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# High Expression of AhR and Environmental Pollution As AhR Linked Ligands Impact on Oncogenic Signaling Pathways in Western Patients With Diffuse Gastric Cancers. A Pilot Study

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# Impact of High Expression of AhR and Environmental Pollution as AhR Linked Ligands on Oncogenic Signaling Pathways in Western Patients with Diffuse Gastric Cancers. A Pilot Study

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**Abstract:** Diffuse type of gastric cancer (GC) has an increasing prevalence worldwide, especially in Western countries, is usually diagnosed at advanced stages, and has no efficacious treatment options. Epidemiological studies have reported an increased mortality from GC after occupational exposure to well-studied pro-carcinogen that are metabolically activated by Cyp P450 enzymes through aryl hydrocarbon receptor (AhR). Substantial studies support the involvement of AhR in gastric carcinogenesis. However, little is known about the role of AhR in diffuse GC, as compared to intestinal GC. In a cohort of 29 gastric tumors, we described a significantly increased *AhR* protein and mRNA expression levels in GCs, independently of subtypes and clinical parameters. *AhR* and *RhoA* mRNA expression were correlated in diffuse GC. Further, our study characterized how AhR affects gene expression in diffuse GC. Using qRT-PCR, we compared the expression levels of *AhR*, *Cyp1A1* and *Cyp1B1* to the expression of genes in a panel previously described. In diffuse GC, *Cyp1A1* expression correlated with genes involved in IGF signalling, EMT (*VIM*), migration (*MMP2*). In an in vitro assay using the poorly differentiated KATOIII epithelial cell line, two well-known ligands for AhR (TCDD and BaP) induced mRNA expression of *CYP1A1, IL1b*, as well as *UGT1, NQO1* and *AhRR* to a lower extent. We also observed a strong increase in *Cyp1B1* expression in diffuse GC, along with a lower TCDD-increased *Cyp1B1* expression as compared to *Cyp1A1* in KATOIII cells, and immunostaining in stromal cells. In intestinal GC, *Cyp1B1* inversely correlated with several genes including *IDO1* (generating endogenous kynurenin-e AhR ligand). Our data provide evidence for a major role of AhR in GC, as an environmental xenobiotics receptor, through different mechanisms and pathways in diffuse and intestinal GC. Our results support continued efforts to clarify the identities of exogenous AhR ligands in diffuse GC in order to define new therapeutic strategies.

**Keywords:** gastric cancers; aryl hydrocarbon receptor (AhR); diffuse-gastric subtype; intestinal-gastric subtype; benzo[a]pyrene (BaP); 2,3,7,8-tetrachlorodibenzeno-p-dioxin (TCDD or dioxin); xenobiotic metabolism; tryptophan metabolism

## 1. Introduction

Gastric cancer (GC) still remains the fourth leading cause of cancer mortality [1–3], with high heterogeneity in different subtypes according to the classification proposed by the World Health Organization [4]. Regardless of the country, the majority of gastric tumors are adenocarcinomas,

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which can be more histologically classified into diffuse-and intestinal- sub types according to the Lauren classification [5]. While intestinal- subtype GC is well differentiated and related to *Helicobacter pylori* infection, the diffuse- subtype is poorly differentiated, and can be seen in familial (germline mutation in the *CDH1* gene) or sporadic settings, as an infiltrating and scattered type [6,7] with unknown origin. The prevalence of the diffuse type is increasing worldwide especially in Western countries [8]. The majority of patients with diffuse- GC are usually diagnosed at advanced stages, with positive axillary nodes (83%) and peritoneal carcinomatosis (18.6%) [6,9,10].

The hypothesis of a link between exposure to persistent organic pollutants (POPs including dioxin) and cancers, including GC, is supported by several epidemiological studies of accidentally exposed populations in Seveso [11]. It is also known that polycyclic polyhalogenated hydrocarbons (like dioxins and PCBs) and polycyclic aromatic hydrocarbons (PAHs) including benzo[a]pyrene from tobacco-smoke and biomass burning) may link to relatively high health risk including cancers. We have previously shown that environmental chemicals such as persistent organic pollutants (POPs) are involved in breast cancers [12], and may accumulate in the Omentum adipose tissue of patients with GC [13]. In addition to TCDD, the prototypical and most potent known environmental ligand in animals and humans, other widespread environmental POPs contaminants bind aryl hydrocarbon receptor (AhR, a basic helix-loop-helix transcription factor) with strong affinities [14,15] and may chronically activate AhR in cancer agression.

For decades, AhR has been studied for its role in environmental chemical toxicity and a mediator of unintended consequences of human pollution characterized as a regulator of xenobiotic metabolic enzymes such as cytochromes P450 Cyp1A1 and Cyp1B1 [16]. Numerous studies have now demonstrated that AhR is involved in important cellular and pathological processes such as tumor initiation and progression including control of proliferation and migration, angiogenesis and control of the immune system [12,15,17–23]. However, the expression of AhR and AhR-signaling pathways has not yet been investigated for Western patients with diffuse GCs.

Various animal experimental data have provided substantial support for an association between AhR expression/function and GCs (23-28). Transgenic mice that express a constitutively active dioxin/AhR mutant (CA-AhR), due to a deletion in the ligand binding domain of AhR, rapidly developed stomach cancers [24,25]. Several tissues of CA-AhR mice have shown life-long continuous low-level activity of AhR, and this model is useful to mimic the dioxin exposure of humans in the general population [26]. Mice exposed to the chemical carcinogen BaP [27] also developed gastric tumors. In humans, elevated AhR expression has been reported in GC [28,29].

In this study, we have analyzed the expression of *AhR* in a series of diffuse- and intestinal-subpopulations of gastric tumors, the expression of xenobiotic metabolic enzymes such as cytochromes P450 *(Cyp1A1* and *Cyp1B1)* and the expression of a large panel of genes known to be regulated by AhR in several cancers. We have also analyzed the effect of exogenous environmental Ahr ligands on 2 gastric cell lines *in vitro*.

## 2. Materials and Methods

### *2.1. Patients and Tissue Samples*

A total of 29 patients underwent partial gastrectomy for histopathologically-confirmed gastric adenocarcinoma primary tumor tissue in the Lariboisiere Hospital (Paris, France) from 2005 to 2014. All patients provided written informed consent prior to their inclusion in the study. Biopsies (provided before 2014) were taken for diagnostic and research purposes, and analysis was permitted by the Ethical Committee of Lariboisiere Hospital (Paris). Eligibility criteria included (1) gastric carcinoma identified by histopathological examination, (2) no other malignancy, (3) no pre-operative chemotherapy or radiotherapy, and (4) availability of complete clinical, histological and biological data. Normal (non-malignant) samples refer to samples harvested from the stomach, from sites distant from the tumor. Immediately after surgery, fresh gastric tumors and their matched normal mucosa were stored in liquid nitrogen until mRNA extraction; other tumor samples and their adjacent normal tissues were routinely fixed in 10% buffered formalin and embedded in paraffin for

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histological analysis. As previously described [10], the population was divided into two groups according to the histological status of GC: diffuse-subtype adenocarcinoma (a poorly differentiated, infiltrating and scattered type) or intestinal-subtype adenocarcinoma (a well differentiated and clustered subtype), according to the Lauren Classification (see Table 1).The malignancy of infiltrating carcinomas was scored according to the TNM staging system (Stage I to IV) as previously described [10]: first according to AJCC7 [30], revised from IGCA [31,32] and AJCC8 [33]. This TNM staging includes T scores for the primary tumor (T1-T4), N scores (lymph node metastasis) and M scores (metastasis).

