

Impairment of aryl hydrocarbon receptor signalling promotes hepatic disorders in cancer cachexia

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Abstract

Background The aryl hydrocarbon receptor (AHR) is expressed in the intestine and liver, where it has pleiotropic functions and target genes. This study aims to explore the potential implication of AHR in cancer cachexia, an inflammatory and metabolic syndrome contributing to cancer death. Specifically, we tested the hypothesis that targeting AHR can alleviate cachectic features, particularly through the gut–liver axis.

Methods AHR pathways were explored in multiple tissues from four experimental mouse models of cancer cachexia (C26, BaF3, MC38 and APC^{Min/+}) and from non-cachectic mice (sham-injected mice and non-cachexia-inducing [NC26] tumour-bearing mice), as well as in liver biopsies from cancer patients. Cachectic mice were treated with an AHR agonist (6-formylindolo(3,2-*b*)carbazole [FICZ]) or an antibody neutralizing interleukin-6 (IL-6). Key mechanisms were validated in vitro on HepG2 cells.

Results AHR activation, reflected by the expression of *Cyp1a1* and *Cyp1a2*, two major AHR target genes, was deeply reduced in all models (C26 and BaF3, $P < 0.001$; MC38 and APC^{Min/+}, $P < 0.05$) independently of anorexia. This reduction occurred early in the liver ($P < 0.001$; before the onset of cachexia), compared to the ileum and skeletal muscle ($P < 0.01$; pre-cachexia stage), and was intrinsically related to cachexia (C26 vs. NC26, $P < 0.001$). We demonstrate a differential modulation of AHR activation in the liver (through the IL-6/hypoxia-inducing factor 1 α pathway) compared to the ileum (attributed to the decreased levels of indolic AHR ligands, $P < 0.001$), and the muscle. In cachectic mice, FICZ treatment reduced hepatic inflammation: expression of cytokines (*Ccl2*, $P = 0.005$; *Cxcl2*, $P = 0.018$; *Il1b*, $P = 0.088$) with similar trends at the protein levels, expression of genes involved in the acute-phase response (*Apcs*, $P = 0.040$; *Saa1*, $P = 0.002$; *Saa2*, $P = 0.039$; *Alb*, $P = 0.003$), macrophage activation (*Cd68*, $P = 0.038$) and extracellular matrix remodelling (*Fga*, $P = 0.008$; *Pcolce*, $P = 0.025$; *Timp1*, $P = 0.003$). We observed a decrease in blood glucose in cachectic mice ($P < 0.0001$), which was also improved by FICZ treatment ($P = 0.026$) through hepatic transcriptional promotion of a key marker of gluconeogenesis, namely, *G6pc* (C26 vs. C26 + FICZ, $P = 0.029$). Strikingly, these benefits on glycaemic disorders occurred independently of an amelioration of the gut barrier dysfunction. In cancer patients, the hepatic expression of *G6pc* was correlated to *Cyp1a1* (Spearman's $\rho = 0.52$, $P = 0.089$) and *Cyp1a2* (Spearman's $\rho = 0.67$, $P = 0.020$).

Conclusions With this set of studies, we demonstrate that impairment of AHR signalling contributes to hepatic inflammatory and metabolic disorders characterizing cancer cachexia, paving the way for innovative therapeutic strategies in this context.

Keywords CYP1A1; CYP1A2; fibroblast growth factor 21; HIF1 α ; TIPARP

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Introduction

The aryl hydrocarbon receptor (AHR) has received much attention in recent years due to its functional role in liver homeostasis and disease.¹ Apart from its well-established regulatory role in hepatic xenobiotic metabolism,² AHR has also been implicated in liver acute-phase response (APR), fibrosis, immunological processes³ and inhibition of the production of fibroblast growth factor 21 (FGF21), a protein that influences adipose and muscle biology.^{4,51} By binding to the AHR nuclear translocator (ARNT), the complex AHR/ARNT will foster the transcription of its canonical target genes, which include the cytochrome P4501A (*Cyp1a*) family and in particular *Cyp1a1*.⁵

AHR-activating ligands can derive from various endogenous or exogenous sources. The gut microbiome is a major source of endogenous AHR ligands: several tryptophan-derived compounds, including indole derivatives, are formed through microbial metabolism of tryptophan and have been shown to possess AHR-activating properties in vitro.^{6,52} A vast amount of AHR ligands is also present in the liver besides these tryptophan metabolites and include bilirubin, biliverdin and modified low-density lipoproteins.^{7,8} Although much evidence is accumulating, the role of AHR in liver diseases remains complex. Hepatic AHR activation can promote (in acute acetaminophen-induced hepatotoxicity, liver infection and hepatocellular carcinoma) or attenuate (in alcohol-related liver disease and acute immune-mediated hepatitis) liver disease pathogenesis.³ In liver fibrosis and nonalcoholic steatohepatitis, it remains controversial, with evidence for both beneficial and detrimental effects of AHR signalling.³

Intestinal AHR activation may also improve liver condition through the gut–liver axis.^{3,9} AHR is widely expressed in the intestinal epithelium, where it promotes barrier integrity by up-regulation of tight junction proteins^{10,11} and downregulation of inflammatory markers.¹² Along these lines, treatment with an AHR agonist improves intestinal barrier dysfunction and reduces hepatic impairments in metabolic syndrome.¹³ Mice with AHR deficiency specifically in intestinal epithelial cells show disruption of the intestinal epithelial barrier and aggravation of alcohol-related liver disease,¹⁴ highlighting an essential role of intestinal AHR in regulating liver disease.

To our knowledge, no previous investigation has been conducted to discover the potential implication of AHR in

the alterations observed in cancer cachexia. This multifactorial syndrome is characterized by involuntary and pathological weight loss, mainly due to skeletal muscle wasting,¹⁵ resulting in a decrease in patients' quality of life, response to cancer treatments and survival.¹⁶ Cachexia is a multiorgan syndrome affecting not only the skeletal muscle but also the gut, adipose tissue and liver.¹⁷ Due to the pathophysiological complexity of this clinical syndrome, there is currently no effective treatment for cancer cachexia. Alterations in liver metabolism are often overlooked and range from the activation of the liver APR, observed in pancreatic cancer patients,¹⁸ to alterations in mitochondrial function,¹⁹ hepatic steatosis,²⁰ reduction in glycolysis and gluconeogenesis,²¹ and a hepatic collagen deposition,²² reported in rodent models of cachexia. Recent work from our team shows increased gut permeability,²³ accelerated intestinal transit and increased levels of tryptophan in the caecal content of cachectic mice,²¹ raising the possibility of an implication of AHR in the cachexia syndrome through the gut–liver axis.

In the present work, we uncover a differential modulation of AHR activation in the liver compared to the ileum and to other organs. We also reveal a key role of AHR as a master switch between hepatic inflammatory and glycaemic disorders in cancer cachexia, independently of the gut permeability.

Experimental procedures

Cell culture

Cachexia-inducing colon carcinoma 26 (C26) cells, non-cachexia-inducing colon carcinoma 26 (NC) cells (TKG0518) and HepG2 cells (HB-8065) were maintained in high-glucose Dulbecco's modified Eagle's medium supplemented with 10% foetal bovine serum (Capricorn Scientific, Brazil), streptomycin and penicillin (Thermo Fisher, Belgium) at 37°C with 5% CO₂.

