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# Study of the toxic effect of the estrogenic mycotoxin Zearalenone on the small intestine

Tarek Lahjouji

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Université  
de Toulouse

# THÈSE

En vue de l'obtention du

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M. TAREK LAHJOUJI

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Etude de l'effet toxique de la mycotoxine oestrogénique Zéaralénone sur l'intestin grêle

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Toxicologie Alimentaire (ToxAlim)

**Directeur(s) de Thèse :**

MME ISABELLE OSWALD

MME LAURA SOLER-VASCO

**Rapporteurs :**

Mme NADIA EVERAERT, GEMBLoux AGRO-BIO TECH

M. PASCAL FROMENT, PRC NOUZILLY

**Membre(s) du jury :**

M. JEAN-DENIS BAILLY, ECOLE NATIONALE VETERINAIRE DE TOULOUSE, Président

Mme ISABELLE OSWALD, INRA TOULOUSE, Membre

Mme LAURA SOLER-VASCO, Laboratoire de toxicologie alimentaire, Membre

Mme MARIE-LOUISE SCIPPO, UNIVERSITE DE LIEGE, Membre

# Résumé

La zéaralénone (ZEN) est l'une des mycotoxines produites par les champignons du genre *Fusarium*, qui contaminent les aliments à base de céréales ainsi que les aliments destinés aux animaux. Après une exposition par voie orale, la ZEN est métabolisée dans divers tissus, y compris l'intestin. Les principaux métabolites sont  $\alpha$ -Zearalenol ( $\alpha$ -ZEL) et  $\beta$ -Zearalenol ( $\beta$ -ZEL). La ZEN est connue pour avoir des effets œstrogéniques en raison de sa structure chimique similaire à l'œstradiol, ce qui lui permet l'interaction directe avec les récepteurs des œstrogènes ER $\alpha$  et ER $\beta$ .

Les effets de ZEN sur la reproduction ont été bien décrits chez plusieurs espèces. En revanche, l'impact de la ZEN sur d'autres organes reste toujours peu connu. C'est le cas pour l'intestin, qui représente le premier organe cible des contaminants alimentaires. Cette étude qui utilise un modèle d'explants jéjunaux porcins, nous étudions les changements de l'expression des gènes et de l'abondance des protéines induites par une exposition aiguë à la ZEN. Dans un deuxième temps, nous avons exploré l'effet combiné de la ZEN et de l' $\alpha$ -ZEL sur l'intestin en terme d'additivité, de synergie ou d'antagonisme, en utilisant comme modèle *in vitro*, les cellules épithéliales intestinales humaines Caco-2.

Les résultats indiquent que la ZEN peut moduler la voie de signalisation Wnt /  $\beta$  - caténine et induire l'expression de biomarqueurs de prolifération. Ceci est associé à une accumulation des ER $\alpha$  dans l'intestin, à une modulation de l'expression des récepteurs des adipokines et de l'activité antimicrobienne sans induire l'inflammation.

La ZEN et son métabolite  $\alpha$ -ZEL ont un effet cytotoxique dose-dépendant sur les cellules épithéliales intestinales Caco-2. Nos résultats indiquent une synergie de l'effet cytotoxique quand les deux toxines sont présentes simultanément. En

conséquence, la co-exposition au ZEN et à l' $\alpha$ -ZEL pourraient entraîner un effet plus fort que celui résultant de l'exposition à chaque toxine.

L'ensemble de nos résultats montrent clairement que l'intestin est une cible de la mycotoxine ZEN, comme l'explique les mécanismes moléculaires observés.

# Summary

Zearalenone (ZEN) is one of the stable mycotoxins produced by fungi of the genus *Fusarium*, which contaminate grain-based foods and animal feeds. Following oral exposure, ZEN is metabolized in various tissues, including the intestine. The major metabolites are  $\alpha$ -Zearalenol ( $\alpha$ -ZEL) and  $\beta$ -Zearalenol ( $\beta$ -ZEL). ZEN is known to have estrogenic effects due to its estrogen-like chemical structure, which allows it to interact directly with the estrogen receptors ER $\alpha$  and ER $\beta$ .

The reproductive effects of ZEN have been well elucidated in several species. However, the impact of ZEN on other organs is still poorly understood. As is the case for the intestine, which is the first organ affected by food contaminants. In the present study, and using a porcine jejunal explant model, we are studying the changes in gene expression and protein abundance induced by acute exposure to ZEN.

Next, we explored the combined effect of ZEN and  $\alpha$ -ZEL on the gut in terms of additivity, synergy or antagonism, using an in vitro model, human intestinal epithelial cells Caco-2.

The results indicate that ZEN can modulate the Wnt /  $\beta$  - catenin signaling pathway and induce the expression of proliferation biomarkers. This is associated with an accumulation of ER $\alpha$  in the gut, modulation of adipokine receptor expression and antimicrobial activity without inducing inflammation.

We observed that ZEN and its metabolite  $\alpha$ -ZEL have a dose-dependent cytotoxic effect on Caco-2 intestinal epithelial cells. Our results also indicate a synergistic cytotoxic effect when the two toxins are present in a mixture. As a result, co-exposure to ZEN and  $\alpha$ -ZEL could result in a stronger effect than that resulting from exposure to each toxin alone.

All our results clearly show that the intestine is a target for the mycotoxin ZEN, as well as the molecular mechanisms that explain these effects.

*A*

*mes parents*

*ma femme Salma*

*ma fille Yakout*

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# Abbreviations

| <b>Abbreviation</b> | <b>Signification</b>  |
|---------------------|---|
| <b>AdipoR2</b>      | Adiponectin receptor 2  |
| <b>AFs</b>          | Aflatoxins  |
| <b>AJs</b>          | Adherens junctions  |
| <b>ALP</b>          | Alkaline Phosphatase  |
| <b>ALT</b>          | Alanine Aminotransferase                                      |
| <b>AMP</b>          | Antimicrobial products  |
| <b>ANXA2</b>        | Annexin A2  |
| <b>Ang4</b>         | Angiogenin-4  |
| <b>APC</b>          | Adenomatous polyposis coli                                    |
| <b>ASK1</b>         | Apoptosis signal-regulating kinase1                           |
| <b>AST</b>          | Aspartate Aminotransferase                                    |
| <b>BMPs</b>         | Bone morphogenetic proteins                                   |
| <b>BTRC</b>         | Beta-Transducin Repeat Containing E3 Ubiquitin Protein Ligase |
| <b>CAP1</b>         | Adenylyl cyclase-associated protein                           |
| <b>CAT</b>          | Catalase  |
| <b>CBC</b>          | The columnar base cells                                       |
| <b>CCND1</b>        | Cyclin D1   |
| <b>CD44</b>         | CD44 Molecule (Indian Blood Group)                            |
| <b>CHOP</b>         | Homologous protein  |
| <b>CFL1</b>         | Cofilin-1   |
| <b>CI</b>           | Combination index   |
| <b>CK1</b>          | Casein kGase 1  |
| <b>CMKLR1</b>       | Chemerin-like receptor1                                       |
| <b>COMT</b>         | Catechol-O- methyltransferase                                 |
| <b>Dkk1</b>         | Dickkopf-related protein 1                                    |
| <b>DNA</b>          | Deoxyribonucleic acid   |
| <b>DON</b>          | Deoxynivalenol  |
| <b>E2</b>           | Estradiol   |
| <b>EDs</b>          | Endocrine disruptors  |
| <b>EFSA</b>         | European Food Safety Authority                                |
| <b>EGF</b>          | Epidermal growth factor                                       |
| <b>ER</b>           | Reticulum endoplasmic   |
| <b>ERE</b>          | Estrogen -responsive element                                  |
| <b>ERK</b>          | Extracellular signal-regulated kinase                         |
| <b>ESR1</b>         | Estrogen receptor 1   |
| <b>FBs</b>          | Fumonisin   |
| <b>FSH</b>          | Follicle-stimulating hormone                                  |
| <b>FZD</b>          | Frizzled family receptors                                     |
| <b>GI</b>           | Gastrointestinal  |
| <b>GnRHr</b>        | Gonadotropin-releasing hormone receptor                       |
| <b>GPx</b>          | Glutathione peroxidase  |

|                                  |   |
|----------------------------------|---|
| <b>GSH</b>                       | Glutathione   |
| <b>GSK3<math>\beta</math></b>    | Glycogen synthase kinase 3 $\beta$                        |
| <b>GST</b>                       | Glutathione S-transferase                                 |
| <b>HepG2</b>                     | Human Hepatoma cells                                      |
| <b>HL60</b>                      | Human promyelocytic leukemia                              |
| <b>HO1</b>                       | Heme Oxygenase1   |
| <b>HSD</b>                       | Hydroxysteroid dehydrogenase                              |
| <b>IBD</b>                       | Inflammatory bowel disease                                |
| <b>Ig</b>                        | Immunoglobuline   |
| <b>INF<math>\gamma</math></b>    | Interferon gamma  |
| <b>IPEC-1</b>                    | Porcine epithelial cells                                  |
| <b>ISC</b>                       | Intestinal stem cells                                     |
| <b>IUPAC</b>                     | International Union of Pure and Applied                   |
| <b>JNK</b>                       | Jun amino terminal kinase                                 |
| <b>LCN2</b>                      | Lipocalin 2   |
| <b>LEF</b>                       | Lymphoid enhancer-binding factor                          |
| <b>LH</b>                        | Luteinizing hormone                                       |
| <b>LPO</b>                       | Lipid peroxidation  |
| <b>LRP5/6</b>                    | Low-density lipoprotein receptor-related protein 5/6      |
| <b>MAPK</b>                      | Mitogen-activated protein kinases                         |
| <b>MCF-7</b>                     | Michigan Cancer Foundation – 7 cells                      |
| <b>MCM3</b>                      | Minichromosome Maintenance Complex Component 3            |
| <b>MDA</b>                       | Malondialdehyde   |
| <b>MMP</b>                       | Mitochondrial membrane potential                          |
| <b>MMPs</b>                      | Matrix Metalloproteinases                                 |
| <b>NLRP3</b>                     | NLR Family Pyrin Domain Containing 3                      |
| <b>NO</b>                        | Nitrogen oxide  |
| <b>OTA</b>                       | Ochratoxin A  |
| <b>PBMC</b>                      | Mononuclear peripheral blood cells                        |
| <b>PCDH11X</b>                   | Protocadherin 11 X-linked                                 |
| <b>PI3K</b>                      | Phosphatidyl-inositol 3-kinases                           |
| <b>PLC</b>                       | Phospholipase C   |
| <b>PPAR<math>\gamma</math></b>   | Peroxisome proliferator-activated receptor $\gamma$       |
| <b>PXR</b>                       | Pregnane X receptor                                       |
| <b>qPCR</b>                      | Quantitative PCR  |
| <b>RegIII<math>\gamma</math></b> | Regenerating the protein derived from islets III $\gamma$ |
| <b>ROS</b>                       | Reactive oxygen species                                   |
| <b>SAA3</b>                      | Serum amyloid A3  |
| <b>SOD</b>                       | Superoxide dismutase                                      |
| <b>TA</b>                        | Transit amplifying  |
| <b>TCF</b>                       | Transcription factor                                      |
| <b>TDI</b>                       | Tolerable daily intake                                    |
| <b>TGF-<math>\beta</math></b>    | Transforming growth factor $\beta$                        |
| <b>TJs</b>                       | Tight junctions   |

|                                  |   |
|----------------------------------|---|
| <b>TLR1-10</b>                   | Toll like receptors(1-10)               |
| <b>TNF-<math>\alpha</math></b>   | Tumor necrosis factor $\alpha$          |
| <b>U937</b>                      | Human promonocytic                      |
| <b>UDPGA</b>                     | Uridine 5'- diphosphate glucuronic acid |
| <b>VIM</b>                       | Vimentin                                |
| <b>WHO</b>                       | World Health Organization               |
| <b>Wnt</b>                       | Wingless integration                    |
| <b>ZEN</b>                       | Zearalenone                             |
| <b>ZO-1</b>                      | Zonula occludens-1                      |
| <b><math>\alpha</math>-1 AGP</b> | Alpha-1acid glycoprotein                |
| <b><math>\alpha</math>-ZEL</b>   | Alpha-Zearalenol                        |
| <b><math>\beta</math>-ZEL</b>    | Beta- Zearalenol                        |



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# Introduction

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## 1.1. Intestine

The gastrointestinal tract is formed by a continuous tubular structure including the stomach, the small intestine (duodenum, jejunum, ileum) and the large intestine. The basic functions is to transport any ingested food, digest it, absorb the micronutrients, electrolytes and water, and waste excretion (Cheng *et al.*, 2014).

The gastrointestinal tract represents the largest area of exchange between the body and the outside environment. The digestive tract is also the first surface exposed to food but also to xenobiotics, while maintaining a defense against intraluminal toxins, bacteria, and antigens, which the humans and animals are exposed in life daily. This digestive tract capacity highlights the importance of the intestinal barrier. The first line of defense in the gastrointestinal tract is found at the intestinal lumen, where microorganisms and antigens are degraded in a nonspecific fashion by pH and gastric, pancreatic and biliary secretions (Salvo-Romero *et al.*, 2015). The second line constitute the ecological barrier which corresponds to the normal intestinal flora that participates in the establishment and maintenance of the function of the intestinal barrier. Furthermore, the epithelial cells are the strongest determinants of the physical intestinal barrier, is composed of the epithelium that ensures the selective passage of molecules and secretions from epithelial cells such as mucus and antimicrobial peptides. Transport of molecules is regulated through the presence of junctional complexes. The three most important complexes are tight junctions (TJs), adherens junctions (AJs) and desmosomes (Groschwitz and Hogan, 2009). The immunological barrier consists of immune cells, which secrete immunoglobulins and cytokines necessary for the establishment of immune responses against potentially dangerous microorganisms (Spahn and Kucharzik, 2004). The mesenchyme is a tissue for physical maintenance and nutrition of the intestinal epithelium by the intervention of blood vessels passing through it and which supply all the tissues with oxygen and nutrients.

The small intestinal mucosa is arranged into two fundamental structures: villi and crypts. Villi are projections into the lumen covered predominantly with mature, absorptive enterocytes, along with occasional mucus-secreting goblet cells. These cells live only for 3 to 5 days, die and are shed into the lumen to become part of the ingesta to be digested and absorbed. Crypts (of Lieberkuhn) are invaginations of the epithelium around the villi, and are lined largely with younger epithelial cells. Toward the base of the crypts are stem cells, which continually divide and provide the source of all the epithelial cells in the crypts and on the villi. The intestinal epithelium is constantly renewing itself. This rapid cell renewal depends on the intestinal epithelial pluripotent stem cells, the columnar base cells (CBC) crypts, reside deep within the crypts and generate cells that migrate to the upper villi where final differentiation takes place (Booth and Potten, 2000). These cells depend on signals and growth factors provided by local niche cells to support their function and self-renewal (Kaestner, 2019). Different types of cells grow from intestinal stem cells (ISC) to form the epithelial barrier (Figure 1). Small intestinal villi epithelium contains mainly absorptive enterocytes that also secrete hydrolytic enzymes to facilitate the efficient breakdown of partly digested food exiting the stomach and the absorption of nutrients. Goblet cells, which secrete mucus, together with much smaller populations of enteroendocrine cells, which produce hormones are also present. Goblet cells are most prevalent in the epithelium of the short villi of the distal small intestine, where they provide the extra lubrication needed to facilitate the passage of increasingly compact stool towards the colon.

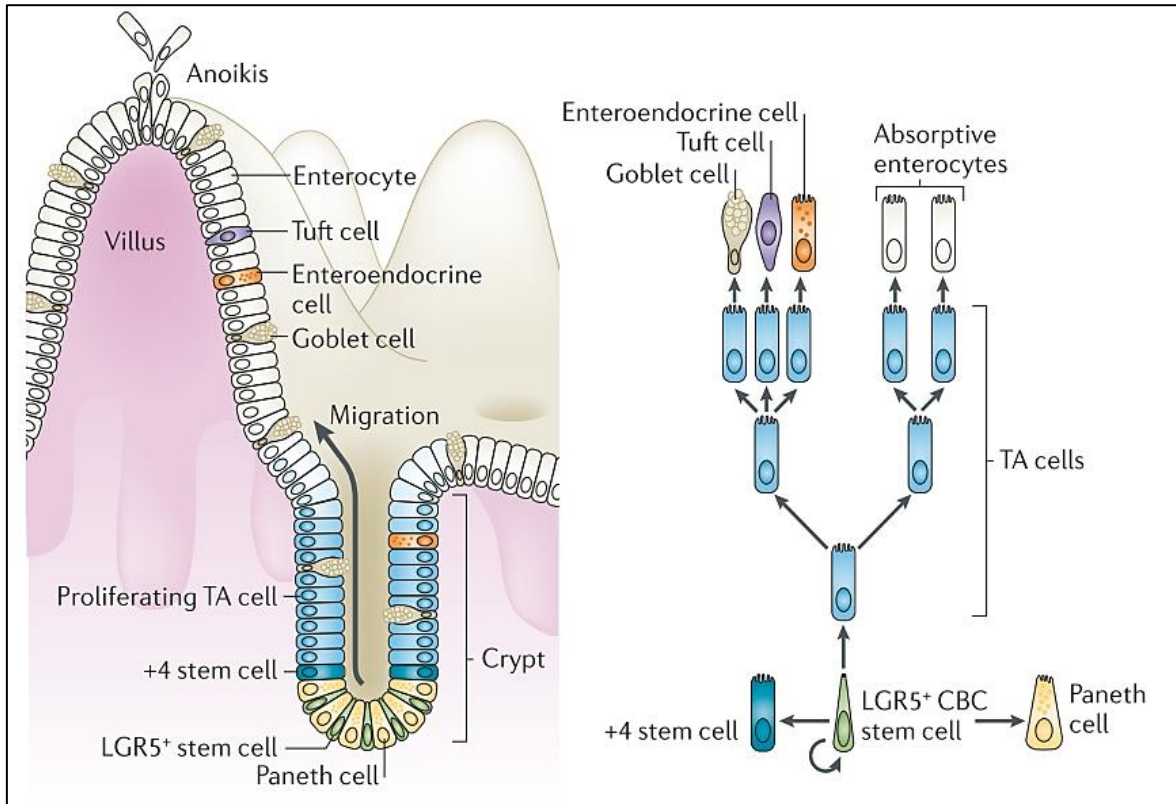


Figure 1 : **Epithelial self-renewal in the intestinal epithelium.** In the small intestine LGR5+(Leu-rich repeat-containing G protein-coupled) crypt base columnar (CBC) stem cells are intercalated with Paneth cells at the crypt base (middle panel). These stem cells continuously generate rapidly proliferating transit-amplifying (TA) cells, which occupy the remainder of the crypt. TA cells differentiate into the various functional cells on the villi (enterocytes, tuft cells, goblet cells and enteroendocrine cells) to replace the epithelial cells being lost at the villus tip. This differentiating hierarchy is shown in the tree on the right panel. Epithelial turnover occurs every 3–5 days. New Paneth cells are supplied from the TA cells every 3–6 weeks. (Barker, 2013).

Paneth cells exist exclusively at the bottom of the crypts. They are responsible for the production of a variety of antimicrobial agents including lysozyme,  $\alpha$ -defensins and phospholipase A2. In humans, Paneth cells express two types of  $\alpha$ -defensins, human defensin HD5 and HD6 (Clevers and Bevins, 2013). They also secrete other antimicrobial products (AMP), such as lysozyme, secretory phospholipase A2 and regenerating the protein derived from islets III $\gamma$  (RegIII $\gamma$ ) (Clevers and Bevins, 2013). These antimicrobial peptides and proteins are packaged and secreted via Paneth cell granules, in particular, angiogenin-4 (Ang4), RegIII $\gamma$  and  $\alpha$ 1-antitrypsin (Holly and Smith, 2018). Paneth cells also serve as an important

source of growth factors for CBC cells, to which they are physically associated, thus governing the abovementioned epithelial tissue renewal.

To summarize, the functions of the gastrointestinal tract include digestion, absorption, excretion, and protection. In order to maintain the normal function and integrity of the intestinal epithelium, it is necessary for this tissue to have a high in order to replace lost or damaged apical cells. These cells depend on signals and growth factors provided by local niche cells to support their function and self-renewal.

## **1.2. Essential crypts signaling pathways**

The intestinal epithelium ensures the absorption of nutrients. The epithelium and the mesenchyme cooperate to generate the dynamic complex observed in the intestinal crypts. Together, they provide the key signals that regulate the determination of intestinal fate. In fact, multiple molecular pathways are involved in the regulation of intestinal epithelial cell polarity including: Wnt, Notch, EGF and BMP signaling (Figure 2) (Sancho *et al.*, 2004; Gehart and Clevers, 2019) .

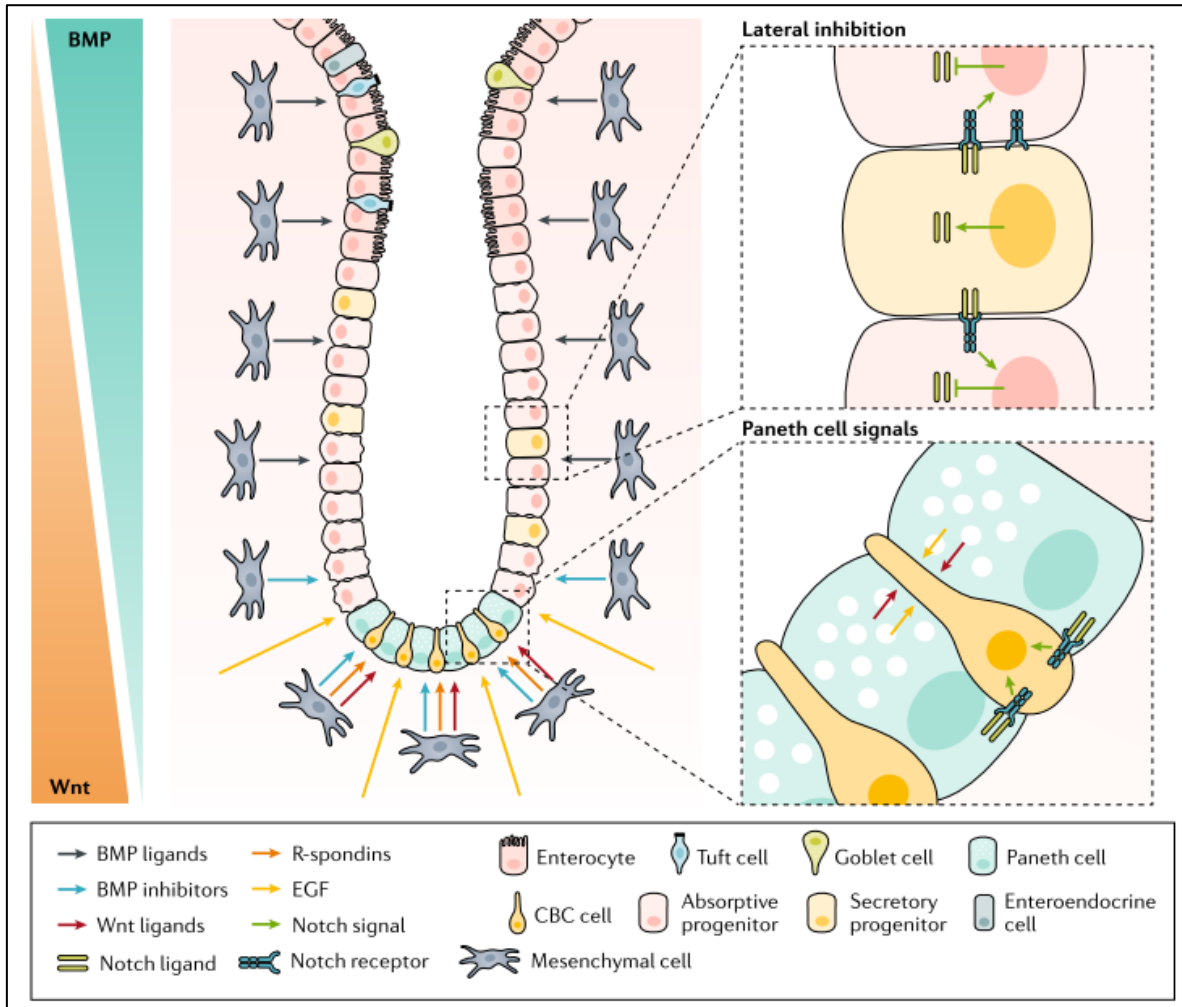


Figure 2: **Main regulatory signals and their sources in the intestinal crypt.** Two counteracting gradients run along the crypt–villus axis: Wnt and bone morphogenetic protein (BMP) signaling. At the bottom of the crypt, Wnt and the Wnt potentiator R-spondin are secreted by mesenchymal and Paneth cells, which induces high Wnt pathway activity in CBC cells. A gradient of increasing BMP activity stands in direct opposition to Wnt. Intercrypt and intervillus mesenchymal cells secrete BMPs that induce maturation in ascending epithelial cells (Gehart and Clevers, 2019).

### 1.2.1. TGF- $\beta$ signaling pathway

The transforming growth factor (TGF- $\beta$ ) signaling regulates embryonic development, wound healing, proliferation, and cell differentiation (Blobe *et al.*, 2000; Shi *et al.*, 2011). The TGF- $\beta$  family of cytokines comprises bone morphogenetic proteins (BMPs), and activin (Sancho *et al.*, 2004). Signaling components of TGF- $\beta$  are expressed in the differentiated compartment of the intestine. Signaling is induced by binding of the TGF $\beta$ 1 ligand dimer to the TGF- $\beta$  receptor type-2 (TGF $\beta$ R2) promotes dimerization of TGF $\beta$ R2 with TGF $\beta$ R1 and results in

transphosphorylation of TGF $\beta$ R1. The activated TGF- $\beta$  receptor type-1 activates R-SMADs (SMAD2 and SMAD3) via phosphorylation. SMAD2 and SMAD3 trimerize with a co-SMAD (SMAD4). The SMAD trimer enters the nucleus to activate gene transcription and promote cell growth and survival such as Snail family of transcriptional repressors and matrix metalloproteinases (Jeon *et al.*, 2013) (Figure 3).

In the intestine, TGF- $\beta$  cytokine family members are secreted by stromal cells and enterocytes and engage in different autocrine and paracrine signaling along the crypt-villus axis, cooperating with others pathways to regulate enterocyte proliferation, differentiation and migration (Hasson *et al.*, 2014). Signaling by TGF- $\beta$  superfamily members is disrupted in various intestinal hyperproliferative states, including colorectal cancer (Fearon and Vogelstein, 1990).

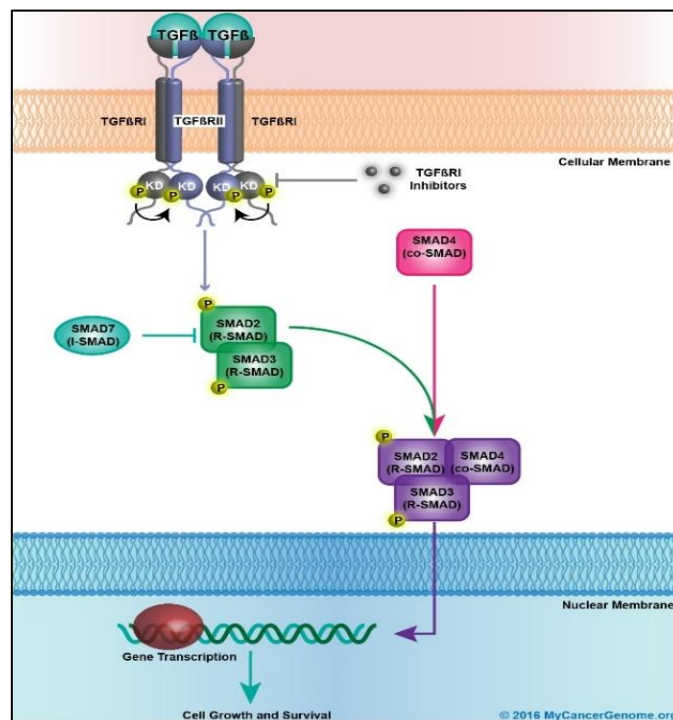


Figure 3 : **TGF- $\beta$  signaling pathway.** Binding of the TGF- $\beta$  ligand dimer to the TGF-beta receptor type-2 (TGFBR2) promotes dimerization of TGFBR2 with TGFBR1 and results in transphosphorylation of TGFBR1. The activated TGF-beta receptor type-1 activates R-SMADs (SMAD2 and SMAD3) via phosphorylation. SMAD2 and SMAD3 trimerize with a co-SMAD (SMAD4). The SMAD trimer enters the nucleus to activate gene transcription and promote cell growth and survival.

### 1.2.2. Wnt/ $\beta$ -catenin signaling pathway

The canonical Wnt/ $\beta$ -catenin signaling pathway is the major player involved in the establishment of tissue architecture during development and is important for the homeostasis of a variety of adult tissues, including the intestine. Numerous studies conducted both *in vivo* and *in vitro* have firmly established the role of Wnt signaling in the preservation of stem cell proliferation and pluripotency (Krausova and Korinek, 2014). Wnt controls three major signaling pathways: the Wnt/calcium pathway, the planar cell polarity pathway, and the canonical Wnt pathway (Deitrick and Pruitt, 2016), being the latter the most important for intestinal physiology. Canonical Wnt signals are transduced through Frizzled (FZD) family receptors and LRP5/LRP6 coreceptor to the  $\beta$ -catenin signaling cascade (Figure 4). In the absence of Wnt ligands (A),  $\beta$ -catenin complexed with Adenomatous polyposis coli (APC) and scaffolding protein AXIN is phosphorylated by casein kinase 1 (CK1) and glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ), which is polyubiquitinated by Beta-Transducin Repeat Containing E3 Ubiquitin Protein Ligase (BTRC or BTRCP2) complex for the following proteasome mediated degradation. When Wnt ligand is present (B), FZD is bound by Wnt and sequesters AXIN, thereby inhibiting the formation of the  $\beta$ -catenin destruction complex.  $\beta$ -catenin accumulates in the cytoplasm and it is allowed to translocate to the nucleus, where it binds to TCF/LEF co-transcription factors which interact with DNA to activate the transcription of target genes, including Myc Proto-Oncogene Protein, Cyclin D1 (CCND1) and Dickkopf1 (Dkk1) (Degirmenci *et al.*, 2018; Chen *et al.*, 2018; Tan and Barker, 2018). DKK-1 encodes a secreted Wnt antagonist that binds to LRP5/6 and induces its endocytosis, leading to inhibition of the canonical pathway (González-Sancho *et al.*, 2005). This pathway is tightly linked with stem cell maintenance, differentiation and, upon deregulation, cancer in the intestinal epithelium (Basu *et al.*, 2016; Gehart and Clevers, 2019).



In brief, the regulation of intestinal epithelial homeostasis is crucial for the maintenance of the structure of the mucosa and the defensive barrier functions. The molecular pathway as TGF- $\beta$  and Wnt/ $\beta$ -catenin signaling pathways, among others, are able to control intestinal epithelial homeostasis, and so their disruption seems to be connected with the development of intestinal chronic diseases and cancer.