**Table 1.** Clinicopathological characteristics of gastric carcinoma patients accordingly with histopathological subtype.

| | Total GC (n=29) | Diffuse/poorly cohesive GC* (n=13) (45%) | Intestinal-subtype GC** (n=16) (55%) | P-value [a] |
|---|---|---|---|---|
| **Gender, n (%)** | | | | |
| **Male** | 13/29 | 6/13 (46%) | 7/16 (43%) | 0.90 (NS) |
| **Female** | 16/29 | 7/13 (54%) | 9/16 (56%) | |
| **Age (years, median)** | 63 +/-17 | 57 (27-71) | 75 (59-82) | **0.0004** [b] |
| **Smoking** | | | | |
| **Negative** | 12/22 | 4/12 | 8/12 | 0.77 (NS) |
| **Positive** | 10/22 | 3/10 | 7/10 | |
| **Tumor size (mm), n (%)** | | | | |
| **<50** | 10/27 | 4/11 (36 %) | 6/16 (37 %) | 0.10 (NS) [b] |
| **>=50** | 17/27 | 7/11 (64 %) | 10/16 (63 %) | 0.95 (NS) |
| **Depth tumor invasion (T)** | | | | |
| **T1-T2** | 6/29 | 2/13 (15%) | 4/16 (33 %) | 0.66 (NS) |
| **T3-T4** | 23/29 | 11/13 (85%) | 12/16 (67 %) | |
| **Lymphatic invasion (N)** | | | | |
| **Negative** | 11/28 | 1/13 (7%) | 10/15 (67 %) | **0.0014** |
| **Positive** | 17/28 | 12/13 (92%) | 5/15 (33%) | |
| **Metastasis (M), n (%)** | | | | |
| **Negative** | 24/29 | 9/13 (69%) | 15/16 (94%) | 0.14 (NS) |
| **Positive** | 5/29 | 4/13 (31%) | 1/16 (6%) | |
| **TNM status** | | | | |
| **I-II** | 16/29 | 5/13 (38.5%) | 11/16 (69%) | 0.10 (NS) |
| **III-IV** | 13/29 | 8/13 (61.5%) | 5/16 (31%) | |
| **Vascular invasion, n + (%)** | | | | |
| **Negative** | 9/29 | 3/13 (23%) | 6/16 (38%) | 0.67 (NS) |
| **Positive** | 20/29 | 10/13 (77%) | 10/16 (62%) | |
| **Neural invasion, n (%)** | | | | |
| **Negative** | 23/29 | 2/13 (15%) | 4/16 (25%) | 0.66 (NS) |
| **Positive** | 6/29 | 11/13 (68%) | 12/16 (75%) | |

*poorly cohesive adenocarcinoma/diffuse-type carcinoma; ** intestinal-type adenocarcinoma. a, Chi-square test, Yates' continuity corrected chi-square test or Fisher's exact test if appropriate; b, Mann Whitney. NS= not statistically different.

*2.2. Total RNA Preparation and qRT-PCR*

The conditions for total RNA extraction, complementary cDNA synthesis and qRT-PCR conditions were as previouly described [12,34]. Primers for *AhR*, *AhRR* and other genes were selected using the Oligo 6.0 program (National Biosciences, Plymouth, MN) [12,29]. Each sample was normalized on the basis of its *TBP* content as previously described [10,12]. Results, expressed as N-fold differences in target gene expressions relative to the *TBP* gene (and termed "*Ntarget*"), were

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determined as *Ntarget* = $2^{\Delta Ctsample}$, where the ΔCt value of the sample was determined by subtracting the average Ct value of the specific target gene from the average Ct value of the *TBP* gene. *Ntarget* values of the samples were subsequently normalized so that the median of N*target* values for normal gastric tissues (n=11) was 1. Target gene expression was normalized to their transcription level of house keeping genes TBP, P0 and PPIA. Preliminary analysis of gene expression did not indicate changes in median basal levels in normal samples in the same patients (with either diffuse- or intestinal- GC subtypes). For each gene expression, normalized RNA values of 3 (or more) were considered to represent gene overexpression in tumor samples, and values 0.33 (or less) represented gene underexpression.

### *2.3. Immunohistochemistry*

Immunohistochemical labeling (IHC) was performed on paraffin sections (4 μm), as previously described [12,29]. Immunohistochemical staining for AhR and Cyp1B1 (Santa Cruz, dilution 1/50 and 1/200 respectively*)* were performed using the Ventana Autostainer (USA). Specificity was checked by control staining performed in the absence of primary antibody and with positive tissue [12] The antigen-antibody complex was visualized using DAB as chromogen. Immunostaigning was analysed blindly in duplicate by two specialists including a certified pathologist.

### *2.4. Cell Culture*

The human gastric cell lines, KATO-III obtained from a poorly differentiated gastric adenocarcinoma, and AGS from moderately differentiated GC were acquired from ATCC (Manassas, VA, USA). Cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum, 0.5% penicillin-streptomycin, and 2 nM of L-glutamine (Gibco, Saint Aubin, France). Cells were grown at 37°C in a humidified 5% CO2/95% air atmosphere. Exponentially growing cells were trypsinized and seeded in flasks; the medium was replaced every 24h; when cells reached 70-80% confluence by microscopic examination, the medium was changed, and compounds (2, 3, 7, 8-tetrachlorodibenzeno-p-dioxin or TCDD (1-30 nM) and BaP (10 μM) (gift from P Balaguer, Montpellier, France) were added for 16-24h. Control experiments included the addition of CHH 223191 (10 μM), a full AhR antagonist (a gift from P Balaguer)

### *2.5. Statistical Analysis*

As the mRNA levels of gene expression did not fit a Gaussian distribution, the relative expression of genes was characterized by the median and the range rather than their mean values and coefficient of variation [10,12,29]. For each gene, differences of expression between tumors versus normal tissues (fold change) were analyzed as previously described [10,12]. Differences in the number of samples that over- (>3-fold) or and under- (<3-fold) expressed were analyzed using the Chi2-square test [29]. The relationships between expressions of genes in GC were determined using non parametric Spearman's rank correlation test. Relationships between expression levels and clinical parameters were analyzed using non parametric Kruskal-Wallis (or Mann-Whitney) and Chi-square tests, as indicated in each Table. Statistical analyses were performed using Prism 5.03 software (GraphPad, San Diego, CA, USA). Differences were considered significant at confidence levels greater than 95% ($p<0.05$).

## **3. Results**

### *3.1. Patient Characteristics*

The clinical characteristics of the patients are shown in Table 1. Patients with diffuse-subtype GC were younger as than patients with intestinal-subtype (57 [27–71] years and 75 [59-82)]) years respectively, *p=0.0004*). Both subtypes of carcinoma had large tumors (>50 mm) and tumor invasion (T3-T4) [10]. Within each subtype, half of the patients smoked. Patients with diffuse adenocarcinoma had more lymphatic invasion (*p=0.0014*) and metastasis (31% vs 6%) than patients with intestinal-

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subtype. Most diffuse GC was TNM stage III-IV, while patients with intestinal-subtype were stage I, II and III. Vascular and neural invasion did not differ among different GC subtypes.

*3.2. High AhR Expression in Gastric Tumors Both at the mRNA and Protein Levels*

The cohort of GC specimens were first used to assess AhR mRNA expression levels. As compared to normal gastric tissue samples, *AhR* expression was significantly increased in gastric tumors (x1.94, *p=0.002*), both diffuse-and intestinal- subtypes GC (x2.12, *p=0.001* and x1.60, *p=0.003*, respectively) (Table 2). Moreover, *AhR mRNA* expression was independent of classical clinical parameters in GCs and their subtypes, *i.e.*, gender, age, tumor grade, lymphatic invasion, metastasis status, TNM stage, vascular or neural invasion (Supplementary Table S1**)**.

**Table 2.** Statistical analysis of mRNA expression levels of *AhR, CYP1B1, CYP1A1* and *AhRR* in gastric cancers (all GC, diffuse- and intestinal-subtypes).

| Genes | nontumoral gastric tissues (n=11) | *All tumors (n=29)* | *p-value*[a] | Diffuse-GC vs PT (n=13) | *p*-value[a] | Intestinal-GC vs PT (n=16) | *p*-value[a] | *p*-value (GC sub-types) |
|---|---|---|---|---|---|---|---|---|
| ***AhR and target genes*** | | | | | | | | |
| **AhR** | 1 (0.37-1.64) | **1.94 (0.55-3.53)** | **0.002** | **2.12 (0.55-3.35)** | **0.001** | **1.60 (0.65-3.53)** | **0.003** | 0.13 (NS) |
| **CYP1B1** | 1 (0.52-2.90) | 1.45 (0.13-4.90) | 0.91 (NS) | **1.62** (0.43-4.90) | **0.014** | 1.22 (0.13-4.0) | 0.82 (NS) | 0.19 (NS) |
| **CYP1A1*** | 0 (0-5.6) | | | 1.37 (0-86) | 0.26 (NS) | 0.43 (0-30) | 0.73 (NS) | NS |
| **AhRR** | 1 (0.23-1.66) | 1.25 (0.19-3.93) | >0.999 (NS) | 2.65 (0.74-3.96) | **0.007** | 0.89 (0.19-3.85) | 0.88 (NS) | **0.017** |

Median (range) of gene mRNA expression levels; p value (Mann Whitney). Significant p value in bold. Comparative basal levels of genes in normal gastric tissue (x1) are as follows: *AhR* (71), *Cyp1A1* (0), *Cyp1B1* (372), *AhRR* (21). NS= not statistically different.