Mouse experiments

The main model used to study cancer cachexia is the well-established C26 model, characterized by body weight

and fat mass loss as well as muscle atrophy.⁵³ After 1-week acclimatization, male CD2F1 mice (7 weeks old, Charles River Laboratories, Italy) were assigned to experimental groups based on their body weight and were subcutaneously injected with a saline solution, C26 or NC cells (1×10^6 cells in 0.1-mL saline). For the 6-formylindolo(3,2-*b*)carbazole (FICZ) experiment, mice were injected intraperitoneally with FICZ (Enzo Life Sciences, Switzerland, 1 µg per mouse) or vehicle (dimethyl sulfoxide [DMSO] 6.7%) at days 1, 5 and 9 after cell injection. Food intake and body weight were recorded. Ten days after cell injection, mice were fasted for 6 h. Blood glucose levels were determined using a glucose metre (Roche Diagnostics, Indianapolis, IN, USA), and blood collected from the tip of the tail prior to necropsy. Blood samples from portal vein and vena cava were harvested following anaesthesia (isoflurane gas, Abbott, Belgium). Tissues were weighed and frozen in liquid nitrogen. All samples were stored at -80°C until further analyses (described in the supplementary experimental procedure). Additional mouse experiments are described in the supplementary experimental procedure.

Analyses of human liver biopsies

Liver biopsies from cancer patients, obtained during surgery, were used to analyse the gene expression of key markers identified in our preclinical work, as detailed in the supplementary experimental procedure.

Statistical analyses

Statistical analyses were carried out using GraphPad Prism® for Windows (v.8.0.1, La Jolla, CA, USA). All data were checked for normality using the D'Agostino and Pearson omnibus normality test. Data were analysed using Student's *t* test when comparing two groups, one-way analysis of variance (ANOVA) with Bonferroni's post hoc tests or two-way ANOVA with Bonferroni's post hoc tests when appropriate. Data determined to be non-normal were analysed using a Mann–Whitney *U* test or Kruskal–Wallis test with Dunn's post-tests. Outliers were excluded using Grubbs' test ($\alpha = 0.05$). We considered $P \leq 0.05$ to be statistically significant.

Results

AHR activation is decreased in preclinical models of cancer cachexia

We observed increased levels of tryptophan in the faeces of cachectic mice (Figure 1A), accompanied by a drop in tryptophan-derived metabolites (Figure 1B,C). The levels of several indole derivatives and AHR agonists (Figure 1D,E) were

decreased in the faeces of cachectic mice. Concordantly, the expression of *Cyp1a1*, a major AHR target gene,⁵ was significantly reduced in cachectic caecal tissue (Figure 2A). This reduction in *Cyp1a1* transcripts was also found in the other intestinal segments (ileum and colon) and in peripheral tissues, namely, the liver, the *gastrocnemius* muscle (GAS), the brown adipose tissue (BAT) and the subcutaneous adipose tissue (SAT) (Figure 2A). As for *Cyp1a2*, another member of the Cyp1a family reflecting AHR activation, its mRNA expression displayed a seven-fold reduction in cachectic liver (Figure 2B). The reduction in *Cyp1a1* and *Cyp1a2* expression was confirmed in other models of cancer cachexia, namely, BaF3 mice, MC38 mice and APC^{Min/+} mice (Figure 2C). When looking at the kinetics of events, it appears that *Cyp1a1* transcripts were decreased in the liver before the occurrence of body weight loss and anorexia whereas the reduction in *Cyp1a1* in the ileum and GAS occurs concomitantly (Figures 2D and S1). To isolate the impact of anorexia, two groups of healthy mice were food restricted to the amount of food consumed by either the CT group (FR-CT mice) or the C26 group (FR-C26 mice). The reduced food intake did not fully recapitulate the modulation, in cachectic mice, of caecal and hepatic *Cyp1a1* (Figure 2E). Next, we wished to determine whether *Cyp1a1* decreased expression was related to cachexia or, more generally, to the tumoural presence. For this purpose, we compared mice inoculated with cachexia-inducing C26 cells (C26 mice), non-cachexia-inducing C26 cells (NC mice) and sham-injected mice (CT mice). We found that the modulation of ileal and hepatic *Cyp1a1* is intrinsically related to cachexia and not only due to the presence of the tumour (Figure 2F).

Altogether, these results highlight an anorexia-independent reduction of AHR activation in preclinical models of cancer cachexia, with an early occurrence in the liver.

AHR activation is differently modulated in the ileum, liver and other organs of cachectic mice

Decreased bacterial production of AHR agonists is a logical explanation for the modulation of AHR activation in the gut. However, we did not observe such a decrease in AHR agonists in the vena cava, portal vein and liver (Figure S2), which led us to explore other hypotheses to explain the decrease in *Cyp1a* transcripts in peripheral organs and particularly in the liver.

Aryl hydrocarbon receptor repressor (AHRR) and TCDD-inducible poly(ADP-ribose) polymerase (TIPARP) have been described as negative regulators of AHR signalling.¹⁰ TIPARP mono-ADP-ribosylates AHR, leading to its proteolytic degradation, whereas AHRR reduces the transactivation of AHR.^{10,54} We put aside AHRR, as its expression remained unchanged in the liver of cachectic mice (Figure S3A). We found an induction of *Tiparp* in the liver of cachectic mice (Figure S3B–E). However, AHR protein expression remained unaltered in cachectic liver (Figure S3F). We thus concluded that