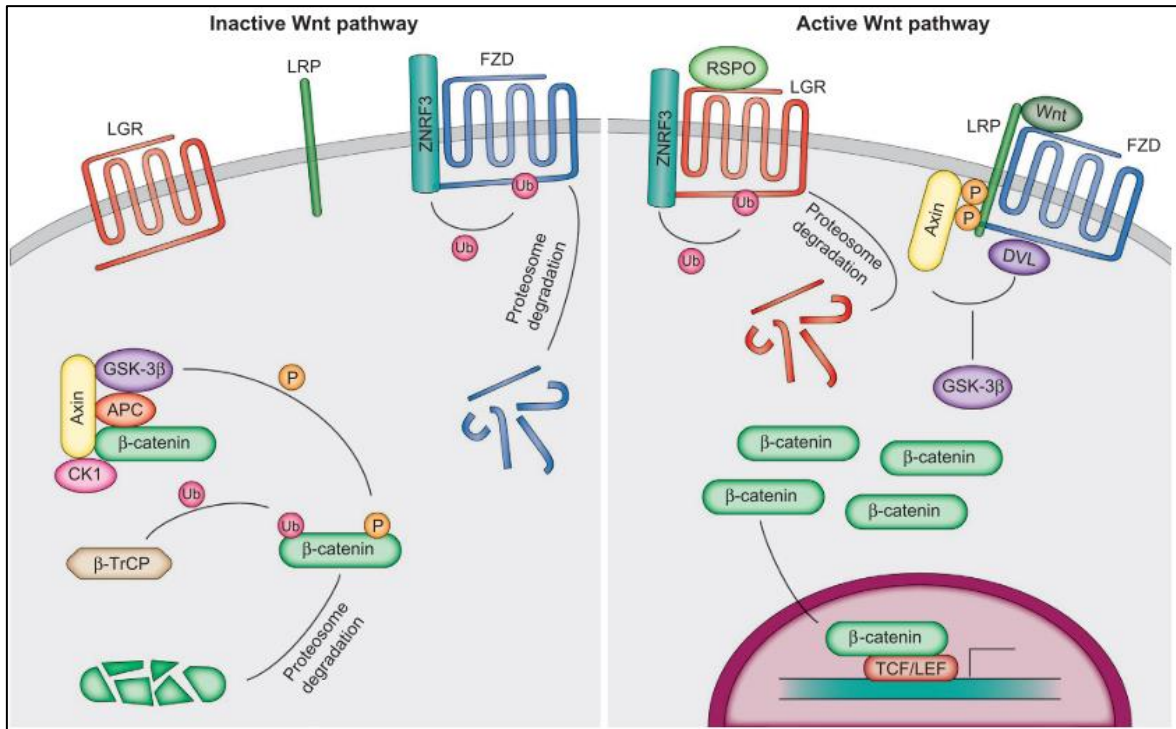


Figure 4 : **Wnt/ $\beta$ -catenin signaling cascade.** In the absence of Wnt ligand, the Wnt pathway is inactive. ZNRF3 associated with FZD receptors targeting them for ubiquitination and proteosomal degradation. The  $\beta$ -catenin degradation complex, composed of AXIN, CK1, APC and GSK3 $\beta$ , phosphorylates  $\beta$ -catenin and targets it for ubiquitination and proteosomal degradation. This leads to low concentrations of FZD on the cell membrane, little  $\beta$ -catenin in the cytoplasm and virtually none in the nucleus. When WNT ligand is present, FZD is bound by WNT and sequesters AXIN, thereby inhibiting the formation of the  $\beta$ -catenin destruction complex.  $\beta$ -catenin accumulates in the cytoplasm and is allowed to translocate to the nucleus where it binds to TCF/LEF co-transcription factors which interact with DNA, activating expression of WNT target genes. RSPO when present, binds to LGR on the cell surface which recruits ZNRF3 away from FZD receptors. This leads to LGR degradation and a buildup of FZD receptors on the cell membrane, increasing cellular sensitivity to extracellular WNT ligand (Czerwinski *et al.*, 2018).

## 1.3. Interaction between estrogen and Wnt / $\beta$ -catenin signaling

### 1.3.1. Estrogenic activity

Estrogens are the primary female sex hormones and play important roles in both reproductive and non-reproductive systems. Estrogens exist in three naturally occurring forms: estrone,  $17\alpha$  and  $17\beta$ -estradiol, and estriol. Among them,  $17\beta$ -estradiol (E2) represents the major physiological estrogen (Morselli *et al.*, 2018). Estrogens are produced primarily in the ovaries, corpus luteum, and placenta, although a small but significant amount of estrogens can also be produced by nongonad organs, such as the liver, heart, skin, and brain (Cui *et al.*, 2013; Liu *et al.*, 2019). In males, testes produced only ~20% of circulating estrogens, and acts locally to regulate normal male gonadal development and spermatogenesis, in particular spermiogenesis (Cooke *et al.*, 2017). Estrogens That play major roles in regulation of the female reproductive system, such as pubertal onset, fertility, and estrous cycle. In addition, they play a very important role in organs not linked to reproduction, in particular in the maintenance of homeostasis of glucose, bone tissue, of the cardiovascular system, and in the liver, immune system and brain (Couse and Korach, 1999; Patel and Bihani, 2018). In fact, estrogens exert a variety of actions on many regions of the nervous system that influence higher cognitive function, pain mechanisms, fine motor skills, mood, and susceptibility to seizures (Mcewen, 2001). In bones, estrogen promote the acquisition of bone mass during puberty and help to maintain it thereafter (Almeida *et al.*, 2017). Moreover, they promote liver glucose storage via increasing glucose transporters and glycogen synthesis and suppress liver glucose production via decreasing gluconeogenesis. Estrogens also actively participate in maintaining lipid and cholesterol balance and play protective roles against hepatic lipid accumulation, via suppressing lipogenesis, lipid uptake, and cholesterol synthesis and promoting lipolysis and cholesterol removal. Interestingly,

estrogens increase both cholesterol synthesis and secretion (Shen and Shi, 2015). E2 enhances the expansion of hematopoietic pluripotent stem cells, the differentiation of monocytes to macrophages and the maturation of double positive cells (CD4+ and CD8+ T lymphocytes) (Maglione *et al.*, 2019).

Estrogens act through two types of receptors: classical nuclear receptors (ER $\alpha$  and ER $\beta$ ) and novel cell surface membrane receptors (GPR30 and ER-X). ER $\alpha$  is the main regulator of estrogen-dependent genes and its activation lead to proliferative effects. ER $\beta$ , when it is co-expressed with ER $\alpha$ , has a tendency to restrain ER $\alpha$  activity and its activation inhibits cell proliferation (Paterni *et al.*, 2014). The mechanisms of action of estrogens on target tissues are triggered either after attachment of the ligand to the receptor or independently of the receptor. Ligand-dependent signaling pathways can take place via the genomic pathway or involve a non-genomic mechanism (Heldring *et al.*, 2007). They first regulates the transcription of target genes through binding of estrogen and ER complex directly to the regulatory DNA elements (estrogen-responsive element (ERE) recruiting additional factors involved in transcriptional regulation (see figure 5, pathway 1). An alternative ER-dependent mechanism is the “membrane-initiated” pathway, in which estrogen-induced signaling is initiated in the membrane or cytoplasm and the downstream effects are only partially dependent on translation or transcription (see figure 5, pathway 2).

Estrogens can exert antioxidant effects and suppress oxidative stress in an ER-independent pathway (Haas *et al.*, 2012) (see figure 5, pathway 3). ER can also be activated in a ligand-independent manner by a variety of factors, including dopamine (Power *et al.*, 1991), epidermal growth factor (EGF) and insulin-like growth factor-1 (IGF-1) (Ignar-Trowbridge *et al.*, 1992; Klotz *et al.*, 2002). In this pathway (ligand-independent genomic actions), growth factors (GF) activate protein-kinase cascades, which activates several cellular signaling pathways such as activation of MAP kinases and phosphatidyl-inositol 3-kinases (PI3K),

phospholipase C (PLC) among others, and leading to phosphorylation (P) and activation of nuclear ER at ERE (see figure 5, pathway 4).

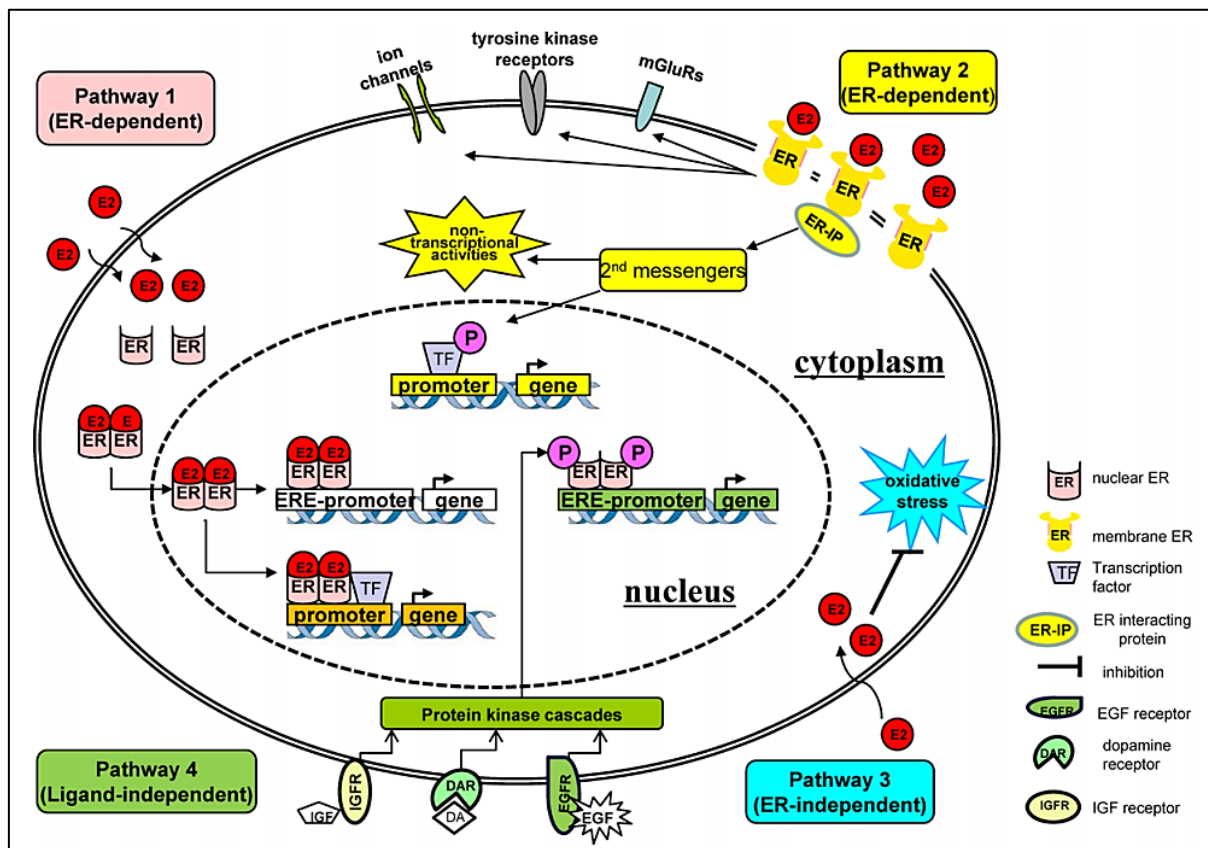


Figure 5: **Signaling pathway mediated by E2 and ERs.** There are 4 estrogen and estrogen receptor signaling pathways. Pathway 1: The nuclear initiated estrogen signaling mediated through classical ERs leads to the transcriptional changes in estrogen-responsive genes with or without EREs. Pathway 2: The membrane initiated estrogen signaling leads to diverse cytoplasmic effects, including the regulation of membrane-based ion channels, regulation of second messenger systems, and modification of transcription factors or other membrane receptors. Pathway 3: Estrogen can also exert antioxidant effects in an ER-independent manner. Pathway 4: Ligand-independent genomic actions. Growth factors (GF) activate protein-kinase cascades, leading to phosphorylation (P) and activation of nuclear ERs at EREs (Cui *et al.*, 2013).

In the intestine, the presence of each ERs is distributed differently along the crypt–villus axis, ER $\alpha$  being more abundant in the crypt and ER $\beta$  more abundant in the villi (Hasson *et al.*, 2014; Cho *et al.*, 2007). Estrogen signaling interplays with other pathways important for the intestinal homeostasis and its disruption seems to be connected with the development of intestinal chronic diseases and cancer (Gao *et al.*, 2013; Filardo *et al.*, 2000). Recently, the interactions between Wnt/ $\beta$ -catenin

signaling pathway and estrogen signaling pathways have been reported in many tissues (Gao *et al.*, 2013; Zhang *et al.*, 2016; Yin *et al.*, 2015; Varea *et al.*, 2010; Armstrong *et al.*, 2007). Estradiol is responsible for increasing cytosolic  $\beta$ -catenin protein levels and activated the transcriptional activity of Wnt/ $\beta$ -catenin signaling in primary cortical neurons and neuroblastoma (Varea *et al.*, 2010). Also, in bone cells, the transcriptional activity of Wnt/ $\beta$ -catenin signaling pathway was activated by binding between estrogen receptor and the transcription factor (TCF) (Westendorf *et al.*, 2004). Therefore, estrogen can regulate the Wnt/ $\beta$ -catenin signaling pathway.

In short, estrogen has a pivotal role in many biological functions in both reproductive and non-reproductive organs, mediating actions through its receptors, estrogen receptor ER $\alpha$  and ER $\beta$ . Estrogen signaling is important for intestinal homeostasis, it can interact with Wnt/ $\beta$ -catenin signaling pathway and its disruption seems to be connected with the development of intestinal pathogenesis.

#### **1.4. The mycotoxin Zearalenone as an endocrine disruptor**

Mycotoxins are a diverse group of toxic compounds that are produced by many filamentous fungi (Alshannaq and Yu, 2017). From about 300 identified filamentous fungi, the most prevalent toxigenic species belong to the genera *Aspergillus*, *Fusarium*, *Penicillium*, and *Alternaria* (Escriva *et al.*, 2015). Mycotoxins are gaining worldwide attention due to their adverse health effects in human and animals, and have a negative economic impact on agriculture. Currently, a large number of mycotoxins have been identified with different chemical structures and biological properties. Among them, aflatoxins (AFs), ochratoxin A (OTA), fumonisins (FBs), T-2 toxin, HT-2 toxin, deoxynivalenol (DON) and zearalenone (ZEN) are the most well-known and regulated mycotoxins.

Zearalenone (ZEN, also known as ZEA/ZON, Figure 6), is a mycotoxin produced by certain species that belong to the genus *Fusarium* such as *graminearum*,

*culmorum, cerealis, crookwellense and semitectum* (Zinedine *et al.*, 2007; Kowalska *et al.*, 2016; Rai *et al.*, 2019). ZEN is a macrocyclic  $\beta$ -resorcylic acid lactone. According to the nomenclature of the International Union of Pure and Applied Chemistry (IUPAC), its chemical name is: (3S,11E) 3, 4, 5, 6, 9, 10 – hexahydro – 14 , 16 – dihydroxy – 3 – methyl - 1H - 2 – benzoxacyclotetradecin -1, 7(8H) dione. ZEN was isolated for the first time in 1962 on corn contaminated by *Gibberella* (Stob *et al.*, 1962), and its chemical structure is determined in 1966 (Urry *et al.*, 1966) and 1967 (Kuo *et al.*, 1967).

ZEN is one of the most frequent and potent contaminants of grains and cereal products that possess serious threat to food safety (Rai *et al.*, 2019). The chemical structure of ZEN is similar to that of the estrogenic hormone 17- $\beta$ -estradiol (Braicu *et al.*, 2016a; So *et al.*, 2014; Diekman and Green, 1992) (Figure 6), which allow its ability to activate ERs. ZEN is a total agonist of ER $\alpha$  and a partial agonist-antagonist of ER $\beta$ . The main target tissues are those rich in these receptors like the uterus, mammary glands, liver and hypothalamus, leading to a sequence of events comparable to the one preceding estrogenic stimulation, and causing a functional and morphological change in the reproductive system in animals and humans (Tatay *et al.*, 2016; Wang *et al.*, 2014). Reproductive issues related to ZEN exposure were observed in various animal species. ZEN is, therefore, considered as an endocrine disruptor, because of its capability of interacting with the endocrine system, and consequently capable of producing an effect on it and of affecting estrogen-responding organs and tissues (EFSA, 2013; Wang *et al.*, 2014; Kowalska *et al.*, 2016; Yip *et al.*, 2017; Zinedine *et al.*, 2007).

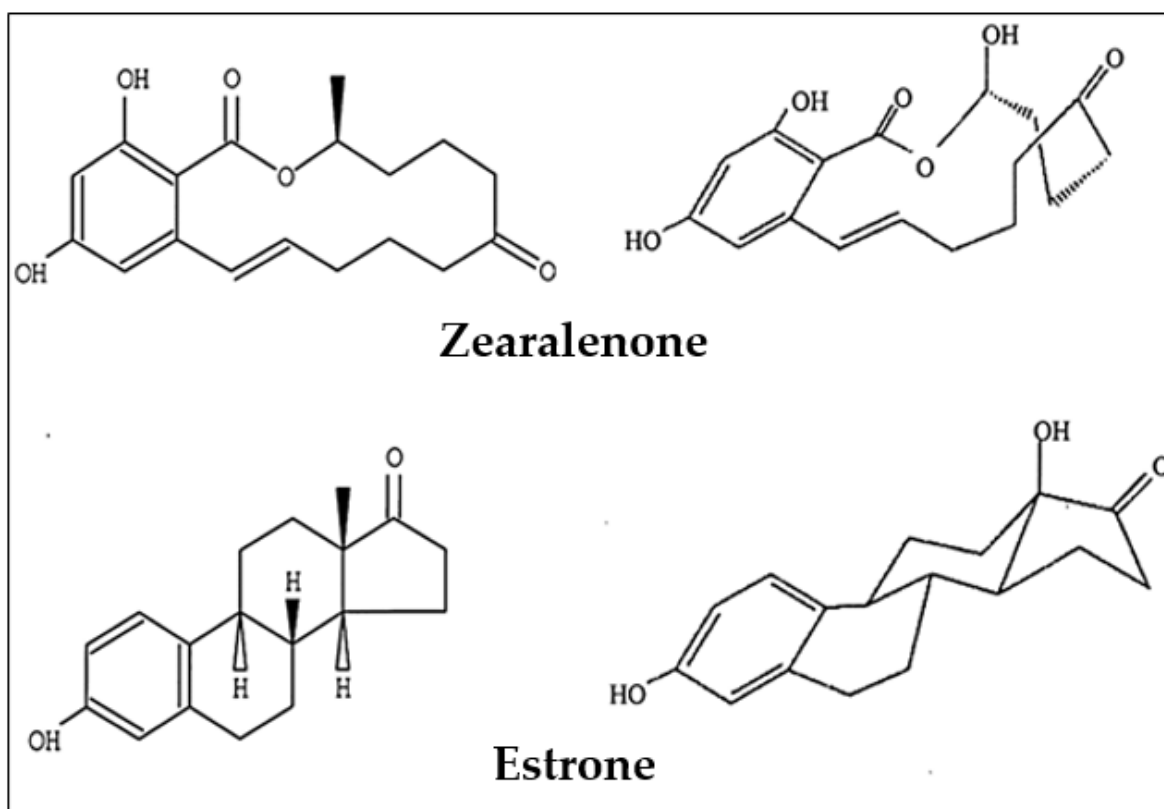


Figure 6: **Chemical structure (left) and perspective drawing (right) of ZEN and estrone.** The structure of ZEN consists of a resorcinol moiety fused to a 14-member macrocyclic lactone ring, which includes a trans double bond, a ketone and a methyl branch. The structure is clearly flexible enough to allow ZEN to adopt a conformation of 17 $\beta$ -estradiol, estriol and estrone.

## 1.5. Toxicokinetics of ZEN

The toxicokinetics of ZEN have been reported in several species following different routes of administration (Prelusky *et al.*, 1989; Mallis *et al.*, 2003; Dänicke *et al.*, 2005; Shin *et al.*, 2009a,b,c; Devreese *et al.*, 2015). In this section, the absorption, distribution, metabolism and elimination of ZEN following an oral exposure is described (Figure 7).

### 1.5.1. Absorption and distribution

Following ingestion with food or feed, ZEN is rapidly absorbed from the gastrointestinal tract (Videmann *et al.*, 2008; Dailey *et al.*, 1980). Studies indicates that absorption is quite fast in pigs, rabbits and humans (Kuiper-Goodman *et al.*,

1987; Zinedine *et al.*, 2007). As an example, following a signal dose of 10 mg ZEN/kg b.w to 15-25kg pigs, the total absorption was estimated to be 80-85 % (Biehl *et al.*, 1993). Once absorbed, ZEN is largely distributed to various tissues, mainly the liver, intestine and kidneys (Bernhoft *et al.*, 2001). ZEN is also found in organs with estrogen receptors such as the uterus, ovarian follicles, and testes (Rai *et al.*, 2019; Kuiper-Goodman *et al.*, 1987; Shin *et al.*, 2009; Liang *et al.*, 2015; Mally *et al.*, 2016). The distribution to the tissues is very fast with half-lives between 0.14 and 1 h observed after intravenous dosing of ZEN (Schelstraete *et al.*, 2020), and 7 hours following oral administration in bile duct-cannulated rats (Shin *et al.*, 2009). In male rats, zearalenone is extensively distributed to tissues other than the gastrointestinal tract, including kidney, liver, adipose, lung, heart, spleen, muscle, brain, and testes (EFSA. 2011).

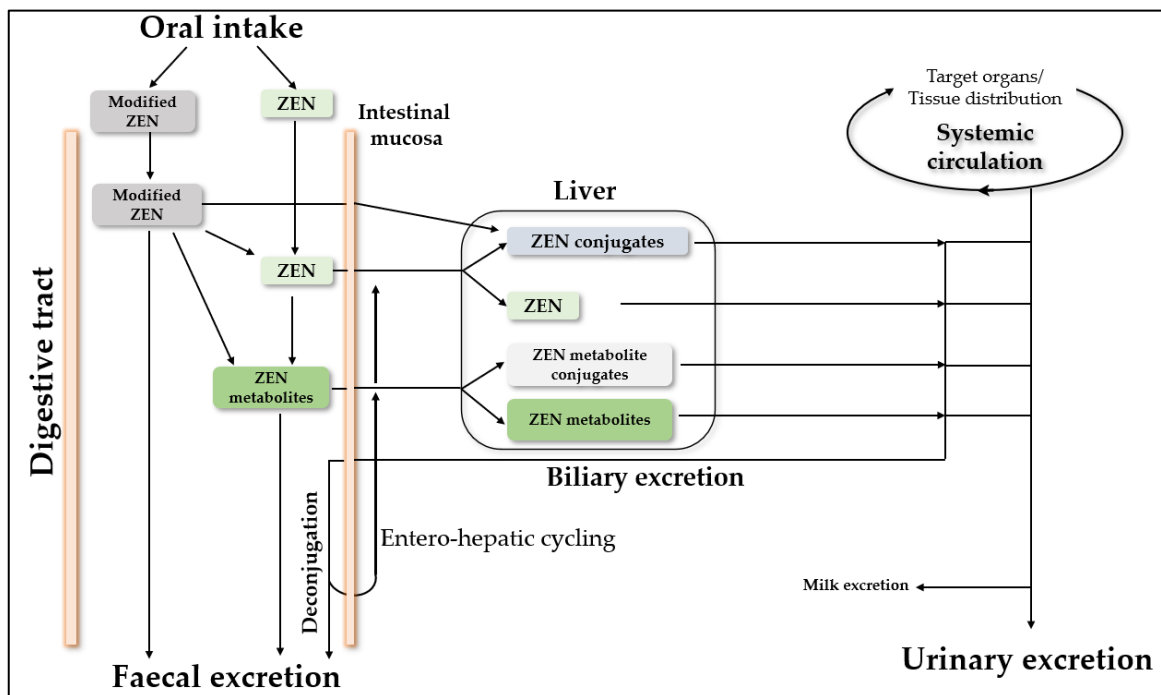


Figure 7: **Principal pathways and metabolism of ZEN and its modified forms** (adapted from Dänicke and Winkler. 2015). Following oral ingestion of ZEN and/or its modified forms, they may undergo metabolism in the intestine, giving rise to metabolites of ZEN. Then, ZEN, the metabolites of ZEN and the modified forms of ZEN cross the intestinal barrier and reach the liver where they undergo a second metabolism giving rise to conjugated forms of ZEN and its metabolites. These conjugated forms can either gain blood circulation to reach the target tissues, and subsequently be eliminated via the urine or via



breast milk, or undergo an enterohepatic cycle, undergo a conjugation to be eliminated via the faeces or regain again liver.

### 1.5.2. Metabolism and excretion

Liver and intestine are the main organs of ZEN metabolism. Once entered in the body, ZEN can be metabolized by intestine as well as the liver, which seems to be one of the main targets of the toxin (Zinedine *et al.*, 2007; Jiang *et al.*, 2012; Koraichi *et al.*, 2012). Indeed, two major phases of metabolism are identified:

#### 1.5.2.1. Phase-I metabolism:

The biotransformation of ZEN into the different metabolites occurs in the intestinal lumen (pre-absorptive stage, microbial) and mucosa, as well as in the liver (Olsen *et al.*, 1987; Fink-Gremmels and Malekinejad, 2007; Zinedine *et al.*, 2007; Dänicke and Winkler, 2015). The biotransformation of ZEN is mainly conducted by reduction, and is done by two  $3\alpha$ - and  $3\beta$ -hydroxysteroid dehydrogenases,  $3\alpha$ -HSD and  $3\beta$ -HSD (Dänicke and Winker, 2015) that produce  $\alpha$ - and  $\beta$ -ZEL, respectively. The presence and activity of these enzymes is species-dependent (Gupta *et al.*, 2018). Rats and chicken predominantly use  $3\beta$ -HSD to transform ZEN mainly in  $\beta$ -ZEL in liver (Malekinejad *et al.*, 2006), while pigs and humans predominantly use  $3\alpha$ -HSD, and so the main metabolite is  $\alpha$ -ZEL (Figure 8).  $\alpha$ -ZEL has been shown to have higher affinity for the estrogen receptors (Fitzpatrick, 1989) than the parent molecule, whereas  $\beta$ -ZEL is much less estrogenic. This explains in part the sensitivity of human and pigs for this mycotoxin.

ZEN is also monohydroxylated by recombinant human cytochromes P450 (EFSA, 2011) and the stereoisomers zearalanone (ZAN),  $\alpha$ - and  $\beta$ -ZAL are obtained (Figure 8). The estrogenic activities of ZEN and its metabolites are different and can be ranked as follows:  $\alpha$ -ZEL= $\alpha$ -ZAL > ZAN > ZEN= $\beta$ -ZAL >  $\beta$ -ZEL in human breast cancer cells in vitro (Parveen *et al.*, 2009). Figure 8 summarizes the major metabolic pathways of zearalenone.

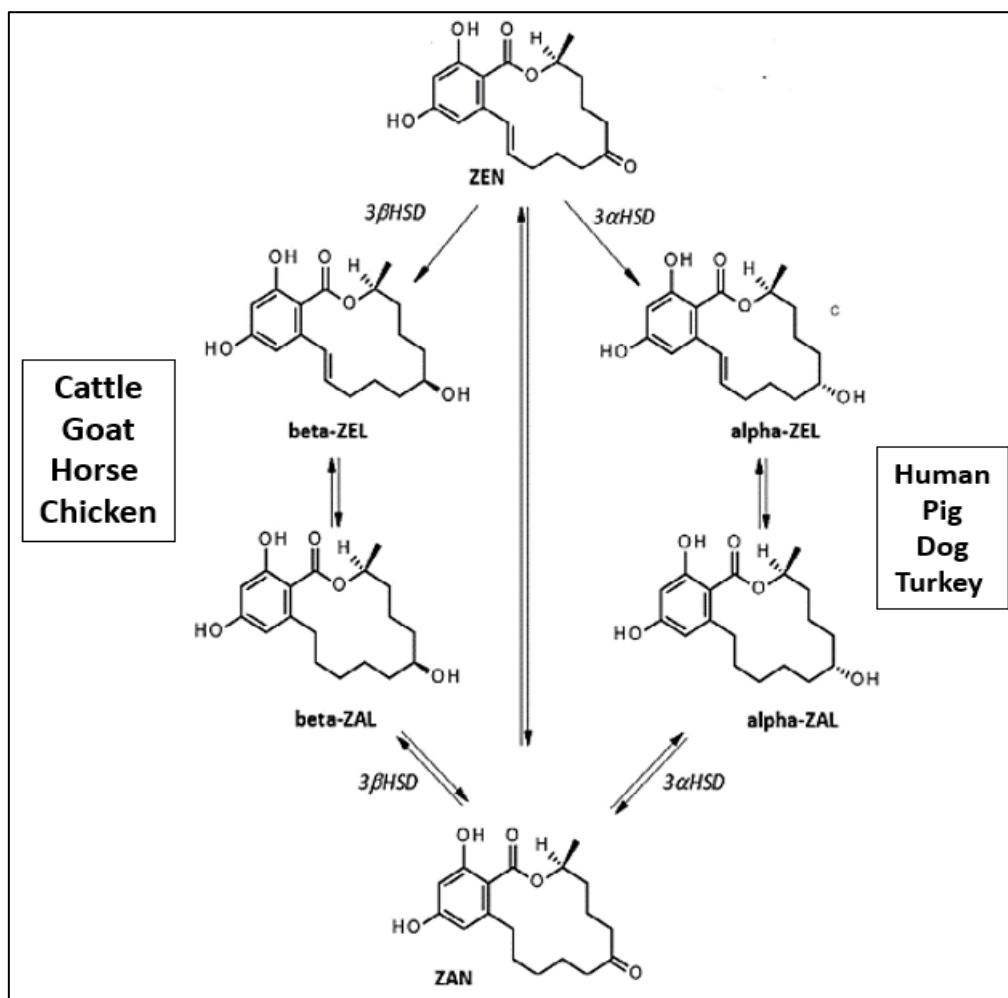


Figure 8: Major phase I reductive metabolism is mediated by  $3\alpha$ - and  $3\beta$ -hydroxysteroid dehydrogenases (HSDs) (EFSA, 2011). The reduction of keto group in ZEN structure leads to two stereoisomeric metabolites  $\alpha$  and  $\beta$  isomers. Common derivatives of ZEN include alpha-Zearalenol (alpha-ZEL); beta-Zearalenol (beta-ZEL); Zearalanone (ZAN); alpha-Zearalanol (alpha-ZAL) and beta-Zearalanol (beta-ZAL). The  $\alpha$ -derivatives seem to prevail in human, pig, dog, and turkey while the  $\beta$ -derivatives appear to be more abundant in cattle, goat, horse, and chicken.

#### 1.5.2.2. Phase-II metabolism:

ZEN metabolites derived from phase-I are can be sulfated and glucuronidated in the liver (Zheng *et al.*, 2019; EFSA, 2016). These conjugated derivatives can then be eliminated in urine (main route of excretion in most species), but also in bile predominantly as glucurono-conjugated compounds. The conjugation of ZEN and its reductive metabolites  $\alpha$ - and  $\beta$ -ZEL with glucuronic acid represents a major metabolic pathway in human and animals, and facilitates their elimination (Biehl *et*

*al.*, 1993; Dänicke *et al.*, 2005; Dong *et al.*, 2010; Mirocha *et al.*, 1981; Olsen *et al.*, 1985; Zöllner *et al.*, 2002).

Besides the main metabolites of ZEN, this mycotoxin can be modified by microorganisms and plants, and these modified forms can be completely converted into ZEN by the animal or human intestinal microbiota (Zheng *et al.*, 2019; EFSA, 2016, 2017) (Figure 9).