Most importantly at the protein expression levels, strong nuclear AhR staining was observed in GC (Figure 1B) in epithelial and stromal cells, including fibroblasts, endothelial and immune cells [29] as compared to the weak cytoplasmic and nuclear staining observed in epithelial cells in non-tumoral tissue ((Figure 1A).

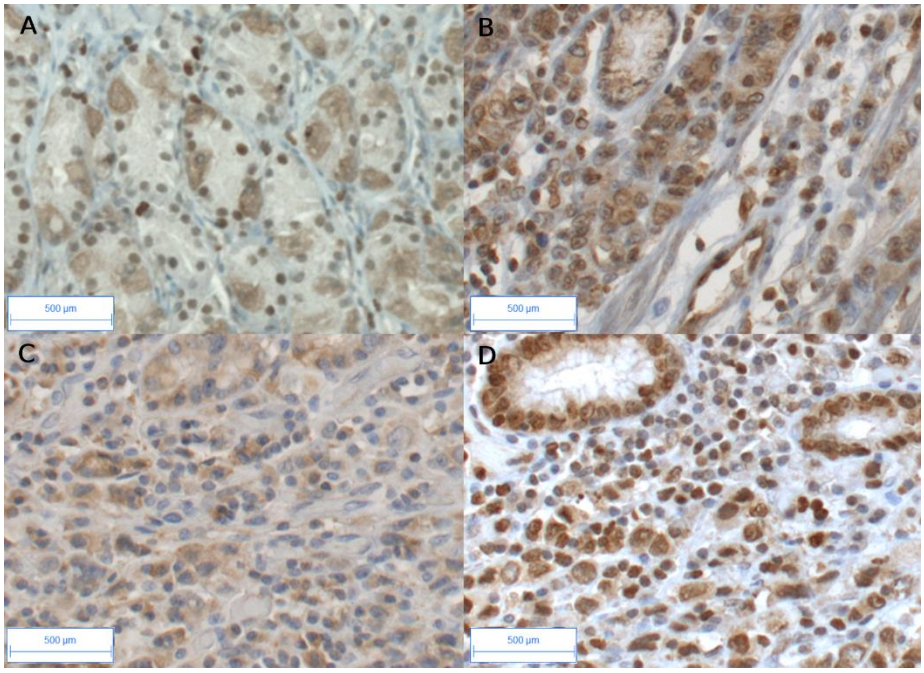


**Figure 1. Immunohistochemical staining AhR and Cyp1B1 in peritumoral and diffuse GCs.** AhR in peritumoral gastric tissue (A); weak cytoplasmic and/or nuclear staining were observed in glandular tissue and stroma. In tumoral tissue (B and D), strong AhR immunostaining is observed in

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most cells, both epithelial and stromal compartments. Cyp1B1 (C) and AhR (D) immunostaining are shown on the same tumor (diffuse GC). Cyp1B1 was mainly observed in the stromal compartment in diffuse GC (C). Original magnification x20. Bar scale, 500 μm.

Altogether, these results indicate that AhR (mRNA and protein) is significantly increased in GCss, both in diffuse- and intestinal- adenocarcinoma, as compared to control samples.

*3.3. Expression of AhR-Target Genes Encoding Xenobiotic Metabolizing Enzymes in Gastric Cancers*

As GC express high AhR expression levels (mRNA and protein), we underwent to analyse changes in the expression of classic target genes of AhR, such as *CYP1A1* and *CYP1B1*, two genes involved in xenobiotic metabolism [16]. *CYP1A1* was expressed at a low level in normal tissues (Table 2). As compared to the non-tumoral tissue, enhanced expression of *Cyp1A1* was attained with an increase of more than 3-fold observed in 5/29 of GC cases (17%) and in 23% diffuse GC. *CYP1B1* expression was significantly increased in diffuse GC (*p=0.014*, 92% lymphatic invasion, Tables 2-3). At the protein level, Cyp1B1 was mainly observed in the stromal compartment in diffuse GC (Figure 1C). In all tumors, *CYP1B1* expression was independent of clinical parameters (gender, age, smoking, tumor grade (T), except for an increase with lymphatic invasion (*p<0.02*) and TNM (*p<0.05* respectively) (Table 3).

**Table 3.** Relationship between *CYP1B1, CYP1A1* and *AhRR* expression with clinical parameters in gastric adenocarcinomas (including sub-populations).

| | **All tumors, n=29** | | | | **Diffuse sub-type GC, n=13** | | | | **Intestinal sub-type GC, n=16** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | ***AhRR*** | ***Cyp1B1*** | ***Cyp1A1*** | **n=** | ***AhRR*** | ***Cyp1B1*** | ***Cyp1A1*** | ***n=*** | ***AhRR*** | ***Cyp1B1*** | ***Cyp1A1*** |
| **Gender** | p= 0.75 | p= 0.47 | *p= 0.063* | | p= 0.42 | p= 0.92 | p= 0.83 | | p= 0.88 | p= 0.67 | *p= 0.058* |
| **Male (n=13)** | 1.63 (0.29-3.96) | 1.62 (0.13-4.03) | 1.37 (0-30.1) | 6 | 2.2 (1.23-3.96) | 1.76 (1.15-2.32) | 1.38 (0-4.01) | 7 | 0.93 (0.29-2.38) | 1.45 (0.13-4) | 0.76 (0-30.1) |
| **Female (n=16)** | 1.18 (0.29-3.85) | 1.34 (0.32-4.9) | 0 (0-86) | 7 | 2.65 (0.74-3.53) | 1.47 (0.43-4.9) | 0 (0-86) | 9 | 0.85 (0.19-3.85) | 1.16 (0.32-2.5) | 0 (0-1.39) |
| **Age** | p= 0.27 | p= 0.18 | p= 0.97 | | p= 0.27 | p= 0.49 | p= 0.16 | | ND | ND | ND |
| **<60 years (n=9)** | 1.66 (0.74-3.96) | 1.47 (1.23-4.9) | 0.67 (0-30.1) | 8 | 1.64 (0.74-3.96) | 1.68 (1.23-4.9) | 0.33 (0-4.01) | 1 | 2.38 | 1.45 | 30.1 |
| **>60 years(n=20)** | 1.17 (0.19-3.85) | 1.21 (0.13-4) | 0.65 (0-86) | 5 | 3.17 (1.15-3.66) | 1.62 (0.43-2.44) | 1.51 (0-86) | 15 | 0.85 (0.19-3.85) | 1.16 (0.13-4) | 0.25 (0-3.31) |
| **Smoking** | p= 0.54 | | p= 0.42 | | p= 0.46 | p= 0.23 | | | p= 0.84 | *p= 0.054* | p= 0.86 |
| **Negative (n=12)** | 1.21 (0.42-1.67) | p= 0.34 | 0.33 (0-1.98) | 4 | 1.43 (0.74-1.66) | 1.78 (1.31-2.32) | p= 0.40 | | 1.02 (0.42-1.67) | 1.63 (0.36-4) | 0.38 (0-1.98) |
| **Positive (n=10)** | 1.65 (0.29-3.85) | 1.63 (0.36-4) 0.98 (0.13-5) | 0.43 (0-86) | 3 | 3.17 (0.74-3.53) | 2.44 (1.85-4.9) | 0.33 (0-1.37) 62.55 (0-86) | 8 7 | 0.93 (0.29-3.85) | 0.49 (0.13-3) | 0.25 (0-30.1) |
| **Tumor** | p= 0.11 | p= 0.18 | p= 0.63 | | ND | ND | | | **p= 0.002** | *p= 0.055* | p= 0.36 |
| **invasion (T)** | 0.43 (0.19-3.5) | 0.94 (0.13-2.44) | 0.47 (0-86) | | 3.35 (3.17-3.53) | 2.14 (1.85-2.44) | ND | | 0.31 (0.19-0.53) | 0.46 (0.13-1.2) | 0.12 (0-0.7) |
| **T1-T2 (n=6)** | 1.31 (0.42-3.87) | 1.47 (0.32-4.9) | 0.67 (0-30.1) | 2 | 1.66 (0.74-3.96) | 1.47 (0.43-4.9) | 74.3 (62.5-86) | 4 | 1.22 (0.42-3.85) | 1.52 (0.32-4) | 0.68 (0-30.1) |
| **T3-T4 (n=23)** | | | | 11 | | | 0.67 (0-4.01) | 12 | | | |
| **Lymphatic** | | | | | | | | | p= 0.25 | **p= 0.019** | |
| **invasion** | **p= 0.032** | | | | ND | ND | | | 0.73 (0.19-3.85) | 0.52 (0.13-2.5) | **p= 0.04** |
| **negative (n=11)** | 0.74 (0.19-3.85) | **p= 0.014** | *p= 0.06* | 1 | 1.63 | 2.32 | ND | 10 | 1.31 (0.42-2.38) | 1.59 (1.27-4) | 0 (0-1.39) |
| **positive (n=17)** | 1.67 (0.42-3.96) | 0.5 (0.3-2.5) 1.6 (0.4-4.9) | 0 (0-1.39) 1 (0-86) | 12 | 2.7 (0.74-3.96) | 1.54 (0.43-4.9) | 1.37 1.03 (0-86) | 5 | | | 1 (0-30.1) |
| **Metastasis** | p= 0.59 | p= 0.55 | p= 0.59 | | **p= 0.006** | p= 0.82 | *p= 0.06* | | ND | ND | ND |
| **(M)** | 1.28 (0.19-4.0) | 1.39 (0.13-4) | 0.68 (0-86) | 9 | 3.17 (1.23-3.96) | 1.62 (1.15-2.44) | 1.51 (0-86) | 15 | 0.85 (0.19-3.85) 2.38 | 1.16 (0.13-4) | 0.25 (0-3.31) |
| **negative (n=24)** | 1.15 (0.74-2.38) | 1.45 (0.43-4.9) | 0 (0-30.1) | 4 | 0.94 (0.74-1.63) | 1.86 (0.43-4.9) | 0 (0-1.37) | 1 | | 1.45 | 30.1 |