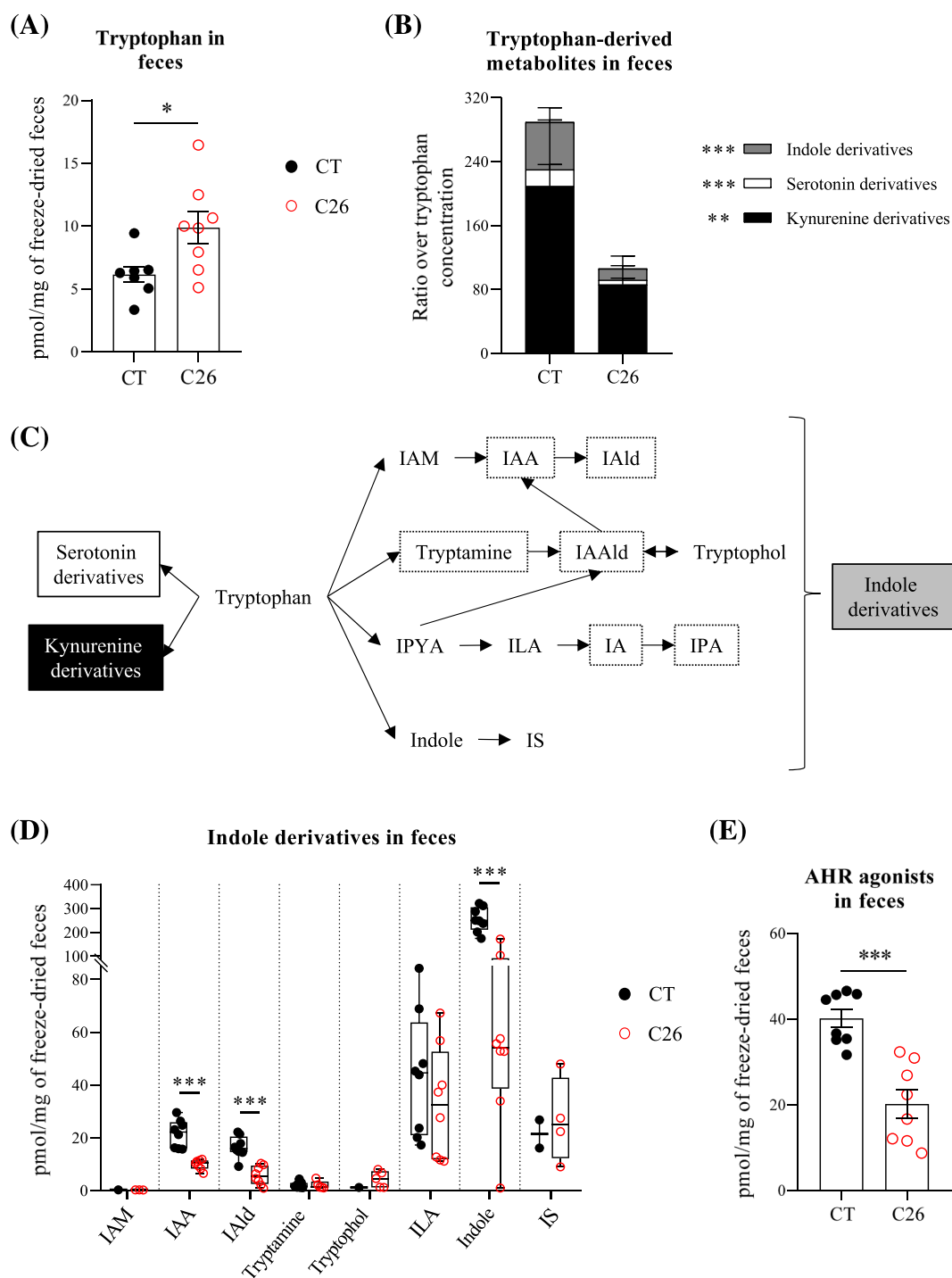


Figure 1 Aryl hydrocarbon receptor (AHR) agonists levels are decreased in the faeces of C26 cachectic mice. (A) Faecal level of tryptophan in sham-injected mice (CT) and mice injected with cachexia-inducing C26 colon carcinoma cells (C26). (B) Stacked plot displaying the sum of the metabolites of indole, serotonin or kynurenine pathway over tryptophan concentration in the faeces of CT and C26 mice. $n = 8$ per group. (C) Schematic view of tryptophan metabolism, with particular emphasis on indole/AHR pathway and AHR agonists (framed in dotted lines). From top left to bottom right: indole-3-acetamide (IAM), indole acetic acid (IAA), indole-3-aldehyde (IAld), indole-3-acetaldehyde (IAAla), indole-3-pyruvate (IPYA), indole-3-lactic acid (ILA), indoleacrylic acid (IA), indole-3-propionic acid (IPA) and indole-sulfonic acid (IS). (D) Quantification of indole pathway metabolites in the faeces of CT and C26 mice. (E) AHR agonist quantification in the faeces of CT and C26 mice. A list of AHR agonists was made based on a literature search,^{9,11,516} and the levels of AHR agonists detected in C26 mice (IAA, IAld and tryptamine) were summed up. In (A) and (E), data are presented as mean $\pm$ SEM. In (D), data are presented with inter-quartile range (IQR) box and min-to-max whiskers. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$.

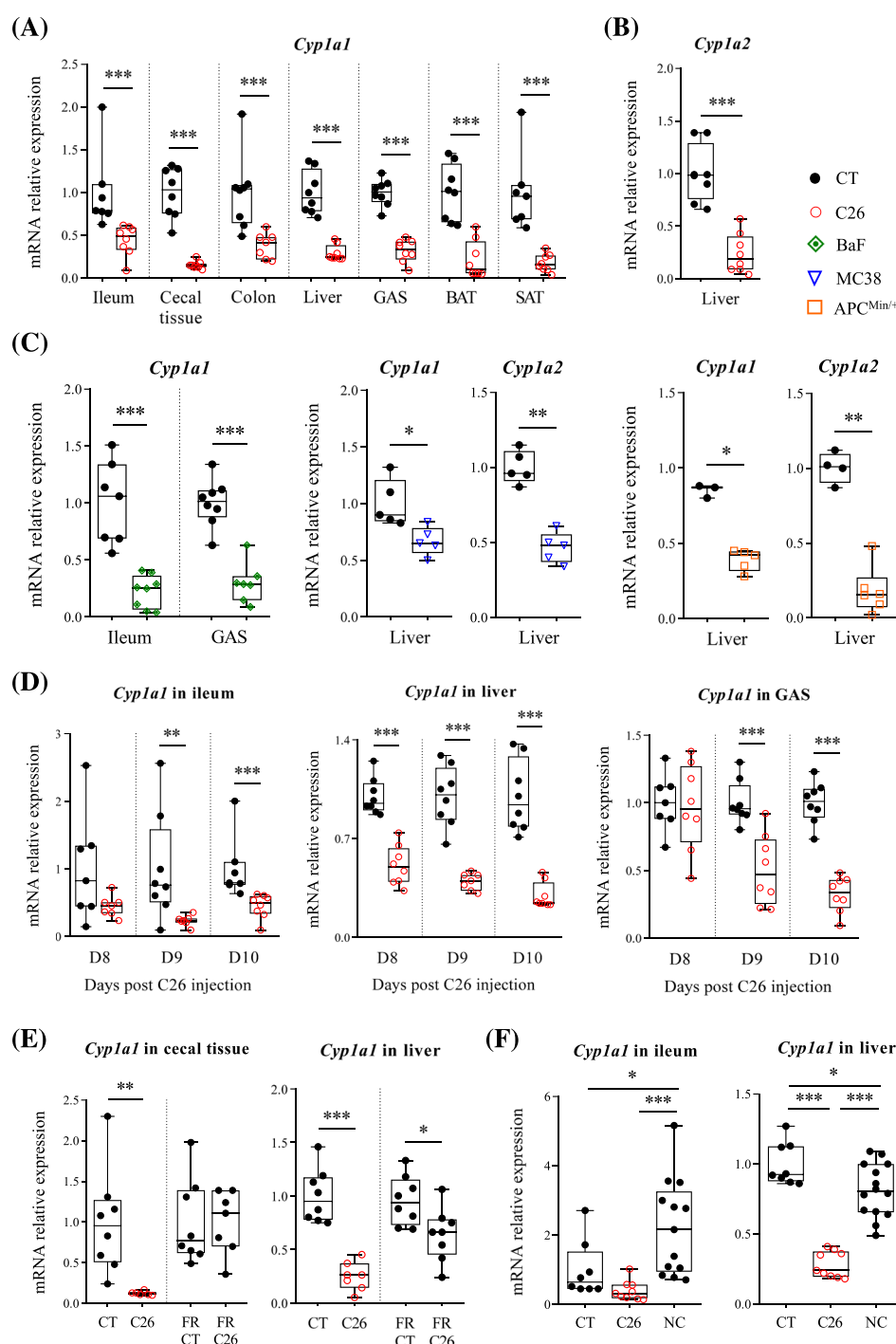


Figure 2 Decreased aryl hydrocarbon receptor (AHR)-regulated CYP1A gene transcripts in preclinical models of cancer cachexia. (A) mRNA expression levels of cytochrome P450 family 1 subfamily A member 1 (*Cyp1a1*) in the ileum, caecal tissue, colon, liver, *gastrocnemius* muscle (GAS), brown adipose tissue (BAT) and subcutaneous adipose tissue (SAT) of sham-injected mice (CT) and mice injected with cachexia-inducing C26 colon carcinoma cells (C26). (B) Hepatic mRNA expression levels of cytochrome P450 family 1 subfamily A member 2 (*Cyp1a2*) in CT and C26 mice. (C) mRNA expression levels of *Cyp1a1* in the ileum and GAS of CT and leukaemic BaF mice; hepatic mRNA expression levels of *Cyp1a1* and *Cyp1a2* in CT mice and mice injected with MC38 colon carcinoma cells (MC38) and in CT mice and APC^{Min/+} mice, which are predisposed to intestinal adenoma formation. (D) Evolution of *Cyp1a1* mRNA expression levels in the ileum, liver and GAS of CT and C26 mice, 8, 9 and 10 days after injection. (E) mRNA expression levels of *Cyp1a1* in the caecal tissue and liver of CT mice, C26 mice and healthy mice sham injected and food restricted to the amount of food consumed by either the CT group (FR-CT mice) or the C26 group (FR-C26 mice). (F) mRNA expression levels of *Cyp1a1* in the ileum and liver of CT mice, C26 mice and mice injected with non-cachexia-inducing C26 colon carcinoma cells (NC). Data are presented with inter-quartile range (IQR) box and min-to-max whiskers. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$.