## 1.6. Exposure and regulation of ZEN

Humans and animals can be exposed to the ZEN directly through the consumption of contaminated plants products, or indirectly through products derived from animals exposed to mycotoxins (Figure 10). ZEN occurs mainly in cereal crops and foodstuffs in the Europe, Asia and Africa (Cano-Sancho *et al.*, 2012; Schollenberger *et al.*, 2006; Pietri *et al.*, 2004; Scudamore and Patel, 2009; Ma *et al.*, 2018; Zinedine *et al.*, 2006; El-Maghraby, 1995). Based on survey results from more than 74,000 samples from 100 countries over 10 years, ZEN is one of the top three mycotoxins in complete animal feed and feedstuffs, with occurrence in 45% of all samples and 55 µg/kg median concentration among the positive samples (Gruber-Dorninger *et al.*, 2019). ZEN contamination is common in ingredients used in livestock animal feed occurring in corn dried distillers grains co-products (distiller's dried grains with solubles; 75%), soybean meal (61%), corn (44%), wheat (33%) and barley (20%) (Gruber-Dorninger, *et al.*, 2019; Liu and Applegate, 2020). Furthermore, a high concentrations of ZEN (up to 600 mg/kg) in wheat and rice, and 38.4 mg/kg in corn have been reported in India and Egypt, respectively (Gao *et al.*, 2018; Kowalska *et al.*, 2016; Zinedine *et al.*, 2007; El-Maghraby *et al.*, 1995). In food, occurrence of ZEN. up to 12.5 µg/kg in milk was reported from Egypt, USA, UK and China (Flores-Flores *et al.*, 2015). Besides, In living organisms (i.e. plants, microbes, animals), the most relevant modification of ZEN leads to the formation of its main reduced metabolites  $\alpha$ - and  $\beta$ -zearalenol (EFSA. 2017). In a study released

in cereal-based products in Belgium, the ZEN metabolites  $\alpha$ -ZEL and  $\beta$ -ZEL accounted for up to 58% and 21% of ZEN, respectively (De Boevre *et al.*, 2013). Additionally, previous research demonstrate that ZEN and its reductive metabolites can be detected in both animal tissues and products. In fact, ZEN and its metabolites were founds in milk samples collected in China, one single sample contained 0.37  $\mu\text{g/L}$  ZAN and 3.03  $\mu\text{g/L}$   $\alpha$ -ZAL (Xia *et al.*, 2009). Moreover, previous research demonstrate that ZEN and its reductive metabolites can be detected in animal tissues (Döll *et al.*, 2003; Shreeve *et al.*, 1979; Hagler *et al.*, 1980; Zöllner *et al.*, 2002; Dong *et al.*, 2010; Dänicke *et al.*, 2002). Feeding pigs with 0.7 mg/kg for 18 days, researchers found that the carry-over into liver and muscle (Zöllner *et al.*, 2002). Whereas ZEN and ZAN were below the detection limit in both tissues, detectable levels of ;  $\alpha$ -ZEL (up to 12  $\mu\text{g/L}$  in liver and 14.5  $\mu\text{g/L}$  in muscle),  $\beta$ -ZEL (up to 4.8  $\mu\text{g/L}$  in liver) . $\alpha$ -ZAL (up to 13.3  $\mu\text{g/L}$  in liver); as well as  $\beta$ -ZAL (traces in muscle) were detected (Zöllner *et al.*, 2002). In another study in boiler chicken, after exposure to ZEN (1.2 mg/kg b.w./ single oral bolus), ZEN and its metabolites were found in different tissues, such as liver (ZEN 3.52  $\mu\text{g/L}$ ;  $\alpha$ -ZEL 7.84-105.2  $\mu\text{g/L}$ ,  $\beta$ -ZEL 24.4-30.9  $\mu\text{g/L}$ ), in kidney (ZEN 3.55  $\mu\text{g/L}$ ;  $\alpha$ -ZEL 1.63-77.99  $\mu\text{g/L}$ ,  $\beta$ -ZEL 4.8-36.6  $\mu\text{g/L}$ ) and muscle ( $\alpha$ -ZEL 2.55  $\mu\text{g/L}$ ,  $\beta$ -ZEL 2.40  $\mu\text{g/L}$ ) (Buranatragool *et al.*, 2015).

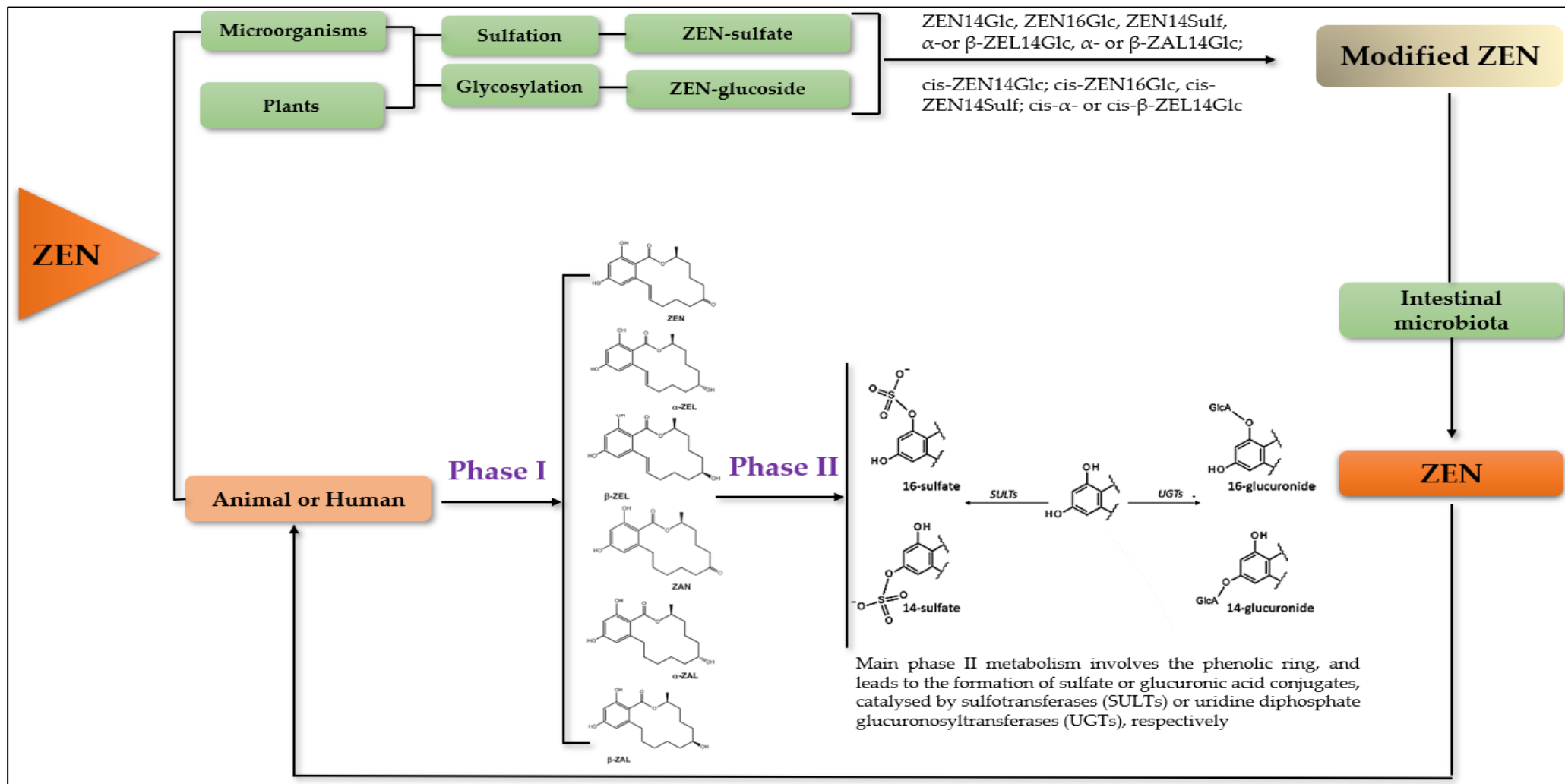


Figure 9: **Scheme of mycotoxin modification and metabolism applied to ZEN** (Adapted from EFSA, 2017 and Zheng et al., 2019). ZEN may contaminated animal and huaman, Its also can be modified from plants and microorganisms. used for fermentation may conjugate ZEN, leading to the formation of phase II metabolites (modified ZEN: ZEN14Glc: ZEN-14-O-β-glucoside; ZEN16Glc: ZEN-16-O-β-glucoside; ZEN14Sulf: ZEN-14-sulfate). In addition, ZEN may undergo cis-isomerisation due to sunlight exposure. The isomeric parent compound, namely cis-ZEN. these modified forms can be completely converted into ZEN by the animal or human intestinal microbiota. Then, ZEN can be transformed by phase I and II metabolism.

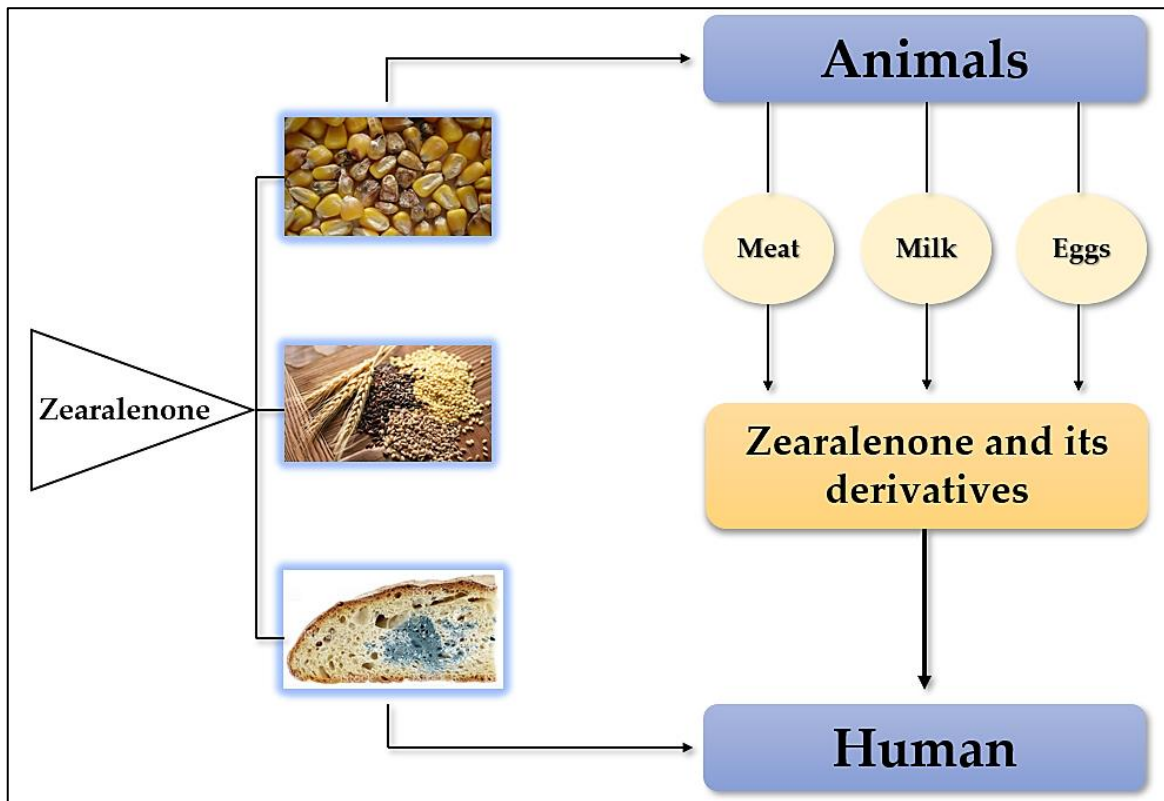


Figure 10: **Human Exposure to ZEN.** The main way for human and animals exposure to ZEN is consuming the cereal grains and derived products which may be contaminated by toxigenic fungi species of *Fusarium* in field or during food production,. ZEN and its derivatives can be occurred via consuming the animal-origin food such as eggs, milk, and meat (Zheng *et al.*, 2018).

Considering the consumer health risk, specific regulations for ZEN in foodstuffs have been formulated. The regulatory guideline for ZEN varies among different national authorities and regulatory organizations. The maximum tolerated levels of ZEN in food for human consumption is established at 20  $\mu\text{g}/\text{kg}$  in food intended for babies and infants, 50 $\mu\text{g}/\text{kg}$  in maize-based snacks and breakfast cereals, and 200  $\mu\text{g}/\text{kg}$  in unprocessed maize and certain maize products (Table 1; EFSA, 2011). The current tolerable daily intake (TDI) for ZEN of 0.25  $\mu\text{g}/\text{kg}$  body weight (bw) per day established by the European Food Safety Authority (EFSA) Panel for Contaminants in the Food Chain (CONTAM Panel) in 2011, 2016 and 2017. Limits to human consumption of ZEN have also been established by the European Commission (Table 1; European Commission, 2007) as well as for animal feed (Table 2; European Commission, 2006).

**Table 1: Limits related to human consumption of ZEN (European Commission, 2007); ppb-parts per billion**

| Source   | Maximum level (ppb) |
|--|---------------------|
| <b>Unprocessed cereals without maize</b>   | 100                 |
| Unprocessed maize  | 350                 |
| Cereals intended for direct human consumption, cereal flour, bran and germ as end product for direct human consumption   | 75                  |
| Maize intended for direct human consumption, maize based snacks and maize based breakfast cereals  | 100                 |
| Maize intended for direct human consumption, maize-based snacks and maize-based breakfast cereals  | 50                  |
| Processed cereal-based foods (excluding processed maize-based foods) and baby foods for infants and young children   | 20                  |
| Milling fractions of maize with particle size >500 micron falling within CN code 1103 13 or 1103 20 40 and other maize milling products with particle size >500 micron not used for direct human consumption falling within CN code 1904 10 10 | 200                 |

**Table 2 : Limits of ZEN related to animal feed (European Commission, 2006); ppm- parts per million**

| Source intended for animal feed                                 | Guidance level (ppm) |
|---|----------------------|
| Cereals and cereal products with exception of maize by-products | 2                    |
| Maize by-products   | 3                    |
| Complementary and complete feeding stuff for:                   |                      |
| – piglets and gilts   | 0.1                  |
| – sows and fattening pigs                                       | 0.25                 |
| – calves, dairy cattle, sheep and goat                          | 0.5                  |

## 1.7. Mode of action of ZEN and induction of cell proliferation and cell death (Figure 11)

Evidences in literature describe that ZEN can exert different mechanisms of toxicity depending on the cell types and the exposure conditions. ZEN toxicity can induce mainly two types of effects: it can be either pro-apoptotic at high, acute doses, and pro-anabolic at low doses during chronic exposure (Zheng *et al.*, 2018; Kowalska *et al.*, 2016).

At lower doses, the toxic effect of ZEN is more dependent on the activation of estrogen receptor (ER) signaling pathway, which has anti-apoptotic and proliferating effect (Metzler *et al.*, 2010; Zheng *et al.*, 2018; Belli *et al.*, 2010; Kowalska *et al.*, 2016). Under these conditions, ZEN passively crosses the cell membrane and binds to oestrogenic receptors (ERs). The ER/ZEN complex is immediately translocated to the nucleus where it binds to oestrogen-responsive elements (ERE) in target genes that will be expressed (Gupta *et al.*, 2018; EFSA, 2004, Metzler *et al.*, 2010). Comparing ZEN to 17- $\beta$ -estradiol, it does bind the ERs in the target tissues and cells at <1–10% (Frizzell *et al.*, 2011). Moreover, ZEN functions to activate both ERs however it is seen as a partial agonist of ER $\beta$  and a full agonist of ER $\alpha$  (Fink-Gremmels and Malekinejad, 2007). In consequence, the biological response derived from ZEN exposure varies according to tissue-specific ratio of estrogen receptor (ER)  $\alpha$  vs  $\beta$ , as well as the density of these receptors (Böttner *et al.*, 2014).

Besides its binding to ERs, the endocrine effects of ZEN involve other mechanisms. Indeed, ZEN and its reduced metabolites are competitive substrates for 3 $\alpha$ - and 3 $\beta$ -hydroxysteroid dehydrogenases (HSDs), two enzymes involved in the synthesis of steroid hormones (Fink-Gremmels and Malekinejad, 2007). In steroidogenic tissues HSDs catalyze the final steps in androgen, estrogen, and progesterone biosynthesis (Penning and Byrns, 2009). ZEN can also act on the hypothalamic hypophysial axis, which can affect the production of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), that are vital hormones



for activating and controlling the synthesis and transport of sex hormones from the gonads into the blood (Zheng *et al.*, 2019; Gupta *et al.*, 2018). In addition, ZEN can activate the pregnane X receptor (PXR), a human xenobiotic receptor member of ligand activated nuclear transcription factors (Ding *et al.*, 2006).

Furthermore, a new insights into the toxicological mechanisms of ZEN proved that this toxin might promote cell proliferation through the activation of MAPK and RAS-RAF-MEK-ERK signaling pathways, with low concentration of ZEN (Zheng *et al.*, 2019). In contrast, at high concentrations, ZEN toxicity is independent of estrogen signaling, and is characterized by a high oxidative state, cell membrane disruption, DNA oxidative damage and induced apoptosis .

The ability of ZEN to stimulate cell proliferation has been observed in different cell lines. In that regard, it has been demonstrated that ZEN promote proliferation in human cancer MCF-7 cells at dose ranging from 6.25 to 25 $\mu$ M (Tatay *et al.*, 2017). Furthermore, to understand the mechanism of ZEN estrogen-like action, a study conducted by Parveen and his collaborators (2009), using breast cancer MCF-7 cells which were treated with 10 nM of E2 or ZEN compounds, they found that all of the ZEN compounds stimulated the growth of MCF-7 cells, as much as E2 and showed similar expression profiles to that of E2, and the effect of ZEN compounds was likely mediated by estrogen-receptor-dependent Erk1/2-signaling. Other studies have also shown that ZEN is able to promote proliferation, colony formation and migration in the human colon carcinoma cell line HCT116 (Abassi *et al.*, 2016). ZEN tend to accumulate in reproductive organs and could significantly contribute to the proliferation of tumor cells in the uterus (Pajewska, 2018). A recent study revealed that the miRNAs might participate in ZEN promoted cell proliferation at low concentrations, through MAPK and RAS-RAF-MEK-ERK pathways. This study provides a molecular basis and new insights into the toxicological mechanisms of ZEN using Leydig cell line TM3 (Zheng *et al.*, 2019).

At higher doses, however, ZEN is able to cause cell death through inducing oxidative stress, endoplasmic reticulum stress and apoptosis. Oxidative stress can disturb the normal redox state of cells by production of peroxides and free radicals that damage biomolecules including proteins, lipids and DNA (Sabatini *et al.*, 2009). *In vitro*, treatment with ZEN can induce mitochondrial damage by reducing antioxidant enzyme activities, accumulation of reactive oxygen species (ROS), and decreasing mitochondrial membrane potential in swine small intestine IPEC-J2 cells (Fan *et al.*, 2017). In human promyelocytic leukemia HL60 and human promonocytic U937, ZEN induced cells apoptosis through an activation of mitochondrial release of cytochrome C through mitochondrial transmembrane potential reduction, activation of caspase-3 and -8, production of reactive oxygen species and induction of endoplasmic reticulum stress (Banjerdpongchai *et al.*, 2010). After exposure to 100  $\mu$ M of ZEN for different incubation times (2, 4, 8, 24, 30, 48 and 60 h), the ROS, DNA fragmentation and cell-cycle arrest were increased in Human Hepatoma HepG2 cells. These events begin after only 2 h of mycotoxin exposure and are earlier than those implicated in the execution of apoptosis (Gazzah *et al.*, 2010). ZEN can induce apoptosis in RAW 264.7 macrophages through the endoplasmic reticulum (ER) stress pathway (Chen *et al.*, 2016). ER stress induced cell apoptosis by activating pro-apoptotic molecules including transcriptional factor C/EBP homologous protein (CHOP), apoptosis signal-regulating kinase 1 (ASK1)/c-Jun amino terminal kinase (JNK), and caspase 12 (Iurlaro and Munoz-Pinedo, 2016). *In vivo*, a recent study has shown a significant induction in oxidative stress indices (ROS, LPO and NO) and reduction in antioxidant defense system, Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx), glutathione S-transferase (GST) and glutathione (GSH) in zebrafish embryos after treatment with zearalenone (Muthulakshmi *et al.*, 2018). However, higher concentrations (1.1–3.2 ppm) of ZEN in the diet were also able to reduce SOD activity in spleen of post-weaning gilts (Jiang *et al.*, 2011) as well in liver and serum of weanling piglets (Jiang *et al.*, 2012).

As a conclusion, ZEN exerts different mechanisms of toxicity in different cell types at different doses. At low doses, it can stimulate cell proliferation mediated by estrogen-receptor-dependent Erk1/2- signaling, MAPK and RAS-RAF-MEK-ERK pathways whereas a high dose of ZEN can cause cell death through inducing cell cycle arrest, oxidative stress, DNA damage, mitochondrial damage, and apoptosis.

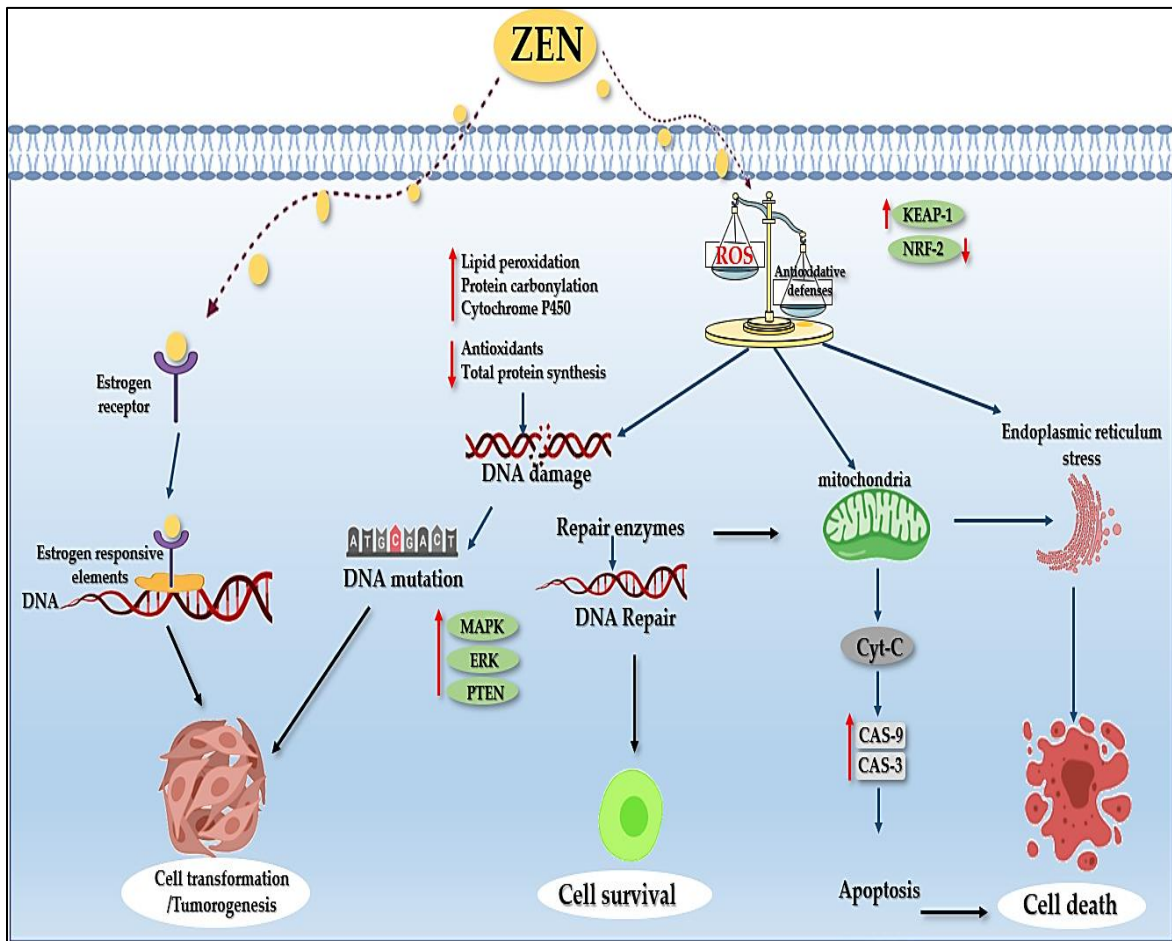


Figure 11: **Intracellular effects of ZEN** (Adapted from Rai *et al.*, 2019). There are several possible mechanisms of ZEN toxicity. ZEN may behave as an endocrine disruptor because of its estrogenic effects. The proliferation activities of ZEN may be due to its hormonal activity. If DNA damage caused by ZEN gets repaired by unscheduled or SOS repair, mutations might take place, which can further lead to cell proliferation. Besides, ZEN can induce oxidative damage, DNA damage, apoptosis, necrosis and finally cell death. ZEN-induced ROS production decreases the mitochondrial membrane potential, making it permeable and inhibiting the function of cytochrome c, and increasing caspase-3 and caspase-9 expressions. Inactivated cytochrome c leaches out from mitochondria into the cytosol, making the cell prone to apoptosis. Furthermore, ZEN-induced apoptosis via endoplasmic stress.

## 1.8. Toxicity of ZEN

### 1.8.1. Reproductive toxicity of ZEN

As mentioned above, ZEN and its metabolites can competitively bind to estrogen receptor due to the structural similarity with  $17\beta$ -estradiol, and activate the transcription of estrogen responsive genes in many organs, especially in the gonads (Bovee *et al.*, 2004; Etienne and Dourmad, 1994; Metzler *et al.*, 2010; Mirocha *et al.*, 1981). Therefore, it induces estrogenic effects as well as fertility and reproductive disorders, often reported as hyperestrogenism (Bielas *et al.*, 2017). The hyperestrogenic effects of ZEN have been reported in domestic species, laboratory animals as well as humans (Rai *et al.*, 2019). In humans, ZEN has been found in the endometrium of women having endometrial adenocarcinoma, hyperplasia and normal proliferative endometrium (Tomaszewski *et al.*, 1998). Furthermore, it was also indicated that ZEN can induce precocious puberty in girls (Zheng *et al.*, 2019). Indeed, ZEN and their metabolites have been implicated in a number of incidences involving precocious puberty among young girls in various countries (Mukherjee *et al.*, 2014).

In animals, the effects of ZEN in female laboratory species such as mice, rats, guinea pig, pigs and rabbits illustrate its estrogenic activity. They include decreases in fertility, reproductive tract alterations, swelling of the uterus and vulva, increases in embryonic resorptions, reductions in litter size, changes in weight of adrenal, thyroid and pituitary glands, and changes in serum levels of progesterone and  $17\beta$ -estradiol (EFSA, 2011). Pigs are considered as the most sensitive animal for ZEN because of their metabolization of ZEN into its more estrogenic form  $\alpha$ -ZEL. In pigs ZEN induces infertility, reduced litter size, and fetal resorption (Fink-Gremmels and Malekinejad, 2007; Tiemann and Danicke, 2007; Chang *et al.*, 1979). The main clinical sign associated with dietary ZEN consumption is the increase in the length and width of the vulva in gilts (Fu *et al.*, 2016). ZEN is also reprotoxic to male pigs. ZEN led to a decrease in the relative weight of testes (Jiang *et al.*, 2012), decrease in

testosterone levels, impaired spermatogenesis, sexual desire inhibition and feminization (EFSA, 2004).

### **1.8.2. Hepatotoxicity**

Apart from reproductive organs, liver is the main target organ for ZEN toxicity. In fact, ZEN can induce hepatotoxicity (Hassen *et al.*, 2005; Rai *et al.*, 2019; Zhou *et al.*, 2017). Exposure to ZEN (40 mg/kg bwt) can induce damage in mice liver after 48 hours of exposure (Abbès *et al.*, 2006). ZEN is cytotoxic to HepG2 cell viability through the induction of oxidative stress and related damage (Hassen *et al.*, 2007). Recently, Yoon and his co-workers demonstrated that ZEN can induce cell death, oxidative stress, ER stress, and autophagy in human liver cells. In addition, cells exposed to high concentrations of ZEN (e.g., 40 µg/mL) were revealed to have a decrease in the protein levels of the phase I/II xenobiotic enzymes, which may impact on the endogenous metabolism of mycotoxins (Yoon *et al.*, 2019).

### **1.8.3. Genotoxicity**

ZEN can induce oxidative damage (Maaroufi *et al.*, 1996; Zorgui *et al.*, 2009) and shows genotoxic effects, including induction of DNA adducts (Hassen *et al.*, 2007), chromosome aberrations, micronuclei and DNA fragmentation, (El Golli *et al.*, 2006; Ouanes *et al.*, 2003). Lioi *et al.* (2004) investigated the cytogenetic and cytotoxic effects of ZEN in cultured bovine lymphocytes treated with 0, 0.1, 0.5, 1 and 2 µM of the toxin. This study provides evidence of significant increases of structural chromosome aberrations and sister chromatid exchanges at all four concentrations. In addition, intra peritoneal dose of 2mg/kg of ZEN was found to cause DNA adduct formation in the kidney and liver of female Balb-c mice (Grosse, *et al.*, 1997). On the other hand, the amelioration of some genotoxic effects of ZEN by antioxidants were consistent with an oxidative stress mechanism (EFSA, 2011).

#### 1.8.4. Immunotoxicity

Many studies have reported the immunotoxic effects of ZEN. The production of IL-2 and IL-5 was significantly increased in the presence of ZEN at 5 and 10  $\mu\text{g/mL}$  using T cells of the EL-4 murine thymoma cell line (Marin *et al.*, 1996). Abbès *et al.* (2006) demonstrated that oral administration of a single dose of ZEN (40 mg/kg) to mice resulted in several immunotoxicological effects within 24 h of oral ingestion. Indeed, a significant difference was found between ZEN-treated mice and the control group regarding the serum concentration of IgG and IgA. In this sense, other findings have been demonstrated that ZEN, induced a significant decrease of the IgG, IgA, IgM levels, tumor necrosis factor (TNF)- $\gamma$  and IL-8 synthesis of several peripheral blood mononuclear cell (PBMC), at concentrations higher than 5  $\mu\text{M}$  affecting humoral and cellular immune response (Marin *et al.*, 2011). Vlata *et al.* (2006) investigated the *in vitro* effects of ZEN (at concentrations of 0.1, 1, 5, 10 and 30  $\mu\text{g/mL}$ ) on freshly isolated human PBMCs. ZEN was found to totally inhibit T and B lymphocytes proliferation, and this inhibitory effects of zearalenone were further related to cell necrosis/apoptosis ZEN also influences cytokine secretion and lymphocyte percentages in ileocaecal lymph nodes. In fact, after exposure pigs to (5  $\mu\text{g}$  ZEN/kg bw per day of ZEN during 45 days) decreased percentages of CD21<sup>+</sup> B cells were observed, while changes in percentages of CD2<sup>+</sup> lymphocytes, CD4<sup>+</sup> CD8<sup>-</sup> helper thymocyte (T) lymphocytes, CD8<sup>+</sup> CD4<sup>-</sup> cytotoxic T cells, and CD21<sup>+</sup> B cells and increases in cytokine (IL-2, IL-12 and IFN- $\gamma$ ) concentrations did not differ significantly to the control group (Obremski *et al.*, 2015).

In summary, ZEN binds competitively to estrogen receptors in a number of *in vitro* or *in vivo* models in various animal species as well as humans, causing significant alterations in the reproductive tract of laboratory and domestic animals. Furthermore, ZEN has also been known to exhibit hepatotoxicity, immunotoxicity and genotoxicity.

## 1.9. Intestinal toxicity of ZEN

The gastrointestinal tract is the first physiological barrier against ZEN, as well as the first target for this mycotoxin. ZEN is absorbed and partially metabolized in the intestine (Olsen *et al.*, 1987; Videmann *et al.*, 2008; Dänicke *et al.*, 2005; Pfeiffer *et al.*, 2011). Concerning the effects of ZEN on intestinal morphology, and according to *in vivo* studies, Przybylska-Gornowicz *et al.* (2018) revealed that administration of ZEN (40 µg/kg BW/6 weeks) did not influence the architecture of the mucosa and submucosa in the large intestine, but decreased the number of goblet cells in the cecum and descending colon. ZEN at the daily dose of 200 and 400µg/kg BW (pigs), increased in the number of Paneth cells at the bottom of the intestinal crypts (Obremski *et al.*, 2005). The histological results showed that ZEN resulted in edema in the lamina propria, leucocyte infiltration, necrosis of epithelial cells, blood capillary hyperemia, and goblet cell hyperplasia in the proximal intestine (PI), mid intestine (MI), and distal intestine (DI) of fish exposed to ZEN (Wang *et al.*, 2019). ZEN had no effect on the epithelial barrier function integrity as indicated by an absence of effect on the transepithelial electrical resistance (TEER) on differentiated IPEC-1 cells exposed to ZEN (Marin *et al.*, 2015). The influence of ZEN on mucus was assessed *in vitro*, using HT29-MTX cells alone or in co-culture with Caco-2 cells (Wan *et al.*, 2014), the results shown that ZEN significantly modulated MUC5AC and MUC5B mRNA and protein, and total mucin-like glycoprotein secretion.

ZEN has an anabolic effects at low doses during chronic exposure (Wan *et al.*, 2013). Abassi *et al.* (2016) demonstrated that with low concentration (1 to 1000nM), ZEN is able to promote proliferation, colony formation and migration in in the human colon carcinoma cell line HCT116. Another study also showed that ZEN (10µM/24h) downregulated the expression of tumor-suppressor genes (PCDH11X, DKK1, and TC5313860) in intestinal cells IPEC-1 (Taranu *et al.*, 2015). These findings suggest that this mycotoxin exhibits carcinogenesis-like properties at intestinal level.