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| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **positive (n=5)** | | | | | | | | | | |
| **TNM** | p= 0.35 | **p= 0.045** | p= 0.10 | | p= 0.50 | p= 0.68 | p= 0.83 | | p= 0.22 | **p= 0.038** | |
| **I-II (n=16)** | 1.06 (0.19-3.96) | 1.19 (0.13-3) | 0.12 (0-4.01) | 5 | 2.65 (1.23-3.96) | 1.47 (1.23-2.17) | 0.67 (0-4.01) | 11 | 0.74 (0.19-3.85) | 0.52 (0.13-3) | p= 0.097 |
| **III-IV (n=13)** | 1.63 (0.42-3.66) | 1.62 (0.43-4.9) | 1.37 (0-86) | 8 | 2.19 (0.74-3.66) | 1.73 (0.43-4.9) | 1.38 (0-86) | 5 | 1.31 (0.42-2.38) | 1.59 (1.27-4) | 0 (0-3.31) 1 (0-30.1) |
| **Vascular invasion, negative (n=9)** | p=0.09 | p= 0.39 | p= 0.45 | | p= 0.84 | p= 0.57 | p>0.9999 | | p= 0.17 | p= 0.53 | p= 0.41 |
| | 0.74 (0.2-4) | 1.27 (0.36-2.32) | 0.61 (0-4.01) | 3 | 1.63 (0.74-3.96) | 1.9 (1.4-2.32) | 1.37 (0-4.01) | 6 | 0.62 (0.19-1.31) | 0.94 (0.36-1.6) | 0.30 (0-1) |
| **positive (n=20)** | 1.65 (0.3-3.8) | 1.54 (0.13-4.9) | 0.71 (0-86) | 10 | 2.70 (0.74-3.66) | 1.54 (0.43-4.9) | 1.03 (0-86) | 10 | 1.09 (0.29-3.85) | 1.56 (0.13-4) | 0.5 (0-30.1) |
| **Neural invasion** | p= 0.38 | p= 0.41 | p= 0.38 | | ND | ND | ND | | p= 0.12 | p= 0.52 | p= 0.76 |
| **negative (n=6)** | 0.79 (0.19-3.53) | 1.76 (0.72-2.51) | 1.04 (0-86) | 2 | 3.35 (3.17-3.53) | 2.14 (1.85-2.44) | 74.3 (62.5-85.9) | 4 | 0.63 (0.19-0.85) | 1.41 (0.72-2.5) | 0.35 (0-1.39) |
| **positive (n=23)** | 1.31 (0.29-3.96) | 1.4 (0.13-4.9) | 0.61 (0-30.1) | 11 | 1.66 (0.74-3.96) | 1.47 (0.43-4.9) | 0.67 (0-4.01) | 12 | 1.22 (0.29-3.85) | 0.89 (0.13-4) | 0.43 (0-30.1) |

Median (range) of gene mRNA expression levels; p value (Mann Whitney). Significant p value in bold; tendancy in italic. ND= not determined.

### *3.4. AhR Ligands such as Environmental Ligands Induced mRNA Expression of CYP1A, IL1β, UGT1A1 and AhRR in Gastric Epithelial Cell Lines*

We further examined the effect of two AhR ligands: TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin or dioxin), the most potent known environmental AhR ligand, and BaP (benzo[a]pyrene, a prototypical polycyclic aromatic hydrocarbon found in combustion processes and cigarette smoke) on mRNA expression of several genes in two epithelial gastric cell lines (KATO III and AGS) using qRT-PCR. These cells are poorly (KATO III) and moderately (AGS) differentiated, with high *AhR* expression.

In KATO III cells**,** TCDD (30 nM) strongly increased expression *of CYP1A1* as compared to control cells (x15, *p<0.0001*) (Figure 2d). The TCDD-induced *CYPA1* expression was reversed by CHH 223191, a full AhR antagonist (*p<0.001*) (Figure 2d). BaP (10 µM), a well-studied pro-carcinogen, also increased *CYP1A* expression (x5, *p<0.001)* (Figure 2d). TCDD (30 nM) also significantly stimulated the expression of *IL1B* (x5.5, p<0.0001), *UGT1A* and *AhRR* (x2, p<0.0001) (Figure 2c, e,f). We did not detect significant effects of TCDD (or BaP) on *AhR* and *ARNT* expression in KATO III cells (Figure 2a, b). In contrast to *CYP1A1*, basal and TCDD-increased levels of *CYP1B1* were low in epithelial KATO III cells (Figure 3A) or undetectable (qPCR threshold >CT50) in AGS cells (not shown). Studies in AGS cells also showed that *CYP1A*, but not *AhR* or *AhRR*, was significantly increased following TCDD treatment (Figure 3B a-c).

