OCCs) and endometriosis in experimental models (*in vivo* and *in vitro*).

We are considering the following sub-objectives:

a) to create a comprehensive narrative synthesis of the current body of knowledge from experimental studies (*in vivo* and *in vitro*) evaluating the association between the exposure to OCCs and the pathogenesis of endometriosis; and
b) to appraise the quality of evidence with a confidence rating and establish a level of evidence for the presence or absence of endometriosis with respect to exposure to OCCs in experimental data.

2. Methods

This SR framework has been developed to follow the guidelines established in NTP/OHAT Handbook for Conducting a Literature-Based Health Assessment, which provides a standardised methodology to implement the Grading of Recommendations Assessment, Development and Evaluation (GRADE) approach to environmental health assessments (OHAT, 2015a; Rooney et al., 2014). The NTP/OHAT framework adapted to the specific research question will thus guide the structure of this systematic review. The workflow for this SR is displayed in Fig. 1, with the corresponding supporting platforms. Selection, data extraction, data synthesis and risk of bias assessment will be performed and managed using Health Assessment Workspace Collaborative (HAWC), an open-source, modular web-based content management system with a user interface (https://hawcproject.org/), developed by Andy Shapiro et al. in 2014 in collaboration with EPA National Center for Environmental Assessment (EPA NCEA) and National Institute of Environmental Health Sciences/National Toxicology Program (NIEHS/NTP) (Shapiro, 2015). HAWC provides transparency throughout the entire assessment process, from literature search and review, data extraction and evidence synthesis, endpoint analysis, risk of bias assessment, data visualisation, and even meta-analysis, should that be performed. This protocol has been developed in accordance with the reporting guidelines established by the Preferred Reporting Items for Systematic reviews and Meta-Analyses for Protocols (PRISMA-P) 2015 and PRISMA for Abstracts (PRISMA-A) (Moher et al., 2015), and registered in the International Prospective Register of Systematic Reviews on July 17th 2018 (PROSPERO) (registration number CRD42018102618). The results will be structured and presented in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (Moher et al., 2009).

2.1. Eligibility criteria

In order to be eligible for inclusion, studies will need to comply with the elements of the PECO (**P**opulation, **E**xposure, **C**omparators and **O**utcomes) statement below, summarised in Table 1.

- *Population*: The population of interest of this review will focus on experimental models, both *in vivo* and *in vitro*.
  - o *In vivo* models will include non-human primates (*e.g.* baboons, rhesus monkeys, cynomolgus monkeys), rodents (*e.g.* rats and mice regardless of strain), and any other experimental animal model in which endometriosis may be surgically induced.
  - o For *in vitro* studies, we will include endometrial and endometriotic primary cells (stromal and epithelial) isolated from human biopsies and immortalised endometriotic cell lines (*e.g.* epithelial cell line 12Z; stromal cell line 22B). Co-cultures, three-dimensional models and explants of endometrial tissues will also be included. Endometrial cancer cell lines (*e.g.* Ishikawa) will be excluded.
- *Exposure:* The exposure of interest will be organochlorine chemicals, a group of organic compounds with chlorinated aromatic molecules which are recognised to have an adverse impact on human health. This group of chemicals includes dioxins, furans, PCBs, pesticides, and other industrial chemicals.
  This review will exclude pharmaceuticals and therapeutic drugs, hormone treatments, and phytoestrogens or flavones/flavonoids.
- *Comparators*: Reference groups will comprise any animal or cell-treatment group not exposed to OCCs or treated only with the vehicle control. Studies without a reference group will be excluded.
- *Outcomes*:
  - o *In vivo* models
    - ■ Primary outcomes of interest will be 1) the onset or aggravation of endometriosis in animal models where spontaneous endometriosis can occur and 2) the proliferation or growth of surgically induced endometriotic lesions in all other models. Endometriosis is the presence of endometrial tissue outside the

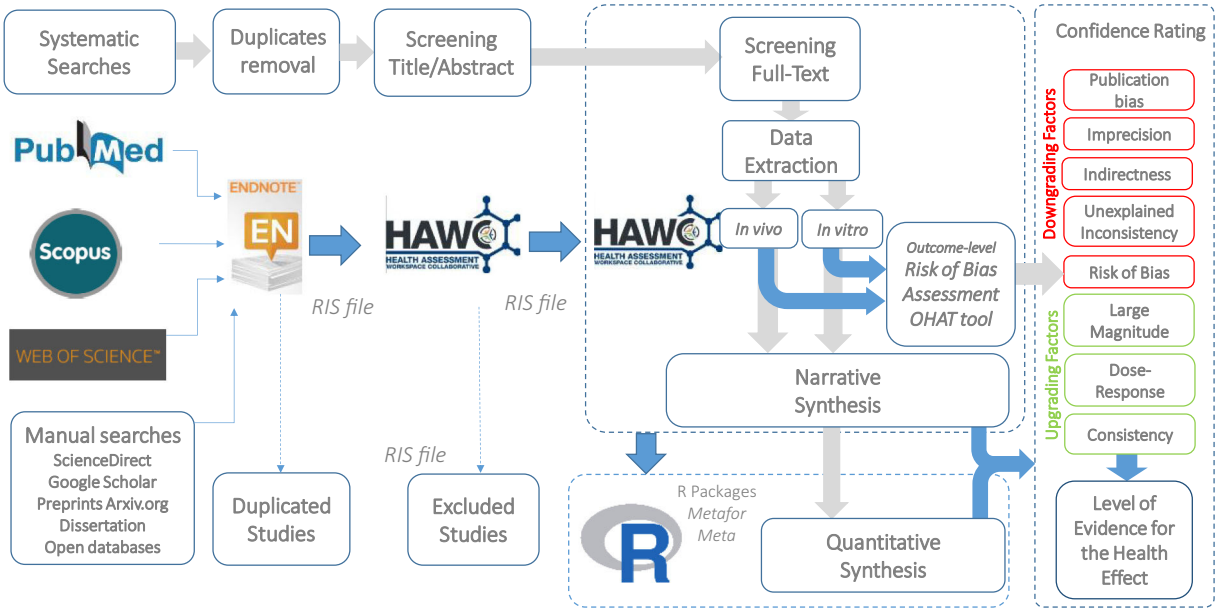

Fig. 1. Workflow diagram for the systematic review framework. Reasons for exclusion must be recorded at "Screening Full-Text" step.

Table 1
PECO statement.