ZEN has, in turn, pro-apoptotic effects at high doses on intestine cells (Taranu *et al.*, 2015; Kouadio *et al.*, 2005; Shen *et al.*, 2019; Abid-Essefi *et al.*, 2009). Oxidative stress appears to be a key determinant of toxicity induced by ZEN, and the imbalanced oxidative system will increase the accumulation of ROS. Using swine small intestine IPEC-J2 cell line, ZEN at high doses (8 and 7  $\mu\text{g}/\text{mL}$ ) can significantly increase the malondialdehyde levels and decrease antioxidant enzymes activities after 48 h of exposure. Meanwhile, the ROS accumulation increased in high dose ZEN treated groups after 2 h treatment, but decreased due to the ROS-induced mitochondrial damage and the caused cell apoptosis after 48 h of high does ZEN treatment (Fan *et al.*, 2017). Another study investigated the cytotoxicity of ZEN on IPEC-J2, after incubation for 48h ZEN, a significant increase in cell viability at 10 $\mu\text{M}$  was observed, then a significant reduction of cell viability at 40  $\mu\text{M}$  (Wan *et al.*, 2013). Also, Taranu *et al.* (2015) studied the effects of ZEN (10  $\mu\text{M}$ ) on gene expression of IPEC-1, the results showed that genes coding for glutathione peroxidase enzymes (GPx6, GPx2, GPx1) were up-regulated, which provides further evidence for this toxin inducing oxidative damage. An *in vivo* study was carried out in order to investigate the effect of ZEN on the intestine of rats (that are sensible to ZEN), with diets containing ZEN 0.3 mg/kg, 48.5 mg/kg, 97.6 mg/kg or 146.0 mg/kg respectively. The results revealed that ZEN induced oxidative stress, and different effects on the expression of cytokines were also observed (Liu *et al.*, 2014).

ZEN can induce intestinal inflammation in mouse colon, which is mediated by enhancing of the NLR Family Pyrin Domain Containing 3 (NLRP3), pro-IL-1b, pro-IL-18, IL-1 $\beta$  and IL-18 mRNA expression (Fan *et al.*, 2018). However, ZEN had no effect on the expression of IL-8 and IL-10 in concentrations of 10 and 20 $\mu\text{M}$  in IPEC-1 (Marin *et al.*, 2015). The expression of the toll like receptors (TLR1-10), and certain cytokines involved in inflammation (e.g. TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8) or responsible for the recruitment of immune cells (IL-10, IL-18) were increased in IPEC-1 (Taranu *et al.*, 2015). Furthermore, data demonstrate that ZEN has a complex effect at



duodenum level in pigs. ZEN is able to modulate molecules involved in immune response, also can modulate MAPK pathways, Toll-Like Receptors (TLRs) and down regulated of IL-1 $\beta$ , IL-8, IL-4 at protein level (Braicu *et al.*, 2016a). Other study in IPEC-1, shown that ZEN altered gene expression pattern involved in proliferation, cytokine production and inflammatory response (Braicu *et al.*, 2016b). A study conducted by Wan *et al.* (2013), was carried out to elucidate the effect of ZEN (10 or 40 $\mu$ M/48h), on the mRNA expression and protein secretion of porcine  $\beta$ -defensins 1 and 2 (pBD-1 and pBD-2) using IPEC-J2. The results show an upregulation of pBD-1 and pBD-2 mRNA expression. However, no significant increase in secreted pBD-1 and pBD-2 protein levels was observed. More effects of ZEN on the intestine were summarized in the Table 3.

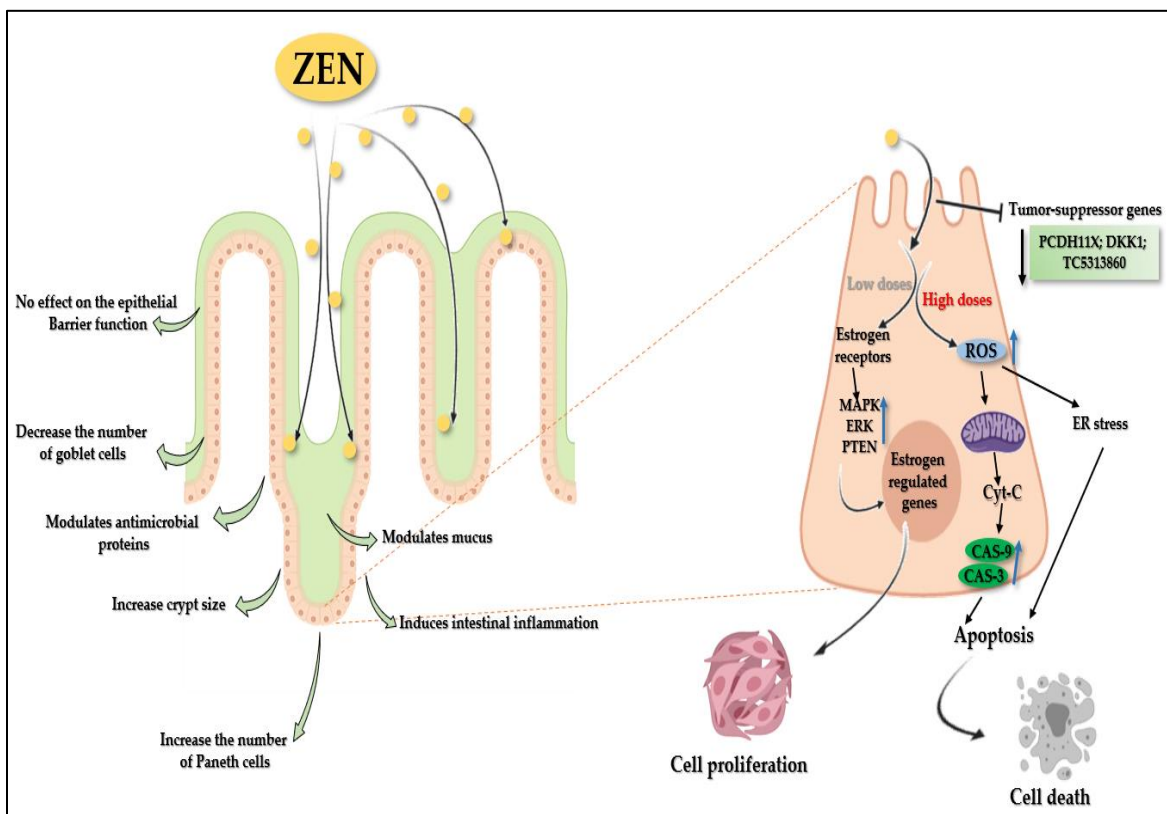


Figure 12: **Effects of ZEN on the intestine.** ZEN did not influence the architecture of the mucosa and submucosa in the large intestine, but decreased the number of goblet cells. ZEN increased in the number of Paneth cells and crypt size, modulates antimicrobial proteins and induced intestinal inflammation in the small intestine. At low doses, ZEN is able to promote proliferation through estrogen signaling, while at high doses ZEN inhibits tumor-suppressor genes, and induces ROS production, decreases the mitochondrial membrane potential making it permeable and inhibiting the function of cytochrome c, and

increased caspase-3 and caspase-9 expression. Inactivated cytochrome c leaks from mitochondria into cytosol promoting apoptosis. Furthermore, ZEN induced apoptosis via endoplasmic stress

In short, ZEN has different effects on intestinal level: it can decrease the number of goblet cells and increase the number of Paneth cells at the bottom of the intestinal crypts without altering the structure of the intestinal epithelium. At the cellular level, ZEN can induce oxidative injury, cell apoptosis, as well as induce inflammation without affecting the epithelial barrier function integrity (Figure 12).

**Table 3: In vitro and in vivo effects of ZEN**

| <b>Mycotoxin</b> | <b>Dose exposure</b> | <b>Exposure period</b> | <b>Animal or cell type</b>                            | <b>Effects</b>   | <b>References</b>            |
|------------------|----------------------|------------------------|---|--|------------------------------|
| ZEN              | 25 µM                | 24h                    | IPEC-1<br>(Intestinal Porcine Epithelial Cell line-1) | Altered gene expression pattern involving in (Transcription factor, Signaling, Cell signaling, Proliferation, Cytokine, Interleukin, Inflammatory response, and Growth factor  | Braicu <i>et al.</i> , 2016a |
| ZEN              | 0–100µM<br>10µM      | 24h<br>24h             | IPEC-1  | Cytotoxic at high concentration<br><br>Modulate the cell proliferation and MAPKs signaling pathway<br>Higher expression of the cytokines (TNF-α, IL-1b, IL-6, IL-8, MCP-1, IL-12p40, CCL20)<br>Increase of TLR(1-10) | Taranu <i>et al.</i> , 2015  |
| ZEN              | 25µM                 | 1h                     | IPEC-1  | Increase of TLR-8 and a decreased of TLR-4 gene expression.  | Taranu <i>et al.</i> , 2015  |

|     |   |                |   |   |                            |
|-----|---|----------------|---|---|----------------------------|
|     |   |                |   | A slight increase of TLR-9 and TLR-10   |                            |
| ZEN | 10–100 $\mu$ M<br><br>10 and 25 $\mu$ M/L | 24h<br><br>24h | IPEC-1  | No effect on trans-epithelial electrical resistance<br><br>No effect on the expression of IL-8 and IL-10  | Marin <i>et al.</i> , 2015 |
| ZEN | 0, 6.125, 12.5, 25, 50 $\mu$ g/mL         | 48 h           | IPEC-J2<br>(Intestinal Porcine Epithelial Cell line-J2) | MDA concentrations were significantly increased<br>Damage antioxidant enzyme system, increased ROS production, aggravate the injury of mitochondria<br>Reduce the activity of GSH-PX, CAT and T-SOD | Fan <i>et al.</i> , 2017   |
| ZEN | 40 $\mu$ M<br>10 $\mu$ M                  | 48 h           | IPEC-J2   | Up-regulation of pro-inflammatory cytokine mRNA expression (IL1 $\alpha$ , IL1 $\beta$ , IL6, IL8, TNF $\alpha$ )   | Wan <i>et al.</i> , 2013   |
| ZEN | 10 or 40 $\mu$ M                          | 48h            | IPEC-J2   | Upregulation in mRNA expression of porcine $\beta$ -defensins 1 and 2 (pBD-1 and pBD-2)   | Wan <i>et al.</i> , 2013   |

|     |                                      |  |   |   |                                  |
|-----|--------------------------------------|--|---|---|----------------------------------|
| ZEN | 8µg/mL<br>4.5 mg/kg body weight      | 24 h<br>1 week                         | IPEC-J2<br>24 Balb/c mice   | Enhance the NLRP3 inflammasome, pro-IL-1b, pro-IL-18, IL-1β and IL-18 mRNA expression<br><br>Increase the production of IL-1β, IL-18 and levels myeloperoxidase (MPO) | Fan <i>et al.</i> , 2018         |
| ZEN | 0, 6, and 8µg/mL<br>8µg/mL           | 24h<br>0 h, 12 h, 24h, 36 h, and 48 h. | IPEC-J2   | Induce autophagy<br>Accumulation of ROS<br><br>Increase in cytochrome P450 reductase  | Shen <i>et al.</i> , 2019        |
| ZEN | 1–150µM<br>10, 20 and 40µM<br>5–40µM | 72 h<br>24h<br>24 h                    | Caco-2<br>(Cancer coli-2; from a human colorectal adenocarcinoma) | Reduce cells viability<br><br>Inhibit protein and DNA syntheses<br><br>Increase significantly the production of MDA   | Kouadio <i>et al.</i> , 2005     |
| ZEN | 0 to 100 µM                          | 48h                                    | Caco-2  | Cell death and an increase in MDA<br>DNA fragmentation<br>caspase-3 activation  | Abid-Essefi <i>et al.</i> , 2009 |
| ZEN | 1 to 60µM<br>10, 20 and 40µM         | 72h<br>24h                             | Caco-2  | Inhibit cell viability<br>Affect cell cycle<br>Decrease in protein and DNA syntheses  | Abid-Essefi <i>et al.</i> , 2004 |

|     |   |  |   |  |   |
|-----|---|--|---|--|---|
|     |   |  |   | Lipid peroxidation   |   |
| ZEN | 0–320 $\mu$ M<br>1 to1000 nM<br>0.64 to 200mM | 48h<br>After24, 48, 72, 96 and<br>120 h<br>30 days | HCT116<br>(human colorectal<br>carcinoma cell line)   | Enhances cell<br>proliferation,<br>increases colony<br>formation and fastens<br>cell migration   | Abassi <i>et al.</i> , 2016                   |
| ZEN | 200 and 400 $\mu$ g/kg b.w                    | 7 days   | Gilts<br>(Large white polish)<br>Small intestine  | Increased activity of<br>the goblet cells,<br>No change in<br>intestinal morphology<br>Increased activity of<br>endocrine cells<br>Presence of Paneth<br>cells on the bottom of<br>intestinal crypts | Obremski <i>et al.</i> , 2005                 |
| ZEN | 40 $\mu$ g/kg BW per day                      | 1, 3, and 6 weeks                                  | Gilts<br>(White Polish Big $\times$<br>Polish White Earhanging)<br>cecum and<br>the ascending and<br>descending colon | Decrease number of<br>goblet cells<br>Increase the number<br>of lymphocytes and<br>plasma cells in the<br>large intestine  | Przybylska-<br>Gornowicz <i>et al.</i> , 2018 |
| ZEN | 0.5 to 1.5 mg/kg                              | 35 days  | Gilts<br>(LandraceYorks-hire $\times$<br>Duroc)<br>Jejunum  | Decrease antioxidant<br>enzyme activity and<br>increase MDA<br>concentration<br>Accumulations of<br>Nrf2, GPX1, HO1,<br>NQO1, and GCLM in<br>the jejunum   | Cheng <i>et al.</i> , 2019                    |

|     |                      |                                   |  |  |   |
|-----|----------------------|-----------------------------------|--|--|---|
| ZEN | 40 mg/kg BW          | 1–6 weeks                         | Gilts<br>(White Polish Big xPolish<br>White Earhanging)<br>Duodenum            | Increase the number<br>of lymphocytes and<br>plasma cells in lamina<br>propria.<br>Change in the<br>thickness of the<br>submucosa  | Lewczuk <i>et al.</i> , 2016                  |
| ZEN | 40 g/kg/d            | 7, 14, 21, 28, 35, and 42<br>days | Gilts<br>(Duodenum, the jejunum,<br>and the ascending and<br>descending colon) | Changes in micrRNA<br>(involved in cell<br>proliferation and<br>survival expression in<br>the descending colon   | Brzuzan <i>et al.</i> , 2015                  |
| ZEN | 0.1mg/kg<br>per day  | 42 days                           | Pig<br>(Polish Large White)<br>Ileum   | Modify the<br>percentages of T and<br>B lymphocyte<br>subpopulations in<br>Peyer's patches<br>Increase the<br>concentration of IL-12<br>and IL-1<br>Increase of<br>neuropeptides VIP in<br>the ileum | Obremski <i>et al.</i> , 2015                 |
| ZEN | 0.1 mg/kg feed / day | 14,28 and 42 days                 | Pig<br>(Polish Large White)<br>Ileum   | Decrease levels of IL-<br>2, IFN- $\gamma$ , IL-4 and IL-<br>10<br>Increase in ZEN<br>concentrations in<br>Peyer's patches   | Obremski, 2014                                |
| ZEN | 100 $\mu$ k/kg       | 14, 28<br>and 42 days             | Pig<br>(Polish Large White)<br>Ileal Peyer's patch                             | High frequency of<br>apoptosis in the<br>germinal centers of<br>ileal Peyer's patch.   | Obremski and<br>Poniatowska-Broniek.,<br>2015 |

|     |  |               |  |  |                              |
|-----|--|---------------|--|--|------------------------------|
| ZEN | 100 ppb  | 30 days       | Pig<br>(Landrace × Large White)<br>× (Duroc × Pietrain)<br>Duodenum  | Gene expression considerably affected by ZEN at duodenum level<br>Alteration of molecules involved in immune response<br>Alteration in MAPK pathways or Toll-Like Receptors (TLRs)<br>Down regulation of IL-1 $\beta$ , IL-8, IL-4 at protein level  | Braicu <i>et al.</i> , 2016b |
| ZEN | 2.77mg/kg  | 35 to 70 days | 18 pregnant sows (Yorkshire sows)<br>(pregnant sows and offspring on postnatal days (PD) 1, 21 and 188)<br>Jejunum | Increase in MDA (pregnant sows and PD1 and PD21 piglets)<br>Increase GPx activity induce an oxidative damage (offspring on PD1 and PD21)<br>Increase the production IL-1 $\alpha$ , IL-1 $\beta$ , IL-6 and TNF- $\alpha$ (newborn piglets)<br>Changes in the structure of jejunum and alterations of the bacterial numbers in cecal digesta | Liu <i>et al.</i> , 2018     |
| ZEN | 0, 535, 1041, 1548, 2002, and 2507 $\mu$ g/kg diet | 10 weeks      | juvenile grass carp  | Accumulated in the intestine and caused  | Wang <i>et al.</i> , 2019    |



|  |  |  |  |   |  |
|--|--|--|--|---|--|
|  |  |  |  | <p>histopathological lesions (Edema in the lamina propria, leucocyte infiltration, necrosis of epithelial cells, blood capillary hyperemia, and goblet cell hyperplasia in the proximal intestine, mid intestine, and distal intestine)</p> <p>Oxidative injury associated with Nuclear factor-erythroid 2-related factor 2 (Nrf2)</p> <p>Apoptosis associated with p38 mitogen activated protein kinases (p38MAPK)</p> <p>Breached tight junctions associated with myosin light chain kinase (MLCK) signaling pathways</p> |  |
|--|--|--|--|---|--|

ZEN : Zearalenone; MAPK : Mitogen-activated protein kinases; TNF- $\alpha$ : Tumor necrosis factor; IL : Interleukin ; MCP-1 : Monocyte chemoattractant protein-1 ;CCL20 : Chemokine ligand 20 ; TLR : Talk like receptor ; CAT : Catalase ; T-SOD : Superoxide Dismutase ; NLRP3 : NLR Family Pyrin Domain Containing 3 ; pro-IL : pro-Interleukin; ROS: Reactive oxygen species; DNA: deoxyribonucleic acid; Nrf2: Nuclear factor (erythroid-derived 2)-like 2; GPX1: Glutathione Peroxidase 1; HO1: Heme Oxygenase 1; NQO1: NAD(P)H Quinone Dehydrogenase 1; GCLM : Glutamate-Cysteine Ligase Modifier Subunit.

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# **Aims of the thesis**

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ZEN is a mycotoxin synthesized by selected fungi of the genus *Fusarium* that frequently contaminates cereal-based food and feed. The structure and shape of ZEN resembles endogenous estrogen, the 17 $\beta$ -estradiol. ZEN is hence considered an endocrine disruptor (Kowalska *et al.*, 2016; Rai *et al.*, 2019).

The intestine is the first organ targeted by dietary mycotoxins (Pinton and Oswald, 2014). The toxic effect of ZEN in the intestine has been investigated in the past, and some studies suggested that ZEN might stimulate molecular changes related to the activation of early carcinogenesis, as well as modifying its metabolic and immune status. However, the molecular mechanisms behind these changes are not clear.

On the other hand, following administration, ZEN is metabolized by intestine giving rise to different derivatives such as  $\alpha$ -ZEL.  $\alpha$ -ZEL is the predominant metabolite in humans, rat and pig (Abid-Essek *et al.*, 2009; EFSA, 2016). In addition, studies reported the co-occurrence of ZEN,  $\alpha$ - and  $\beta$ -ZEL in cereal-based products (De Boevre *et al.*, 2013; Nathanail *et al.*, 2015). Both ZEN and  $\alpha$ -ZEL are cytotoxic at high concentrations. Most toxicological studies concerning the cytotoxic effects of ZEN and  $\alpha$ -ZEL effects in the intestine, took into account the exposure to a mycotoxin alone not in combination. However, it is important to know whether there will be an interaction between these toxins as well as the nature of that interaction.

The aim of the present study was to assess the toxic effect of ZEN on the intestine. This research will pursue two objectives:

1. To investigate the molecular mechanisms related to the apparent ZEN-dependent activation of signaling related with early carcinogenesis, metabolic changes and immune status changes in the intestine (Chapter 1).

2. To elucidate the individual and combined cytotoxicity of ZEN and  $\alpha$ -ZEL, as well as evaluating the nature of the possible interaction observed between studied mycotoxins (synergistic, antagonistic or additive effect) in intestinal cells (Chapter 2).

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# Experimental work

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# Chapter 1

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## **Acute Exposure to Zearalenone Disturbs Intestinal Homeostasis by Modulating the Wnt/ $\beta$ -Catenin Signaling Pathway.**

*The content of this chapter was published in the Volume 12, Issue 2 of the journal "Toxins" in February 2020 under the title "Acute Exposure to Zearalenone Disturbs Intestinal Homeostasis by Modulating the Wnt/ $\beta$ -Catenin Signaling Pathway" and was authored by Tarek Lahjouji, Aurora Bertaccini, Manon Neves, Sylvie Puel, Isabelle P. Oswald, and Laura Soler ( page 113; doi: 10.3390/toxins12020113.)*

## Introduction

Food contaminants, including mycotoxins, are able to compromise intestinal pathophysiology. The mycoestrogen ZEN is known to promote molecular changes related to the activation of early carcinogenesis, as well as modifying its metabolic and immune status. Here, we investigated the Wnt/ $\beta$ -catenin and TGF- $\beta$  signaling pathways, two pathways that are known to play a key role in the toxic effect of ZEN in different cells types, and are essential for the intestinal epithelial physiology. The effect of ZEN in the expression on the immune status and metabolism-related genes was also investigated.

## Abstract

The mycotoxin zearalenone (ZEN), which frequently contaminates cereal-based food human and animal feed, is known to have an estrogenic effect. The biological response associated with exposure to ZEN has rarely been reported in organs other than the reproductive system. In the intestine, several studies suggested that ZEN might stimulate molecular changes related to the activation of early carcinogenesis, but the molecular mechanisms behind these events are not yet known. In this study, we investigated gene expression and changes in protein abundance induced by acute exposure to ZEN in the jejunum of castrated male pigs using an explant model. Our results indicate that ZEN induces the accumulation of ER $\alpha$  but not ER $\beta$ , modulates Wnt/ $\beta$ -catenin and TGF- $\beta$  signaling pathways, and induces molecular changes linked with energy sensing and the antimicrobial activity without inducing inflammation. Our results confirm that the intestine is a target for ZEN, inducing changes that promote cellular proliferation and could contribute to the onset of intestinal pathologies.

## 2.1. Introduction

Zearalenone (ZEN) is a mycotoxin synthesized by some fungi of the *Fusarium* genus that frequently contaminate crops such as corn, barley, wheat, rice, oats and sorghum, particularly in temperate regions (Zinedine *et al.*, 2007). ZEN is classified as an endocrine disruptor because it can bind and activate estrogen receptors with hyper-estrogenic effects (Kowalska *et al.*, 2016). Like other endocrine disruptors, ZEN has a dual toxicity: pro-apoptotic effects at high, acute doses, and anabolic effects at low doses during chronic exposure (Zheng *et al.*, 2018). At the cellular level, the toxic effect of high concentrations of ZEN is independent of estrogen signaling, and is characterized by a high oxidative state that induces apoptosis (Zheng *et al.*, 2018). Indeed, ZEN provokes a dose-dependent increase of reactive oxygen species levels, oxidative DNA damage, alteration of the mitochondria,



membrane disruption and lipid peroxidation (Gazzah *et al.*, 2010; Zheng *et al.*, 2017). These processes can activate a pro-inflammatory cascade (Liu *et al.*, 2014; Fan *et al.*, 2018), resulting in the expression of inflammatory cytokines, which has been reported in several tissues exposed to ZEN (Fan *et al.*, 2018; Marin *et al.*, 2013) . At lower doses, the in vitro toxic effect of ZEN is more dependent on the activation of the estrogen receptor (ER) signaling pathway, which has an anti-inflammatory (Liu *et al.*, 2014; Marin *et al.*, 2013; Braicu *et al.*, 2016 ), anti-apoptotic and proliferating effect (Zheng *et al.*, 2018; Metzler *et al.*, 2010; Belli, et al., 2010). ER is a ligand-activated transcriptional factor, and signaling is mainly activated directly upon DNA binding in the estrogen response elements located in target genes, but also indirectly (not involving DNA binding) through interaction with other signaling pathways (Kuiper *et al.*, 1996; Paech *et al.*, 1997). There are two types of ERs, ER $\alpha$  and ER $\beta$ , whose roles differ. ER $\alpha$  is the main regulator of estrogen-dependent genes and its activation has proliferative effects. ER $\beta$ , when co-expressed with ER $\alpha$ , has a tendency to restrain ER $\alpha$  activity and its activation inhibits cell proliferation (Paterni *et al.*, 2014). ZEN is able to activate both ERs but is a partial agonist of ER $\beta$  and a full agonist of ER $\alpha$  (Fink-Gremmels *et al.*, 2007). Consequently, the biological response to exposure to ZEN varies depending on the tissue-specific ratio of the estrogen receptor (ER)  $\alpha$  vs  $\beta$ , as well as on the density of these receptors (Böttner *et al.*, 2014). In the intestine, the presence of each ER is distributed differently along the crypt–villus axis, ER $\alpha$  being more abundant in the crypt (where cell proliferation occurs) while ER $\beta$  is more abundant in the villi (composed of differentiated enterocytes) (Hasson *et al.*, 2014; Cho *et al.*, 2007). Estrogen signaling interacts with other pathways that are important for intestinal homeostasis and its disruption seems to be connected with the development of chronic intestinal diseases and cancer (Gao *et al.*, 2013; Matsuda *et al.*, 2001 ). As the intestine is an estrogen-responding organ, it is important to understand the molecular effect of natural endocrine disruptors such as ZEN. Moreover, due to its ability to induce

both cell proliferation and oxidative DNA damage, a link has been suggested between ZEN and cancer promotion in the literature (Zheng *et al.*, 2013).

The toxic effect of ZEN in the intestine has been investigated in the past, mostly using pig as model for humans due to their similar sensitivity and toxicity (Kuiper-Goodman *et al.*, 1987). The main reason for ZEN sensitivity is that pigs, like humans, convert ZEN into the more estrogenically active  $\alpha$ -zearalenol (Binder *et al.*, 2017). In vivo, whereas some authors found no morphologic changes (Obremski *et al.*, 2005; Przybylska-Gornowicz *et al.*, 2018). Others found that chronic exposure of pigs to ZEN led to transient morphological modifications in the small intestine during the first weeks of exposure (Przybylska-Gornowicz *et al.*, 2018), or to an increase in the number of Paneth cells at the bottom of the intestinal crypts (Obremski *et al.*, 2005). At the cellular and molecular level, several reports have described changes in the gene expression of pro-inflammatory cytokines, genes implicated in the induction of a proliferative state such as Dickkopf-related protein 1 (DKK1),  $\beta$ -catenin or the proto-oncogene c-Myc (Liu *et al.*, 2014; Braicu *et al.*, 2016; Taranu *et al.*, 2015). Taken together, these results suggest that ZEN can induce transient proliferation in the small intestinal crypt, which could be connected with pro-cancerogenic changes in wingless-type MMTV integration site family (Wnt) / $\beta$ -catenin and transforming growth factor  $\beta$  (TGF- $\beta$ ) signaling pathways. The two latter pathways are known to play a key role in the toxic effect of ZEN in ovarian (Yang *et al.*, 2018), uterine and prostatic cancer cells (Kowalska *et al.*, 2018), and to be implicated in the onset and progression of intestinal proliferative/cancerous events (Spit *et al.*, 2018), but the activation of these pathways by ZEN has not been investigated to date.

In this study, we investigated the activation/repression of Wnt/ $\beta$ -catenin and TGF- $\beta$  signaling pathways, the immune status and metabolism by ZEN to understand the effect of this toxin on the intestine of castrated male pigs. Our results confirmed that the small intestine is a target for ZEN, and that its toxic effect could

contribute to the development or aggravation of severe intestinal pathologies like cancer.

## **2.2. Materials and Methods**

### **2.2.1. Toxin**

Purified zearalenone (ZEN) was purchased from Sigma (St Quentin Fallavier, France), and dissolved in dimethylsulfoxide (DMSO; Sigma) to obtain a concentration of 20 mM, aliquoted and stored at  $-20^{\circ}\text{C}$ .

### **2.2.2. Animals and culture of pig jejunal explants**

Six castrated male, 5-week old piglets, were provided by a local farm (Gaec de Calvignac, St. Vincent d'Autejac, France). The experiment was conducted under the guidelines of the French Ministry of Agriculture for animal research. The Ethics Committee of Pharmacology-Toxicology of Toulouse-Midi-Pyrénées approved all animal experimentation procedures (Toxcométhique; N°: TOXCOM/0136/ PP). The collection of jejunum tissue and the preparation of explant cultures was as described previously (Lucioli *et al.*, 2013; García *et al.*, 2018). The only modification being changing the culture medium to Williams phenol red-free (Sigma) supplemented with 1% of insulin transferrin-selenium (Sigma), 1% penicillin (Eurobio, Courtaboeuf, France), 1% alanine-glutamine (Sigma), 0.5% gentamycin (Eurobio) and 50 mL of glucose (Sigma). Explants were exposed to ZEN (100  $\mu\text{M}$ ) or vehicle only (DMSO; Sigma) and incubated in the same conditions for a period of 1 h or 4 h (Figure 13). After treatment, tissues were snap frozen in liquid nitrogen, and the culture medium containing proteins leaked from explants was recovered. All samples were stored at  $-80^{\circ}\text{C}$  until analysis.

### 2.2.3. RNA extraction and real-time quantitative PCR (RT-qPCR)

#### (Figure 13)

The pig jejunal explants were homogenized using 2 mL plastic bead tubes (MT Biomedicals, Illkirch, France) in 1 mL of Extract-All reagent (Eurobio) in a Precellys Evolution tissue homogenizer (Bertin Technologies, Montigny-le-Bretonneux, France). ARN isolation and RT-qPCR were performed as described elsewhere (Alassane-Kpembé *et al.*, 2017; Pierron *et al.*, 2016). Data analysis was carried out using LinRegPCR freeware (Ruijter *et al.*, 2009), and normalized against two reference genes, TATA-Box Binding Protein (TBP) and Hypoxanthine guanine phosphoribosyl transferase 1 (HPRT1). Primers are described in supplemental table S2.