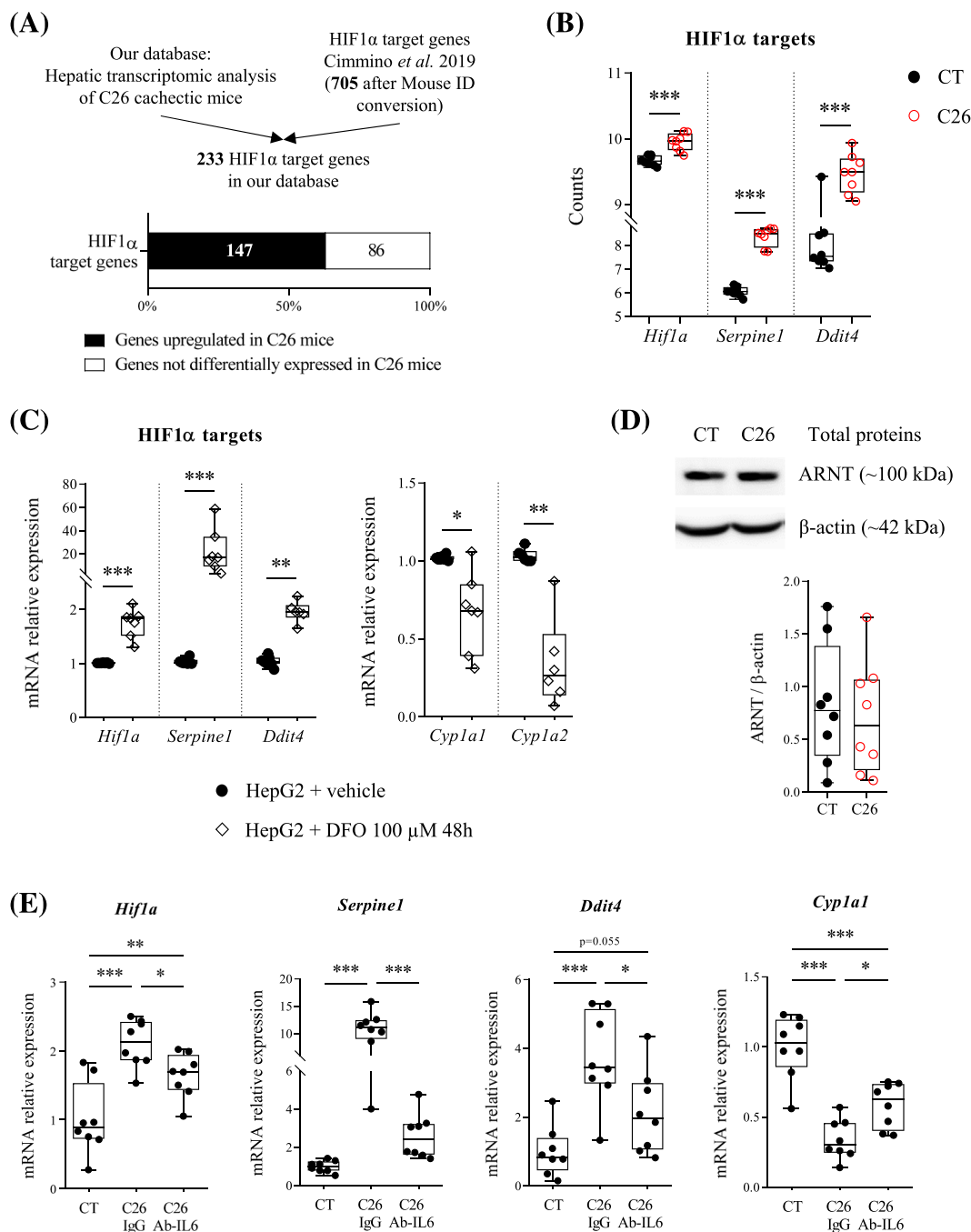


Figure 3 Interleukin-6 (IL-6)/hypoxia-inducing factor 1α (HIF1α) pathway is involved in the decrease in aryl hydrocarbon receptor (AHR) activation in the liver of C26 cachectic mice. (A) Stacked plot displaying HIF1α target genes identified in an existing experimentally determined dataset²⁵ upregulated or not differentially expressed in the liver of C26 mice.²⁶ Data were analysed with Fisher's exact test on a 2 × 2 contingency table. (B) Hepatic RNAseq data expressed in counts of three HIF1α targets in sham-injected mice (CT) and mice injected with cachexia-inducing C26 colon carcinoma cells (C26). Hypoxia-inducible factor 1, alpha subunit (*Hif1a*) (log₂ fold change [L2FC]: 0.42); serine (or cysteine) peptidase inhibitor, clade E, member 1 (*Serpine1*) (L2FC: 3.92); and DNA-damage-inducible transcript 4 (*Ddit4*) (L2FC: 1.91). (C) mRNA expression levels of *Hif1a*, *Serpine1*, *Ddit4*, cytochrome P450 family 1 subfamily A member 1 (*Cyp1a1*) and cytochrome P450 family 1 subfamily A member 2 (*Cyp1a2*) in HepG2 cells treated with or without 100-μM desferrioxamine (DFO), a hypoxia-mimetic agent inducing HIF1α, for 48 h. (D) Total protein expression levels of aryl hydrocarbon receptor nuclear translocator (ARNT) in the liver of CT and C26 mice, normalized to β-actin. (E) mRNA expression levels of *Hif1a*, *Serpine1*, *Ddit4* and *Cyp1a1* in the liver of sham-injected mice (CT), cachectic mice treated with a neutralizing antibody targeting IL-6 (C26 Ab-IL6) or an isotype control (C26-IgG). Data are presented with inter-quartile range (IQR) box and min-to-max whiskers. **P* < 0.05. ***P* < 0.01. ****P* < 0.001.

TIPARP is not responsible for the decrease in AHR activation by inducing its degradation.

The hypoxia-inducing factor 1 α (HIF1 α) can compete with AHR for binding to ARNT, leading to a disruption of the AHR/ARNT complex and the inhibition of AHR transcriptional activity.^{24,55} We therefore investigated the HIF1 α pathway as a candidate to explain the decrease in *Cyp1a* transcripts in the liver. We performed an overrepresentation analysis using an experimentally determined list of HIF1 α target genes²⁵ and found an enrichment of HIF1 α target genes among the genes upregulated in the C26 hepatic transcriptome (Figure 3A). Accordingly, we observed an increased expression of key HIF1 α targets (*Hif1a*, *Serpine1* and *Ddit4*) in cachectic liver that was intrinsically related to cachexia (Figures 3B and S4A). This hepatic modulation of HIF1 α target expression was also validated in other animal models (MC38 and APC^{Min/+}, Figure S4B,C). To determine if the upregulation of the HIF1 α pathway could be responsible for the decrease in AHR activation in the liver, we treated HepG2 cells, one of the most used human liver-based in vitro models, with desferrioxamine (DFO). This iron chelator mimics HIF1 α activation, through inhibition of its protein degradation.²⁷ We observed an increased expression of HIF1 α target genes and a decreased expression of *Cyp1a1* and *Cyp1a2* (Figure 3C) in treated cells. These results demonstrate that HIF1 α is a modulator of *Cyp1a1* and *Cyp1a2* expression, thereby of AHR activation. Interestingly, ARNT protein expression remained unaltered in C26 liver (Figure 3D), which is in accordance with the hypothesis of a disruption of the AHR/ARNT complex rather than a degradation of ARNT. To explore the direct interaction between AHR and ARNT, several co-immunoprecipitation assays were carried out. However, these assays were proven inconclusive due to the low level of endogenous expression of AHR in mouse liver.

The interleukin-6 (IL-6)/signal transducer and activator of transcription 3 (STAT3) pathway can promote the activation of HIF1 α signalling.²⁸ Consistently, we observed increased STAT3 phosphorylation in the liver of cachectic mice (Figure S4D), evidencing an activation of STAT3 pathway. To evaluate the role of IL-6 in the induction of HIF1 α signalling, cachectic mice were treated with a neutralizing IL-6 antibody. Such treatment counteracted the expression levels of the three HIF1 α targets and partially restored *Cyp1a1* expression level (Figure 3E). These results pinpoint the IL-6/HIF1 α pathway as a key mediator regulating *Cyp1a1* gene expression, thereby AHR activation in the liver of cachectic mice.