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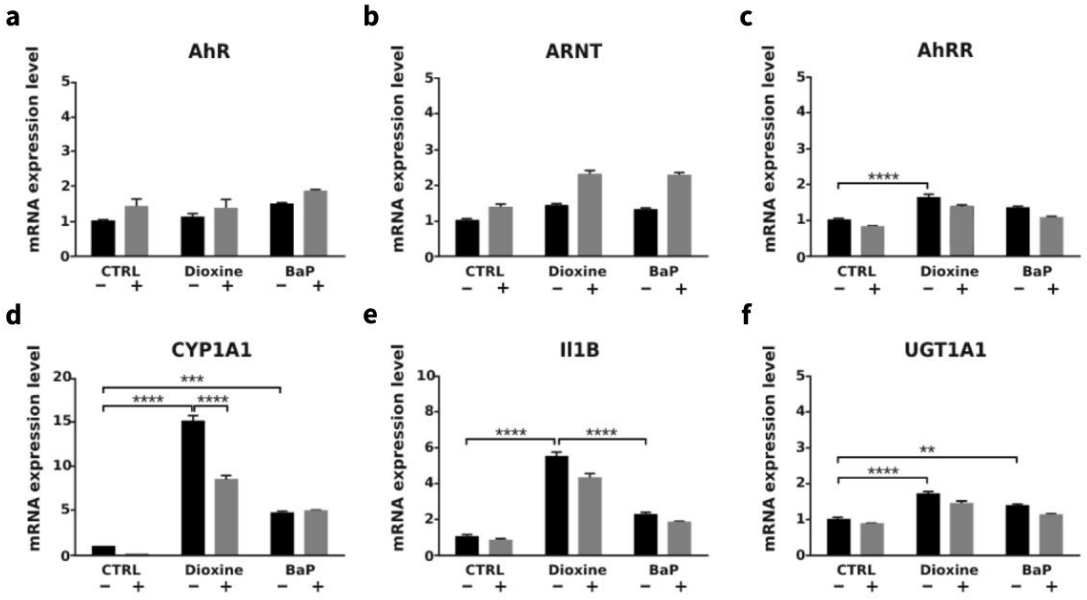


**Figure 2.** mRNA expression levels of *AhR, ARNT, Cyp1A1, IL1B* and *UGT1A1* in KATO III gastric cells treated with two different ligands. KATO III cells were cultivated in the absence (Ctrl) or presence of TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin i.e., dioxin, 30 nM) or BaP (benzo[a]pyrene) (10µM) for 16h. Cells were incubated with (grey column) or without (black column) CHH22319. Expression of genes *AhR* (a), *ARNT* (b), *AhRR* (c), *CYP1A1* (d), *IL1β* (e) and UGT1A1 (f) were determined by qRT-PCR. All experiments were performed in triplicate. Results were expressed as means +/- S.E.M and normalized so that the mean of the control cells was 1. Three levels of statistical significance are distinguished: **p-value<0.04; ***p-value<0.001, ****p-value<0.0001.

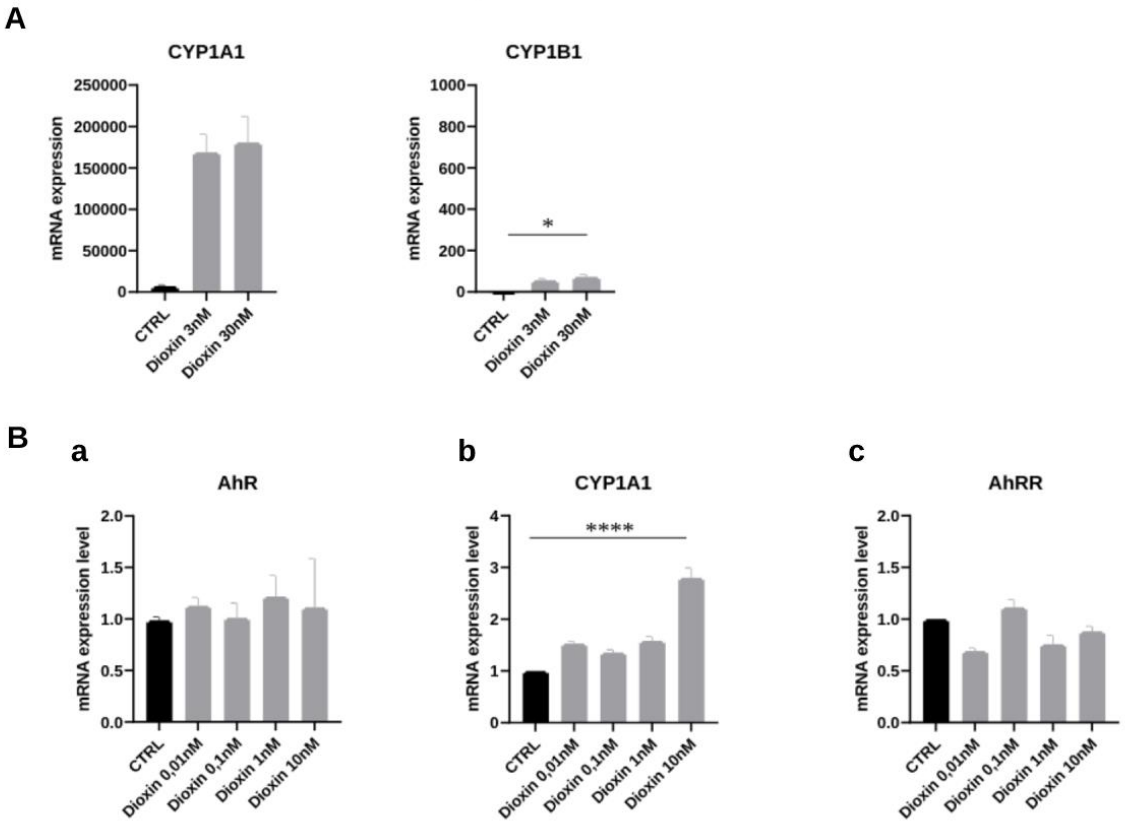

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**Figure 3. mRNA expression levels of *AhR, Cyp1A1, Cyp1B1* and *AhRR* in gastric epithelial cancer cells treated with TCDD.** Figure 3A: Kato III cells were cultivated in the presence or absence of dioxin. Expression levels of *Cyp1A1* and *Cyp1B1* were determined by qRT-PCR in the same experiment. Results were expressed as arbitrary values (means +/- S.E.M) vs CT35 for *Cyp1A1* and *CYP1B1*; basal level of *CYP1B1* was undetectable in AGS cells (CT>50). Figure 3B: AGS cells were cultivated in the absence (Ctrl in black) or presence of TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin) (0.01-10 nM, in grey). Expression levels of *AhR, CYP1A1, AhRR* were determined by qRT-PCR in the same experiment All experiments were performed in triplicate. Results were expressed as means +/- S.E.M and normalized so that the mean of the control cells was 1 (Fig. B a-c). ***p-value<0.001.

### *3.5. Correlations of AhR, Cyp1A1 and Cyp1B1, AhRR, Expression with a Panel of Genes Involved in AhR-Related Signaling Pathways*

AhR is known to activate several signalling pathways governing proliferation, epithelial-mesenchymal transition (EMT), cell migration, inflammation, immunity and angiogenesis in cancers [12,15,17–23]. We then compared the mRNA expression levels of *AhR*, *CYP1A1* and *CYP1B1* with the expression of 36 genes involved in EMT, cell proliferation and migration, immunity and angiogenesis, that we previously described in our cohort of GCss [10,29]. *AhR* expression was correlated with *RHOA* expression in both GC subtypes (Table 4**).** In diffuse GC**,** *CYP1A1* expression correlated with several genes such as growth factors *IGF1 (p=0.001), IGFR2 (p=0.015),* genes involved in EMT such as *VIM (p=0.007*), *SNAI2* and *ZEB2* (*p=0.04*), and migration (*MMP2*, *p=0.01*) (Table 4). *CYP1B1* expression was only inversely correlated with *ERRB2 (p=0.01)*. Moreover, significantly increased *AhRR* expression (x2.65, p<0.01) positively correlated mainly with *IGF1 (p=0.0001), TWIST2* and *ZEB2 (p<0.02), MMP2* (*p<0.002*) and *NRP1* (*p=0.02*) in diffuse, but not intestinal GC (Table 4). In intestinal GC, low expression of *CYP1A1* and *CYPB1* inversely correlated with *IDO1* in intestinal GC (*p=0.014* and *p=0.001*, respectively, Table 4). Low *CYP1B1* expression also correlated with genes that were not significantly increased including growth factors and receptors, EMT (*VIM, SNAI2, TWIST2*), *VEGF* and *NRP1* (Table 4).