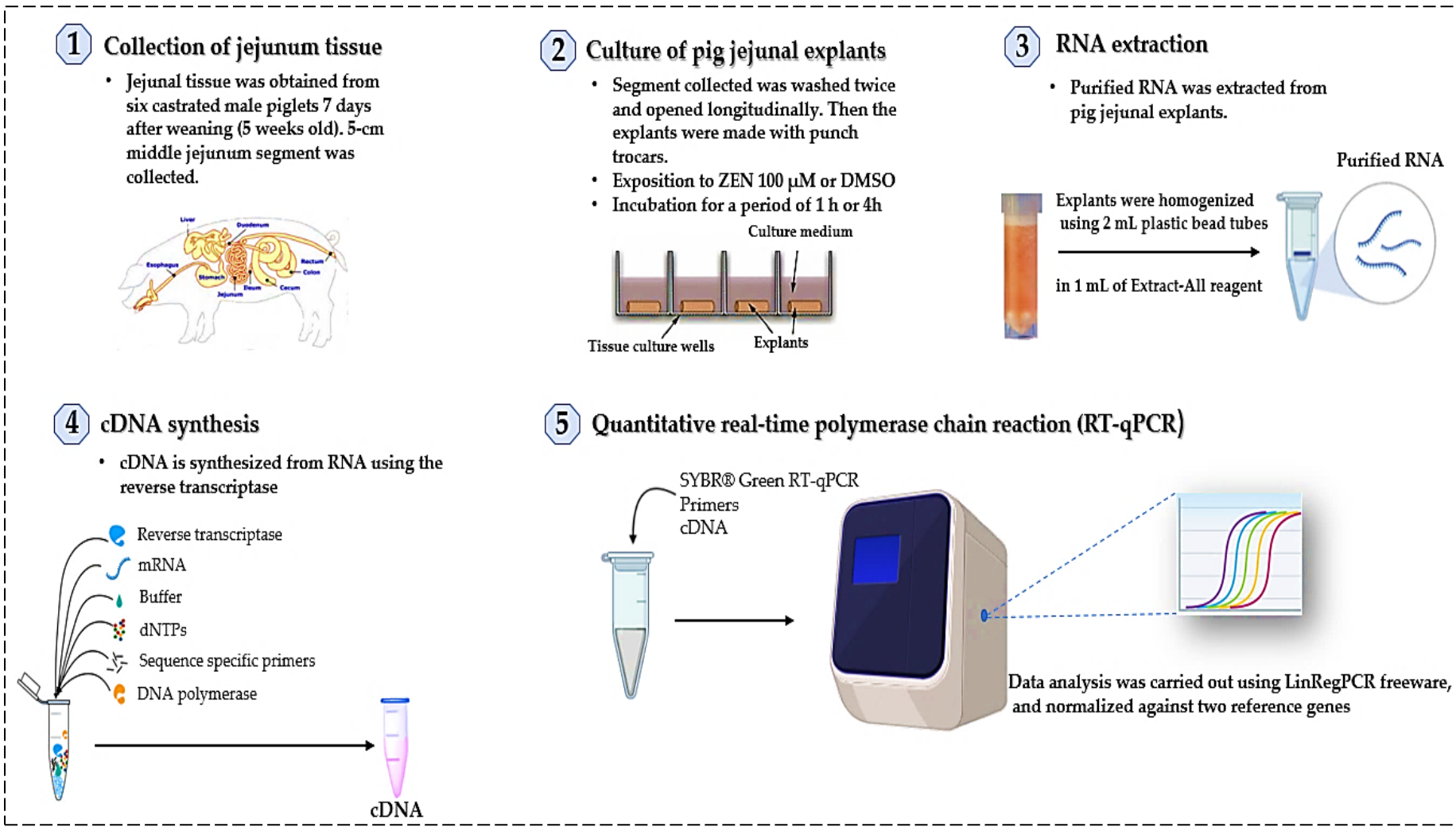


Figure 13: Overview of experimental design to study the effect of ZEN on the intestine. After collection of jejunum explants, they were exposed to ZEN (100  $\mu$ M) for 1 or 4h. RNA from jejunal explants were extracted and cDNA was synthesized. Finally, RT-qPCR was performed to determine the relative mRNA expression levels of the different genes.

#### **2.2.4. Protein extraction and western blot (Figure 14)**

Proteins from jejunal explants were extracted using 2 mL plastic bead tubes (MT Biomedicals) in 0.5 mL of RIPA buffer containing protease- and phosphatase inhibitors (Sigma) in a Precellys Evolution tissue homogenizer (Bertin Technologies). Total protein content was quantified (BCA protein assay, Thermo Fisher Scientific). Total protein extracts (20 µg) were separated in 12.5% or 15% acrylamide-bisacrylamide gels, and transferred onto nitrocellulose membranes. Blots were blocked in RotiBlock (Carl Roth GmbH, Karlsruhe, Germany) and incubated with the corresponding primary antibody at 4 °C overnight under agitation (Supplemental table 3). Detection was achieved using appropriate species-specific fluorescent secondary antibodies (Biotium, Inc., Fremont, CA, USA). Total protein staining of the membrane with SyproRuby blot stain (Thermo Fisher Scientific) prior to blocking served as a loading control in all cases. Images were obtained by scanning Chemidoc (SyproRuby-stained membranes; Bio-Rad) or a Li-Cor Odyssey Infrared Imager (Fluorescent immunoblots; Li-Cor Biosciences, Lincoln, NE, USA). All the images were digitalized and analyzed with Image Studio Lite Software (Li-Cor Biosciences). For each lane, band intensity values were normalized onto the respective overall protein staining.

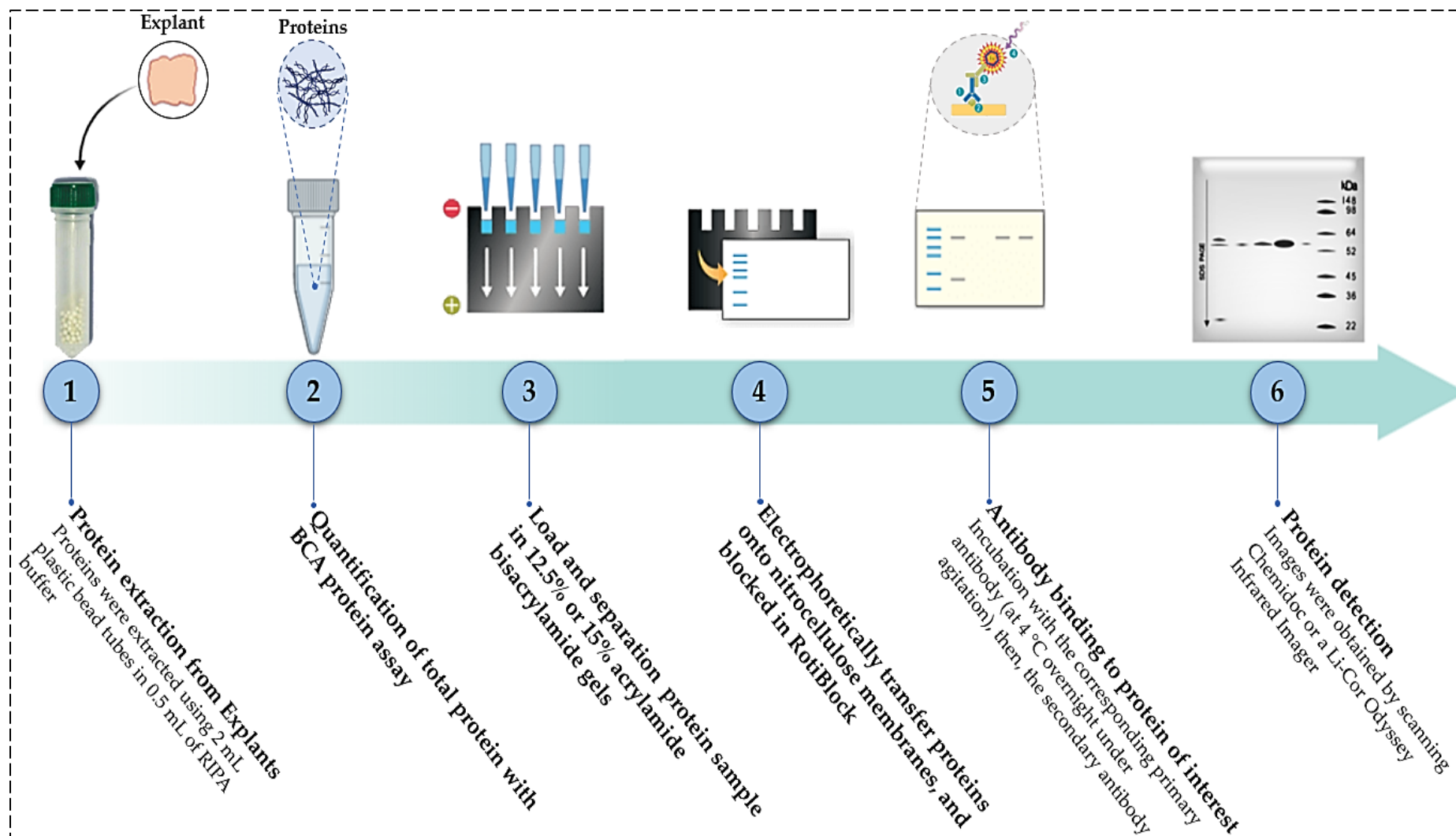


Figure 14: **Protein extraction and western blot.** The total proteins were extracted from explants and quantified. Using the western blot, the proteins were separated by size, then transferred onto nitrocellulose membranes, and marking target protein using a proper primary and secondary antibody to visualize proteins by scanning Chemidoc or a Li-Cor Odyssey Infrared Imager.

### 2.2.5. Gelatin Zymography (Figure 15)

Gelatin zymography is a method used for the detection, identification, and relative quantification of gelatinase enzyme activity in biological samples (Ren *et al.*, 2017; Toth *et al.*, 2012). In brief, samples are separated in a gel electrophoresis under non-reducing conditions in a gel containing gelatin. Once separated, the gelatinase activity of each enzyme contained in the gel will be activated. This activity will be revealed as a clear band after Coomassie blue staining in the gel. The gelatinase activity of gelatinase enzymes, such as the metalloproteinases MMP2 and MMP9 and their proteoforms can be hence identified using an adequate activation buffer according to their migration pattern. In our experiment, the explant culture medium was concentrated (approximately 10-fold) using centrifugal filter units (Amicon Ultra-2 mL Centrifugal Filters, Merck-Millipore, Darmstadt, Germany). Total protein content was quantified (BCA protein assay, Thermo Fisher Scientific) according to the manufacturer's instructions. Protein aliquots (10 µg) were separated in 7.5% acrylamide-bisacrylamide gels containing 0.3 mg/mL gelatin from porcine skin (Sigma) under non-reducing, non-denaturing conditions. Gels were incubated (2 × 30 min) in 2.5% Triton X-100 (Sigma) for protein renaturation and then proteolytic reaction was allowed to take place in 50 mM Tris-HCl, 50 mM NaCl, 10 mM CaCl<sub>2</sub>, pH 7.5, for 20 h at 37 °C, followed by staining with colloidal Coomassie blue stain (Sigma). Gelatinolytic activity was visible as clear zones on a blue background. Images were obtained using a Li-Cor Odyssey Infrared Imager (Li-Cor Biosciences) and analyzed with Image Studio Lite Software (Li-Cor Biosciences).



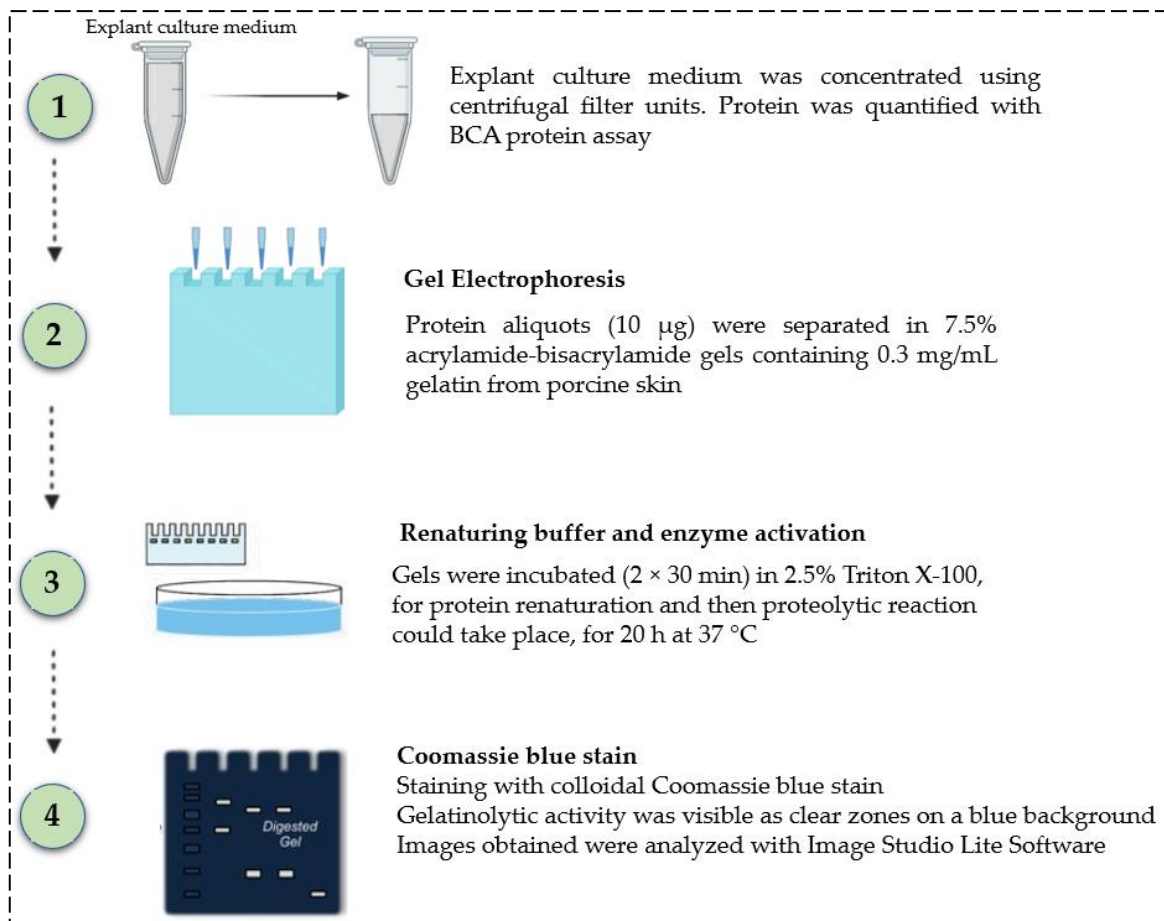


Figure 15: **Gelatin Zymography for MMP9 and MMP2.** Using filter Units, the explant culture medium was concentrated, and the proteins was quantified. Then, the proteins were separated in a gel electrophoresis under non-reducing conditions in a gel containing gelatin. Once separated, the gelatinase activity of each enzyme contained in the gel will be activated. This activity will be revealed as a clear band after Coomassie blue staining in the gel. The gelatinase activity of gelatinase enzymes, such as the metalloproteinases MMP2 and MMP9 and their proteoforms can be hence identified as clear zones on a blue background. Finally, Images obtained were analyzed with Image Studio Lite Software.

## 2.2.6. Statistical analysis

Statistical differences in gene expression and protein abundance were determined using paired t-tests using GraphPad Prism statistical software version 6 (GraphPad Software, San Diego, CA, USA). The significance level was set at  $p < 0.05$ . The false discovery rate (FDR) method was utilized to correct for multiple testing ( Table S4).

### Supplementary Materials:

Table S1: Results of the RT-qPCR of pig intestinal explants exposed for 1 h or 4 h to 100  $\mu$ M ZEN



Regarding protein expression, changes in relative abundance were significant for estrogen receptor alpha (ER $\alpha$ ), cofilin-1 (CFL1),  $\beta$ -catenin, vimentin (VIM), and chemerin receptor (CMKLR1) at 4 hours ( $p < 0.05$ ; Figure 17). Zymography analysis revealed a significant decrease in the gelatinase activity of pro- and active metalloproteinase proteins 2 (MMP2) and active metalloproteinase protein 9 (MMP9) at 4 h of exposure. A description of the results is given on the following paragraphs, and is organized according to the main signaling pathway studied.

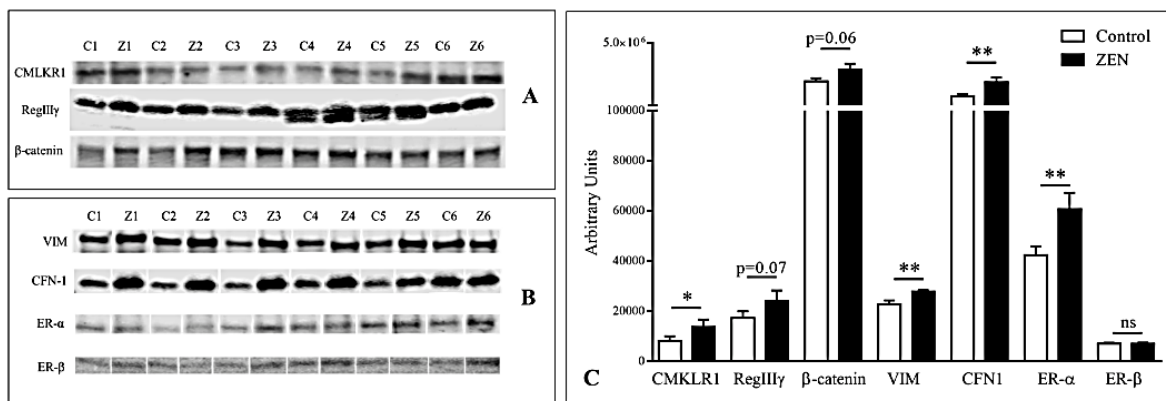


Figure 17: **Immunoblotting analysis of ER $\alpha$  and ER $\beta$** , as well as proteins showing either significant or almost significant differences between six paired pig jejunal explants (1-6) exposed (Z) or not (C) to 100  $\mu$ M ZEN (Z). (A) CMLKR1, RegIII $\gamma$ ,  $\beta$ -catenin, (B) VIM, CFN-1, ER $\alpha$  and ER $\beta$ . (C) Relative quantification of normalized signal (arbitrary units). Values are means with standard errors of the mean represented by vertical bars (n = 6). Asterisks indicate statistical differences (\* $p < 0.05$ ; \*\*  $p < 0.01$ ).

### 2.3.1. Effect of Zearalenone on ER abundance

Relative protein quantification by western blot showed a significant up-accumulation of ER $\alpha$  after 4 h of exposure of ZEN ( $p < 0.01$ ), while ER $\beta$  abundance did not change at any time point.

### 2.3.2. Effect of ZEN on the Wnt/ $\beta$ -catenin signaling pathway

We investigated the effect of ZEN on the activation of the Wnt/ $\beta$ -catenin signaling pathway. Extracellular Wnts activate responding cells by binding to its membrane receptors Frizzled and Low-density lipoprotein receptor-related protein (LRP). This leads to a cascade of events that allows the stabilization of cytoplasmic  $\beta$ -catenin and its translocation of the nucleus through a process that depends on the

activity of glycogen synthase kinase 3 beta (GSK3 $\beta$ ) and is inhibited by the proteins adenomatous polyposis coli (APC) and axins. In the nucleus,  $\beta$ -catenin associates with T-cell factor/lymphoid enhancer-binding transcription factors (TCF/LEF) to induce the expression of target genes, such as Cyclin 1, Zinc Finger E-Box Binding Homeobox 1 and 2 (Zeb1/2), Snail1, Snail2 and c-Myc among others. The whole process is inhibited by DKK-1 (Gregorieff *et al.*, 2005).

Several genes of this signaling cascade were up-regulated by 4 h of exposure to ZEN, namely,  $\beta$ -catenin ( $p < 0.05$ ; showing a similar trend at the protein level, although the up-accumulation was only almost significant;  $p=0.06$ ), Cyclin D1 ( $p < 0.05$ ), c-Myc ( $p < 0.05$ ), Zeb1 ( $p < 0.05$ ) and Snail2 ( $p < 0.01$ ), whereas TCF1 was upregulated at 1 h of exposure ( $p < 0.05$ ). A significant down-regulation was observed at 1 h for TCF4 ( $p < 0.01$ ), and at 4 h ( $p < 0.05$ ) for Wnt11 ( $p < 0.05$ ), DKK1 ( $p < 0.05$ ), APC ( $p < 0.01$ ). Nearly significant downregulation of axin2 ( $p=0.06$ ) and APC ( $p=0.06$ ) at 1 h was also observed. The gene expression of ATPase H<sup>+</sup> transporting accessory protein 2 (ATP6ap2), a facilitation of the transmission of  $\beta$ -catenin signaling (Sihn *et al.*, 2010), was significantly induced at 4 h ( $p < 0.05$ ).

To link the activation of Wnt/ $\beta$ -catenin with cell proliferation, we investigated two markers of cell proliferation, minichromosome maintenance complex component 3 (MCM3;  $p < 0.05$ ) and Vimentin (VIM;  $p < 0.01$ ), whose expression was significantly up-regulated at 4 h of exposure. VIM was also significantly up-accumulated at 4 h of exposure at the protein level ( $p < 0.001$ ).

Among the expected consequences of  $\beta$ -catenin transcriptional activation is the repression of the expression of the tight junctional proteins Zonula occludens-1 (ZO-1), claudins, occludins as well as the adherens junctional proteins cadherins and  $\alpha$ -catenin (Kim *et al.*, 2019). No changes in cadherins were observed at the transcriptional or protein level at any time point. Significant up-regulation of occludin ( $p < 0.05$ ), ZO-1 ( $p < 0.05$ ) and  $\alpha$ -catenin ( $p < 0.05$ ) gene expression was observed at 4 h of exposure. No differences in occludin and ZO-1 were observed at

the protein level, whereas significant up-accumulation of the actin cytoskeleton protein CFL1 was found at 4 h of exposure ( $p < 0.05$ ).

Paneth cells are small-intestine epithelial cells located at the bottom of the crypts. They contain secretory granules containing antimicrobial proteins and peptides, but also play a role in regulating proliferation in the crypt. As their quantity and maturation depends on Wnt/ $\beta$ -catenin signaling (van Es *et al.*, 2005; Farin *et al.*, 2012), we investigated the abundance of several antimicrobial peptides. At 4 h of exposure, we observed an almost significant overexpression of  $\beta$ -defensin 1 (pBD1;  $p=0.05$ ), downregulation of the expression of  $\beta$ -defensin 3 (pBD3;  $p < 0.05$ ) together with a nearly significant overexpression of lysozyme at the transcript level ( $p=0.06$ ). The abundance of regenerating islet-derived protein 3 gamma (RegIII $\gamma$ ) was investigated by western blot, and an accumulation was observed, although it was only nearly significant ( $p=0.07$ ) at 4h.

Taken together, our results provide evidence that ZEN is able to activate the Wnt/ $\beta$ -catenin signaling pathway without affecting the intestinal barrier, and induce the expression of proliferation biomarkers as well as antimicrobial proteins.

### **2.3.3. Effect of ZEN on the TGF- $\beta$ -catenin signaling pathway**

In the intestine, the Wnt signaling pathway interplays with other pathways, including the TGF- $\beta$  signaling pathway. TGF- $\beta$  signaling is mediated through transmembrane receptors that use Smad2 and Smad3 proteins to transduce their signals from the cell surface to the nucleus in a process that requires the presence of Smad4 for DNA binding and transcriptional activation of target genes, and is inhibited by Smad 7 (Matsuda *et al.*, 2001; Derynck *et al.*, 1997).

Transcript levels of TGF- $\beta$ 1 were significantly up-regulated at 1 h of exposure ( $p < 0.05$ ), but no difference was found at 4 h. The expression of all the Smads studied (1,2,3,4 and 7) remained unchanged at all time points, with the exception of Smad2, whose transcript levels were significantly downregulated at 1 h ( $p < 0.05$ ) and 4 h of exposure ( $p < 0.001$ ). We investigated several target genes in this pathway,

and found significant up-regulation of Integrin-alpha 10 at 1 h ( $p < 0.05$ ), no changes in the expression of the CD44 molecule (Indian Blood Group; CD44) nor MMP7, and downregulation of the expression of fibronectin (nearly significant;  $p=0.07$ ), as well as MMP2 and MMP9 ( $p < 0.05$ ) at 4 h of exposure. Gelatinase activity of MMPs leaked to the explant medium was analyzed and whereas no differences were found at 1 h of exposure nor pro-MMP9 at 4 h, the intensity of bands decreased significantly at 4 h of exposure to ZEN for active MMP9 ( $p < 0.05$ ), pro-MMP2 ( $p < 0.01$ ) and active MMP2 ( $p < 0.01$ ; Figure 18).

These results provide no evidence for ZEN-dependent activation of the TGF- $\beta$  signaling pathway.

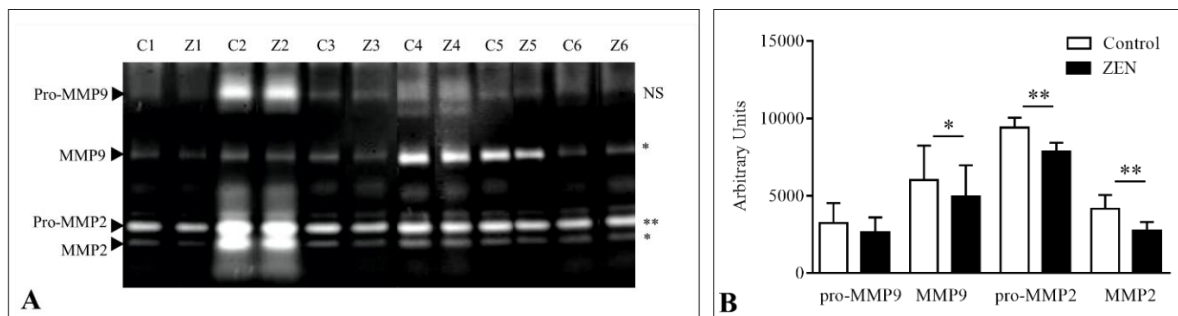


Figure 18: Gelatinolytic activities of jejunal explant culture medium from the control (C) and at 4 h of exposure to ZEN (Z) paired samples from six pigs (1-6). Arrows indicate the position of pro-MMP9, active MMP9, pro-MMP2 and active MMP2. The level of significance of paired t-test analysis of the differences in gelatinolytic activity is given on the right for each band (\* $p < 0.05$ ; \*\*  $p < 0.01$ ; NS: not significant).

#### 2.3.4. Effect of Zearalenone on cytokine, inflammatory markers and adipokine expression

To investigate if ZEN is able to induce intestinal inflammation under our conditions, several cytokines and acute phase proteins were analyzed. No differences were found in the expression or protein abundance of all the inflammatory markers investigated, namely interleukins 8, 17, 18, 22, tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and interferon gamma (IFN $\gamma$ ), protein S100A8, protein S100A12, serum amyloid A3 (SAA3), alpha-acid glycoprotein alpha 1 ( $\alpha$ -1 AGP) and lipocalin 2 (LCN2). Regarding the complex S100A10/AnnexinA2 (ANXA2), no

changes were observed in the gene expression of ANXA2, whereas a marked down-regulation of was significant at 4 h of exposure ( $p < 0.05$ ), and almost significant at 1 h ( $p < 0.06$ ), of protein S100A10.

We investigated the expression of peroxisome proliferator activated receptors (PPARs), since ZEN exposure can increase energy production through an increase in the uptake and  $\beta$ -oxidation of fatty acids, in a processes that has been suggested to be mediated by activation of PPARs in Leydig cells (Li *et al.*, 2014). However, we observed no changes in PPAR $\alpha$  or in PPAR $\gamma$  gene expression at any time-point. The expression of several adipokines and their receptors was also investigated in order to compare it with previous results that produced evidence for regulation on the expression of the chemerin receptor (Taranu *et al.*, 2015). No differences were found in the expression of adiponectin, whereas the mRNA levels of the adipokines receptors for adiponectin (Adiponectin receptor 2; AdipoR2), resistin (cyclase associated actin cytoskeleton regulatory protein 1; CAP1) and chemerin (chemerin chemokine-like receptor 1; CMKLR1, confirmed at the protein level) were significantly upregulated at 4 h of exposure ( $p < 0.05$ ).

These results show that no inflammation was associated with the treatment with ZEN, whereas significant changes were associated with adipokine receptors.

## 2.4. Discussion

Estrogen signaling is important for intestinal homeostasis, but disturbance of the mycoestrogen ZEN in the intestinal molecular pathways that interact with that of estrogen, such as Wnt/ $\beta$ -catenin and TGF- $\beta$ , is not completely understood. Unfortunately, most of the molecular pathways that regulate intestinal physiology cannot be studied *in vitro*, since they are compartmentalized or present as a gradient that can increase or decrease along the crypt-villus axis. Here, an *ex-vivo* model of jejunal explants was used to reflect the complexity of the interaction between the different cell types present in the intestine. The pig is a ZEN-sensitive species (Kuiper-Goodman, *et al.*, 1987) and resemblances in anatomy, physiology and

genetics makes pig the best model for human intestinal studies (Gonzalez *et al.*, 2015). Moreover, the use of control-treated paired explants allowed us to account for individual variations and hence to detect the subtle changes associated with the toxicity of ZEN. Given that the exposure time in this model is restricted to a maximum of 4 h, we used a high dose of toxin that would induce as many molecular changes as possible, while that the short exposure time ensures that cell mortality is low enough so analysis will not be polluted by unspecific events (Rabilloud *et al.*, 2015). As the present study aims at the study of the ZEN toxicity in intestinal explants for the first time, we employed castrated immature males in order to avoid the known influence of the animal hormonal status of exposed animals in ZEN toxicity (Hennig-Pauka *et al.*, 2018).

The small intestinal mucosa is a barrier against food contaminants. It is formed by an epithelial cell lining that is continuously renewed in a process that depends on the division of intestinal stem cells (ISCs), located near the base of the crypt (Wells *et al.*, 2017; Spit *et al.*, 2018 ). Proliferation is governed mainly by the Wnt/ $\beta$ -catenin pathway that is present in an increasing gradient from the base to the bottom of the crypts, whereas TGF- $\beta$  superfamily proteins (including BMPs, GDFs) maintain a state of growth equilibrium and promote epithelial differentiation towards the tips of the villus (Gregorieff *et al.*, 2005). ER activity regulates the Wnt/ $\beta$ -catenin and TGF- $\beta$  pathways (Hasson *et al.*, 2014; Cho *et al.*, 2007). ZEN is known to alter the balance between ER, Wnt/ $\beta$ -catenin and TGF- $\beta$  signaling in prostatic cancer cells as well as ovarian cells, these being linked with the pro-proliferative and cancerogenic effects of ZEN (Yang *et al.*, 2018; Kowalska *et al.*, 2018). Here, we demonstrate that ZEN induces an accumulation of ER $\alpha$ , but no ER $\beta$ , in the intestine, and also modulates the Wnt/ $\beta$ -Catenin and TGF $\beta$  signaling pathways in the intestine.

Under our conditions, significant accumulation of ER $\alpha$  was observed, whereas the levels of ER $\beta$  remained unchanged. The accumulation of ER $\alpha$  can explain other molecular changes observed in the present study such as the activation of the



Wnt/ $\beta$ -catenin signaling pathway (Kuiper *et al.*, 1996; Paech *et al.*, 1997). Significant down-regulation of the inhibiting factors DKK1 and APC was observed, together with up-regulation of  $\beta$ -catenin (with a nearly significant protein up-accumulation), Cyclin D1, c-Myc, Zeb1 and Snail2. We observed down-regulation of the transcription factor TCF4, which seemed to conflict with the fact that TCF4 is major transcriptional mediator of the Wnt signaling pathway. In contrast, we observed up-regulation of the expression of TCF1 after 1 h of exposure. In mice, the epithelial expression of TCF4 is indispensable for cell proliferation and tumor initiation but in humans, the TCF4 role is redundant with the related TCF1 and LEF1 transcription factors (Hrckulak *et al.*, 2018). No information is available on how signaling is regulated in pig, but up-regulation of the most important target genes, such as c-Myc and Cyclin D1, confirms that down-regulation of TCF4 is not essential for  $\beta$ -catenin-mediated transcriptional activation. We also observed significant up-regulation of the expression of ATP6AP2, a protein that is necessary for the facilitation of the transmission of Wnt/ $\beta$ -catenin signaling (Sihn *et al.*, 2010), and whose ZEN-dependent up-regulation has already been observed in the pig duodenum (Braicu *et al.*, 2016). Our results suggest that the activation of Wnt signaling could be mediated by the up-accumulated ER $\alpha$ , since it is known that ER $\alpha$  downregulates the Wnt inhibitor DKK1. The ability of ZEN to suppress DKK1 in intestinal tissues has been already described; in that study, the ability was linked with possible modulation of EGFR signaling, which is closely connected with Wnt signaling (Taranu *et al.*, 2015). ER $\alpha$  can also activate Akt1, which inhibits GSK-3 $\beta$  and favors  $\beta$ -catenin nuclear translocation and subsequent transcription of Wnt target genes (Gao *et al.*, 2013; Shi *et al.*, 2008). The activation of Akt1 by ZEN has also been described in other tissues including granulosa (Zhang *et al.*, 2018), Sertoli (Wang *et al.*, 2018) and Leydig cells (Wang *et al.*, 2019). Among the Wnt transcripts we investigated, we only observed a change in Wnt11. Wnt11 might function as a tumor suppressive gene that can inhibit Wnt signaling (Railo *et al.*, 2008; Wang *et al.*, 2018). According to our results and those of and other authors, ZEN effectively

inhibits the Wnt repression system through repression of DKK1. It would be interesting to investigate if Wnt11 is also down-regulated by ZEN to act as a Wnt repressor. The activation of the pro-proliferative Wnt/ $\beta$ -catenin pathway was further confirmed by the significant up-regulation of proliferation biomarkers MCM3, and VIM, confirmed at the protein level for the two latter markers. Results additionally indicate that ZEN does not remodel tight nor adherens junctions but is able to promote cytoskeletal changes related to enhanced migration, such as the regulation of the expression of  $\alpha$ -catenin and CFN-1.