PECO		Inclusion criteria	Exclusion criteria
Population	*In vivo*	• Experimental animal models	• Humans
	In vitro	• Endometrial epithelial and stromal cells, endometrial tissues, and related co-cultures	• Cells from organs and tissues other than endometrium • Cancer cells
Exposure	*In vivo*	• Organochlorine chemicals • All dose levels, routes of administration, duration and frequency	• Chemicals that are not organochlorine chemicals
	In vitro	• *Above applicable to in vitro*	• *Above applicable to in vitro*
Comparators	*In vivo*	• Has positive, negative, and/or vehicle control group	• Lacks control group
	In vitro	• *Above applicable to in vitro*	• *Above applicable to in vitro*
Outcomes	*In vivo*	• Onset or aggravation of endometriosis • Proliferation or growth of induced endometriotic lesions • Presence-of "endometriotic-like" phenotypes with human reference standard	• Outcomes irrelevant to endometriosis
	In vitro	• Proliferation, migration, and/or invasion of endometrial cells • Other biomarkers of primary outcomes such as gene/protein/receptor/interleukin expression associated with endometriosis	• *Above applicable to in vitro*

uterus and fits to code N80 of the International Statistical Classification of Diseases and Related Health Problems 10th Revision (ICD-10).

- Secondary outcomes. For all animal models, the presence or aggravation of "endometriosis-like" phenotypes will be considered as a secondary outcome. Studies must define "endometriosis-like" phenotypes in comparison with an equivalent human standard to maintain relevance to the research question (*e.g.* endometriosis-like glands or tube formation). Only studies that use a human endometriosis reference standard for endometriosis-like phenotypes *in vivo* will be considered.

o *In vitro* outcomes will be defined to be as inclusive as possible while maintaining relevance to endometriosis and will be considered secondary to outcomes from *in vivo* studies due to their potentially heterogeneous nature, as exact mechanisms of action between OCC exposure and endometriosis are unknown and these mechanistic pathways are not specific to endometriosis.

- Primary outcomes for *in vitro* models will include proliferation, migration, and/or invasion of endometrial and endometriotic cells.
- Secondary outcomes will include the expression and levels of intermediary molecular markers and signalling pathways relating directly with endometriosis, such as estrogen or progesterone receptor activity and expression, endometriosis-related protein or enzyme activity (like aromatase and matrix metalloproteinase expression), endometriosis-related inflammation, and expression or activation of endometriosis-related genes. The mechanistic cell-based experiments should elucidate a direct link between intermediates and apical outcomes through study design and/or cell models. Supplemental Table 2 provides the list of inclusive endometriosis-related genes which were identified through a comprehensive PubMed text-mining approach and validated to be statistically relevant to endometriosis (Liu and Zhao, 2016).

- *Publication types*: Articles that do not contain original data, such as reviews, editorials, or commentaries, or conference abstracts, studies published in languages other than English, and non-experimental studies (*e.g.* human epidemiologic or other observational studies) will also be excluded. All publication years will be considered.

2.1.1. Exclusion criteria prioritisation

The studies will be excluded if those are:

1) Duplicates OR
2) Not primary data OR
3) Irrelevant population (*e.g.* not experimental animal model or not endometrial cells) OR
4) Irrelevant exposure (*e.g.* not organochlorine chemical) OR
5) Irrelevant outcome (*e.g.* not endometriosis-related, as previously defined) OR
6) Not in English

2.2. Search strategy

The literature search will be performed simultaneously in three scientific databases (*i.e.* PubMed, Web of Science, SCOPUS). All retrieved references will be imported into reference management software ENDNOTE® wherein duplicate documents will be manually eliminated from the pool of studies. The remaining studies will then be imported into HAWC for eligibility screening. Additional articles may be added through manual searches through the list of references of included articles.

2.2.1. Databases

The searches will be conducted in the following scientific databases:

- NCBI PubMed - http://www.ncbi.nlm.nih.gov/pubmed
- ISI Web of Knowledge [v.5.29] - https://apps.webofknowledge.com
- SCOPUS - https://www.scopus.com

2.2.2. Search strings

The search string combines the EXPOSURE and OUTCOME elements from the PECO statement, nested through the Boolean operators "AND/OR". Search terms are intended to identify all relevant published evidence on endometriosis and endometriosis-related effects following exposure to organochlorine chemicals and were identified by (1) reviewing PubMed Medical Subject Heading (MeSH) terms and literature tags used by previously identified endometriosis-related epidemiologic studies for relevant and appropriate terms, (2) adapting and expanding on existing lists of pollutants (UNEP, 2017; Wassenaar et al., 2017), and (3) extracting several potential endometriosis-related mechanistic outcomes (Liu and Zhao, 2016). More details about the development of the search string is available in Section 1.1. Literature Search of the Supplementary Material. The search string will be adapted and calibrated to run in Web of Knowledge and SCOPUS as well. No restrictions will be made in either database to limit publication dates, and no other filters will be applied. A test set of relevant studies will be used to calibrate the overall search strategy. The calibration and piloting of this search string can be found in the Supplementary Material.

2.3. Study selection

Two reviewers (KM and GCS) will independently perform the study

Table 2
Body of evidence structure based on major experimental outcomes of endometriosis to guide grouping endpoints and experiments.

Level	Endometriosis-related outcomes	Endpoint/assay examples	Body of evidence grouping examples
Primary/apical outcomes	Spontaneous endometriosis	***In vivo***: onset after chronic/transgenerational exposure in non-human primates	1-Spontaneous endometriosis in animals
	Migration/attachment	***In vivo***: experiments evaluating the invasiveness of implants in rodents or primates ***In vitro***: migration assays in cell models	2- Invasiveness of endometriotic tissue in animals 3 – Invasiveness of endometriotic tissue in cell cultures
	Survival/proliferation/ apoptosis	***In vivo***: experiments on proliferation/expansion of endometriotic lesions in rodents and/or primates ***In vitro***: proliferation/viability/apoptosis cell assays	4 – Survival/proliferation of lesions in animals 5- Proliferation in cell culture
Intermediary /secondary	Progesterone resistance	***In vivo***: PR-B/A expression	6- Progesterone resistance in animals
	Aromatase/steroidogenic pathway	***In vitro***: CYP19A1 expression	7- Disruption of aromatase pathway in cell culture
	Inflammatory cytokines	***In vivo***: IL6 levels	8 - Inflammation in animals
	Other outcomes: immunosuppression, oxidative stress		

selection, and conflicting results will be discussed with a third reviewer (JPA). Study selection will occur in two phases. Phase I will consist of an initial screening of title and abstracts, according to the pre-established inclusion/exclusion criteria (Table 1). Studies whose titles/abstracts do not provide enough information to decide or are otherwise unclear will be automatically moved to the next phase for full text assessment. Retained articles from Phase I will be exported into a Research Information Systems (RIS) file for transparency and uploaded into the HAWC Literature Review module for Phase II.