Regarding the other tissues, the mRNA expression of HIF1 α target genes remained generally unaltered in the ileum and GAS of cachectic mice (Figure S5A). Furthermore, the anti-IL-6 antibody administration showed no effect on *Cyp1a1* expression in both the ileum and GAS (Figure S5B).

Altogether, these results establish a differential modulation of AHR activation in the ileum, most probably by bacterial AHR ligands; in the liver, at least partially through the IL-6/HIF1 α pathway; and in other organs in cachectic mice.

AHR agonist treatment does not alleviate skeletal muscle atrophy and altered intestinal permeability

Next, CT and C26 mice were treated with FICZ, a pharmacological AHR agonist,^{13,56} to delineate the contribution of the AHR pathway to cachectic features. Body weight, food intake, and GAS, SAT and BAT weights were reduced in cachectic mice as compared to CT mice, but FICZ treatment did not affect these parameters (Figure S6). Liver weight remained unchanged between groups (Figure S6).

Various studies have shown high tumoural AHR expression and a tumour-promoter or tumour-suppressor role for AHR, depending on the tumour type.^{29,30} Here, C26 and C26-FICZ mice did not differ in terms of tumour weight and tumoural mRNA expression levels of *Cyp1a1* and pro-inflammatory cytokines well known to be involved in the development of cancer cachexia^{31,32} (Figure S7).

FICZ improved the expression of *Cyp1a1* in the ileum, colon and GAS (Figures 4A,B and S9A) and of *Cyp1a1* and *Cyp1a2* in the liver of cachectic mice (Figure 5A), thus demonstrating the efficacy of the AHR agonist. FICZ treatment did not alleviate the alteration in intestinal permeability (evidenced through an FITC-dextran assay, Figure S8) and the reduced expression of markers of the gut barrier function (Figure 4C,D). It also did not prevent the induction of markers of atrophy and inflammation in the muscle (Figure S9B–F). We did not find signs of intramuscular fat accumulation (myosteatosis) in C26 mice, as triglyceride levels remained unchanged in muscle tissue (Figure S9G). We observed no change in myofibre area, proportion of centrally nucleated fibres or collagen content in the skeletal muscle of C26 mice compared to healthy controls, independently of FICZ treatment (Figure S10).

We also investigated whether FICZ treatment reduced FGF21 levels, considering the ability of the later to activate adipose thermogenesis⁵¹ and promote muscle loss,⁴ two features of cancer cachexia.¹⁷ We did not see any effect of cachexia or FICZ on hepatic *Fgf21* mRNA expression levels, as well as on hepatic and plasma FGF21 levels (Figure S11).

Globally, these results show that the pharmacological activation of AHR by FICZ does not exert a protective effect against skeletal muscle atrophy and gut barrier dysfunction.

AHR agonist treatment improves hepatic inflammation and glycaemic disorders in cachectic mice

We next evaluated the impact of FICZ treatment on hepatic alterations. The expression of genes involved in liver inflammation (*Il1b*, *Ccl2* and *Cxcl2*), in the APR (*Apcs*, *Saa1*, *Saa2* and *Alb*), in macrophage activation (*Cd68*), in extracellular matrix deposition and remodelling (*Fga*, *Pcolce* and *Timp1*) and in neutrophil adhesion (*Icam1*) was improved in C26-FICZ mice as compared to C26 mice (Figure 5B–E). FICZ

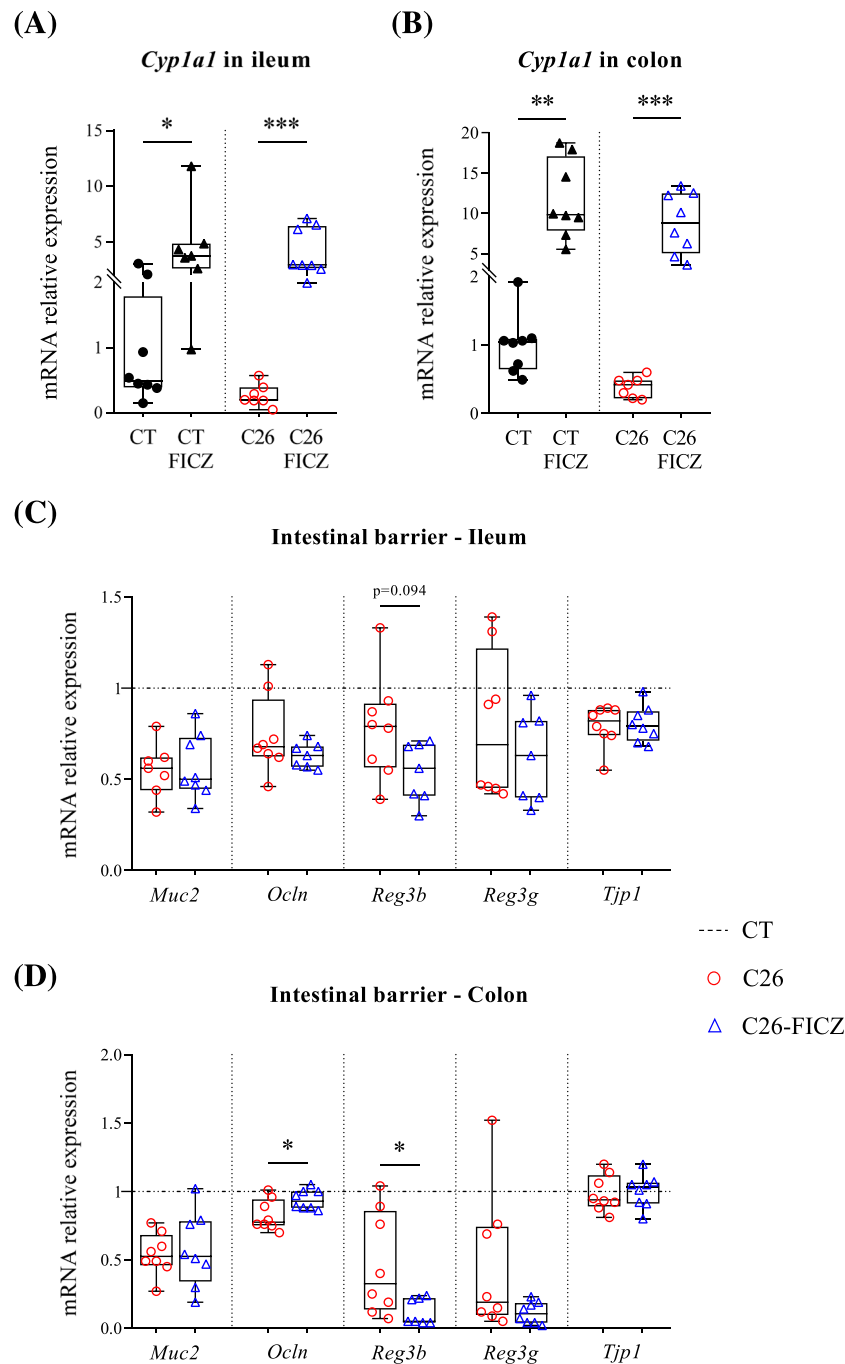


Figure 4 6-Formylindolo(3,2-*b*)carbazole (FICZ) treatment does not improve altered intestinal permeability in C26 cachectic mice. mRNA expression levels of cytochrome P450 family 1 subfamily A member 1 (*Cyp1a1*) in (A) the ileum and (B) colon of sham-injected mice (CT), sham-injected mice treated with FICZ (CT-FICZ), mice injected with cachexia-inducing C26 colon carcinoma cells (C26) and mice injected with cachexia-inducing C26 colon carcinoma cells and treated with FICZ (C26-FICZ). mRNA expression levels of five markers of intestinal barrier: mucin 2 (*Muc2*), occluding (*Ocln*), regenerating islet-derived 3 beta (*Reg3b*), regenerating islet-derived 3 gamma (*Reg3g*) and tight junction protein 1 (*Tjp1*), in (C) the ileum and (D) colon of CT, C26 and C26-FICZ mice. Mean expression of CT mice = 1 (dotted line). Data are presented with inter-quartile range (IQR) box and min-to-max whiskers. * $P < 0.05$. ** $P < 0.01$. *** $P < 0.001$.