There might be a link between the ZEN-induced activation of Wnt/ $\beta$ -catenin signaling and the detected changes in antimicrobial peptides. Although our results were not significant, up-regulation/up-accumulation of the antimicrobial proteins lysozyme and RegIII $\gamma$  produced by the Paneth cells was observed. Previous reports report a ZEN-induced increase in Paneth cells in pig (Obremski *et al.*, 2005). The homeostasis of Paneth cells depends on ER (Hasson *et al.*, 2014) and Wnt/ $\beta$ -catenin (van Es *et al.*, 2005), and dysregulation in their function is related to the development of cancer (Cho *et al.*, 2007) and chronic inflammatory diseases (Sankaran-Walters *et al.*, 2017). More in-depth research is required to investigate the influence of ZEN on the homeostasis of Paneth cells, and consequently on the ISC microenvironment. In the small intestine, BD1 and BD3 are mostly expressed in enterocytes, and are constitutive in the case of BD1 and induced by inflammation in the case of BD3 (Wells *et al.*, 2017). While ZEN induced the expression of pBD1, pBD3 was downregulated. Other than differences in the antimicrobial and chemotactic function (Wells *et al.*, 2017), both defensins appear to play a role in the promotion/inhibition of migration of cancer cells, and the direction of the regulation depends on the tissue concerned (Ghosh *et al.*, 2019). In the human intestine, BD3 inhibits migration (Uraki *et al.*, 2014). Further research is needed to understand the effect of the observed ZEN-induced dysregulation of defensins in pig.

In contrast to the pro-proliferating Wnt/ $\beta$ -catenin signaling, TGF- $\beta$  maintains a state of growth equilibrium and promotes epithelial differentiation towards the tips

of the villus (Mishra *et al.*, 2005). TGF- $\beta$  is an immunosuppressive cytokine that actively participates in the gut immune cell homeostasis through the regulation of the growth and functions of dendritic cells, T natural killer and B cells (Massagué *et al.*, 2012). TGF- $\beta$  is also a potent growth inhibitor of intestinal epithelial cells (Massagué *et al.*, 2012). Our results suggest that TGF- $\beta$  signaling is suppressed after 4 h of exposure. This could be explained by the known ER $\alpha$ -mediated suppression of TGF- $\beta$ -induced activation by interaction with Smad proteins (Matsuda *et al.*, 2001).

The ZEN-mediated induction of inflammation has already been studied in different tissues in immune cells (Marin *et al.*, 2010; Marin *et al.*, 2013), the spleen (Pistol *et al.*, 2015), the liver, (Marin *et al.*, 2013) and the intestine (Liu *et al.*, 2014; Braicu *et al.*, 2016; Taranu *et al.*, 2015; Marin *et al.*, 2015). We observed no signs of inflammation either at the gene expression level (interleukins 8, 17, 18, 22, TNF- $\alpha$ , interferon gamma, Lipocalin 2, S100A8, S100A12) or at the protein level (IL-17 $\alpha$ , IL-8, serum amyloid A3 and alpha-acid glycoprotein alpha 1). Significant down-regulation of inflammatory cytokines has also been reported in the duodenum of pigs exposed to ZEN (Braicu *et al.*, 2016). These results could be linked to the ZEN-induced activation of ER $\alpha$  signaling, which has anti-inflammatory effects (Vegeto *et al.*, 2010; Villa *et al.*, 2015).

We investigated the expression of PPAR $\alpha$ , PPAR $\gamma$  as well as that of other genes linked with adipokines, a family of metabolic sensing proteins, of which some members have been found to be regulated by ZEN in the intestine (Taranu *et al.*, 2015; Li *et al.*, 2014). No differences in the expression of PPAR $\alpha$ , PPAR $\gamma$ , serum amyloid A, lipocalin 2 or adiponectin were found, whereas the mRNA levels of the adipokines receptors for adiponectin (AdipoR2), resistin (CAP1) and chemerin (CMKLR1) were significantly up-regulated. Adipokines are peptides that signal the functional status of adipose tissue to targets in different tissues, including the intestine. Their main roles are the regulation of inflammatory processes and energy balance, mainly glucose metabolism, as well as cell proliferation and differentiation,

through the activation of ERK1/2, Akt, and/or PI3K signaling pathways (Saygin *et al.*, 2017). Resistin can induce the secretion of mucins in the intestine (Kwak *et al.*, 2018). Interestingly, we observed nearly significant up-regulation of mucin-1 in our conditions. The effect of ZEN on the systemic levels and local signaling of adipokines merits further investigation not only in castrated males, but also in prepubertal females and adults from both sexes, since other known effects of ZEN suggest modulation of energy balance. Indeed, the anabolic effects of ZEN are well known and can disturb glucose levels *in vivo* in exposed prepubertal gilts (Kowalska *et al.*, 2016; Rykaczewska *et al.*, 2018; Gajęcka *et al.*, 2016 ). The importance of these findings is the possible connection between these endocrine-disturbing effects and the development of metabolic diseases (Petrakis *et al.*, 2017), a link that has been poorly investigated in the case of ZEN.

## 2.5. Conclusions

Our results provide new knowledge on the toxicity of ZEN and confirm that exposure to ZEN can promote the development of severe intestinal pathologies. ZEN increase ER- $\alpha$ , activate Wnt/ $\beta$ -catenin signaling and repress TGF- $\beta$  in the small intestine of castrated male pigs, inducing a pro-proliferative/pre-cancerous phenotype, characterized by over-expression of MCM3 and VIM. This suggests that ZEN disturbs the ISC microenvironment, promoting a pro-cancerous, proliferative state in the intestine that has been already described in the literature (Braicu *et al.*, 2016; Obremski *et al.*, 2005; Taranu *et al.*, 2015; Marin *et al.*, 2015). Our results also show that ZEN can modulate the energy sensing-related phenotype of intestinal tissues without inducing inflammation. More research including models of different sex and age is needed to understand the influence of ZEN on the immune-metabolic status of exposed individuals.

## Conclusion

Our results indicate that acute exposure to ZEN promotes changes in signaling pathways that are important for the intestinal physiology such as estrogen, Wnt/ $\beta$ -catenin and TGF- $\beta$  as well as changes in the expression of metabolic sensing molecules and antimicrobial proteins (Figure 19).

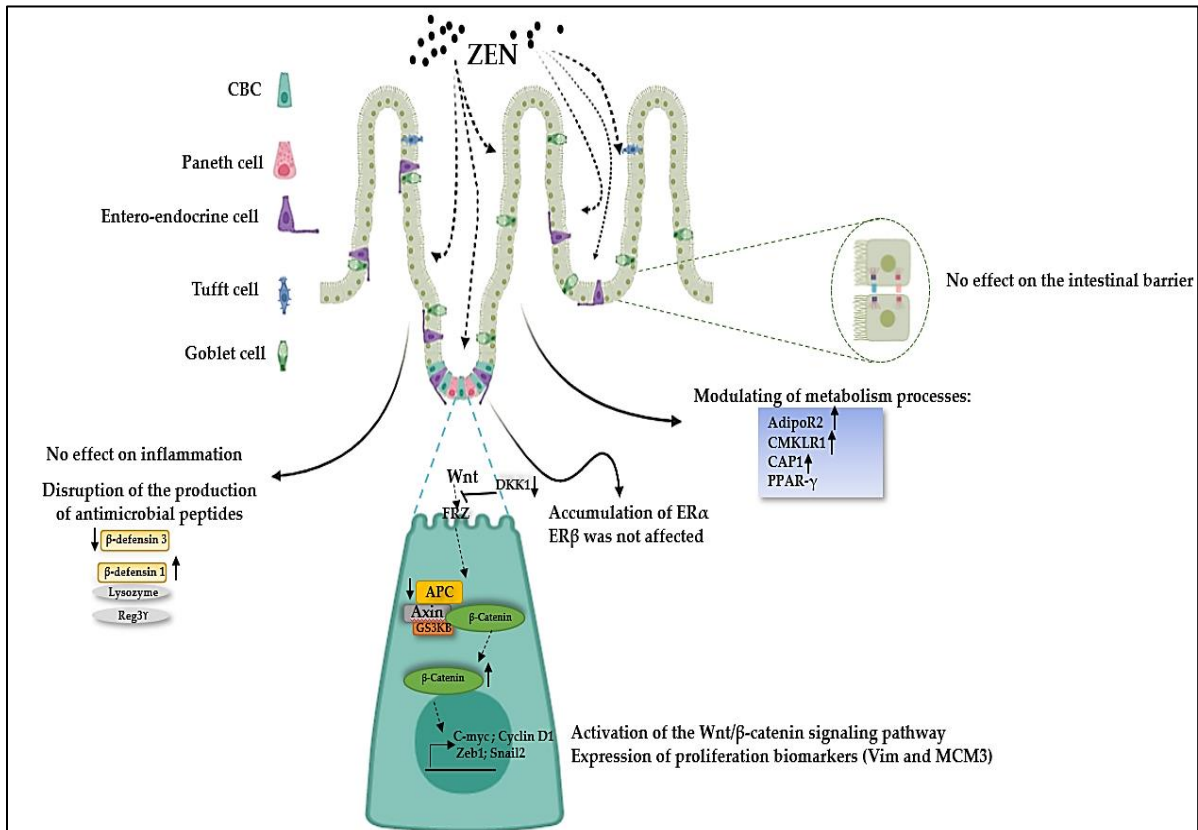


Figure 19 : **Different effects of ZEN on the intestine.** At non-cytotoxic conditions, ZEN can cause different effects at intestinal level. It can modulated the expression of genes related with adipokines and antimicrobial response without inducing inflammation. ZEN can activate the Wnt/ $\beta$ -catenin signaling pathway, and induce the expression of proliferation biomarkers as well as antimicrobial proteins. These effects were associated with a significant up-accumulation of ER $\alpha$  while ER $\beta$  abundance did not change at any time-point.

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# Chapter 2

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**Combined toxicity of  $\alpha$ -Zearalenol and the parent  
mycotoxin Zearalenone on Caco-2 cells**

## Introduction

ZEN is metabolized by human and pigs intestinal cells giving rise to several metabolites mainly  $\alpha$ -Zearalenol ( $\alpha$ -ZEL).  $\alpha$ -ZEL is the predominant form in humans, and is considered as the most cytotoxic form comparing to other ZEN metabolites. The cytotoxicity of individual mycotoxins has been widely studied, several studies established that ZEN and its derivatives cause cell death through apoptosis in dose and time-dependent manner. However, data on the toxicity of mycotoxin mixtures are limited. In this second chapter, we opted to study the cytotoxic effect of ZEN and  $\alpha$ -ZEL alone, or in combination (ZEN +  $\alpha$ -ZEL) to determine whether their combined effect is synergistic, antagonistic or additive., using different doses of mycotoxins and for long exposure (low and high doses for 48h).

## Abstract

The intestine is the first target for ZEN, causing a cytotoxic effects to epithelial intestinal cells at high doses. ZEN can be present in food along with its metabolites, and it can also be metabolized in the epithelial intestinal cells. In consequence, epithelial intestinal cells can be exposed to ZEN and its metabolites simultaneously. The ZEN derivative  $\alpha$ -ZEL is known to be the main metabolite from ZEN in humans, and it is known to be cytotoxic. However, the combined cytotoxic effect of ZEN and  $\alpha$ -ZEL has not been explored. The aim of this study was to assess the individual and the combined effects of the parent compound ZEN and  $\alpha$ -ZEL. The viability of human intestinal epithelial Caco-2 cells was evaluated through the quantification of ATP content after 48h of exposure to increasing doses of ZEN (2.5-160  $\mu$ M) and  $\alpha$ -ZEL (6.25-400  $\mu$ M) alone or in combination. Interactions were assessed using the median-effect method of Chou–Talalay, classifying interaction effects as antagonisms, additive effects, or synergisms. Our results demonstrated that the binary combination of ZEN and  $\alpha$ -ZEL was more cytotoxic for Caco-2 cells than any of the toxins alone, and that their interaction was synergic. Our results suggest that stronger adverse effects should be expected when the intestine is exposed to a mixture of ZEN and  $\alpha$ -ZEL .

## 3.1. Introduction

ZEN is mainly known for being a mycoestrogen, but its toxic effect is independent of estrogen signaling at high concentrations. At high doses, ZEN is able to induce an acute oxidative stress that activate different apoptotic cascades, leading to a cytotoxic effect. Indeed, ZEN provokes a dose-dependent increase of reactive oxygen species levels, oxidative DNA damage, alteration of the mitochondria, membrane disruption and lipid peroxidation (Rai *et al.*, 2019). Other ZEN metabolites that can be produced *in vivo* or *ex vivo* also show this cytotoxic ability, namely the main metabolism product in humans,  $\alpha$ -Zearalenol ( $\alpha$ -ZEL).



Biotransformation of ZEN produces several derivatives of ZEN (EFSA, 2016). ZEN biotransformation includes the phase I metabolites  $\alpha$ - and  $\beta$ -zearalenol ( $\alpha$ - and  $\beta$ -ZEL) through reduction, and zearalenone (ZAN) and  $\alpha$ - and  $\beta$ -zearalanol ( $\alpha$ - and  $\beta$ -ZAL) through oxidation. Humans, pigs and rats are able to metabolize ZEN leading to the formation of  $\alpha$ -ZEL and  $\beta$ -ZEL (Kollarczik *et al.*, 1994; Dänicke and Winkler, 2015; Belhassen *et al.*, 2015; EFSA, 2016).

Recent studies reported the co-occurrence of ZEN,  $\alpha$ - and  $\beta$ -ZEL in cereal-based products (De Boevre *et al.*, 2013; Nathanail *et al.*, 2015). According to Palumbo *et al.* (2020), in wheat, the mean concentrations of ZEN ranged between 24.2–27.0  $\mu\text{g}/\text{kg}$  in food, different modified forms were reported, with  $\alpha$ -ZEL and  $\beta$ -ZEL as those with the highest mean concentrations. Human intestinal Caco-2 cells are able to transform ZEN in  $\alpha$ -ZEL and  $\beta$ -ZEL, being respectively  $40.7 \pm 3.1\%$  and  $31.9 \pm 4.9\%$  of total metabolites.

Most toxicological studies concerning the cytotoxic effects of ZEN and  $\alpha$ -ZEL effects in the intestine, took into account the exposure to a mycotoxin alone not in combination. Multi-exposure may lead to additive, synergistic or antagonistic toxic effects. At the present very little is known about the actual combined health risk from exposure to mycotoxins. The aim of the present study was to explore the combined cytotoxic effect of  $\alpha$ -ZEL and ZEN and to assess the nature of their interaction using human intestinal epithelial cells. Caco-2 cells were employed in this study because they express morphological and functional characteristics of human intestinal cells (Le Ferrec *et al.*, 2001). The individual and combined toxicity was assessed using CellTiter-Glo® Luminescent Assay, based on the measure of ATP content, proportional to the number of viable cells.

## **3.2. Material and methods**

### **3.2.1. Cell culture and reagents**

The human colon carcinoma cell line Caco-2 (Sigma, Passage 48) was maintained in DMEM-glutamax (Gibco, Life Technologies, France) supplemented with 10% FCS, 1% non-essential amino-acids (Sigma, France) and 0.5% gentamycin (Eurobio, Courtaboeuf, France). Cells were incubated in a humidified atmosphere at 37°C and 5% CO<sub>2</sub> in culture flasks and the culture media were changed every 2 days.

ZEN,  $\alpha$ -ZEL (Sigma, France) were dissolved in dimethyl sulfoxide (DMSO; Sigma, St Quentin Fallavier, France) at 10mM to prepare stock solution, and stored at -20°C.

### **3.2.2. Measurement of cell viability**

For studying the cytotoxicity of ZEN and  $\alpha$ -ZEL, Caco-2 cells were subcultured in 96-well plate, with DMEM without phenol red (Invitrogen, France), at a seeding density of 10<sup>4</sup> cells per well, until reaching the confluence of 80%. Then, the cells were treated with different concentrations of each individual toxin and their binary mixture for 48h (ZEN: 2.5–5–10–20–40–80–160 $\mu$ M;  $\alpha$ -ZEL: 6.25–12.5–25–50–100–200–400 $\mu$ M). The concentrations used in our study are based on the cytotoxicity tests realized in our laboratory, the IC<sub>50</sub> was referred for each toxin. Then we opted for the choice of concentrations above this IC<sub>50</sub> and other concentrations below for ZEN and  $\alpha$ -ZEL (triplicates; Figure 20).

### **3.2.3. CellTiter-glo cell viability assay**

Toxicity was assessed using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Charbonnières-les-Bains, France) that determine the number of viable cells based on the quantitation of adenosine triphosphate (ATP). The luminescent signal produced by the luciferase reaction, reflecting the presence of

metabolically active cells, was read using a multiplate reader (TECAN, Lyon, France). The results were obtained by calculating the percentage of viable cells (Mean luminescence in treated samples/luminescence in vehicle samples x100).

### 3.2.4. Analysis of the interaction between mycotoxins ZEN and $\alpha$ -ZEL

Chou and Talalay method can easily be implemented using the web-based CompuSyn software which automatically calculates dose-effect curves and combination indices. Dose-effect studies for single mycotoxins, and binary associations were carried out, based on a series of cytotoxicity tests realized in our laboratory. The inserted data are fraction affected ( $f_a = 100 - \text{Value of viability}$ ) value that obtained in the consequence of cells exposure by one toxin (ZEN,  $\alpha$ -ZEL) or two toxins in combination (ZEN+  $\alpha$ -ZEL). First,  $f_a$  value of serial doses of each toxin inserted into the software (CompuSyn), in order to analyze dose effect and obtain  $IC_{50}$  of corresponding toxin. Then,  $f_a$  value of combined effect of two toxins were inserted respect to their ratios, in order to obtain CI (Combined Index) value and know, how is the interaction. The next step consist to generate the different necessary parameters : effect dose ( $D_m$ ), m Value, r Value, Combination Index(CI) and Isobolograms.

The dose-effect relationships of the individual and combined mycotoxins were biometrically modeled by using the Median-Effect Equation of the Mass Action Law (Chou 2006):

$$f_a/f_u = (D/D_m)^m$$

Where D is the dose of mycotoxin,  $f_a$  and  $f_u$  are the fractions of the cell system affected respectively, unaffected ( $f_a + f_u = 1$ ).  $D_m$  is the median-effect dose which is required to produce 50% inhibition of cell viability, and m is a Hill-type coefficient signifying the shape of the dose-effect relationship, where  $m > 1$ ,  $m = 1$ , and  $m < 1$  indicate hyperbolic, sigmoidal, and negative sigmoidal dose-effect curve, respectively.

- Combination Index(CI)

Toxicological interactions were analyzed by the isobologram and combination-index (CI) methods. For the combinations, CI values were generated over a range of fractions of cell viability affected (fa) from 0.05–0.95 (5–95 % toxicity). CI < 1, =1, and > 1 indicate synergism, additive effect, and antagonism, respectively.

$${}^n(CI)_x = \sum_{j=1}^n \frac{(D)_j}{(D_x)_j}$$

${}^n(CI)_x$ : the combination index for n agents at x% inhibition

(D)<sub>j</sub>: the doses of n agents that exerts x% inhibition in combination

(D<sub>x</sub>)<sub>j</sub>: the doses of each of n agents alone that exerts x% inhibition.

The dose-response relationship analysis for individual and combined effect of mycotoxins on cell viability, were performed with CompuSyn software version 1.0 (Compusyn Inc., USA).

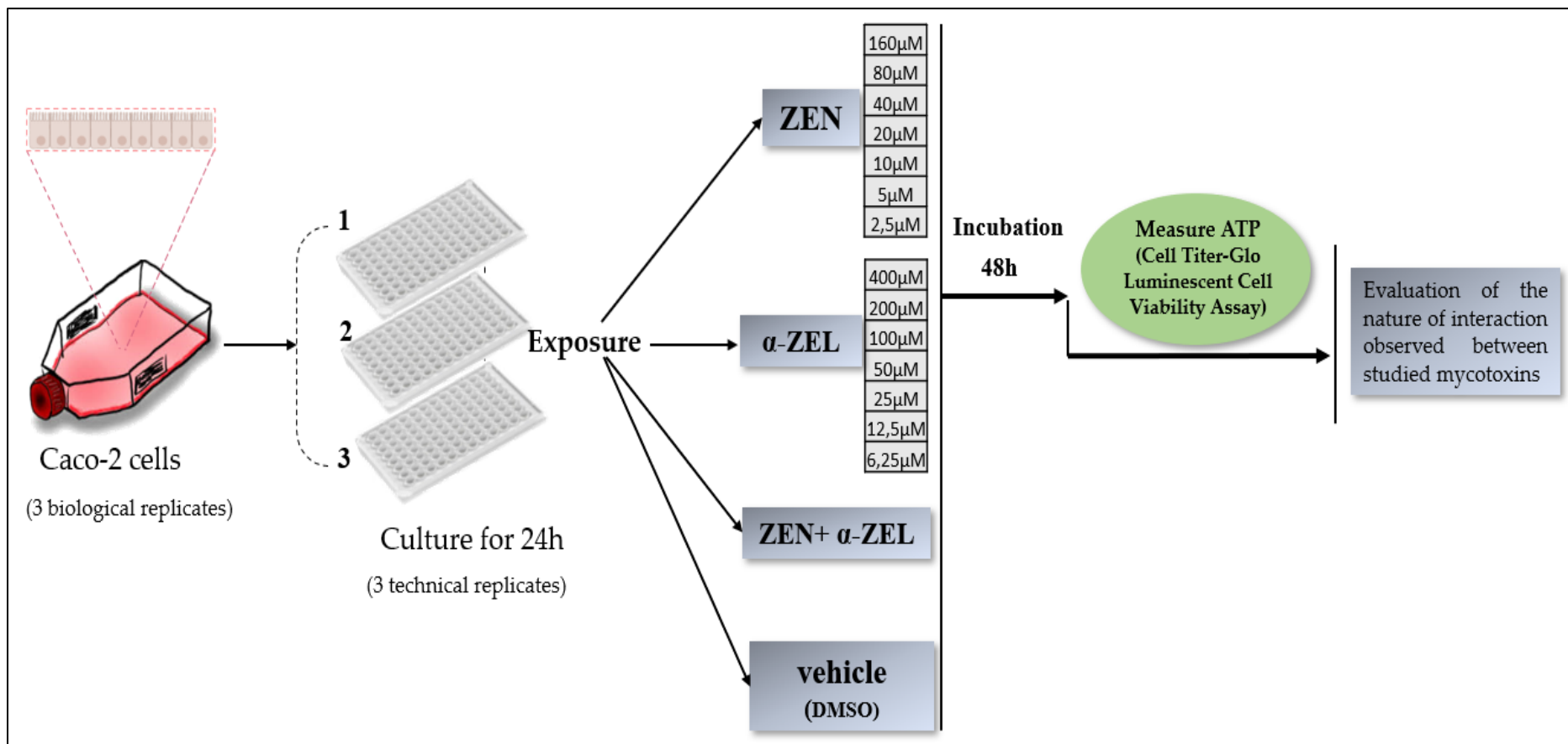


Figure 20: **Experimental design for measurement of cell viability and analysis of the interaction between mycotoxins  $\alpha$ -ZEL and ZEN** (DMSO: Dimethyl sulfoxide). Caco-2 cells were growing in cell culture Flask (3 biological replicates). Then, subconfluent Caco-2 cells were treated with different concentrations of individual toxins and their binary mixture. After 48 h of exposure, 100  $\mu$ L of Cell Titer-Glo was added to each well and the Luminiscence was read using a spectrophotometer. Finally, the nature of the interaction between mycotoxins were analyzed using the Chou and Talalay method (Chou 2006).

### 3.3. Results

#### 3.3.1. Effects of individual and combined mycotoxin exposures on Caco-2 cell viability

Results from the cell viability experiments are illustrated in Figure 21. Analysis of the effect of both individual and binary mixture of  $\alpha$ -ZEL and ZEN on cytotoxicity has shown a dose-dependent reduction of cell viability. When Caco-2 cells were treated with ZEN and  $\alpha$ -ZEL alone or in combination, the cytotoxic effect induced by the mixture  $\alpha$ -ZEL+ZEN was higher than the toxicity of  $\alpha$ -ZEL and ZEN alone.

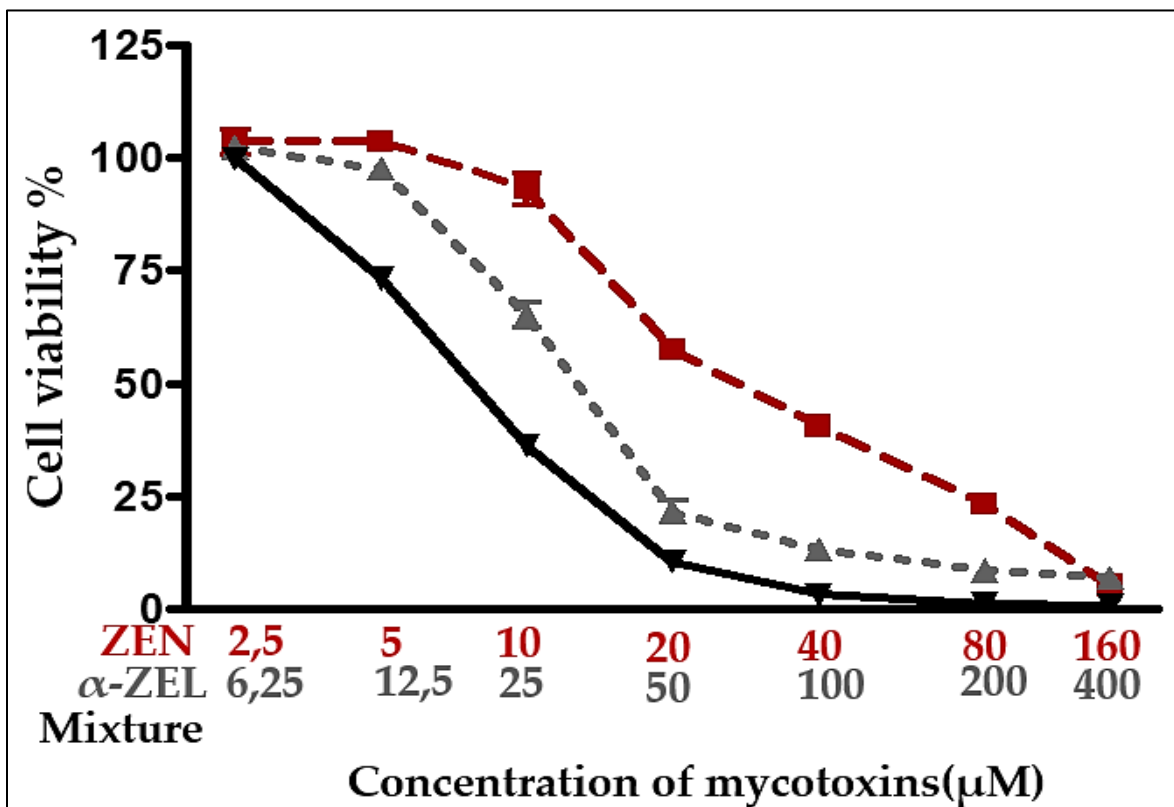


Figure 21: Cytotoxicity effect of  $\alpha$ -ZEL (grey line), ZEN (red line) alone or in mixture (black line, binary mixture  $\alpha$ -ZEL+ ZEN for each concentration) on cell viability of caco-2 cell line evaluated by CellTiter-Glo®; Cells were treated with  $\alpha$ -ZEL or ZEN at indicated concentrations for 48h. The values are expressed as percent of control response and each value is a result of three experiments with three technical replicates. Bars represents standard deviation.

As shown below, table 4 present the IC<sub>50</sub> for individual and binary mycotoxin, The obtained data revealed that the combination of  $\alpha$ -ZEL and ZEN was the most cytotoxic with an IC<sub>50</sub>=15 $\mu$ M comparing with the individual effect of  $\alpha$ -ZEL (IC<sub>50</sub>=31 $\mu$ M) or ZEN ( IC<sub>50</sub>=33 $\mu$ M). Depending on these findings, and based on the IC<sub>50</sub> value, the mycotoxins were ranked for their cytotoxicity against Caco-2 cells as following:  $\alpha$ -ZEL+ZEN >  $\alpha$ -ZEL >ZEN.

### 3.3.2. Analysis of the combined cytotoxicity of ZEN and $\alpha$ -ZEL

The next step was to determine the types of interactions (additivity, synergy, or antagonism) between  $\alpha$ -ZEL and ZEN using isobologram and combination index method (Chou, 2006). The isobologram for the combination of  $\alpha$ -ZEL and ZEN at three different cytotoxicity levels (50, 75 and 90%) is presented in Figure 22A. In this type of graph, the additive combined effect follows the diagonal line between the effective concentrations of each single toxin. If the measured combined effect of two toxins is above or below the diagonal line, it indicates an antagonistic or a synergistic effect of the combination, respectively. The CI values were calculated automatically by the CampuSyn software. The calculation of the combination index values for binary mixtures that responsible for an inhibition level of 50, 75 and 90% and the dose reduction index requested for the decrease of cell viability with the above percentages are presented in Table 4. Concerning the interaction between  $\alpha$ -ZEL+ZEN, all combination indexes values indicating a synergistic effect (CI<1), and the CI values increased with the mycotoxin concentrations. The point position below the diagonal lines (Figure 22B) indicates the synergistic between the binary mixture of the toxins at 50, 75 and 90% of affected fraction.

Table 4: The parameters  $m$ ,  $IC_{50}$  for  $\alpha$ -ZEL and ZEN and their combination for cell viability; Combination index (CI) values for binary toxins combinations affecting 50, 75 and 90% of cell viability. CI values were calculated using CompuSyn software. CI values indicate synergism ( $CI < 0.9$ ), additive effect ( $0.9 < CI < 1.1$ ), and antagonism ( $CI > 1.1$ ).

| Mycotoxin          | IC <sub>50</sub> | m             | CI value         |                  |                  |
|--------------------|------------------|---------------|------------------|------------------|------------------|
|                    |                  |               | CI <sub>50</sub> | CI <sub>75</sub> | CI <sub>90</sub> |
| ZEN                | 33 $\mu$ M       | 1.81 +/- 0.21 |                  |                  |                  |
| $\alpha$ -ZEL      | 31 $\mu$ M       | 1.81 +/- 0.21 |                  |                  |                  |
| ZEN+ $\alpha$ -ZEL | 15 $\mu$ M       | 1.52 +/- 0.14 | 0.48             | 0.54             | 0.61             |

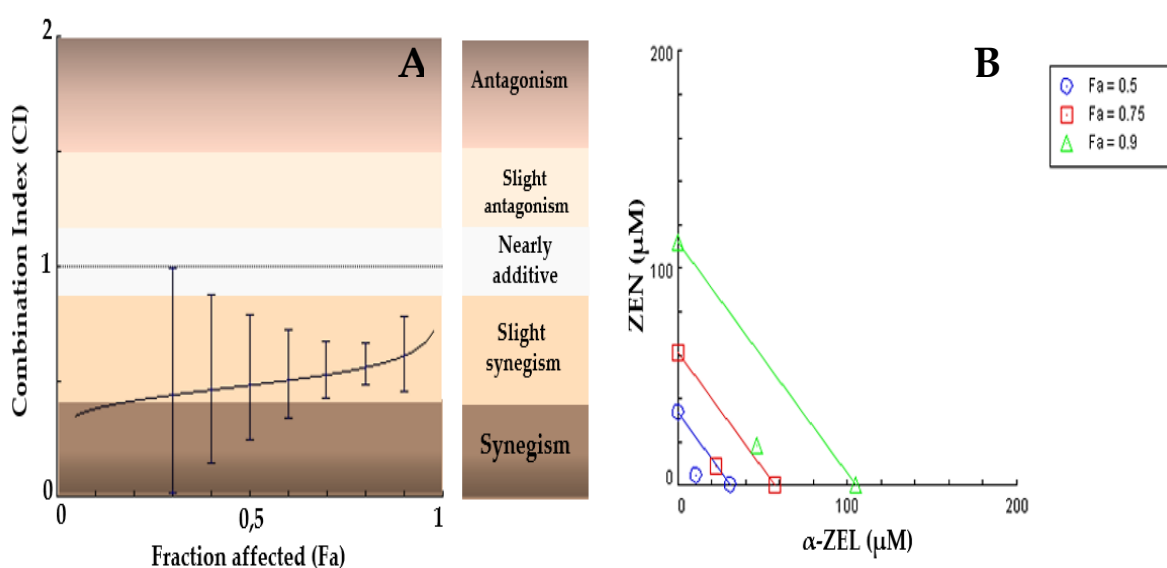


Figure 22: The interactive effects induced by  $\alpha$ -ZEL and ZEN mixtures on cell viability. (A) Combination index fractions affected curves for cell viability for the mixtures:  $\alpha$ -ZEL+ZEN. The fraction affected (fa) is the fraction of the cytotoxic effect induced by the mixture. CI values were calculated from data obtained from three independent experiments on the basis of equipotent mycotoxin combinations. (B) Isobologram illustrating the combined cytotoxic effect of the binary combinations of  $\alpha$ -ZEL and ZEN at concentration responsible for 50% (circle), 75% (square), and 90% (triangle) inhibition of cell viability. The points are mean concentrations of dose–response cytotoxicity curves for each toxin or toxin combination.