Phase II will consist of a comprehensive full-text review of all the articles retained from Phase I. The full text of retained records will be gathered in print or electronic pdf and linked within HAWC for ease of accessibility. In the event that full text to a study is not available, the reason that limited the accessibility to the document will be clearly reported in a tracking document. Exclusion at this stage will also be recorded in the tracking document with a brief justification of exclusion rationale.

2.4. Data extraction

The data extraction method will be piloted in duplicate by two reviewers (KM and GCS) to calibrate consistency using comprehensive data forms specifically designed for animal and *in vitro* studies available in HAWC, adapted from the NTP/OHAT Protocol (OHAT, 2015a). Complete data forms are available in Supplemental Table 4. The following items will be extracted from the studies:

- Study identification information (*i.e.* Title, Author, Journal, Date of Publication)
- Funding Source and Conflicts of Interest
- Type of Study (*i.e.* Animal Bioassay, *In vitro*)
- Exposure (*i.e.* Chemical name, class, purity, vehicle, *etc.*)
- Population (*i.e.* Animal species, strain, sex, source, age; Cell line, tissue culture, source, *etc.*)
- Dosing methodology (*i.e.* Route of exposure, duration, frequency, dose groups, vehicle, *etc.*)
- Controls (positive, negative, vehicle)
- Randomisation or blinding procedure(s)
- Endpoint (*e.g.* onset of endometriosis, severity of endometriosis, lesion diameter, nuclear receptor expression levels, changes in gene expression, effect sizes, *etc.*)
- Diagnostic or method to measure endpoint (*e.g.* laparoscopy; assay name, source, *etc.*)
- Statistical methods (*e.g.* significance levels)

Data provided only on plots will be extracted directly from the image using the WebPlotDigitizer (version 4.0 PLOTCON 2017 - Oakland, CA), a semi-automated web-based tool. Whenever relevant information is missing, the authors will be contacted to provide further details.

2.5. Body of evidence structure

The evidence will be organised in outcome-related groups favouring data synthesis and confidence rating at health outcome level. Evidence grouping by outcomes and/or biological endpoints aligns with regulatory decision-making frameworks based on mode of action (MOE) or adverse outcome pathways (AOPs), where intermediary biological perturbations required for toxicity are identified as key events (Villeneuve et al., 2014; Becker et al., 2015). The criteria to determine the inclusion of specific studies or experiments in each outcome group will consider the evidence stream (*i.e. in vivo*, *in vitro*), animal model (*e.g.* non-human primates, rodent models), health outcomes/endpoints (*e.g.* spontaneous apparition, proliferation of implants) or exposure regime (*e.g.* high or low exposures, type of OCCs, duration). Literature suggests that OCCs may contribute the endometriosis pathogenesis at different apical levels, here identified as primary outcomes; 1) by increasing the spontaneous apparition of endometriotic lesions, 2) increasing the proliferation/gravidity of already established endometriotic lesions and 3) increasing the capacity of endometrial cells to migrate and attach in ectopic tissues. Secondary outcomes are envisaged to represent intermediary endpoints upstream of primary outcomes which may be grouped around specific signalling or functional pathways. Grouping and sub-grouping of experiments in outcomes will be guided by expert judgement considering the significance of overall conclusions. Some anticipated examples of outcome-based groups of evidence are depicted in Table 2.

Sub-group analysis will be conducted considering the nature and heterogeneity of included experiments, including but not limited to the study design, animal models/species, OCC congener, exposure duration, dose level, and assays.

2.6. Internal quality assessment

The internal quality for both streams of evidence (*i.e. in vitro*, *in vivo*) will be evaluated using the Risk of Bias (RoB) Rating Tool developed by OHAT (2015b). The OHAT RoB tool is consistent with the other exising RoB tools (*e.g.* Navigation Guide), with substantial overlapping of RoB domains and/or questions, yet lack of harmonisation has been already

Table 3
Confidence Rating process (OHAT, 2015b).

Initial confidence by key features[a] of study design	Factors decreasing confidence	Factors increasing confidence	Confidence in the body of evidence
High (+ + + +) – 4 features	• Risk of bias	• Large magnitude of effect	High (+ + + +)
Moderate (+ + +) – 3 features	• Unexplained inconsistency	• Dose response	Moderate (+ + +)
Low (+ +) – 2 features	• Indirectness	• Consistency	Low (+ +)
Very low (+) – ≤1 feature	• Imprecision	- Across animal models or species	Very Low (+)
	• Publication bias	- Across dissimilar populations	
		- Across study design types	

[a] Features: (1) controlled exposure, (2) exposure prior to outcome, (3) individual outcome data, and (4) use of comparison group.

recognised (Rooney et al., 2016). The OHAT RoB tool consists of a common set of questions catered to each experimental stream of evidence, addressing main bias domains as follows:

Selection bias

1. Was administered dose or exposure level adequately randomised?
2. Was allocation to study groups adequately concealed?

Performance bias

3. Were experimental conditions identical across study groups?
4. Were the research personnel blinded to the study group during the study?

Attrition bias

5. Were outcome data incomplete due to attrition or exclusion from analysis?

Detection bias

6. Can we be confident in the exposure characterisation?
7. Can we be confident in the outcome assessment?

Selective reporting bias

8. Were all measured outcomes reported?

Other

9. Were there any other potential threats to internal validity?

Each question within the tool will receive one of five possible responses: “Definitely Low Risk of Bias”, “Probably Low Risk of Bias”, “Probably High Risk of Bias”, “Definitely High Risk of Bias” or “Not Reported”, with a statement of justification based on the study text. Reponses will be determined by two independent assessors (KM and GCS) following the pre-specified criteria detailed in Suppl. Mat. 1 Section 3.2.1.1: Risk of Bias Response Criteria, which are specific to each type of evidence stream. Discrepancies will be compared and discussed to reach a consensus, and, if needed, other members of the review team will be consulted to resolve remaining disagreements. In case of incomplete data or dubious reporting, study authors will be contacted for clarification.

Risk of bias will be evaluated at individual and outcome level, meaning that one individual study subject to different outcome-based experiments will receive multiple RoB evaluations. Summaries of RoB will be presented through heatmaps for each stream of evidence and specific outcome. The 3-Tier approach proposed by the NTP/OHAT Handbook will be used to synthesise RoB evaluations across studies included in the same outcome. The tiered approach establishes a Tier for each study considering the RoB responses of every question, from Tier 1 (Most answers including from the key questions, rated as “Definitely” or “Probably” low) to Tier 3 (Most answers including from the key questions, rated as “Definitely” or “Probably” high) (See details in Suppl. Mat. Section 3.2.1: Risk of Bias). The “Key Questions” are those domains which have a greater impact on the overall bias. Randomisation bias, outcome detection bias and performance bias related to blinding of assessors comprise the Key Questions for RoB assessment. The application of the tiered approach here does not seek to exclude Tier 3 (High Risk of Bias) studies but to support decisions for sub-group analysis based on RoB.