**Table 4.** Correlations of selected genes analysed in the study in relation to AhR and "AhR-related signalling pathways" in subtypes GC.

| | Diffuse GC | | | | | | | | Intestinal GC | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Genes** | **AHR** | **AHR** | **AHRR** | **AHRR** | **CYP1B1** | **CYP1B1** | **CYP1A1** | **CYP1A1** | **AHR** | **AHR** | **AHRR** | **AHRR** | **CYP1B1** | **CYP1B1** | **CYP1A1** | **CYP1A1** |
| | **r** | P value | **r** | P value | **r** | P value | **r** | P value | **r** | P value | **r** | P value | **r** | P value | **r** | P value |
| **AHR** | x | | -0.031 | 0.92 | -0.066 | 0.83 | 0.181 | 0.553 | x | | **0.679** | **0.004** | 0.018 | 0.95 | 0.112 | 0.68 |
| **AHRR** | -0.031 | 0.92 | x | | -0.107 | 0.73 | **0.711** | **0.007** | **0.679** | **0.004** | x | | 0.253 | 0.34 | 0.241 | 0.368 |
| **CYP1B1** | -0.066 | 0.83 | -0.107 | 0.73 | x | | 0.342 | 0.253 | 0.018 | 0.95 | 0.253 | 0.34 | x | | **0.655** | **0.006** |
| **Growth factors and receptors (n=10)** | | | | | | | | | | | | | | | | |
| **IGF1** | 0.184 | 0.55 | **0.865** | **0.0001** | 0.155 | 0.71 | **0.846** | **0.001** | -0.113 | 0.68 | 0.168 | 0.53 | **0.765** | **0.001** | 0.371 | 0.16 |
| **IGF1R** | 0.596 | 0.032 | 0.267 | 0.38 | -0.159 | 0.6 | 0.345 | 0.24 | -0.053 | 0.84 | 0.077 | 0.78 | **0.667** | **0.005** | 0.521 | 0.04 |
| **FGFR1** | -0.234 | 0.441 | 0.542 | 0.055 | 0.269 | 0.37 | 0.444 | 0.13 | -0.021 | 0.94 | 0.132 | 0.62 | **0.794** | **<0.0001** | 0.455 | 0.08 |
| **FGF7** | 0 | 1 | 0.576 | 0.04 | -0.055 | 0.86 | 0.424 | 0.15 | -0.127 | 0.64 | 0.047 | 0.86 | **0.721** | **0.002** | 0.401 | 0.12 |
| **IGF2** | 0.041 | 0.89 | 0.119 | 0.7 | -0.033 | 0.91 | -0.043 | 0.69 | -0.093 | 0.73 | 0.144 | 0.59 | **0.774** | **<0.0001** | 0.337 | 0.2 |
| **IGFR2** | 0.562 | 0.046 | 0.375 | 0.21 | -0.06 | 0.84 | **0.655** | **0.015** | 0.169 | 0.53 | 0.018 | 0.95 | 0.524 | 0.037 | 0.364 | 0.15 |
| **IRS1** | -0.259 | 0.39 | 0.457 | 0.12 | -0.121 | 0.69 | 0.384 | 0.19 | 0.056 | 0.84 | 0.471 | 0.07 | **0.915** | **<0.0001** | 0.531 | 0.05 |
| **IRS2** | 0.259 | 0.39 | 0.501 | 0.08 | 0.005 | 0.99 | **0.623** | **0.02** | 0.113 | 0.68 | 0.203 | 0.45 | 0.582 | 0.018 | 0.05 | 0.83 |
| **ERBB2** | 0.341 | 0.25 | 0.529 | 0.06 | **-0.676** | **0.01** | 0.291 | 0.34 | 0.533 | 0.03 | 0.288 | 0.28 | -0.141 | 0.602 | 0.097 | 0.72 |
| **EMT and migration (n=10)** | | | | | | | | | | | | | | | | |
| **VIM** | 0.135 | 0.66 | 0.518 | 0.07 | 0.280 | 0.35 | **0.709** | **0.007** | 0.186 | 0.49 | 0.132 | 0.62 | **0.812** | **<0.0001** | 0.379 | 0.15 |
| **CDH1** | 0.501 | 0.08 | 0.57 | 0.09 | -0.264 | 0.38 | 0.592 | **0.03** | 0.284 | 0.286 | 0.085 | 0.75 | 0.051 | 0.85 | 0.214 | 0.43 |
| **SNAI1** | 0.239 | 0.43 | 0.102 | 0.74 | 0.066 | 0.83 | 0.131 | 0.67 | 0.087 | 0.75 | 0.068 | 0.8 | 0.577 | 0.02 | 0.241 | 0.37 |
| **TGFB1** | 0.297 | 0.32 | 0.182 | 0.55 | -0.511 | 0.83 | -0.165 | 0.59 | 0.21 | 0.39 | -0.091 | 0.74 | 0.453 | 0.08 | 0.118 | 0.66 |
| **RUNX3** | -0.771 | 0.8 | 0.202 | 0.51 | -0.313 | 0.30 | -0.065 | 0.83 | 0.195 | 0.47 | 0.081 | 0.76 | -0.199 | 0.46 | -0.071 | 0.8 |
| **SNAI2** | 0.317 | 0.29 | 0.441 | 0.13 | 0.044 | 0.89 | **0.605** | **0.04** | 0.282 | 0.29 | 0.171 | 0.53 | **0.711** | **0.002** | 0.131 | 0.63 |
| **TWIST2** | -0.005 | 0.99 | **0.667** | **0.013** | 0.115 | 0.71 | 0.504 | 0.08 | 0.121 | 0.66 | 0.109 | 0.69 | **0.827** | **<0.0001** | 0.307 | 0.25 |
| **ZEB2** | 0.033 | 0.91 | **0.661** | **0.014** | -0.038 | 0.90 | **0.602** | **0.04** | 0.133 | 0.62 | 0.242 | 0.37 | 0.477 | 0.001 | 0.462 | 0.07 |
| **RHOA** | **0.600** | **0.034** | 0.176 | 0.56 | -0.203 | 0.50 | 0.484 | 0.09 | **0.693** | **0.003** | 0.324 | 0.22 | -0.056 | 0.84 | 0.171 | 0.53 |
| **RHOB** | -0.215 | 0.48 | 0.295 | 0.23 | 0.429 | 0.14 | 0.444 | 0.13 | -0.094 | 0.73 | 0.041 | 0.88 | 0.559 | 0.02 | 0.201 | 0.45 |