### 3.4. Discussion

At high concentrations, the mycotoxin ZEN and its metabolite  $\alpha$ -ZEL induce a high oxidative state that leads to cytotoxicity. Fungi and plants can produce  $\alpha$ - and



$\beta$ -ZEL from the parent mycotoxin ZEN, so they co-occur in cereal-based products (De Boevre *et al.*, 2013; Nathanail *et al.*, 2015). Recent studies reported the co-occurrence of ZEN,  $\alpha$ - and  $\beta$ -ZEL in cereal-based products (De Boevre *et al.*, 2013; Nathanail *et al.*, 2015). According to Palumbo *et al.* (2020), in wheat, the mean concentrations of ZEN ranged between 24.2–27.0  $\mu\text{g}/\text{kg}$  in food, different modified forms were reported, with  $\alpha$ -ZEL and  $\beta$ -ZEL as those with the highest mean concentrations. Humans, pigs and rats are able to metabolize ZEN leading to the formation of  $\alpha$ -ZEL and  $\beta$ -ZEL (Kollarczik *et al.*, 1994; Dänicke and Winkler, 2015; Belhassen *et al.*, 2015; EFSA, 2016). Human intestinal Caco-2 cells are able to transform ZEN in  $\alpha$ -ZEL and  $\beta$ -ZEL, being respectively  $40.7 \pm 3.1\%$  and  $31.9 \pm 4.9\%$  of total metabolites.

ZEN and  $\alpha$ -ZEL showed a dose-dependent reduction of viability of cells after 48h of exposure. Based on IC<sub>50</sub>,  $\alpha$ -ZEL was slightly more cytotoxic than ZEN. Several studies revealed that ZEN can induce oxidative damage, DNA damage, apoptosis, necrosis leading to cell death (Rai *et al.*, 2019). These findings were in accordance with the study conducted by Tatay *et al.* (2014), who investigated the cytotoxicity of ZEN and its metabolites in ovarian (CHO-K1) cells during 24, 48 and 72 h, and according to the IC<sub>50</sub> values obtained the  $\alpha$ -ZEL showed the highest cytotoxic effect on CHO-K1 cells. Our observations were also in accordance with the results of Ayed-Boussema *et al.* (2011), showing that  $\alpha$ -ZEL is the more cytotoxic metabolite in HeLa (Human adenocarcinoma) and Vero (kidney epithelial cells from green monkey) cells after 24 h of exposure. Other studies indicate that ZEN has the highest cytotoxicity effect over their metabolites (Abid *et al.*, 2009; Marin *et al.*, 2019), suggesting that the effect of ZEN and its metabolites is cell type or tissue dependent.

The interaction between ZEN and  $\alpha$ -ZEL was analyzed using the Chou-Talalay method (Chou, 2006), which allows the evaluation of the type of interactions: synergistic, additive, or antagonistic. In Caco-2 cells, the type of interaction between ZEN and  $\alpha$ -ZEL is synergistic, this synergism decreased with the increase mixture

concentrations, as shown in figure 22, at fraction affected (fa) 0.50 combination index (CI) value was 0.48, For fa = 0.75 , CI was 0.54 and at fa= 0.9 the CI value was 0.61. Our findings are in agreement with previous studies on the combinatory effects of ZEN and  $\alpha$ -ZEL. Wang *et al.*, (2014) investigated the cytotoxicity of combined mycotoxins ZEN and  $\alpha$ -ZEL on human HepG2, the results showed an antagonism at exposure times of 24h and 72h, but an additive effect and synergism were found at 48 h. Similarly, a recent research conducted by Marin *et al.* (2019) consisting in co-exposing HepG2 cells to a mixture of ZEN and  $\alpha$ -ZEL (0-100 $\mu$ M), the binary combination had a synergistic cytotoxic effect (at fa = 0.25, fa = 0.50 and fa = 0.75 the CI value was 0.63, 0.49 and 0.38 respectively). These values indicates that the synergy increased with the increases of mycotoxin concentrations. The synergistic effect of ZEN and  $\alpha$ -ZEL observed especially at high concentration can be explained by the fact that  $\alpha$ -ZEL is the metabolite of ZEN, and they have similar structures and the same mechanism of toxicity (Tatay *et al.*, 2014). Furthermore, this effect might be explained by the potentiation of cytotoxicity due to their lipophilic structures, which allows them to be easily incorporated into cell membranes, and this process lead to an increase of their cytotoxic potential (Marin *et al.*, 2019; Wang *et al.*, 2014).

## Conclusion

The present study demonstrates that the toxicity of a mixture of mycotoxins cannot be predicted solely on the basis of the effect of the individual compounds. The data evidenced in this study showed that  $\alpha$ -ZEL is more cytotoxic than ZEN, and showed an important synergistic effect for these toxins. Therefore, this synergy should be taken into account for regulatory purposes, as the co-exposure to these mycotoxins might results in a strong adverse effect than resulted after the exposure to the mycotoxin alone.

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# General Discussion

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The intestine is the first barrier against food contaminants, as well as the first target for toxicants (Pinton and Oswald, 2014; Alassane-Kpembé *et al.*, 2019). The intestine can be exposed to the mycotoxin ZEN through contaminated food and feed. The effects of ZEN in the intestine has been frequently reported in the past, and several studies suggested that this mycotoxin exhibits carcinogenesis-like properties (Taranu *et al.*, 2015; Abassi *et al.*, 2016). So far, the mechanisms by which ZEN induces these events is still unclear. On the other hand, toxicological studies on the effects of mycotoxin mixtures are of great importance, as they help to determine whether mycotoxins can interact and whether these interactions could result in synergism, an additive effect, or antagonism (Marin *et al.*, 2019; Wang *et al.*, 2014). Therefore, studying the combined effects of ZEN and  $\alpha$ -ZEL is important for human and animal health and safety.

To characterize the gene expression and protein abundance changes induced by exposure to ZEN on the intestine, we used jejunal explant model from pigs, that are considered as the most sensitive animal to mycotoxins. This model has the advantage of being a closer model to the human gut than single *in vitro* cell models (Maresca *et al.*, 2018). In addition, explants contain all cell types normally present in the epithelium, as well as immune cells, myofibroblasts, and cells from the enteric nerve system (Randall *et al.*, 2011). Furthermore, this model can represent an interesting alternative to *in vivo* studies in the context of reducing the number of animals served in experiments for ethical considerations. The main limitation of this *ex vivo* approach is the rapid degeneration, so 4 hours is the maximal exposure time (Kolf-Clauw *et al.*, 2009). For this reason, we used a high dose of ZEN (100 $\mu$ M) that would induce as many molecular changes as possible, while that the short exposure time ensures that cell mortality is low enough so analysis will not be polluted by unspecific events (Rabilloud and Lescuyer, 2015). This dose correspond to 31.8mg/kg (assuming that 1kg=1L of gastrointestinal fluid). This chosen

concentration remains low compared to the large concentrations detected in animal feed. Indeed, in India, a high concentrations of ZEN (up to 600 mg/kg) in wheat and rice have been reported (Gao *et al.*, 2018; Kowalska *et al.*, 2016; Zinedine *et al.*, 2007). Furthermore, corn from Egypt was also found contaminated with high levels of ZEN that ranged from 9.8 to 38.4 mg/kg (El-Maghraby *et al.*, 1995). In Scotland, according to Gross and Robb (1975), high contamination of barley stored (for 3 month to about one year) with ZEN was detected and levels varied between 2.1 and 26.5 mg/kg.

In our study, the abundance of 68 gene transcripts and 14 proteins were evaluated using RT-qPCR and western blot, respectively. First, our findings indicate that ZEN activates the Wnt/ $\beta$ -catenin signaling pathway, and proved no evidence for ZEN-dependent activation of TGF- $\beta$  signaling pathway. Our results are in agreement with those obtained by Yang *et al.* (2018), that proved that ZEN (0.1-1.5 mg/kg) increases the accumulation of Wnt-1 and  $\beta$ -catenin in the ovaries of gilts. The Wnt pathway is essential for intestinal homeostasis (Spit *et al.*, 2018; Gehart and Clevers, 2019), as it is tightly linked with intestinal epithelial cells differentiation and renewal (Czerwinski *et al.*, 2018; Degirmenci *et al.*, 2018). Aberrant signaling of this pathway is highly associated with several cancers. Indeed, hyperactive Wnt pathway can produce tumorigenesis in colon (Fodde and Brabletz, 2007). Furthermore, we demonstrated that ZEN induces the accumulation of ER $\alpha$  and the estrogenic receptor (ER $\beta$ ) was not affected. Estrogen signaling interacts with other pathways (such as Wnt/ $\beta$ -catenin) that are important for intestinal homeostasis and its disruption seems to relate to the development of chronic intestinal diseases and cancer (Hasson *et al.*, 2014; Cho *et al.*, 2007; Caiazza *et al.*, 2015). ER $\alpha$  is known to promote proliferation whereas estrogen action through ER $\beta$  is anti-proliferative (Caiazza *et al.*, 2015). Taken together, with earlier demonstrations describing ZEN's ability to cause DNA damage (Ayed- Boussema *et al.*, 2007; Ouanes *et al.*, 2005; Ouanes *et al.*, 2003), these data suggest that exposure ZEN could participate in the

molecular mechanisms promoting carcinogenesis. Further investigations are needed to determine pro-carcinogenic effects of ZEN in the intestine.

On the other hand, Wnt signaling is required for maturation and differentiation of Paneth cells (Farin *et al.*, 2012; van Es *et al.*, 2005). Indeed, inactivation of Wnt signaling results in the loss of Paneth cells (Krausova and Korinek, 2014). These cells secrete antimicrobial molecules such as defensins or lysozyme (Mayumi and Smith, 2018). Dysfunction of Paneth cell biology contributes to the pathogenesis of chronic inflammatory bowel disease and can disturb crypt cells homeostasis (Clevers and Bevins, 2013). Pigs (animal model used in our study), like many other mammals, produce an impressive range of AMPs, including  $\beta$ -defensin 1,  $\beta$ -defensin 2 and RegIII $\gamma$  (Lam-Yim Wan *et al.*, 2013). ZEN is able to induce an increase in Paneth cells counts in pigs (Obremski *et al.*, 2005). In our study, we observed an almost significant over-expression of pBD1, down-regulation of the expression of pBD3 that are mostly expressed in enterocytes, together with a nearly significant over expression of lysozyme and a nearly significant abundance of RegIII $\gamma$  produced by the Paneth cells. With all, our finding suggest that ZEN can disturb the production of antimicrobial peptides, which could be linked with a possible alteration of Paneth cells' function. Further research is needed to investigate the influence of ZEN on the homeostasis of Paneth cells.

Beside Paneth cells, absorptive enterocytes produce a series of cytokines and chemokines, which play a pivotal role in regulating the diversity of the commensal microorganisms and the immune responses of subjacent mucosal (Wang *et al.*, 2018). Cytokines and chemokines are crucial for the recruitment of the immune cells, and several cytokines are constitutively expressed by intestinal cells playing a role in the influx of immune cells into the mucosa, in cell growth and homeostasis (Stadnyk, 2002). Estrogens are important modulators of the immune response. Estrogen's primary effects are mediated via estrogen receptors (ER $\alpha/\beta$ ) that are expressed on most immune cells, as well as epithelial cells (Cunningham and Gilkeson, 2010). The modulation of inflammation by each estrogen receptor is differential: ER $\alpha$ -

dependent signaling is considered to have anti-inflammatory effects (Vageto *et al.*, 2010; Villa *et al.*, 2015), whereas ER $\beta$  is associated with pro-inflammatory effects (Straub, 2007). A recent study demonstrated recently that, under non-cytotoxic conditions, ZEN is able to induce an ER $\alpha$ -dependent activation of NF- $\kappa$ B cascade, but blocks the pro-inflammatory transcriptional response associated with this activation in the pig intestine (Soler *et al.*, 2020). Exposure conditions to ZEN might be behind the pro- or anti-inflammatory effects of this mycotoxin, since other reports suggested an activation of the inflammatory response of the pig intestine exposed to ZEN (Marin *et al.*, 2015; Taranu *et al.*, 2015; Braicu *et al.*, 2016a).

Then, we investigated the effect of ZEN in expression of different genes involving in metabolism such as adiponectin receptor 2, resistin and chemerin. Adipokines contribute to the regulation of appetite and satiety, fat distribution, insulin secretion and sensitivity, energy expenditure, endothelial function, inflammation, blood pressure, and hemostasis. Alterations in adipokine secretion may contribute to obesity-related diseases (Fasshauer and Blüher, 2015). Actually, a synthetic form of ZEN, Zeranol, is employed as an anabolic growth promoter in animal production, since it is able to significantly increase weight gain and conversion indexes in treated animals. ZEN can change different systemic metabolic processes, including cell membrane metabolism, protein biosynthesis, glycolysis, and gut microbiota metabolism in rats and pigs (Liu *et al.*, 2013). The toxicity of ZEN has been related with its ability to disrupt lipid metabolism and lipid-related signaling. Different studies have described an increased  $\beta$ -oxidation of short fatty acid in a process that could involve the nuclear transcription factors PPAR $\alpha$  and PPAR $\gamma$  (Li *et al.*, 2014). Moreover, ZEN is able to alter ER signaling, thus activating the ERK1/2, Akt, and/or PI3K signaling pathways, which are involved in cell proliferation and induce an anabolic effect. Signaling related with PPAR $\alpha$ , PPAR $\gamma$  as well as ERK1/2 and PI3K/Akt can regulate the expression and function of adipokines, a family of metabolic sensing proteins, of which some members have



been found to be regulated by ZEN in the intestine (Soler *et al.*, 2020) at similar exposure conditions as in the present study.

According to our results, ZEN is able to alter signaling pathways related with epithelial cells dynamics in the intestine. It would be necessary to investigate if ZEN could induce cell proliferation in the small intestinal crypt, which could be connected with pro-cancerogenic changes in Wnt/ $\beta$ -catenin signaling pathways.

In general, ZEN exerts different mechanisms of toxicity depending on the dose and the cell types. It has hence a pleiotropic toxicity depending on the target organ (estrogen-responding or not) and the exposure conditions (low or high dose). A low dose of ZEN with long time exposure, can exert the estrogen-like effects, which can stimulate the proliferation of cells. Different studies has showed that ZEN can stimulate cell proliferation in different cells (Lecomte *et al.*, 2017; Li *et al.*, 2014; Ahamed *et al.*, 2001; Khosrokhavar *et al.*, 2009; Zong *et al.*, 2016; Parveen *et al.*, 2009; Cortinovis *et al.*, 2016; Abassi *et al.*, 2016). Likewise, in our study, changes induced by a short, non-cytotoxic exposure to ZEN in the intestine using pig jejunal explants, showed that the intestine is a target for ZEN, and induce changes related with the promotion of cellular proliferation, also could contribute to the onset of intestinal pathologies such as cancer.

ZEN can cause apoptosis and cell death at high doses. In fact, several studies have indicated that the viability was disturbed by ZEN in different cells after exposure to high doses (3-600 $\mu$ M) for 24h (Zheng *et al.*, 2016; Sang *et al.*, 2016; Zhu *et al.*, 2012; Zhang *et al.*, 2018; Hu *et al.*, 2016; Lin *et al.*, 2015; Abid-Essefi *et al.*, 2003). ZEN can be transformed in other molecules before and after ingestion, and several of these metabolites can also extent cytotoxic effects. Several studies have suggested that the toxicity of ZEN and its metabolites when present together, cannot always be predicted based upon their individual toxicities (Speijers and Speijers, 2004), and it is known that the simultaneous exposure to different mycotoxins may be more toxic than predicted from the mycotoxins alone (Alassane-Kpembé *et al.*, 2014).

In the second part of our research, the individual and the combined toxicity of ZEN and  $\alpha$ -ZEL in the intestinal cytotoxicity were investigated. The data evidenced in this study showed that  $\alpha$ -ZEL is more cytotoxic than ZEN.  $\alpha$ -ZEL is the predominant metabolite in humans, rat and pigs (Abid-Essef *et al.*, 2009; EFSA, 2016). The binary results showed a synergistic effect of these toxins on intestinal level. Our findings are in agreement with previous studies (Tatay *et al.*, 2016; Wang *et al.*, 2014; Marin *et al.*, 2019). This synergism effect can be explained the fact that  $\alpha$ -ZEL is the metabolite of ZEN, and they have similar structures and the same mechanism of toxicity (Marin *et al.*, 2019). ZEN-induced cytotoxicity is promoted by oxidative damage (Hassen *et al.*, 2007). The combined effects of ZEN and  $\alpha$ -ZEL on the viability and on the expression of genes involved in inflammation (IL-1 $\beta$ , TNF- $\alpha$ , and IL-8) of human liver cancer cell line (HepG2) revealed that at low concentrations mixtures have induced a slight to high antagonistic response on inflammatory cytokines. In turn, synergism was observed in high concentrations, proving greater toxicity comparing to the ZEN and  $\alpha$ -ZEL alone.

The synergistic effects of ZEN and its metabolites  $\alpha$ -ZEL might be explained by the fact that due to their lipophilic structure, the toxins are easily and concomitantly incorporated into cell membranes, and this process lead to an increase of their cytotoxic potential (Prosperini *et al.*, 2014). Therefore, ZEN and its derivatives contamination in food and feed, pose a significant health risk to humans and animals worldwide. For that, multiple detoxification strategies for contaminated foods and feeds to reduce or eliminate the toxic effects of ZEN and its metabolites by biological methods that are developed to improve food safety, prevent economic losses, and reclaim contaminated products. Biological detoxification has high specificity, produces harmless products, and can even lead to complete detoxification under suitable conditions. For ZEN, a variety of non-pathogenic microorganisms have been reported that can be used for the detoxification such as *Bacillus subtilis*, *Lactobacillus rhamnosus* and *Saccharomyces cerevisiae* (Cho *et al.*, 2010; Tinyiro *et al.*, 2011; El-Nezami *et al.*, 2004; Armando *et al.*,

2012; Krifaton *et al.*, 2013). Recently, an efficient ZEN-degrading lactonase from *Gliocladium roseum*, named ZENG, was identified and the recombinant enzyme showed a high degrading performance toward ZEN and its toxic derivatives  $\alpha$ -zearalenol ( $\alpha$ -ZEL) and  $\alpha$ -zearalanol ( $\alpha$ -ZAL) (Zhang *et al.*, 2020).

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# Conclusion and perspectives

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The **first conclusion** of the present thesis is that the intestine is a target for ZEN. ZEN intestinal toxicity was characterized by the accumulation of ER $\alpha$  but not ER $\beta$ , as well as the induction of molecular changes related with the intestinal homeostasis, and alteration of antimicrobial response and disruption of energy sensing signaling.

In our study, intestinal explants were used, and the main limitation of this *ex-vivo* approach remains to the fact that the tissue undergoes progressive and rapid degeneration. For that, we suggest to use a three-dimensional *in vitro* model of cell culture such as organoids, that display architectures and functionalities similar to *in vivo* organs (Rossi *et al.*, 2018). In addition, this structure contain most of the cell types normally present in the epithelium, and can be maintained for a long time in culture, allowing long-term exposure to several doses of ZEN (Maresca *et al.*, 2018; Merker *et al.*, 2016). As intestinal organoids restore physiological cellular composition, they represent suitable models to study the mechanism of development and differentiation or the molecular basis of functions in specific types of intestinal epithelial cells IECs (Nakamura and Sato, 2018). The presence of the crypt in this model permit to investigate more about the mechanism action of ZEN on the small intestine and its carcinogenic potential. Our hypothesis is that ZEN can interrupt intestinal homeostasis. To understand the molecular mechanism by which ZEN activates estrogenic receptors, and explaining the link between ZEN-estrogenic receptors and the other pathways in the intestine such as Notch, epidermal growth factor and bone morphogenetic protein pathways, that are the major players in homeostatic control of the adult epithelium (Sancho *et al.*, 2004). In addition, this 3D tissue structure also owns Paneth cells residing at crypt apex (Nakamura and Sato, 2018). As we know, the intestinal homeostasis depend to Paneth cells that are a major secretary of antimicrobial proteins, and supply key developmental and homeostatic signals to intestinal stem cells in the crypt base (Wehkamp *et al.*, 2006; Clevers and Bevins, 2013). Dysfunction of Paneth cell biology contributes to the pathogenesis of chronic inflammatory bowel disease (Clevers and

Bevins, 2013). Previous reports report a ZEN-induced increase in Paneth cells in pig (Obremski *et al.*, 2005). The homeostasis of Paneth cells depends on ER (Hasson *et al.*, 2014) and Wnt/ $\beta$ -catenin (Van *et al.*, 2005), and dysregulation in their function is related to the development of cancer (Cho *et al.*, 2007). In this respect, we expect that ZEN can disrupt the Paneth cells function. Further investigations are needed to understand the mode of action and molecular pathway of ZEN on these cells, and how can it disturb the intestine microenvironment.

On the other hand, it would be interesting to explore the effect of ZEN on the intestine during the prenatal period and the neonatal period, because the ZEN manages to cross the blood-placental barrier. It has demonstrated that fetal exposure and/or during the period of breastfeeding with xenoestrogens alters the intestinal epithelium renewal in the newborn. A chronic administration of xenoestrogens to rats at low doses (5  $\mu$ g/kg per day) during gestation and lactation period induces in the offspring a decrease in cell proliferation in the colon observed as early as ten days of age (Braniste and Houdeau, 2012). According to this, it could be expected that ZEN, as an xenoestrogen, might cause an effect on intestinal maturation.

The **second conclusion** of the present thesis that the simultaneous presence of ZEN and  $\alpha$ -ZEL in food commodities might be responsible of the induction greater intestinal cytotoxicity than predicted from their effects alone.

The presence of synergetic toxic effects in food contaminants represents a significant threat to human and animal health. Further research are needed to assess interactions of ZEN and its metabolites on other organs, the analysis of gene and proteins expression related to the toxicity effects, will help at understanding the specific and commons mechanisms of action of ZEN and its metabolites. In this context, we suggest to use the high content analysis (HCA) as a means of quantifying the toxicity of single and complex mixtures of chemical food contaminants. This technique quantitatively examines chemical-induced toxicity at sub-cellular microscopic resolution, and allows kinetic evaluation of changes.

(Clarke *et al.*, 2015). The combined effect of ZEN and its metabolites might then be evaluated by HCA measuring selected cellular events as they are hallmarks of cell health, including DNA damage markers such as 8-hydroxydeoxyguanosine and phosphorylated histone variant H2AX, mitochondrial membrane potential, caspases, as well as reactive oxygen species (Thompson *et al.*, 2012; O'Brien *et al.*, 2006).

The interaction of effect of ZEN and its derivatives in the activation of selected genes can be performed using Chemical Activated LUciferase gene eXpression (CALUX). Several targets, other than the evidently relevant hormone receptors, could be studied using this system, such as the nuclear receptor aryl hydrocarbon receptor (AhR). AhR is involved in the control of intestinal mucosal homeostasis. This receptor is a ligand-activated transcription factor that can activate the transcription of a variety of target genes that contain dioxin response elements or xenobiotic response elements, including cytochrome P450 CYP1A1 (Beischlag *et al.*, 2008; Koch *et al.*, 2015). AhR has been recently reported to inhibit inflammatory processes in the gut and to have a protective effect against inflammatory bowel disease IBD (Yu *et al.*, 2018). ZEN is known to activate human AhR in primary cultures of hepatocytes mRNA levels, but ZEN activation of AhR in the intestine is still not elucidated to date. It would be interesting to analyze the single and combined effect of ZEN and their metabolites in the activation of AhR and their gene targets using gene reporter approaches such as CALUX.

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# Annex

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# Communications

| Date               | Meeting Title   | Venue  | Type of Presentation             | Presentation title   |
|--------------------|---|--|----------------------------------|--|
| June 16 - 21, 2019 | The 2019 Gordon Research Conference (GRC) on Mycotoxins and Phycotoxins | Stonehill College<br>320 Washington Street<br>Easton, MA, US | Poster                           | <b>Development of a new molecular phenotyping tool for mycotoxin toxicity screening</b><br>SOLER VASCO Laura, LAHJOUJI Tarek, LABAS Valérie, TEIXEIRA-GOMES Ana Paula, BANLIAT Charles, OSWALD Isabelle P  |
| 3-7 Dec 2018       | ProtectED 2nd Summer school 2   | NMBU,<br>OSLO,<br>NORWAY                                     | Poster/Talk                      | <b>Effect of Zearalenone, an estrogenic mycotoxin, alone or in combination, on the small intestine</b><br>Tarek Lahjouji, Laura Soler-Vasco & Isabelle P Oswald  |
| 28 - 29 March 2019 | Club d'études des cellules épithéliales digestives meeting              | Toulouse, France   | Talk                             | <b>Molecular phenotyping of intestinal epithelial cells exposed to mycotoxins.</b><br>SOLER VASCO, Laura ; LABAS Valérie ; TEIXEIRA-GOMES Ana Paula; BANLIAT Charles; LAHJOUJI Tarek ; PINTON Philippe ; TERCIOLO Chloé ; NEVES, Manon ; OSWALD Isabelle |
| 28-31 May 2018     | International conference: Belfast summit on global food integrity-ASSET | Belfast (Ireland)  | Poster (lightning talks session) | <b>In-vitro and ex-vivo combined toxicity of mycotoxins.</b><br>Tarek Lahjouji, Imourane Alassane-Kpembé, Philippe Pinton, Laura Soler-Vasco, Isabelle P Oswald  |
| 10-13 Nov 2019     | ProtectED 3rd Summer school   | Universidad Austral De CHILE, Valdivia Chile                 | Poster                           | <b>Effect of Zearalenone, an oestrogenic mycotoxin, in the small intestine</b><br>Tarek Lahjouji, Laura Soler-Vasco & Isabelle P Oswald  |
| 4-8 June 2018      | ProtectED 1st Summer school   | Belfast (Ireland)  | Talk                             | <b>Effect of Zearalenone, an estrogenic mycotoxin, alone or in combination, on the small intestine</b><br>Tarek Lahjouji, Laura Soler-Vasco & Isabelle P Oswald  |

# Publications

**Lahjouji\***, T.; **Bertaccini\***, A.; Neves, M.; Puel, S.; Oswald, I.P.; Soler, L. Acute Exposure to Zearalenone Disturbs Intestinal Homeostasis by Modulating the Wnt/ $\beta$ -Catenin Signaling Pathway. *Toxins* 2020, 12, 113.

*\* Same level of contribution*

Soler, L., Stella, A., Seva, J., Jose, F., **Lahjouji, T.**, Burlet-Schiltz, O., and Oswald, I.P. (2020). Proteome changes induced by a short , non-cytotoxic exposure to the mycoestrogen zearalenone in the pig intestine. *J. Proteomics* 224, 103842.