The entire assessment will be performed and registered within the Risk of Bias Module in the HAWC platform to ensure the transparency.

2.7. Confidence rating for each body of evidence

The health outcomes from each stream of data (*i.e. in vitro*, *in vivo*) will be considered as independent bodies of evidence. An assessment will be performed for each to determine a confidence rating reflecting the confidence with which the study findings accurately reflect a true effect of given organochlorine chemicals on endometriosis-related outcomes, following the NTP/OHAT Systematic Review framework, based on GRADE guidelines (OHAT, 2015a).

Each body of evidence is given an initial confidence rating, which is subsequently downgraded or upgraded according to factors that decrease or increase confidence in the results. Eventually both confidence ratings will be integrated to deliver a final rating for the overall body of evidence for the exposure of OCCs on endometriosis for both *in vivo* and *in vitro* studies.

Briefly, the initial confidence rating is determined by the presence or absence of four features (*i.e.* controlled exposure, exposure prior to outcome, individual outcome data, and use of comparison group) (Table 3). Both experimental animal studies and *in vitro* studies generally receive an initial “High confidence” rating, as controlled experimental study designs tend to incorporate all four features. This initial rating is then downgraded (*i.e.* Risk of Bias, Unexplained Inconsistency, Indirectness, Imprecision, Publication Bias) or upgraded (*i.e.* Magnitude, Dose Response, Consistency Across Species/Model) upon evaluation of the aforementioned factors (Table 2) (OHAT, 2015a). The confidence rating independently determined for each stream of evidence will then be combined to reflect the entire body of evidence. Full details on the confidence rating process can be found in the Suppl. Mat. 1 Section 3. Rating the Body of Evidence.

2.8. Translation of confidence rating into level of evidence

As the confidence rating in the body of evidence does not reflect the direction of the effect, a final step will be performed to translate the confidence rating into a level of evidence for the health effect as according to NTP/OHAT framework.

OHAT proposes the following descriptors to translate the final confidence rating into the level of evidence for the health effect for each stream of evidence considering the confidence in the body of evidence and direction of the health effect (OHAT, 2015a):

- **High level of evidence:** There is high confidence in the body of

evidence for an association between exposure to the substance and the health outcome(s)

- **Moderate level of evidence:** There is moderate confidence in the body of evidence for an association between exposure to the substance and the health outcome(s).
- **Low level of evidence:** There is low confidence in the body of evidence for an association between exposure to the substance and the health outcome(s), or no data are available.
- **Evidence of no health effect:** There is high confidence in the body of evidence that exposure to the substance is not associated with the health outcome(s).
- **Inadequate evidence:** There is insufficient evidence available to assess if the exposure to the substance is associated with the health outcome(s).

The direction or nature of the effect will be considered in the translation process as following:

If there is evidence of a health effect, confidence in the body of evidence translates directly into one of four category descriptors for level of evidence of the health effect ("High", "Moderate", "Low", or "Inadequate"). Only there is a high confidence in the body of evidence that there is no health effect will the level of evidence be considered that there is evidence of no health effect; otherwise, the level of evidence will be considered inadequate (Table 4). See details in Suppl. Mat. 1 Section 3.5: Translation of Confidence Rating into Level of Evidence.

2.9. Data synthesis

A narrative synthesis will be performed for data from both *in vivo* and *in vitro* studies. Data from each stream of evidence will be synthesised separately, supported by tables and plots summarising the main direction of effects using visualisation tools available in HAWC. Subgroup analyses will be performed as data deems fit. Though exact subgroups depend on the nature of extracted data and thus cannot be precisely anticipated, potential subgroups may include but are not limited to: animal model (*e.g.* primate, rodent-autologous, rodent-heterologous), chemical exposure (*e.g.* dioxins and dioxin-like chemicals, non-dioxin-like chemicals), experimental design (*e.g.* acute, subchronic, chronic, developmental), dosing regime (*e.g.* dosing levels, frequency, timing, duration, route of administration), variation in study risk of bias level (*e.g.* high risk of bias, low risk of bias) for specific domains or overall ratings. Subgroups will be analysed for their impact on not only degree of effect but also direction of effect. An emphasis will be placed on the primary and secondary outcomes of *in vivo* studies, using the evidence provided by *in vitro* studies as support.

Meta-analysis will be conducted only on primary outcome measures when at least two studies provide quantitative and commensurate data of the same endpoint and exposure that may be combined. The main objectives of the meta-analysis will be to evaluate the consistency between studies, to estimate of the overall size and direction of associations and to identify sources of heterogeneity. If a quantitative synthesis is deemed appropriate, for dichotomous outcomes (*e.g.* spontaneous endometriosis onset in non-human primates), we will build contingency tables and calculate odds ratios. For continuous outcomes (*e.g.* proliferation of endometrial lesions) we will estimate the standardised mean difference (SMD) with 95% confidence intervals (CIs) using Hedge's G effect sizes which corrects for small sample size bias (Vesterinen et al., 2014). Between-study variance will be represented by tau-squared (τ^2). Inconsistency will be assessed with the I^2 statistic, which quantifies the heterogeneity and degree of inconsistency among studies, and will be interpreted according the Cochrane's criteria. Potential small study bias will be evaluated by funnel plots and Egger's test (Harbord et al., 2006). Publication bias will be corrected with the "trim and fill" method, which trims the asymmetrical studies to estimate the true centre of the funnel plot, then fills the assumed missing studies (mirror image), allowing for the estimation of adjusted overall confidence intervals (Duval and Tweedie, 2000). The influence of each individual study in the meta-analysis will be investigated by omitting one study at the time, and re-calculating the summary estimates (leave-one-out method). The statistical analysis will be performed with R software. More details on study synthesis and calculations for potential bias adjustments can be found in Suppl. Mat. 1 Section 3: Rating the Body of Evidence.

Table 4
Translation of confidence rating into level of evidence.

Confidence in the body of evidence	Direction of the effect	Level of evidence for the health effect
Health effect		
High	⇨	High
Moderate	⇨	Moderate
Low	⇨	Low
Very low or no evidence	⇨	Inadequate
No health effect		
High	⇨	Evidence of no health effect
Moderate	⇨	Inadequate
Low	⇨	Inadequate
Very low or no evidence	⇨	Inadequate

3. Pilot study

3.1. Search string calibration

The search strings were refined and evaluated through an iterative process by applying targeted search terms to the different search engines. Details on the calibration process are available in the Supplementary Material 2. 658 articles were identified from PubMed, 375 from SCOPUS, and 497 in Web of Science.