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| | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Cell proliferation and migration (n=3)** | | | | | | | | | | | | | | | | |
| **Ki67** | 0.463 | 0.11 | 0.328 | 0.27 | -0.176 | 0.56 | 0.537 | 0.26 | 0.277 | 0.3 | -0.135 | 0.61 | **-0.665** | **0.006** | -0.381 | 0.1 |
| **MMP2** | -0.099 | 0.74 | **0.774** | **0.002** | 0.164 | 0.59 | **0.701** | **0.01** | 0.121 | 0.65 | 0.118 | 0.66 | **0.800** | **<0.0001** | 0.335 | 0.2 |
| **MMP9** | 0.193 | 0.53 | 0.005 | 0.99 | -0.511 | 0.07 | -0.271 | 0.32 | 0.139 | 0.61 | 0.041 | 0.88 | 0.185 | 0.49 | 0.07 | 0.8 |
| **Immunity (n=5)** | | | | | | | | | | | | | | | | |
| **IDO1** | 0.528 | 0.07 | -0.228 | 0.44 | -0.440 | 0.13 | -0.245 | 0.42 | 0.272 | 0.3 | -0.025 | 0.94 | **-0.727** | **0.001** | **-0.601** | **0.014** |
| **TDO2** | 0.267 | 0.38 | 0.330 | 0.27 | -0.280 | 0.35 | 0.048 | 0.87 | 0.046 | 0.87 | 0.103 | 0.7 | 0.218 | 0.41 | 0.163 | 0.54 |
| **PD1** | 0.534 | 0.06 | 0.033 | 0.92 | 0.115 | 0.71 | 0.209 | 0.49 | -0.155 | 0.56 | -0.132 | 0.62 | 0.056 | 0.84 | -0.261 | 0.25 |
| **CD274** | 0.446 | 0.13 | -0.437 | 0.13 | -0.060 | 0.85 | -0.435 | 0.11 | 0.199 | 0.46 | 0.156 | 0.56 | -0.251 | 0.34 | -0.332 | 0.15 |
| **PDL2** | 0.332 | 0.26 | 0.080 | 0.79 | 0.091 | 0.77 | 0.266 | 0.38 | -0.015 | 0.95 | -0.172 | 0.517 | 0.455 | 0.08 | -0.032 | 0.76 |
| **Angiogenesis (n=6)** | | | | | | | | | | | | | | | | |
| **FLT1** | 0.559 | 0.047 | 0.303 | 0.31 | 0.214 | 0.48 | 0.319 | 0.29 | -0.407 | 0.12 | -0.394 | 0.13 | 0.032 | 0.91 | -0.176 | 0.51 |
| **VEGF165** | 0.402 | 0.17 | 0.358 | 0.23 | 0.011 | 0.97 | 0.364 | 0.22 | -0.531 | 0.03 | **-0.641** | **0.01** | -0.321 | 0.23 | -0.013 | 0.68 |
| **VEGF189** | 0.306 | 0.31 | 0.088 | 0.77 | -0.137 | 0.65 | 0.114 | 0.71 | -0.181 | 0.51 | -0.238 | 0.37 | -0.156 | 0.56 | -0.046 | 0.86 |
| **KDR** | -0.187 | 0.54 | -0.151 | 0.62 | -0.368 | 0.22 | -0.387 | 0.19 | -0.081 | 0.77 | -0.079 | 0.77 | 0.482 | 0.06 | 0.061 | 0.82 |
| **VEGFC** | 0.179 | 0.56 | 0.328 | 0.27 | 0 | 1 | 0.199 | 0.51 | 0.01 | 0.88 | 0.132 | 0.63 | **0.788** | **0.0001** | 0.353 | 0.18 |
| **NRP1** | 0.185 | 0.55 | **0.614** | **0.02** | 0.033 | 0.91 | 0.411 | 0.16 | 0.131 | 0.62 | 0.168 | 0.53 | **0.838** | **<0.0001** | 0.324 | 0.22 |

r, Spearman's rank test (relationship between two quantitative parameters). Values in bold type are statistically significant at confidence level greater than 99% (p value<0.01) and r>0.6**.** Cyp1B1 and AhRR were not significantly increased in intestinal GC and statistical correlations should be considered with caution (see Table 2).

## 4. Discussion

Although AhR is known for its role in environmental chemical toxicity, as a mediator of unintended consequences of human pollution, and its involvement in tumor initiation and progression [24–26,28,35], the relationship between AhR expression, pollution linked AhR-dependent function and Western patients with GCs remains unexplored. Because diffuse GC is increasing in prevalence worldwide in Western countries, usually diagnosed at advanced stages, and has no efficacious treatment options, exploration of its cellular and molecular causes is crucial. We reported significant high expression of *AhR* in our Western cohort of GC, independently of their clinical subtypes [29]. A link between exposure to persistent organic pollutants (POPs) and diffuse GC is supported by the study of accidentally exposed populations in Seveso [11]. In the study presented here, we have analyzed the expression of AhR and several AhR-regulated genes in a series of gastric tumors including diffuse- and intestinal-GC. Furthermore, we have studied the impact of two AhR ligands well-known for their critical role in cancer development linked to pollution [26,27].

**Expression of *AhR* and *RHOA*.** Using RT-PCR, we found a correlation between the expression of *AhR* and *RHOA* in GCs independently of their subtypes (Table 4), as previously documented for other types of cancers [12,36,37]. A higher expression of *RHOA* has been found in diffuse GC with 85% overexpression (>3fold) as compared to normal samples, along with 50% in intestinal subtype) [10]. The functional and coordinated role of RhoA in the development of cancers involves several processes such as cell proliferation, migration, invasion and angiogenesis [36–40]. Increase RhoA activity is correlated with worse overall survival in diffuse patients [41]. Interestingly, *RHOA* transcription has recently been shown to be initiated by a ligand-AhR-Arnt complex. Somatic alterations in *RHOA* and *CDH1* have been reported in aggressive diffuse GC and generally associated with familial disease [42,43]. However, our Western cohort of diffuse GC by the Lauren classification did not include familial GC.

**Expression and distribution of *Cyp1A1* and *Cyp1B1*.** Cytochrome P450-1 enzymes are inducible forms of the cytochrome P450 family of xenobiotic metabolizing enzymes [44,45]. In our study, ***Cyp1A1*** was detected at very low level in stomach, but overexpressed (>3) in 23% in diffuse GC as compared to non-tumoral tissue. *Cyp1A1* was also highly induced by TCDD, a non-genotoxic AhR, in undifferentiated diffuse GC (Kato III) cells. ***Cyp1B1*** was the most significantly expressed form in diffuse GC, as previously reported in a wide range of human cancers including breast, colon, lung and others [44]. Cyp1A1 and Cyp1B1 have a central role in tumor development and in the activation step of pro-carcinogen compounds such as benzopyrene (BaP) [16,45–49]. BaP (benzo[a]pyrene) is a

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prototypical polycyclic aromatic hydrocarbon (PAH) found in tobacco and combustion processes such as biomass burning [50,51].

**Our in vitro experiments** using two GC cell lines (KATO III and AGS) indicated that TCDD, and BaP *to a lesser extent*, strongly induced *CYP1A1, UGT1A1* and *NQO1* in KATO III epithelial cells, but low levels of *CYP1B1*, as compared to unexposed cells (Figs 2 and 3A). *Cyp1A1* was also increased following TCDD treatment in AGS cells as compared to unexposed cells. Functional DRE enhancer element has been identified in vitro and in vivo for AhR target genes including *CYP1A1*, *CYP1B1* and *NQO1,* which encode phase I and II xenobiotic metabolizing enzymes [52]. **Our in vivo study** also shows that ***CYP1B1*** expression is significantly increased (*p=0.014*) in diffuse GC, as compared to intestinal GC. At the protein level, Cyp1B1 was mainly observed in the stromal compartment in diffuse GC (Figure 1C). It is well known that stromal cells, such as fibroblasts and macrophages, express *CYP1B1*, but not *CYP1A1,* in response to TCDD or benzopyrene [53,54]. Taken together, our results suggest a cell-specific distribution of Cyp1B1 and Cyp1A1 in diffuse GC. Our results also suggest that activated AhR may contribute to the tumor-stroma interaction (through Cyp1A1 and Cyp1B1) in diffuse GC.

**Funtional role of AhR, Cyp1A1 and Cyp1B1.** Animal and clinical data provide evidence for the role of AhR in gastric tumorigenesis, implicating the receptor in regulation of tumor growth, EMT, migration, invasion and cancer aggression [24,25,28,35]. The connection between EMT, a process allowing cells to transition from epithelial to mesenchymal, and tumorigenesis has been established in human cancers involving several pathways such as activation of Wnt/beta-catenin signaling through Cyp1s [55,56], or hedgehog signaling [57,58]. It is also well known that Cyp1A1 and Cyp1B1 have important roles in both tumor development (cell invasion, migration, and disease progression), in addition to the metabolic activation of BaP related to carcinogenesis. Analysis of *CYP1s* and co-regulated genes using large scale analysis may also be helpful for functional studies [59]. Using RT-qPCR, we compared the expression of *CYP1*s with the expression levels of 36 genes coding for proteins that have been previously studied in the same cohort of patients [10,29]. These genes were selected on the basis of their roles in proliferation, the IGF pathway, the EMT signature, migration, angiogenesis or immunity. In diffuse GC, but not intestinal GC, *CYP1A1* expression was strongly correlated with expression of genes involved in proliferation (*IGF1, p=0.001),* EMT signature *such as VIM, p=0.007*, *SLUG* and *ZEB2* (*p=0.04*), and migration (*MMP2*, *p=0.01*); these genes were previously shown to be correlated with *IGF1* [10]. Interestingly, the promoters of *CYP1A1, VIM, SNAI2/SLUG* contain a xenobiotic responsive element (DRE) sequence that when bound by AhR-ARNT heterodimers (canonical pathway) leads to their transcription. Activation of the AhR pathway by TCDD has been previously shown to enhance cancer cell invasion through metalloproteinases [60,61]. Environmental pollutants have been found to contribute to EMT and mesenchymal markers that provide invasion, migration and subsequent metastasis [61–63]. In contrast to diffuse GC, the low expression of *CYP1A1* and *CYP1B1* in intestinal GC inversely correlated with *IDO1* expression ($p<0.02$). The IDO enzyme mediates the early steps of tryptophan metabolism leading to kynurenine, an endogenous AhR ligand produced in intestinal but not in diffuse GC [29]. Thus, environmental pollutants vs endogenous kynurenin may have differently effects on AhR-dependent gene expression in GCs.