## Supplementary Material (Table S1, S2 and S3)

**Table S1: Results of the RT-qPCR of pig intestinal explants exposed for 1 h or 4 h to 100  $\mu$ M ZEN**

| Gene symbol   | Gene name  | 1h exposition |             | 4h exposition |             |
|---------------|--|---------------|-------------|---------------|-------------|
|               |  | p-value       | ratio ZEN/C | p-value       | ratio ZEN/C |
| CDH1          | E-Cadherin   | 0,18          | 0,66        | 0,08          | 1,4         |
| CDH2          | N-Cadherin   | 0,43          | 0,92        | 0,39          | 0,9         |
| CDH3          | P-Cadherin   | 0,71          | 0,95        | 0,18          | 1,2         |
| Adiponectin   | Adiponectin  | 0,24          | 0,34        | 0,47          | 1,7         |
| ADIPOR2       | Adiponectin Receptor 2                                     | 0,18          | 1,53        | 0,05          | 1,3         |
| CAP1          | Cyclase Associated Actin Cytoskeleton Regulatory Protein 1 | 0,89          | 1,07        | 0,03          | 1,22        |
| CMKLR1        | Chemerin Chemokine-Like Receptor 1                         | 0,88          | 0,91        | 0,04          | 1,33        |
| BD3           | $\beta$ -defensin 3  | 0,51          | 0,77        | 0,01          | 0,51        |
| BD1           | $\beta$ -defensin 1  | 0,7           | 1,19        | 0,06          | 1,27        |
| LYZ           | Lysozyme   | 0,98          | 1,01        | 0,07          | 1,81        |
| PR39          | Protegrin 39   | 0,2           | 0,39        | 0,85          | 1,17        |
| Reg3 $\gamma$ | Regenerating islet-derived protein 3 gamma                 | 0,33          | 1,57        | 0,23          | 1,16        |
| SMAD7         | SMAD Family Member 7                                       | 0,32          | 1,61        | 0,1           | 0,68        |
| ANXA2         | Annexin A2   | 0,76          | 1,22        | 0,6           | 1,44        |
| S100A8        | S100 Calcium Binding Protein A8                            | 0,12          | 0,8         | 0,27          | 0,8         |
| S100A12       | S100 Calcium Binding Protein A12                           | 0,72          | 0,76        | 0,34          | 1,91        |
| S100A10       | S100 Calcium Binding Protein A10                           | 0,05          | 0,76        | 0,03          | 0,74        |
| S100A4        | S100 Calcium Binding Protein A4                            | 0,23          | 1,15        | 0,18          | 1,2         |

|               |  |       |       |      |      |
|---------------|--|-------|-------|------|------|
| FN1           | Fibronectin 1                                    | 0,12  | 0,881 | 0,07 | 0,81 |
| Cyclin D1     | Cyclin D1  | 0,22  | 1,29  | 0,01 | 1,3  |
| LEF1          | Lymphoid Enhancer Binding Factor 1               | 0,94  | 0,07  | 0,91 | 0,9  |
| MCM3          | Minichromosome Maintenance Complex Component 3   | 0,25  | 1,15  | 0,01 | 1,16 |
| TCF1          | Transcriptional activator 1                      | 0,04  | 1,15  | 0,68 | 1    |
| TCF3          | Transcriptional activator 3                      | 0,8   | 1,56  | 0,15 | 1,18 |
| TCF4          | Transcriptional activator 4                      | 0,004 | 0,83  | 0,03 | 0,75 |
| MMP2          | Matrix Metalloproteinase 2                       | 0,11  | 0,86  | 0,02 | 0,73 |
| MMP7          | Matrix Metalloproteinase 7                       | 0,68  | 1,09  | 0,35 | 0,51 |
| MMP9          | Matrix Metalloproteinase 9/                      | 0,39  | 1,19  | 0,03 | 0,67 |
| SNAIL 2       | Snail Family Transcriptional Repressor 2         | 0,13  | 1,28  | 0,01 | 1,2  |
| SNAIL1        | Snail Family Transcriptional Repressor 1         | 0,39  | 1,18  | 0,08 | 1,75 |
| CD44          | CD44 Molecule (Indian Blood Group)               | 0,59  | 1,15  | 0,12 | 0,7  |
| IFNg          | Interferon Gamma                                 | 0,46  | 1,3   | 0,38 | 1,22 |
| IL17          | Interleukin 17                                   | 0,13  | 2,85  | 0,42 | 1,21 |
| IL18          | Interleukin 18                                   | 0,7   | 1,29  | 0,93 | 0,98 |
| IL22          | Interleukin 22                                   | 0,25  | 1,83  | 0,25 | 1,54 |
| IL8           | Interleukin 8                                    | 0,56  | 1,36  | 0,42 | 1,55 |
| LCN2          | Lipocalin 2                                      | 0,89  | 0,94  | 0,28 | 2,41 |
| MUC1          | Mucin 1  | 0,31  | 0,42  | 0,06 | 1,3  |
| TNF $\alpha$  | Tumor Necrosis Factor alpha                      | 0,74  | 1,16  | 0,11 | 1,19 |
| SAA3          | Serum Amyloid A3                                 | 0,33  | 0,5   | 0,11 | 1,91 |
| PPAR $\alpha$ | Peroxisome Proliferator Activated Receptor Alpha | 0,56  | 1,35  | 0,28 | 1,27 |
| PPAR $\gamma$ | Peroxisome Proliferator Activated Receptor Gamma | 0,06  | 0,91  | 0,63 | 0,9  |
| VIM           | Vimentin   | 0,76  | 1,33  | 0,01 | 1,66 |
| DKK1          | Dickkopf WNT Signaling Pathway Inhibitor 1       | 0,16  | 0,68  | 0,04 | 0,5  |

|                |  |        |      |        |      |
|----------------|--|--------|------|--------|------|
| APC            | Adenomatous Polyposis Coli Protein                     | 0,06   | 0,69 | 0,01   | 0,71 |
| Axin1          | Axin 1   | 0,3    | 1,09 | 0,09   | 1,4  |
| Axin2          | Axin 2   | 0,06   | 0,96 | 0,38   | 0,88 |
| C-myc          | Myc Proto-Oncogene Protein                             | 0,16   | 1,29 | 0,04   | 1,38 |
| CTNNA1         | Catenin Alpha 1  | 0,18   | 1,7  | 0,03   | 1,35 |
| CTNNB1         | Catenin Beta 1   | 0,72   | 1,2  | 0,02   | 1,43 |
| GSK3- $\beta$  | Glycogen Synthase Kinase 3 Beta                        | 0,11   | 1,43 | 0,49   | 1,07 |
| ITGA10         | Integrin Subunit Alpha 10                              | 0,04   | 1,98 | 0,43   | 1,4  |
| LRP5           | LDL Receptor Related Protein 5                         | 0,47   | 1,03 | 0,7    | 0,96 |
| LRP6           | LDL Receptor Related Protein 6                         | 0,1    | 0,88 | 0,28   | 0,83 |
| Wnt 11         | Wnt Family Member 11A                                  | 0,81   | 0,94 | 0,03   | 0,66 |
| Wnt 4          | Wnt Family Member 4                                    | 0,4    | 1,24 | 0,07   | 0,8  |
| Wnt1           | Wnt Family Member 1                                    | 0,35   | 1,63 | 0,24   | 1,4  |
| Wnt2b          | Wnt Family Member 2b                                   | 0,64   | 1,03 | 0,23   | 1,17 |
| ZEB1           | Zinc Finger E-Box Binding Homeobox 1                   | 0,16   | 2,19 | 0,03   | 1,48 |
| ZEB2           | Zinc Finger E-Box Binding Homeobox 2                   | 0,18   | 0,76 | 0,19   | 0,86 |
| SMAD1          | SMAD Family Member 1                                   | 0,79   | 0,98 | 0,16   | 1,19 |
| SMAD2          | SMAD Family Member 2                                   | 0,0185 | 0,83 | 0,0002 | 0,65 |
| SMAD4          | SMAD Family Member 4                                   | 0,32   | 1,03 | 0,09   | 1,12 |
| TGF- $\beta$ 1 | Transforming Growth Factor Beta 1/                     | 0,03   | 1,33 | 0,48   | 1,04 |
| SMAD3          | SMAD Family Member 3                                   | 0,3    | 0,8  | 0,49   | 1,12 |
| ATP6AP2        | ATPase H <sup>+</sup> Transporting Accessory Protein 2 | 0,92   | 0,95 | 0,02   | 1,48 |
| OCLN           | Occludin   | 0,29   | 0,42 | 0,04   | 1,2  |
| ZO1            | Zonula Occludens 1                                     | 0,29   | 1,13 | 0,04   | 1,1  |

**Table S2: List of primers used in this study**

| Official Gene Name | Transcript ID ENSEMBL | Sequence 5'-3' |                            | pb | % GC | Amplicon | Tm   |
|--------------------|-----------------------|----------------|----------------------------|----|------|----------|------|
| CDH1               | ENSSSCT00000029834.2  | F              | CATACACGGCCCTCATTATAG      | 21 | 47   | 136      | 60   |
|                    |                       | R              | CTCTGGCAGAAGTCCATATTT      | 21 | 42   |          | 60   |
| CDH2               | ENSSSCT00000004122.3  | F              | GACTGGATTCCCTGAAGATG       | 20 | 50   | 129      | 60   |
|                    |                       | R              | GGTTCGCTGCTTTCATATTG       | 20 | 45   |          | 60   |
| CDH3               | ENSSSCT00000026686.2  | F              | GGTATCCTGACAACCAAGAAG      | 21 | 47   | 101      | 60   |
|                    |                       | R              | GGGAGCTTCACCACAAAG         | 18 | 55   |          | 60   |
| ADIPOQ             | ENSSSCT00000047495.1  | F              | AGGGTGAGAAAGGAGATACA       | 20 | 45   | 141      | 60   |
|                    |                       | R              | GGTAGACATAGGCGCTTTC        | 19 | 52   |          | 60   |
| CAP1               | ENSSSCT00000004068.3  | F              | GCGGAAATGGTCCATACA         | 18 | 50   | 104      | 60   |
|                    |                       | R              | GGTGCCAAACAATTCAGAAAG      | 20 | 45   |          | 60   |
| CMKLR1             | ENSSSCT00000058690.1  | F              | TGAGGACCCTCCATCAG          | 18 | 57   | 99       | 60   |
|                    |                       | R              | TCACTGTTGTGACATCCAAA       | 20 | 40   |          | 60   |
| BD1                | ENSSSCT00005005133.1  | F              | TTCCTCCTCATGGTCCTGTT       | 20 | 50   | 130      | 56   |
|                    |                       | R              | AGGTGCCGATCTGTTTCATC       | 20 | 50   |          | 55   |
| LYZ                | ENSSSCT00000034939.1  | F              | GGTCTATGATCGGTGCGAGTTC     | 22 | 55   | 51       | 66,8 |
|                    |                       | R              | TCCATGCCAGACTTTTTCAGAAT    | 23 | 39   |          | 65,6 |
| Reg3g              | ENSSSCT00000034539.2  | F              | CTCCTGTCTCAGGTCCAA         | 18 | 56   | 116      | 60   |
|                    |                       | R              | GGTGTTATAAACAAGGCATAGC     | 22 | 41   |          | 60   |
| ANXA2              | ENSSSCT00000005057.4  | F              | GCTGAAAGCCTCCATGA          | 17 | 53   | 111      | 59   |
|                    |                       | R              | CTCCTTG TAGACTCTGTTAATTTTC | 24 | 37   |          | 59   |
| S100A8             | ENSSSCT00000036517.2  | F              | GAGACGGAGTGTCTAAGTA        | 20 | 50   | 121      | 60   |
|                    |                       | R              | CCACCTTGATCACCAGTATG       | 20 | 50   |          | 60   |
| S100A12            | ENSSSCT00000038862.1  | F              | AAGGAACTGCCCAACAC          | 17 | 53   | 118      | 60   |
|                    |                       | R              | CCAGGACCACGAATTCTTTA       | 20 | 45   |          | 60   |
| S100A10            | ENSSSCT00000007246.4  | F              | CAAAGGAGGACCTGAGAGTA       | 20 | 50   | 126      | 60   |
|                    |                       | R              | AGCCACTTTCCCATCT           | 17 | 53   |          | 60   |
| S100A4             | ENSSSCT00000033355.2  | F              | CAAGGAGGGTGACAAGTTC        | 19 | 53   | 107      | 60   |
|                    |                       | R              | TCTGGAAAGCAGCTTCATC        | 19 | 47   |          | 60   |



|                   |                      |   |                          |    |    |     |    |
|-------------------|----------------------|---|--------------------------|----|----|-----|----|
| Fibronectine<br>1 | ENSSSCT00000017610.3 | F | AATGCACCAGAACCATCTC      | 19 | 47 | 101 | 60 |
|                   |                      | R | TTCAAGTGGCCTGGAATG       | 18 | 50 |     | 60 |
| CCND1             | ENSSSCT00000046016.1 | F | GAAGTTC AACAGCTTCCT      | 19 | 48 | 106 | 60 |
|                   |                      | R | GCTTCGATCTGCTCCTG        | 17 | 59 |     | 59 |
| LEF1              | ENSSSCT00000010018.2 | F | GATCACACCCATCACACATC     | 20 | 50 | 122 | 61 |
|                   |                      | R | GGTAATCTGTCCAACACCAC     | 20 | 50 |     | 60 |
| MCM3              | ENSSSCT00000031013.2 | F | GACCATAGAGCGACGTTATAC    | 21 | 47 | 110 | 60 |
|                   |                      | R | GGCCATATTCTGTCTCAAGG     | 20 | 50 |     | 60 |
| TCF7/1            | ENSSSCT00000023234.2 | F | TCTACTCCGCCTTCAATCT      | 19 | 47 | 99  | 60 |
|                   |                      | R | AAGGCTGACTGGCTTTG        | 17 | 53 |     | 60 |
| TCF3              | ENSSSCT00000014681.3 | F | TGGCCTCATGCACAAC         | 16 | 56 | 98  | 60 |
|                   |                      | R | TGATCTCACCAGCTCCA        | 17 | 52 |     | 60 |
| MMP2              | ENSSSCT00000003130.3 | F | CGACAAGGATGGCAAGTA       | 18 | 50 | 121 | 59 |
|                   |                      | R | GCAGCTGTTGTAGGATGT       | 18 | 50 |     | 60 |
| MMP9              | ENSSSCT00000008139.3 | F | AACACACACGACATCTTCC      | 19 | 48 | 115 | 60 |
|                   |                      | R | TCACGTAGCCACATAGT        | 18 | 50 |     | 60 |
| MPP7              | ENSSSCT00000016344.3 | F | TGCTCACTTTGATGAGGATG     | 20 | 45 | 98  | 60 |
|                   |                      | R | GACCTAAAGAATGGCCAAGT     | 20 | 45 |     | 60 |
| SNAI2             | ENSSSCT00000048563.1 | F | CGCTCCTTCCTGGTCAA        | 17 | 59 | 84  | 61 |
|                   |                      | R | GGAAACGATCACTGTGTGTG     | 20 | 50 |     | 61 |
| SNAI1             | ENSSSCT00000040909.1 | F | ACGACTTCTCCAGAGTT        | 19 | 47 | 96  | 60 |
|                   |                      | R | AGGCGGTGGGATTGA          | 15 | 60 |     | 60 |
| CD44              | ENSSSCT00000014523.3 | F | CGCCATGGACACCTTT         | 16 | 56 | 133 | 59 |
|                   |                      | R | GCTGTAGCGACCATTCTT       | 18 | 50 |     | 60 |
| IL18              | NM_213997            | F | CGTGTGTTGAGGATATGCCTGATT | 23 | 43 | 107 | 66 |
|                   |                      | R | TGGTTACTGCCAGACCTCTAGTGA | 24 | 50 |     | 72 |
| IL22              | ENSSSCT00000000520.3 | F | CCCAACTCTGATAGATTCCAC    | 21 | 48 | 103 | 60 |
|                   |                      | R | GCTGGTCATCACCCCTAAT      | 19 | 47 |     | 60 |
| TNF-alpha         | NM_214022            | F | ACTGCACTTCGAGGTTATCGG    | 21 | 52 | 118 | 64 |
|                   |                      | R | GGCGACGGGCTTATCTGA       | 18 | 61 |     | 58 |
| IFN-gamma         | NM_213948            | F | TGGTAGCTCTGGGAAACTGAATG  | 23 | 48 | 79  | 68 |
|                   |                      | R | GGCTTTGCGCTGGATCTG       | 18 | 61 |     | 58 |
| IL6               | NM_214399            | F | TTCACCTCTCCGGACAAAACCTG  | 22 | 50 | 122 | 66 |

|            |                      |   |                          |    |    |     |      |
|------------|----------------------|---|--------------------------|----|----|-----|------|
|            |                      | R | TCTGCCAGTACCTCCTTGCTGT   | 22 | 55 |     | 68   |
| IL8        | NM_213867            | F | GCTCTCTGTGAGGCTGCAGTTC   | 22 | 59 | 79  | 70   |
|            |                      | R | AAGGTGTGGAATGCGTATTTATGC | 24 | 42 |     | 68   |
| IL17A      | NM_001005729.1       | F | CCAGACGGCCCTCAGATTAC     | 20 | 60 | 66  | 58,2 |
|            |                      | R | GGTCCTCGTTGCGTTGGA       | 18 | 61 |     | 59,2 |
| LCN2       | ENSSSCT00000006200.3 | F | AGCAAGGCCGGTTTAAG        | 17 | 53 | 109 | 60   |
|            |                      | R | CTGATCCAGTTGTCACAGAG     | 20 | 50 |     | 60   |
| MUC1       | ENSSSCT00000007146.4 | F | GCATTACAAACCTCCAGTTTACCT | 24 | 36 | 123 | 58   |
|            |                      | R | CCCAGAAGCCCGTCTTCTTT     | 20 | 55 |     | 59   |
| PPAR-alpha | ENSSSCT00000000007.3 | F | GTGGCACTGAACATCGAAT      | 19 | 47 | 108 | 61   |
|            |                      | R | CCGAATGGTTCTCCGAAAG      | 19 | 53 |     | 60   |
| PPAR-gamma | ENSSSCT00000012672.3 | F | GTCAGTACTGTCGGTTTCAG     | 20 | 50 | 99  | 60   |
|            |                      | R | CCAACAGCTTCTCCTTCTC      | 19 | 53 |     | 60   |
| VIM        | ENSSSCT00000065218.1 | F | AAGCCGAGAGCACTTTG        | 17 | 53 | 60  | 60   |
|            |                      | R | CGTGCCAAAGAAGCATTG       | 18 | 50 |     | 60   |
| DKK1       | ENSSSCT00000011413.3 | F | TGACAACATCAGCCATACC      | 20 | 45 | 112 | 59   |
|            |                      | R | GCAGGCTAGGCAGATTT        | 17 | 53 |     | 59   |
| APC        | ENSSSCT00000046118.1 | F | ACTTCTTGCTGACCTTGAC      | 19 | 47 | 131 | 60   |
|            |                      | R | GTCTGGTCATATCTGTCTGTAAG  | 23 | 43 |     | 60   |
| Axin1      | ENSSSCT00000023524.2 | F | TGACAGCCTATCCCTCAC       | 18 | 56 | 90  | 60   |
|            |                      | R | AGGCTCTCCTGCATCTC        | 17 | 59 |     | 60   |
| Axin2      | ENSSSCT00000055385.1 | F | CAATGGCCAAGTGTCTCTAC     | 20 | 50 | 133 | 61   |
|            |                      | R | GGTCTCCAATTCCAGCTTC      | 19 | 53 |     | 60   |
| C-MYC      | ENSSSCT00000006548.4 | F | CTGGATTCCTTCGGATAGTG     | 21 | 47 | 94  | 60   |
|            |                      | R | AGTCGTAGTCGAGGTCATAG     | 20 | 50 |     | 60   |
| CTNNA1     | ENSSSCT00000015666.3 | F | GGAGAGTCAGTTTCTCAAGG     | 20 | 50 | 81  | 60   |
|            |                      | R | GCAGCACTCTTCATCAAATC     | 20 | 45 |     | 59   |
| CTNNB1     | ENSSSCT00000012342.3 | F | GCTCTCTGTCTGCTCTAGTA     | 20 | 50 | 97  | 60   |
|            |                      | R | AAGACGTTGACTTGATCTG      | 20 | 45 |     | 60   |
| GSK3-beta  | ENSSSCT00000013006.3 | F | GGATAGTGGTGTGGATCAAT     | 20 | 45 | 92  | 59   |
|            |                      | R | CTGTGTAGTTTGGGTTTCATTC   | 22 | 40 |     | 60   |
| ITGA10     | ENSSSCT00000007326.3 | F | TCATCCCTCACCTGTTCT       | 18 | 50 | 74  | 60   |
|            |                      | R | GTGATGCACGTCCAGATTA      | 19 | 47 |     | 60   |

|          |                      |   |                           |    |    |        |      |
|----------|----------------------|---|---------------------------|----|----|--------|------|
| ITGB1    | ENSSSCT00000012148.3 | F | GTTGGTAAACAGCGCATATC      | 20 | 45 | 104    | 59   |
|          |                      | R | ACATTCCTCCAGCCAATC        | 18 | 50 |        | 59   |
| LRP5     | ENSSSCT00000026471.2 | F | GCTCATCCAGCACCAAG         | 17 | 59 | 112    | 60   |
|          |                      | R | GGAATGTTGGAGGAGTAGAAC     | 21 | 48 |        | 60   |
| LRP6     | ENSSSCT00000039050.1 | F | GCAGTGACAAATCGGATGA       | 19 | 47 | 98     | 60   |
|          |                      | R | GGTGACAATTACGCCAATAAC     | 21 | 42 |        | 60   |
| Wnt4     | ENSSSCT0000003912.3  | F | GGAAGTGTCCACCCT           | 16 | 63 | 89     | 61   |
|          |                      | R | AGAGATGGCGTACACGAA        | 18 | 50 |        | 61   |
| Wnt11    | ENSSSCT00000024218.2 | F | GTGTGCTATGGCATCAAGT       | 19 | 47 | 139    | 60   |
|          |                      | R | GCATGAGCTCCAGGTTG         | 17 | 59 |        | 60   |
| Wnt2B    | ENSSSCT00000007426.3 | F | GGCCGTTCTTGACAAA          | 17 | 52 | 125    | 60   |
|          |                      | R | CGAATCACAGCCACAGAT        | 18 | 50 |        | 60   |
| ZEB2     | ENSSSCT00000065884.1 | F | ACACACAGTGATCGTTTCC       | 19 | 47 | 109    | 60   |
|          |                      | R | GGTCCACACCGCAATG          | 16 | 63 |        | 60   |
| ZEB1     | ENSSSCT00000012067.3 | F | GAAGGAAGGACAAGAAATCCT     | 21 | 43 | 81     | 60   |
|          |                      | R | GAGTCGATTTCGTCATCTT       | 19 | 47 |        | 60   |
| SMAD2    | ENSSSCT00000004977.3 | F | GGTTCTGCCTAGGTTTACTC      | 20 | 50 | 108    | 60   |
|          |                      | R | AAACTTCACCGCCTATGTAA      | 20 | 40 |        | 60   |
| SMAD4    | ENSSSCT00000004997.2 | F | CCCACCTAATTTGCCTCAC       | 19 | 52 | 118    | 61   |
|          |                      | R | AGAAATTGGAGGCTGGAATG      | 20 | 45 |        | 61   |
| SMAD1    | ENSSSCT00000009900.3 | F | CAGCCCATGGACACAAA         | 17 | 52 | 95     | 60   |
|          |                      | R | CAGTGTTGGGTTTCCTCATA      | 20 | 45 |        | 60   |
| SMAD7    | ENSSSCT00000004979.4 | F | CCTCCTCCTTACTCCAGATAC     | 21 | 52 | 105    | 60   |
|          |                      | R | GGCCAGATAATTCGTTCCC       | 19 | 53 |        | 60   |
| TGF-beta | ENSSSCT00000036469.2 | F | GGATACCAACTACTGCTTCAG     | 21 | 47 | 102    | 60   |
|          |                      | R | GGTTCATGAATCCAATTCCA      | 20 | 45 |        | 60   |
| SMAD3    | ENSSSCT00000005464.2 | F | TCACCCAAGTGAACCAGCG       | 20 | 55 | 62     | 66   |
|          |                      | R | CCTGGTGGGATCTTGACAGA      | 19 | 58 |        | 66,4 |
| ATP6AP2  | ENSSSCT00000013393.3 | F | GAACTGCAGGTGCTACAT        | 18 | 50 | 125    | 60   |
|          |                      | R | CCGTAACGTTTCCCAATTTC      | 20 | 45 |        | 60   |
| OCLN     | U79554               | F | AGCTGGAGGAAGACTGGATCAG    | 22 | 55 | 91 pb  | 68   |
|          |                      | R | TGCAGGCCACTGTCAAAATT      | 20 | 45 |        | 58   |
| ZO1      | AJ318101             | F | ATAACATCAGCACAGTGCCTAAAGC | 25 | 44 | 116 pb | 72   |

|         |                      |   |                       |    |    |     |    |
|---------|----------------------|---|-----------------------|----|----|-----|----|
|         |                      | R | GTTGCTGTAAACACGCCTCG  | 21 | 52 |     | 64 |
| ADIPOR2 | ENSSSCT00000026686.2 | F | TGTTCCGCCACCCCTCAGTAT | 20 | 55 | 71  | 58 |
|         |                      | R | AATGATTCCACTCAGGCCCA  | 20 | 50 |     | 57 |
| TCF4    | ENSSSCT00035106376.1 | F | ATCAGCAAGCACTGCCGACT  | 20 | 55 | 167 | 60 |
|         |                      | R | CCAACATTCTCCGTAGCCA   | 20 | 55 |     | 57 |
| BD3     | ENSSSCT00005005099.1 | F | CCTTCTCTTTGCCTTGCTCTT | 20 | 47 | 163 | 55 |
|         |                      | R | GCCACTCACAGAACAGCTACC | 21 | 57 |     | 58 |
| PR39    | NM_214450.1          | F | AGCAGTCCTCGGAAGCTAATC | 21 | 52 | 215 | 57 |
|         |                      | R | GTCATTGGATGGGTTCAAGGT | 21 | 48 |     | 55 |
| TBP     | ENSSSCT00000029370.1 | F | GCCCGAAATGCTGAATATAA  | 20 | 40 | 82  | 60 |
|         |                      | R | ATATCAGTGCGGTGGTC     | 17 | 53 |     | 60 |
| HPRT1   | ENSSSCT00000051852.1 | F | CTGACCTGCTGGATTACA    | 18 | 50 | 73  | 60 |
|         |                      | R | CCCGTTGACTGGTCATTA    | 18 | 50 |     | 61 |

**Table S3: List of primary antibodies used in this study**

| <b>Protein name</b>                | <b>MW</b> | <b>Purchased from</b>                            | <b>Antibody reference</b> |
|------------------------------------|-----------|--|---------------------------|
| RegIIIγ                            | 16 kDa    | In-house produced antibody                       |                           |
| Serum Amyloid A                    | 15k Da    | In-house produced antibody                       |                           |
| IL-17A                             | 32 kDa    | KingFicher Biotech, St Paul, MN, USA             | KP0498S                   |
| IL8                                | 11.6 kDa  | R&D Systems, Inc. Minneapolis, MN, USA           | MAB5351                   |
| E-cadherin                         | 97.7 kDa  | Cell Signaling Technology, Inc, Danvers, MA, USA | 3195                      |
| Zonula occludens 1                 | 120kDa    | Invitrogen, Carlsbad, CA, USA                    | 61-7300                   |
| Acid-1-alpha glycoprotein          | 47 kDa    | In-house produced antibody                       |                           |
| Occludin                           | 64 kDa    | Invitrogen, Carlsbad, CA, USA                    | 71-1500                   |
| β-catenin                          | 85.5 kDa  | Abcam Ltd., Cambridge, UK                        | ab22656                   |
| Vimentin                           | 53 kDa    | Abcam Ltd., Cambridge, UK                        | ab8069                    |
| Cofilin 1                          | 20 kDa    | Abcam Ltd., Cambridge, UK                        | ab42824                   |
| Chemerin Chemokine-Like Receptor 1 | 45 kDa    | AB Clonal, Woburn, MA, USA                       | A15237                    |
| Estrogen Receptor alpha            | 68 kDa    | Abcam Ltd., Cambridge, UK                        | ab3575                    |
| Estrogen Receptor beta             | 56 kDa    | Santa Cruz Biotechnology, Dallas, TX, USA        | sc-390243                 |

**Table S4: False discovery rate correction**

| Gene symbol   | P-Value<br>1h exposition | P-Value<br>4h exposition | FDR_1h             | FDR_4h              |
|---------------|--------------------------|--------------------------|--------------------|---------------------|
| CDH1          | 0.18                     | 0.08                     | 0.5321739130434782 | 0.1813333333333332  |
| CDH2          | 0.43                     | 0.39                     | 0.6799999999999999 | 0.49111111111111116 |
| CDH3          | 0.71                     | 0.18                     | 0.8742857142857142 | 0.306               |
| Adiponectin   | 0.24                     | 0.47                     | 0.5862068965517241 | 0.5462295081967212  |
| ADIPOR2       | 0.18                     | 0.05                     | 0.5321739130434782 | 0.14782608695652175 |
| CAP1          | 0.89                     | 0.03                     | 0.9310769230769231 | 0.12                |
| CMKLR1        | 0.88                     | 0.04                     | 0.9310769230769231 | 0.12363636363636364 |
| BD3           | 0.51                     | 0.01                     | 0.7539130434782608 | 0.09714285714285714 |
| BD1           | 0.7                      | 0.06                     | 0.8742857142857142 | 0.1632              |
| LYZ           | 0.98                     | 0.07                     | 0.98               | 0.17                |
| PR39          | 0.2                      | 0.85                     | 0.5666666666666668 | 0.8757575757575757  |
| Reg3 $\gamma$ | 0.33                     | 0.23                     | 0.5905263157894737 | 0.36372093023255814 |
| SMAD7         | 0.32                     | 0.1                      | 0.5905263157894737 | 0.20606060606060606 |
| ANXA2         | 0.76                     | 0.6                      | 0.8759322033898306 | 0.6580645161290322  |
| S100A8        | 0.12                     | 0.27                     | 0.5321739130434782 | 0.3885714285714286  |
| S100A12       | 0.72                     | 0.34                     | 0.8742857142857142 | 0.4624000000000001  |
| S100A10       | 0.05                     | 0.03                     | 0.4533333333333333 | 0.12                |
| S100A4        | 0.23                     | 0.18                     | 0.5862068965517241 | 0.306               |
| FN1           | 0.12                     | 0.07                     | 0.5321739130434782 | 0.17                |
| Cyclin D1     | 0.22                     | 0.01                     | 0.5862068965517241 | 0.09714285714285714 |
| LEF1          | 0.94                     | 0.91                     | 0.9540298507462687 | 0.9235820895522389  |
| MCM3          | 0.25                     | 0.01                     | 0.5862068965517241 | 0.09714285714285714 |
| TCF1          | 0.04                     | 0.68                     | 0.4533333333333333 | 0.7225              |
| TCF3          | 0.8                      | 0.15                     | 0.8883870967741936 | 0.2756756756756757  |
| TCF4          | 0.004                    | 0.03                     | 0.272              | 0.12                |
| MMP2          | 0.11                     | 0.02                     | 0.5321739130434782 | 0.12                |
| MMP7          | 0.68                     | 0.35                     | 0.8742857142857142 | 0.46666666666666666 |
| MMP9          | 0.39                     | 0.03                     | 0.646829268292683  | 0.12                |
| SNAIL 2       | 0.13                     | 0.01                     | 0.5321739130434782 | 0.09714285714285714 |
| SNAIL1        | 0.39                     | 0.08                     | 0.646829268292683  | 0.1813333333333332  |
| CD44          | 0.59                     | 0.12                     | 0.8187755102040816 | 0.22666666666666666 |
| IFN $\gamma$  | 0.46                     | 0.38                     | 0.7102222222222222 | 0.48754716981132074 |
| IL17          | 0.13                     | 0.42                     | 0.5321739130434782 | 0.5099999999999999  |
| IL18          | 0.7                      | 0.93                     | 0.8742857142857142 | 0.93                |
| IL22          | 0.25                     | 0.25                     | 0.5862068965517241 | 0.37777777777777777 |
| IL8           | 0.56                     | 0.42                     | 0.7933333333333334 | 0.5099999999999999  |
| LCN2          | 0.89                     | 0.28                     | 0.9310769230769231 | 0.3885714285714286  |
| MUC1          | 0.31                     | 0.06                     | 0.5905263157894737 | 0.1632              |
| TNF $\alpha$  | 0.74                     | 0.11                     | 0.8759322033898306 | 0.21371428571428572 |
| SAA3          | 0.33                     | 0.11                     | 0.5905263157894737 | 0.21371428571428572 |
| PPAR $\alpha$ | 0.56                     | 0.28                     | 0.7933333333333334 | 0.3885714285714286  |

|                                |        |          |                    |                      |
|--------------------------------|--------|----------|--------------------|----------------------|
| <b>PPAR<math>\gamma</math></b> | 0.06   | 0.63     | 0.4533333333333333 | 0.6799999999999999   |
| <b>VIM</b>                     | 0.76   | 0.01     | 0.8759322033898306 | 0.09714285714285714  |
| <b>DKK1</b>                    | 0.16   | 0.04     | 0.5321739130434782 | 0.12363636363636364  |
| <b>APC</b>                     | 0.06   | 0.01     | 0.4533333333333333 | 0.09714285714285714  |
| <b>Axin1</b>                   | 0.3    | 0.09     | 0.5905263157894737 | 0.19125              |
| <b>Axin2</b>                   | 0.06   | 0.38     | 0.4533333333333333 | 0.48754716981132074  |
| <b>C-myc</b>                   | 0.16   | 0.04     | 0.5321739130434782 | 0.12363636363636364  |
| <b>CTNNA1</b>                  | 0.18   | 0.03     | 0.5321739130434782 | 0.12                 |
| <b>CTNNB1</b>                  | 0.72   | 0.02     | 0.8742857142857142 | 0.12                 |
| <b>GSK3-<math>\beta</math></b> | 0.11   | 0.49     | 0.5321739130434782 | 0.5462295081967212   |
| <b>ITGA10</b>                  | 0.04   | 0.43     | 0.4533333333333333 | 0.5129824561403509   |
| <b>LRP5</b>                    | 0.47   | 0.7      | 0.7102222222222222 | 0.7323076923076923   |
| <b>LRP6</b>                    | 0.1    | 0.28     | 0.5321739130434782 | 0.3885714285714286   |
| <b>Wnt 11</b>                  | 0.81   | 0.03     | 0.8883870967741936 | 0.12                 |
| <b>Wnt 4</b>                   | 0.4    | 0.07     | 0.6476190476190476 | 0.17                 |
| <b>Wnt1</b>                    | 0.35   | 0.24     | 0.6102564102564102 | 0.3709090909090909   |
| <b>Wnt2b</b>                   | 0.64   | 0.23     | 0.8704000000000001 | 0.36372093023255814  |
| <b>ZEB1</b>                    | 0.16   | 0.03     | 0.5321739130434782 | 0.12                 |
| <b>ZEB2</b>                    | 0.18   | 0.19     | 0.5321739130434782 | 0.3151219512195122   |
| <b>SMAD1</b>                   | 0.79   | 0.16     | 0.8883870967741936 | 0.28631578947368425  |
| <b>SMAD2</b>                   | 0.0185 | 2,00E-04 | 0.4533333333333333 | 0.013600000000000001 |
| <b>SMAD4</b>                   | 0.32   | 0.09     | 0.5905263157894737 | 0.19125              |
| <b>TGF-<math>\beta</math>1</b> | 0.03   | 0.48     | 0.4533333333333333 | 0.5462295081967212   |
| <b>SMAD3</b>                   | 0.3    | 0.49     | 0.5905263157894737 | 0.5462295081967212   |
| <b>ATP6AP2</b>                 | 0.92   | 0.02     | 0.9478787878787879 | 0.12                 |
| <b>OCLN</b>                    | 0.29   | 0.04     | 0.5905263157894737 | 0.12363636363636364  |
| <b>ZO1</b>                     | 0.29   | 0.04     | 0.5905263157894737 | 0.12363636363636364  |