3.2. Workflow feasibility pilot

A preliminary pilot was performed to test the feasibility of the proposed tools and protocol, and consisted of a literature search and subsequent study selection and data extraction process for a subset of studies published between 1996 and 1997. The above calibrated search string was applied to PUBMED, SCOPUS and WOS with dates filters limiting the studies between 1 and 1-1996 and 31–12-1997. No other filters were applied. Results of this feasibility pilot aim to provide clarity and transparency for the workflow of this review process (from literature review to data selection, risk of bias assessment, and data synthesis preparation) and are available in Supplementary Material 1 Annex 1.

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Conflict of interest

The authors declare no other conflict of interests.

Authors contributions

JPA and GCS performed problem formation and gathered the review team. KM and GCS coordinated protocol development. KM wrote the draft protocol, and SP, XC, MK, BLB and JPA have made substantial contributions and edits on the protocol draft version. Expertise on systematic review methodology, risk of bias and statistics was provided by JPA, GCS and KM; expertise on endocrinology, gynaecology and

pathophysiology was led by SP and MK; molecular biology and experimental modelling was covered by SC, MK and GCS; technical advice on endocrine disruption, exposure and risk assessment was provided by JPA, BLB and GCS.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2018.12.063.

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Annexe 2 : Poster Prix Kerner de vulgarisation scientifique JJC ARC 2018

Meriem KOUAL I Hôpital Européen Georges Pompidou - PARIS

4

DIOXINE DE SEVESO

UN POLLUANT QUI BOOSTE LE CANCER DU SEIN

La dioxine de Seveso fait partie des polluants organiques persistants, les POPs, relargués dans l'environnement par l'agriculture intensive, les procédés industriels, les pots d'échappement des voitures, la fumée de cigarette, la combustion du bois ou encore les éruptions volcaniques. Oui, et alors ? Et bien, cette dioxine, déjà reconnue comme perturbateur endocrinien, dope l'agressivité et la résistance des cellules malignes dans leur course au cancer du sein. Rencontre avec le Dr Meriem Koual, chirurgien gynécologue, doctorante à l'Université de Boston et chargée de l'étude.

Pourquoi la dioxine de Seveso se retrouve-t-elle incriminée dans le cancer du sein ?

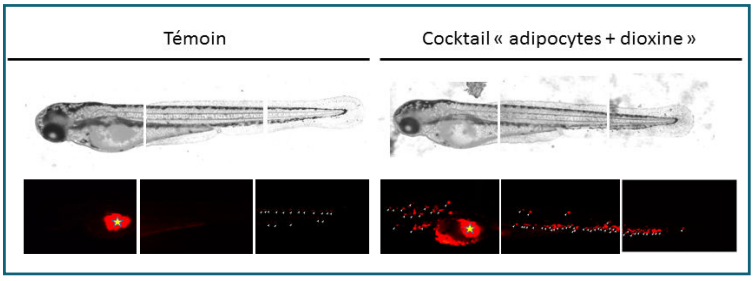
Les POPs sont des polluants persistants, bioaccumulables, toxiques et mobiles, potentiellement cancérigènes pour l'organisme qui les ingère. La dioxine de Seveso est l'un des plus puissants. Elle est connue pour ses capacités à perturber le système hormonal et à s'accumuler dans les adipocytes, les cellules graisseuses qui constituent le terrain de jeu du cancer du sein. Ces dernières années, de nombreuses études se sont intéressées au rôle de ce polluant et d'autres POPs dans l'apparition du cancer du sein. Nous avons pris le taureau par une autre corne : pour la première fois, nous nous sommes demandé si les POPs, et en particulier la dioxine de Seveso, n'étaient pas aussi des facteurs aggravants du cancer du sein. La sanction a été sans appel, car c'est exactement ce que nous avons prouvé !

Comment avez-vous mené votre étude sur la dioxine de Seveso ?

Nous avons reconstitué les conditions réelles de la compétition. Pour représenter la tumeur dans son microenvironnement, nous avons mis en culture des cellules mammaires cancéreuses avec des cellules graisseuses saines, puis exposé le tout à la dioxine de Seveso. La réponse des cellules cancéreuses a été examinée sous toutes les coutures : expression génique, expression protéique, modification morphologique, prolifération, migration... Nous avons ainsi démontré que l'association des adipocytes et de la dioxine de Seveso entraîne des modifications biologiques majeures qui démultiplient le potentiel agressif et métastasique des cellules. *In vitro*, la présence du polluant dans les adipocytes est corrélée à une augmentation de la migration des cellules cancéreuses, à l'expression de différents gènes de mauvais pronostic et à l'acquisition d'un caractère « cellule souche cancéreuse » favorable à la formation de métastases, ces cellules malignes qui s'échappent de la tumeur pour coloniser d'autres territoires tissulaires. Pour vérifier ces résultats *in vivo*, nous avons greffé des cellules cancéreuses exposées à la dioxine à des embryons de poisson zèbre, un modèle classique en toxicologie. Les cellules cancéreuses gagnent bien du terrain : de nombreuses métastases se forment dans d'autres organes du poisson, notamment le cerveau. Et ce n'est pas tout ! La dioxine associée aux adipocytes préserve les efforts des cellules cancéreuses en provoquant la perte de l'expression du récepteur aux œstrogènes, alors que sa présence offre aux patientes une possibilité thérapeutique supplémentaire avec la prescription d'un traitement anti-oestrogénique. Sous dioxine, les cellules mammaires cancéreuses deviennent donc plus agressives, plus performantes, mais aussi plus résistantes face aux traitements. Nous sommes d'ailleurs sur le point de montrer leur capacité à également développer une résistance aux chimiothérapies les plus classiques, mise en œuvre pour ralentir, devancer, voire même disqualifier la maladie.

Quelle suite allez-vous donner à votre étude ?

Notre objectif est maintenant de décrypter les mécanismes moléculaires sous-jacents aux effets dopants de la dioxine de Seveso sur les cellules mammaires cancéreuses dans le tissu adipeux. Ces informations sont cruciales pour développer des pistes médicamenteuses, comme pour alerter davantage les pouvoirs publics et les autorités de santé sur la dangerosité des POPs et la nécessité de mieux les contrôler. Nous espérons aussi apporter des réponses sur les risques de l'autogreffe de tissus adipeux dans le cadre des reconstructions mammaires chez les patientes ayant un antécédent de cancer du sein. En somme, nos recherche visent à détrôner le cancer du sein du podium des maladies les plus meurtrières chez la femme, en particulier celle touchée par l'obésité, bien plus exposée que les autres à l'association dangereuse des adipocytes et de la dioxine de Seveso.



Embryons de poissons zèbre, au sein desquels nous avons injecté (zone marquée par une étoile) des cellules mammaires cancéreuses exposées ou non au cocktail toxique « adipocytes + dioxine de Seveso ». Les cellules mammaires cancéreuses, marquées au fluorescent rouge, migrent (flèches blanches) dans le poisson contaminé par le polluant, provoquant l'apparition de métastases dans d'autres organes, notamment au niveau du cerveau.