treatment was also efficient in reducing hepatic levels of interleukin-1 β (IL-1 β) and had a positive effect on hepatic levels of IL-6 and CXCL2, although not significant (Figure 5F). We hypothesized that nuclear factor- κ B (NF- κ B) or STAT3 might

mediate the modulation of *Icam1* gene expression by FICZ as they have been described as activators of *Icam1* transcription.⁵⁷ However, FICZ treatment had no effect on STAT3 and NF- κ B nuclear translocation and phosphorylation

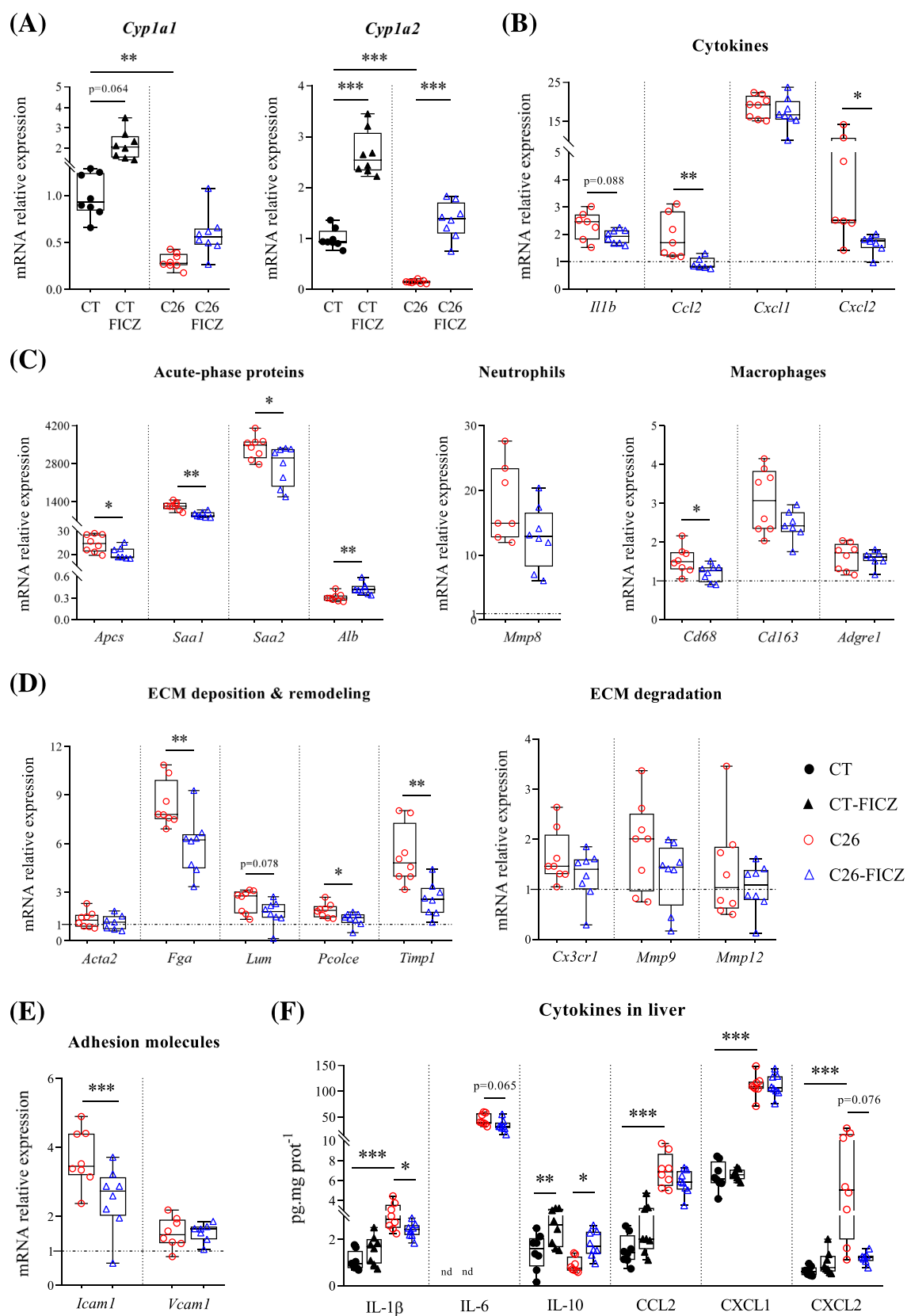


Figure 5 6-Formylindolo(3,2-*b*)carbazole (FICZ) treatment improves hepatic inflammation in C26 cachectic mice. (A) Hepatic mRNA expression levels of cytochrome P450 family 1 subfamily A member 1 (*Cyp1a1*) and cytochrome P450 family 1 subfamily A member 2 (*Cyp1a2*) in sham-injected mice (CT), sham-injected mice treated with FICZ (CT-FICZ), mice injected with cachexia-inducing C26 colon carcinoma cells (C26) and mice injected with cachexia-inducing C26 colon carcinoma cells and treated with FICZ (C26-FICZ). Hepatic mRNA expression levels of (B) one cytokine: interleukin 1 beta (*Il1b*), and three chemokines: chemokine (C–C motif) ligand 2 (*Ccl2*), C–X–C motif chemokine ligand 1 (*Cxcl1*) and C–X–C motif chemokine ligand 2 (*Cxcl2*); (C) three positive and one negative acute-phase proteins: amyloid P component, serum (*Apcs*), serum amyloid A 1 (*Saa1*), serum amyloid A 2 (*Saa2*) and albumin (*Alb*); one marker of neutrophil recruitment: matrix metalloproteinase 8 (*Mmp8*); three markers of macrophages: CD68 antigen (*Cd68*), CD163 antigen (*Cd163*) and adhesion G protein-coupled receptor E1 (*Adgre1*); (D) one marker of hepatic stellate cell activation: actin alpha 2, smooth muscle, aorta (*Acta2*); four markers of extracellular matrix deposition and remodelling: fibrinogen alpha chain (*Fga*), lumican (*Lum*), procollagen C-endopeptidase enhancer protein (*Pcolce*) and tissue inhibitor of metalloproteinase 1 (*Timp1*); three markers of extracellular matrix degradation: chemokine (C–X3–C motif) receptor 1 (*Cx3cr1*), matrix metalloproteinase 9 (*Mmp9*) and matrix metalloproteinase 12 (*Mmp12*); and (E) two adhesion molecules: intercellular adhesion molecule 1 (*Icam1*) and vascular cell adhesion molecule 1 (*Vcam1*) in CT, C26 and C26-FICZ mice. Mean expression of CT mice = 1 (dotted line). (F) Hepatic levels of cytokines and chemokines in CT, CT-FICZ, C26 and C26-FICZ mice. IL-6, interleukin-6; IL-10, interleukin-10; nd, not detected. Data are presented with inter-quartile range (IQR) box and min-to-max whiskers. **P* < 0.05. ***P* < 0.01. ****P* < 0.001.