Whether or not the increase of *CYP1s* expression that we observed in diffuse as compared to intestinal GC is due to exposure to a specific or to multiple POPs remains to be established [64]. We have previously reported the significant and widespread increase of a substantial set of POPs such as polychlorinated dioxins (PCDDs/PCDFs), polychlorobiphenyls (PCBs) and polybrominated flame retardants (such as PBDE 209, a carcinogenic intermediate of BaP) in human omental tissue (fat deposits) from French patients with diffuse-GC, as compared to control biopsies [13]. Coexposure of TCDD and PBDE209 was observed in 33% of the omentum from patients with diffuse GC. Interestingly, an increased incidence of hepatocellular carcinomas was observed in rodents upon exposure to PBDE-209 as well as an increased *CYP1A1* mRNA expression levels in Caco-2 cells [65].

**Expression of *AhRR*.** Our results reveal that *AhRR* mRNA expression is strongly increased in the cohort of diffuse GC when compared to normal tissues of the same anatomical origin (x2.65,

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*p=0.007*) (Table 2). The effect of dioxin on *Cyp1A1* and *AhRR* expression in the undifferentiated KATO III as compared to AGS gastric epithelial cells also support a role of xenobiotic compounds *in vivo*. Expression of *AhRR* correlated with *Cyp1A1* (*p=0.007*), *IGF1* (*p<0.0001*), and with genes involved in EMT (*TWIST2, ZEB2*) and migration (*MMP2, p=0.002)*. Our observations in patients with metastatic diffuse GC further indicate a significant decrease of *AhRR* expression. Our results suggest that *AhRR* may represent an independent prognosis factor in diffuse GC, as we previously reported for breast cancer [12]. Poor prognosis was previously correlated with a decreased expression of *AhRR* in GCs from an Asian gastric cohort, but without discrimination between subtypes [66]. Moreover, loss of *AhRR* correlates with an aggressive tumorigenic phenotype in several tumors including colon, cervical, and ovarian carcinoma [67].

**In conclusion,** this pilot study explores two forms of GCs, diffuse- and intestinal- GC that lead to metastases in the peritoneal cavity. The induction of *CYP1s* through AhR activation may potentially serve as a biomarker for exposure to xenobiotics in diffuse GC. In vitro experiments indicate that TCDD strongly induce *CYP1A1* in epithelial cells. Expression of *Cyp1A1* strongly correlated with that of *IGF*, genes involved in EMT and migration. Increased expression of *Cyp1B1* was observed in diffuse GC. Cyp1B1 activates a large number of pollutants which may result in activation of pro-cancer signaling pathways. AhR may contribute to the tumor-stroma interaction (through Cyp1A1 and Cyp1B1) in diffuse GC. Whether clinical factors such as smoking are prognostic factors remain to be investigated in GCs. We argue that reduction of exposure to subsets of environmental ligands could be important to prevent primary diffuse GC. A recent study revealed that exposure to environment pollutants such as POPs and BaP may reduce the efficacy of chemotherapy [68].

We acknowledge that our study has limitations. Because of the relatively low sample size in this report (n=29), certainly the results need to be confirmed using a larger cohort of gastric tumor samples with different clinical characteristics (including early and advanced stages). Nonetheless our pilot study shed light on the impact of AhR and related signaling pathays in Western patients with GCs. In addition, it will be interesting to extend the coverage to different geographical population settings. This will allow us to understand if the signaling pathways identified in GC subtypes are characteristic only in Western patients or can also be observed in patients from other geographical distributions. Further in vitro and in vivo studies with a larger cohort of gastric tumor samples will provide a better understanding of the complexity of the effect of different ligands on the regulation of the AhR pathway and may contribute to the development of novel clinically relevant agonists or antagonists.

As summarized in **Figure 4**, the present study provides new insights about the diversity of AhR functions in the development of cancer including GCs. It is likely that binding of various ligands is central to this carcinogenesis. The gastric epithelium is constantly exposed to exogenous AhR ligands such as dietary compounds and environmental toxins (PAH and other dioxin-like compounds), which enable to strong activation of AhR. Furthermore, the endogenous AhR kynurenine is produced through metabolism of tryptophan by IDO1 which is induced in stromal cells, or by TDO2 which can be up-regulated in tumor cells and in the tumor stroma. Our findings merit further studies with a larger cohort of gastric tumor samples with different clinical characteristics, including early and advanced tumor stages.

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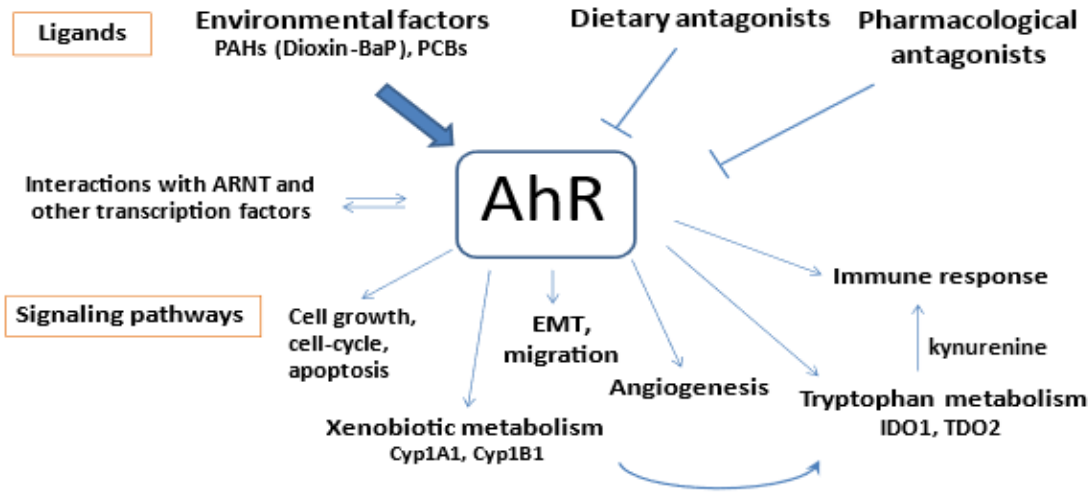


**Figure 4.** AhR role in cancer biology; environmental compounds at the crossroads of toxicity and several signaling pathways.

**Supplementary Materials:** The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Table S1: Relationship between *AhR* mRNA expression and clinical biological parameters in a series of 29 gastric cancers including diffuse- and intestinal subtypes.

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**Authors' Contributions:** Conceptualization: MP-A; Methodology: CP, SV, MP-A; Software: CP, M P-A, VB, Data curation: MP collected the clinical samples and data from patients. Writing original draft: MP-A; rewriting: VB. Funding acquisition: MP-A, MP; All authors have read and agreed to the published version of the manuscript.

**Availability of data and materials**. A total of 29 patients underwent partial gastrectomy for histopathologically-confirmed gastric adenocarcinoma primary tissue in the lariboisiere Hospital (Paris, France) from 2005-2014.

**Ethics approval and consent to participate:** The studies involving human participants were approved by the Ethical Commitee of Lariboisiere Hospital (Paris, France). The patients/participants provided their written informed consent to participate in scientific studies. Written informed consent was obtained from the participants before the collection of any samples, and the specimens were irreversibly de-identified.. All experiments involving the handling of human tissues were performed in accordance with Tenets of the Declaration of Helsinki. Biopsies from patients underwent partial gastrectomy for gastric adenocarcinoma tumor tissue from 2005-2014. The cohort of patients was previously described in Perrot-Applanat et al., *Oncol Letters 2019, 18:674-686.*

**Patient consent for publication**. The patients/participants provided their written informed consent to participate in this study and associated publications.

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