<u>Annexe 3:</u> Article 5 Intégrité scientifique

“Formation à l’éthique et l’intégrité scientifique au sein des écoles doctorales françaises : Un premier état des lieux en 2018*”*. Médecine/sciences 2019.

Au cours de cette formation doctorale, j’ai participé à plusieurs séminaires organisés afin de sensibiliser les doctorants aux manquements à l’intégrité scientifique en pratique biomédicale et en recherche. Dans la continuité de cette formation, les organisateurs nous ont sollicité pour la rédaction d’un article court sur le sujet.

J’ai réalisé une étude auprès des écoles doctorales de France afin de connaître les modalités de cette formation sur le territoire français en 2018. Les résultats de ce travail ont été publiés dans médecine/sciences.

Je remercie Mr Christian Hervé, Mr Jacques Haïech et Mr Claude Forest pour leur aide dans la publication de ce travail.

médecine/sciences 2019 ; 35 : 353-5

Formation à l'éthique et l'intégrité scientifique au sein des écoles doctorales françaises

Un premier état des lieux en 2018

Meriem Koual[1-3]

> La formation à l'éthique de la recherche et à l'intégrité scientifique des doctorants au cours de leur formation, toutes spécialités confondues, est obligatoire depuis 2016. Elle est confiée aux écoles doctorales qui sont libres d'organiser cet enseignement comme elles le souhaitent. Nous avons réalisé une étude auprès de l'ensemble des écoles doctorales de France afin de connaître les modalités de cette formation en 2018. Il apparaît que ces écoles sont bien engagées dans cet enseignement sur l'ensemble du territoire et que la tendance est à son renforcement, ce qui témoigne d'une prise de conscience générale de l'importance de ces sujets pour la recherche de demain. <

La déclaration de Singapour sur l'intégrité scientifique publiée en 2011 [1] a entraîné une forte mobilisation de la communauté scientifique internationale contre les manquements à l'intégrité scientifique ou les méconduites dans la recherche. En France, les initiatives dans ce sens se sont multipliées avec la signature en janvier 2015 de la « chartre de déontologie des métiers de la recherche » par la Conférence des Présidents d'Universités et par les institutions de recherche française (Inserm, CNRS, IRD, INRA, INRIA, CIRAD) [2], et la remise du rapport du Professeur Pierre Corvol intitulé « Bilan et propositions de mise en œuvre de la chartre nationale d'intégrité scientifique » [3], qui a abouti à la fondation de l'Office français de l'intégrité scientifique en mars 2017. Dans ce contexte, des initiatives ont vu le jour dans les universités et au sein des écoles doctorales (ED), afin de sensibiliser les futurs chercheurs aux grands enjeux de l'intégrité scientifique. Depuis mai 2016, la formation à l'éthique de la recherche et à l'intégrité scientifique des doctorants est entrée dans un cadre légal [4]. Cette mission a été confiée aux ED, chargées d'assurer cette formation auprès de tous les doctorants, quelle que soit leur discipline, selon des modalités qu'elles choisissent. L'objectif de notre étude était de faire un état des lieux sur les conditions de cette formation au sein des ED de France.



Vignette (Photo © Inserm- Koulikoff, Frédérique/Pinci, Alexandra).

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L'enquête

Nous avons réalisé une enquête auprès des 269 écoles doctorales accréditées qui sont répertoriées sur le site

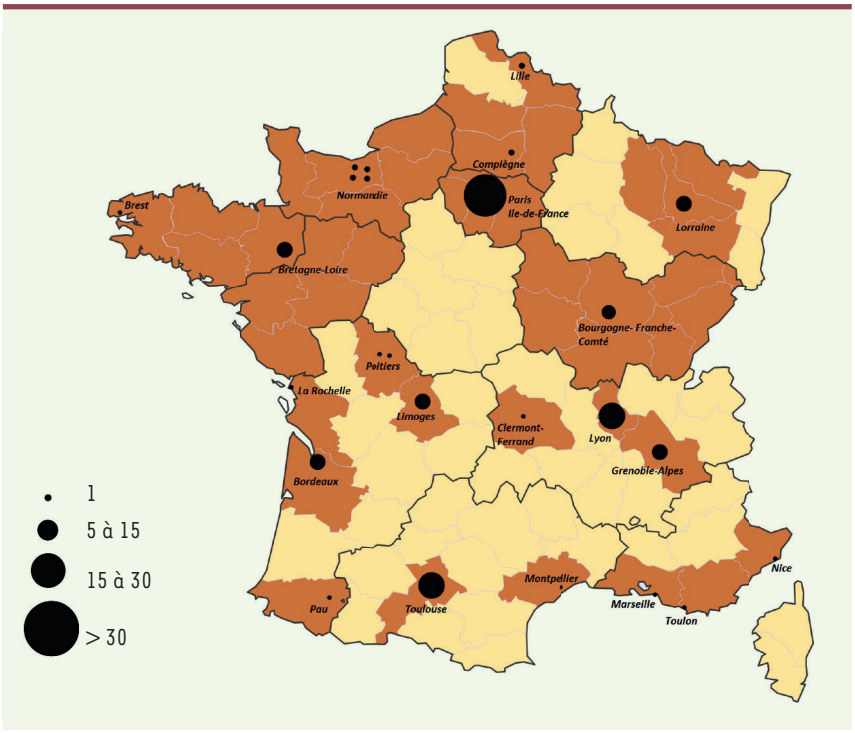

Figure 1. ***Répartition des écoles doctorales prises en compte dans l'étude selon le nombre et la ville ou région.***

data.gouv.fr[1]. Un questionnaire simple (formulaire Google), comportant six questions portant sur les modalités de la formation des doctorants à l'éthique de la recherche et à l'intégrité scientifique a été adressé par mail aux secrétariats et aux directeurs des écoles doctorales en août 2018, avec un rappel un mois plus tard. Les questions étaient les suivantes :

1) Existe-t-il une formation spécifique à l'intégrité scientifique au sein de votre école doctorale ?

2) Si une telle formation existe, sous quelle forme se présente-t-elle ?

3) Cette formation est-elle obligatoire ?

4) Quel volume horaire représente-t-elle ?

5) Y-a-t-il des évolutions ou projets en cours dans le cadre de cette formation ?

6) Question ouverte : les participants avaient également la possibilité de laisser des commentaires, remarques ou suggestions sur le sujet s'ils le souhaitaient.