(Figure S12). By contrast, the expression of other genes involved in liver inflammation (*Cxcl1*), in neutrophil adhesion and recruitment (*Vcam1* and *Mmp8*), in macrophage markers (*Cd163* and *Adgre1*), in hepatic stellate cell activation (*Acta2*) and in extracellular matrix degradation (*Cx3cr1*, *Mmp9* and *Mmp12*) was not changed in C26-FICZ mice (Figure 5B–E). Consistently, the positive area for the F4/80 protein, a major marker of murine macrophages encoded by the *Adgre1* gene, on liver sections was increased in C26 mice, as compared to CT mice, but remained the same in C26-FICZ mice (Figure S13A,B). Nonetheless, cachectic mice did not display overt liver inflammation and fibrosis upon haematoxylin and eosin (H&E) and Sirius Red-based histological examination (Figure S13C–G), which is coherent with an early stage of hepatic inflammation.⁵⁸ We measured plasma levels of pro-inflammatory cytokines and observed an increase in IL-1 β , IL-6, interleukin-10 (IL-10) and C–X–C motif chemokine ligand 1 (CXCL1) (mouse orthologue of IL-8) in cachectic mice compared to CT mice (Figure S14). FICZ treatment significantly lowered plasma levels of CXCL1 only, suggesting that the anti-inflammatory effect of AHR activation is largely limited to the liver. Overall, these data show that FICZ treatment partially counteracts liver inflammation in cachectic mice. These results were confirmed in a second independent in vivo experiment, demonstrating the robustness of our findings (Figure S15).

Previous work from our team has shown downregulation in hepatic gluconeogenesis, leading us to propose that amino acids are mainly used for acute-phase protein synthesis by the liver rather than to fuel gluconeogenesis.²¹ Concordantly, we observed a decrease in blood glucose, associated with an impairment of the expression of key genes involved in gluconeogenesis (*G6pc* and *Pck1*) in cachectic mice. FICZ treatment increased blood glucose and hepatic *G6pc* mRNA expression, with both parameters being significantly correlated (Figure 6A–C). *G6pc* expression was also impaired in the liver of MC38 mice and APC^{Min/+} mice, where it strongly correlated with AHR activation markers, *Cyp1a1* and *Cyp1a2* (Figure S16A–D), highlighting

the close relationship between AHR activation and gluconeogenesis.

We explored several hypotheses to explain the effect of AHR activation by FICZ treatment on gluconeogenesis in C26 mice. First, an analysis of the mouse *G6pc* promoter was conducted in silico using CiiIDER. We did not find AHR-binding sites in the promoter region of mouse *G6pc*, arguing against this hypothesis. Second, as HIF1 α activation reduces AHR activation and AHR activation promotes *G6pc* expression, we postulated that HIF1 α activation would reduce *G6pc* expression. Concordantly, the treatment of HepG2 cells by DFO tended to decrease *G6pc* mRNA expression (Figure S16E), revealing a link between HIF1 α and hepatic gluconeogenesis, probably through modulation of AHR activation. Third, as overexpression of AMPK has been associated to suppression of hepatic gluconeogenesis,⁵⁹ we investigated whether it could mediate the modulation of *G6pc* expression by FICZ, but we found no effect of the treatment on protein expression levels of AMPK and its phosphorylated form (Figure S17).

Next, we verified that the inflammatory and metabolic changes occurring in cachectic mice and corrected by FICZ were intrinsically related to cachexia (Figure S18). Taken together, this last set of analysis clearly demonstrates that AHR activation plays a therapeutic effect in the liver of cachectic mice and that the alterations of the AHR pathway strongly contribute to hepatic inflammatory and glycaemic alterations in cancer cachexia.

AHR activation correlates with the expression of a gluconeogenic marker in liver biopsies from cancer patients

Finally, to explore the translational value of our findings, we analysed the expression of *Cyp1a1*, *Cyp1a2* and *G6pc* in human liver biopsies collected from cancer patients. We found a strong correlation between these markers (Figure 6D).

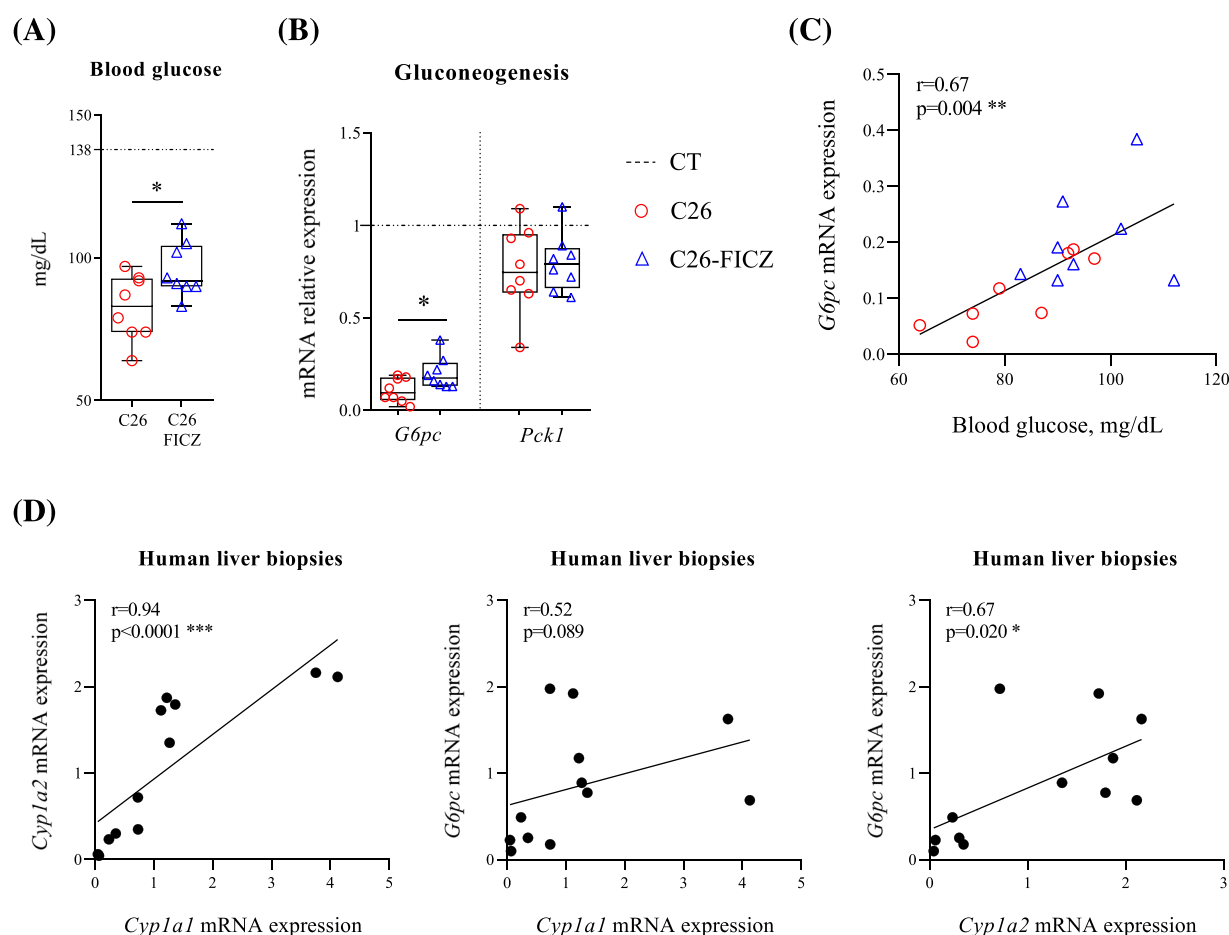


Figure 6 6-Formylindolo(3,2-*b*)carbazole (FICZ) treatment improves glycaemic disorders in C26 cachectic mice through regulation of hepatic gluconeogenesis. Concordantly, aryl hydrocarbon receptor (AHR) activation and gluconeogenesis markers correlate together in liver biopsies from cancer patients. (A) Blood glucose levels in sham-injected mice (CT), mice injected with cachexia-inducing C26 colon carcinoma cells (C26) and mice injected with cachexia-inducing C26 colon carcinoma cells and treated with FICZ (C26-FICZ). Mean blood glucose level of CT mice = 138 (dotted line). (B) Hepatic mRNA expression levels of two markers of gluconeogenesis: glucose-6-phosphatase (*G6pc*), catalytic; phosphoenolpyruvate carboxykinase 1 (*Pck1*), cytosolic in CT, C26 and C26-FICZ mice. Mean expression of CT mice = 1 (dotted line). Data are presented with inter-quartile range (IQR) box and min-to-max whiskers. $*P < 0.05$. (C) Pearson correlation between hepatic mRNA expression levels of *G6pc* and blood glucose levels in C26 and C26-FICZ mice. (D) Spearman correlations between mRNA expression levels of cytochrome P450 family 1 subfamily A member 1 (*Cyp1a1*), cytochrome P450 family 1 subfamily A member 2 (*Cyp1a2*) and *G6pc*, measured in liver biopsies from cancer patients.