Il était précisé pour chaque répondant le nom de l'école doctorale, la ville ainsi que le domaine de recherche. Les réponses ont été extraites puis analysées. Les écoles doctorales ont été regroupées par grands domaines de recherche (sciences humaines et sociales, sciences technologiques, biologie/santé), et la répartition géographique sur le territoire des répondants a été étudiée. Les commentaires faits en réponse de la question ouverte ont été analysés afin d'en extraire les opinions les plus fréquemment exprimées et les plus pertinentes. L'offre de formation disponible en ligne sur les différents sites des écoles doctorales (cours en ligne, programmes de cours...) a été analysée afin d'en évaluer le contenu.

[1] Un tableau Excel comportant les différents contacts d'ED disponibles est accessible. https://www.data.gouv.fr/fr/datasets/liste-des-ecoles-doctorales-accreditees/

Ce qui existe et ce qui pourrait exister

L'échantillon examiné

Cinquante-cinq réponses ont été obtenues, dont 9 formulées au nom de collèges doctoraux qui regroupent plusieurs ED par grande ville ou par région. Cela a été le cas pour les collèges doctoraux des universités de Bordeaux (8 ED), de Toulouse (15 ED), de Lyon (17 ED), de Bourgogne Franche-Comté (6 ED), de Bretagne-Loire (11 ED), de Grenoble-Alpes (14 ED), de Limoges (8 ED), de Lorraine (8 ED) et de Paris-Saclay (20 ED). L'échantillon des ED qui ont répondu s'est avéré représentatif des ED de France : 153 ED sur 269 au total se sont en effet manifestées, ce qui représente 57 % des ED françaises. La répartition de ces ED sur le territoire est superposable à celle des ED répertoriées *(Figure 1)*. La répartition des domaines de recherche a également été respectée : 33 % d'entre elles proposent une formation en sciences humaines et sociales (dont droit, économie et gestion) ; 31 % en sciences technologiques ; 25 % en biologie, médecine et santé ; et 11 % sont multidisciplinaires.

La formation proposée

Une formation spécifique à l'intégrité en recherche est proposée aux doctorants dans 93 % des écoles doctorales analysées. Dans les cas où il n'existe pas de formations spécifiques, les doctorants sont informés de la nécessité de participer à une telle formation et sont orientés vers des offres externes. Cette formation est obligatoire dans 75 % des cas, mais elle ne fait l'objet d'une évaluation que de façon exceptionnelle. Concernant le type de formation proposée, 37 % des ED proposent des cours en présentiel, 35 % des conférences/séminaires et 23 % des cours en ligne. Le manque de formateurs a été souligné à plusieurs reprises, rappelant ainsi que la sensibilisation des chercheurs à ces domaines est récente et doit encore être développée. Le volume horaire d'enseignement est très faible : le plus souvent (dans 62 % des cas), il est compris entre 2 et 10 heures. Il dure entre 10 et 20 heures dans 21 % des cas, plus de 20 heures pour 4 % des ED, mais aussi moins de 2 heures pour 13 % d'entre elles. Ces résultats sont présentés sous forme graphique dans la *Figure 2*.

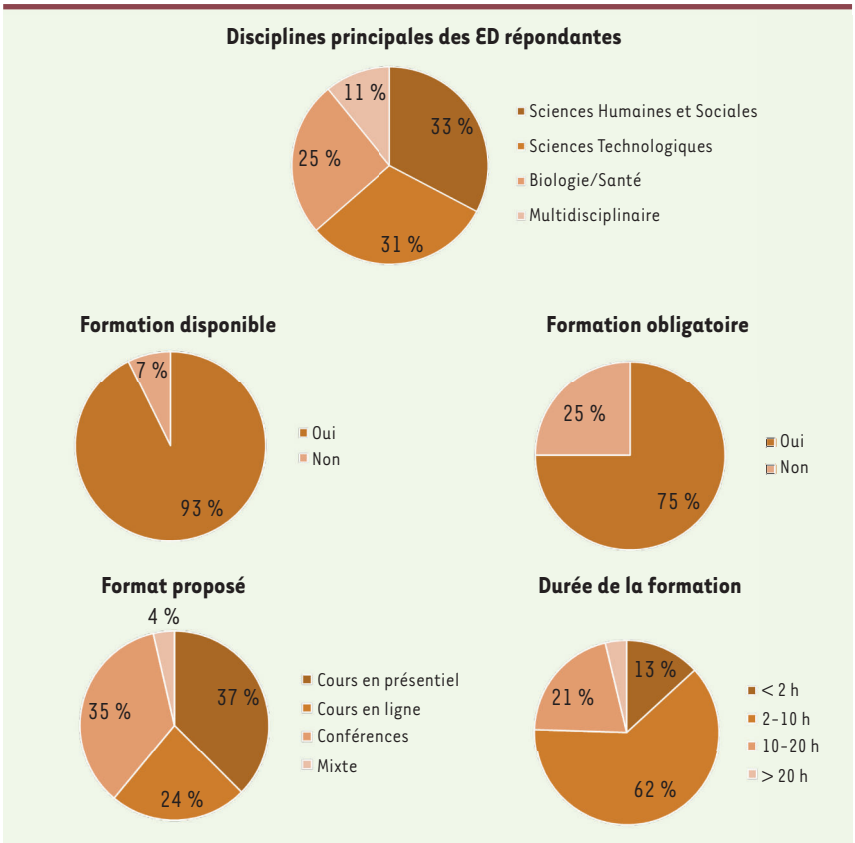

Figure 2. ***Formation à l'intégrité scientifique dans les écoles doctorales françaises en 2018.***

Les sujets récurrents abordés au cours des formations couvrent bien les questions d'intégrité scientifique : les manquements à l'intégrité, les enjeux, la prévention des inconduites, la sensibilisation au plagiat, etc. *A contrario*, l'éthique de la recherche, qui peut être abordée de manière très différente selon que l'on parle d'éthique médicale ou d'éthique en anthropologie ou dans d'autres disciplines, n'est que rarement présentée dans les programmes des cours. Les écoles doctorales/collège doctoraux proposent en effet souvent un enseignement unique à tous leurs doctorants, sans prise en compte des spécificités de chaque discipline et spécialité. Cinquante-deux % des ED qui ont répondu ont déclaré que la formation qu'elles prodiguaient faisait l'objet de projets en cours d'élaboration ou d'évolution, avec des perspectives de renforcement. Et elles ont précisé leur volonté d'impliquer davantage les doctorants : développement de MOOC (*massive open online course*)[2], mise en place d'ateliers et d'activités interactives, tels que des quizz et des jeux de rôle. La nécessité de proposer une offre de formation en anglais pour les étudiants non francophones a également été soulignée. Un travail dans ce sens est déjà en cours dans plusieurs ED ou collèges d'ED.

Conclusion

Désormais, depuis 2017, chaque étudiant en thèse doit signer à son inscription la charte d'éthique du doctorat [6]. Pourtant, il ignore bien souvent les enjeux de l'intégrité scientifique et beaucoup d'erreurs ou de méconduites dont il sera l'auteur auront pour origine une méconnaissance des bonnes pratiques de laboratoire et de recherche.

Le travail que nous présentons ici, première photographie en 2018 de l'offre de formation à l'éthique en recherche et à l'intégrité scientifique au sein des écoles doctorales françaises, nous permet d'affirmer que ces écoles sont globalement bien sensibilisées et impliquées dans la formation des doctorants et qu'une dynamique positive est enclenchée. Cependant, même si proposer une offre générale/globale avec de plus en plus d'uniformisation des enseignements semble plus simple à réaliser pour les écoles doctorales, cette formation devra être adaptée aux différents domaines de recherche et ne prendre en compte les spécificités des spécialités. Il est également important de souligner que la sensibilisation du doctorant aux manquements à l'éthique et à l'intégrité scientifique nécessite l'engagement quotidien de l'équipe qui l'encadre, notamment celui de son directeur de thèse, qui est en général malheureusement encore peu sensibilisé au sujet et à la nécessité de la transmission d'un savoir sur l'éthique en recherche et l'intégrité scientifique[3]. La sensibilisation des jeunes chercheurs en formation pourrait permettre de mobiliser les chercheurs seniors eux-mêmes, à qui ces formations pourraient être ouvertes, comme cela est parfois fait. De façon plus générale, une formation aux enjeux cruciaux que représentent l'éthique en recherche et l'intégrité scientifique devrait être donnée aux étudiants le plus tôt possible, dès « le plus jeune âge », au sein des universités au cours de leur cursus (licences, masters, IUT, BTS, école d'ingénieur, etc.), voire même au lycée. ◊

SUMMARY

Training PhD students in ethics and research integrity in France in 2018

Since 2016, the national framework for doctoral studies in France states that doctoral schools from the

[2] Cours en ligne ouvert et massif (CLOM) : un type de formation à distance ouverte s'adressant via internet à un grand nombre de participants.

3 Les directeurs de thèse, le plus souvent formés il y a de nombreuses années, sont en général peu formés à l'éthique et à l'intégrité telles que définies par la charte. La transmission des bonnes pratiques de laboratoire et de recherche aux jeunes chercheurs peut donc poser problème.

French universities should ensure that each doctoral student receives a training in research ethics and integrity. The doctoral schools can organize the training as they want. We have conducted a study among all French doctoral schools to know the modalities of this training in 2018. Our inquiry shows that the doctoral schools are well engaged in this effort of training on the whole territory and are on the verge of reinforcing it. Overall, our inquiry shows a general awareness by the doctoral schools of the importance of these topics for tomorrow research. ◊

LIENS D'INTÉRÊT

L'auteure déclare n'avoir aucun lien d'intérêt concernant les données publiées dans cet article.

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6. Exemple de charte du doctorat: file:///C:/Users/3283887/Downloads/Charte%20du%20doctorat%20USPC.pdf

TIRÉS À PART

M. Koual

Annexe 4 : Classification OMS 2012 (4ème édition)
des carcinomes mammaires infiltrants (26)

TYPE HISTOLOGIQUE	SOUS-TYPE HISTOLOGIQUE
Carcinome invasif de type non spécifique	Carcinome pléomorphe Carcinome avec cellules géantes stromales ostéoclastes-like Carcinome avec aspects choriocarcinomateux Carcinome avec aspect mélanocytaire
Carcinome lobulaire invasif	Carcinome lobulaire classique Carcinome lobulaire solide Carcinome lobulaire alvéolaire lobulaire Carcinome pléomorphe tubulo-lobulaire Carcinome lobulaire mixte
Carcinome tubulaire	
Carcinome cribriforme	
Carcinome mucineux	
Carcinome avec aspect médullaires	Carcinome médullaire Carcinome médullaire atypique Carcinome invasif non spécifique avec aspects médullaires
Carcinome apocrine	
Carcinome à cellules en bague à châton	
Carcinome micro-papillaire invasif	
Carcinome métaplasique	
Carcinome avec aspects neuroendocrines	
Carcinome secretoire	
Carcinome papillaire invasif	
Carcinome acineux	
Carcinome muco-épidermoïde	
Carcinome polymorphe	
Carcinome oncocytaire	
Carcinome riche en lipides	
Carcinome à cellules claires riches en glycogènes	
Carcinome sébacé	
Carcinome de type glande salivaire/annexiel	Cylindrome Hidradénome à cellules claires

Annexe 5 : Classification TNM de l'AJCC/ UICC 2018 (27)

T : Tumeur primitive

Tis : carcinome in situ
T0 : pas de signe de tumeur primitive
T1 : tumeur de 2 cm ou moins dans sa plus grande dimension

T1a : ≤ 0,5 cm
T1b : > 0,5 cm et ≤ 1 cm pT1c : > 1 cm et ≤ 2 cm

pT2 : tumeur dont le plus grand diamètre est compris entre 2 et 5 cm
pT3 : tumeur de plus de 5 cm dans sa plus grande dimension
pT4 : tumeur de toute taille avec extension directe à la paroi thoracique ou à la peau

pT4a : avec fixation à la paroi thoracique.
pT4b : avec œdème, infiltration ou ulcération de la peau, présence de nodules cutanés satellites limités au même sein
pT4c : T4a + T4b.
pT4d : carcinome inflammatoire

N : Adénopathies régionales

pN0 : absence d'atteinte ganglionnaire
pN1 : micrométastases ou métastases dans 1 à 3 ganglions axillaires homolatéraux et/ ou mammaires internes

pN1mi : micrométastases (> 0,2mm et < 2mm)
pN1a : atteinte de 1 à 3 ganglions axillaires (dont au moins 1 > 2mm) pN1b : atteinte ganglions mammaires internes détectés par biopsie du GS pN1c : pN1a + pN1b

pN2 :
pN2a : atteinte de 4 à 9 ganglions axillaires (dont au moins 1 > 2mm)
pN2b : métastases ganglions mammaires internes détectées en imagerie en l'absence d'atteinte ganglionnaire axillaire

pN3 :
pN3a : atteinte de 10 ou plus ganglions axillaires (dont au moins 1 > 2mm) ou atteinte ganglions sous-claviculaires
pN3b : métastases ganglions mammaires internes détectées en imagerie et atteinte ganglionnaire axillaire ou atteinte de plus de 3 ganglions axillaires et de ganglions mammaires internes avec micro ou macrométastases détectés par la biopsie du GS pN3c : métastase ganglion sus-claviculaire homolatéral

M : Métastases à distance

pMx : détermination impossible de l'extension métastatique pM0 : absence de métastases à distance
pM1 : présence de métastases à distance