Discussion

Cancer cachexia is a major public health concern worldwide, particularly as cancer rates rise. Patients' quality of life and survival are reduced, and response to cancer treatments is endangered because this complex multifactorial syndrome remains undertreated. Apart from the skeletal muscle and adipose tissue, the gut and liver are also deeply affected in cancer cachexia but are still overlooked in scientific research and clinical practice. Here, we show for the first time that AHR activation is deeply impaired in multiple models of cancer cachexia. This reduction occurs independently of food intake and is intrinsically related to cachexia. We report a differential modulation of AHR activation in the liver, ileum and

other organs, as well as the beneficial effects of AHR activation on hepatic inflammation and glycaemic disorders. Interestingly, this last aspect is supported by our pilot clinical analyses.

Strikingly, the reduction in hepatic alterations is not associated with an improved gut barrier function. On the one hand, these results are consistent with previous observations from our lab showing that indole administration, an AHR activator, had anti-inflammatory and hepatoprotective properties, away from the gut.^{33,34} On the other hand, this is dissimilar to previous studies that reported that FICZ treatment was sufficient to correct the intestinal barrier dysfunction associated with inflammatory bowel disease,³⁵ metabolic syndrome¹³ and intestinal obstruction.³⁶ The discrepancy

could be linked to the source of altered gut barrier function. Indeed, intestinal inflammation and HIF1 α have been described as key regulators of intestinal epithelial tight junction proteins.³⁷ However, C26 cachectic mice do not display local intestinal inflammatory response²³ nor do they show an increase in HIF1 α target genes in the ileum, thus arguing against an involvement of both factors. Our study raises the likelihood of another source of altered gut barrier function in cancer cachexia, where AHR is not involved, and that needs to be further explored, as restoring gut barrier function may provide benefits.³⁸

Our results identify the IL-6/HIF1 α pathway as a key mediator of AHR activation in the liver of cachectic mice. ARNT has been described as a DNA-binding partner for both AHR and HIF1 α . Therefore, the upregulation of the HIF1 α pathway in cachectic liver could be responsible for the decrease in AHR activation by competing with AHR for binding to ARNT. Treating C26 mice with FICZ partially counteracts the hepatic expression levels of *Serpine1* and *Ddit4*, two HIF1 α targets (Figure S19). FICZ treatment seems to shift the balance, probably leading to a disruption of the HIF1 α /ARNT complex and the reduction of HIF1 α transcriptional activity, which is in accordance with our hypothesis. As HIF1 has been proposed as a promising target for cancer chemotherapy,^{39,510,511} it would be interesting to evaluate whether a treatment with a pharmacological inhibitor of HIF1 could also foster AHR activation and alleviate cachectic features, beyond an anti-cancer effect.

The reduction of hepatic damage by AHR agonist treatment appears to be attributable to its direct effect on the liver. This is consistent with previous reports of the dampening effects of AHR activation on liver inflammation.³ Interestingly, we also observed an effect on liver macrophages and cytokines involved in macrophage recruitment following indole supplementation in metabolic fatty liver disease.³³ We now propose that AHR acts as a master switch between hepatic inflammatory and glycaemic disorders in cancer cachexia. Muscle proteins are massively degraded in cachexia, which results in a significant amino acid efflux to the circulation reaching the liver.⁵¹² We previously determined that these amino acids are mainly used for acute-phase protein synthesis rather than to sustain hepatic gluconeogenesis.²¹ As AHR activation can directly repress cytokine-induced APR in the liver,⁴⁰ such AHR-mediated repression could promote the use of amino acids to fuel gluconeogenesis and counteract hypoglycaemia. Altogether, these results suggest a metabolic shift towards the use of amino acids to produce acute-phase proteins at the expense of gluconeogenesis, in which AHR plays a central role. This mechanism is specific to cachectic mice as we did not observe anti-inflammatory effects or hyperglycaemia in CT mice treated with the AHR agonist. Further studies will be needed to clarify how AHR improves hepatic gluconeogenesis in cancer cachexia.

A recent review outlined the fact that AHR pathway may have cell type-specific regulatory effects in liver disease.³

Using an existing dataset,⁴¹ we found that AHR protein is primarily expressed in hepatic stellate cells and also in smaller quantities in liver sinusoidal endothelial cells, Kupffer cells, cholangiocytes and hepatocytes. This differential expression of AHR in liver cell subtypes might be an explanation as to why FICZ treatment improved the hepatic expression of some genes involved in liver inflammation but not others.

Our findings corroborate those of Narasimhan et al., who reported a significant inhibition of AHR signalling in the SAT of cachectic patients with pancreatic ductal adenocarcinoma as compared to non-cancer patients,⁴² thus confirming the relevance of impaired AHR signalling in the context of human cachexia. Our results provide the basis for further studies that will aim to explore the potential implication of AHR in the cachexia syndrome. Of note, an AHR-mitochondria crosstalk has been reported in the literature.⁴³ Briefly, it was shown that the basal mitochondrial reactive oxygen species (ROS) levels were lower in AHR^{-/-} mice compared to wild-type mice and that the removal of damaged mitochondria by mitophagy was reduced by AHR knockdown in vitro. Studies have also suggested that AHR is localized within the mitochondria, inside the intermembrane space in murine hepatoma cells, but probably only in the absence of ligands.⁴³ In the cachexia syndrome, several clinical and preclinical studies demonstrated mitochondrial alterations in the skeletal muscle¹⁵ and the liver,¹⁹ but the influence of different AHR modulators on mitochondria in the context of cachexia has never been explored. Given that AHR is widely repressed in all models of cachectic mice, differentially modulated in tissues and has pleiotropic functions and target genes, this may represent a promising axis of research to further delineate the cell-specific role of AHR pathways in cachectic development.

Correcting AHR activation deficiency improves hepatic cachectic features, with no effect on muscle atrophy in cachectic mice. However, we cannot rule out a beneficial impact of FICZ on muscle function. Of note, our mice would be considered moderately cachectic (10% loss of baseline body mass) according to the current literature.^{513,514} This 'moderately cachectic' stage may explain why we found no obvious changes in muscle histology and triglyceride content.⁵¹⁵

Because skeletal muscle wasting is a key feature of cancer cachexia, at this point, our results do not support a benefit from the administration of a direct AHR agonist alone to cachectic patients. However, recent therapies for the cachectic syndrome involve a multimodal approach combining nutritional support with exercise and pharmacological agents. As it has been shown that *Lactobacillus reuteri*, known to produce AHR agonists, or other direct AHR ligands, tryptophan derivatives and indoles, can improve metabolic syndrome¹³ and alcohol-induced liver injury⁹ in animal models, a probiotic approach or nutritional advice that boosts AHR activation could be a new therapeutic component in the multimodal approach to tackle cancer cachexia.

In conclusion, our study shows that AHR activation is deeply impaired and differentially modulated in the liver compared to other organs in cancer cachexia. Pharmacological activation of AHR was sufficient to mediate an improvement in hepatic inflammation and glycaemic disorders in cachectic mice, independently of an amelioration of the gut barrier dysfunction. As there is currently no approved treatment that reverses cancer cachexia, we believe these findings are of great importance for the development of new strategies aimed at tackling cancer cachexia. Boosting the AHR activation using a probiotic or a nutritional approach may hold promise in the development of novel complementary therapeutic tools to treat or prevent cancer cachexia.

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Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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