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FRONTIER

Inflammatory diseases modelling in zebrafish

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Abstract

The ingest of diets with high content of fats and carbohydrates, low or no physical exercise and a stressful routine are part of the everyday lifestyle of most people in the western world. These conditions are triggers for different diseases with complex interactions between the host genetics, the metabolism, the immune system and the microbiota, including inflammatory bowel diseases (IBD), obesity and diabetes. The incidence of these disorders is growing worldwide; therefore, new strategies for its study are needed. Nowadays, the majority of researches are in use of murine models for understand the genetics, physiopathology and interaction between cells and signaling pathways to find therapeutic solutions to these diseases. The zebrafish, a little tropical water fish, shares 70% of our genes and conserves anatomic and physiological characteristics, as well as metabolical pathways, with mammals, and is rising as a new complementary model for the study of metabolic and inflammatory diseases. Its high fecundity, fast development, transparency, versatility and low cost of maintenance makes the zebrafish an interesting option for new researches. In this review, we offer a discussion of the existing genetic and induced zebrafish models of two important Western diseases that have a strong inflammatory component, the IBD and the obesity.

Key words: Zebrafish; Western diseases; Inflammatory disorders; Obesity; Inflammatory bowel diseases

Core tip: The western lifestyle with a high fat and carbohydrates diet, lack of physical activity and stress, is a trigger for different diseases with complex interactions between the host genetics, the metabolism, the immune system and the microbiota, as the inflammatory bowel

disease (IBD), obesity and diabetes. The zebrafish has 70% homology with our genes, shares anatomic and physiological characteristics with mammals, and emerges as a new model for the study of metabolic and inflammatory diseases. In this review, we examine the existing genetic and induced zebrafish models of two important Western diseases with strong inflammatory component, IBD and obesity.

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INTRODUCTION

In the last decades, the standard of living of the western world has had consequences that affect human health. Factors such as a diet that is high in carbohydrates and fats, a sedentary lifestyle, and stress trigger a state of chronic systemic low-grade inflammation, insulin resistance, and changes in the microbiota[1-3], which lead to the so-called Western diseases. Some of these diseases include inflammatory bowel disease (IBD), obesity, type 2 diabetes and heart disease, among others, and they are an issue of global significance, because of the high incidence of such disorders in western society. It is estimated that IBD affects approximately 1-1.3 million people in the United States[4] and in the same country 9.3% of the population has diabetes[5]. Additionally, more than two-thirds (68.8%) of United States adults are considered as overweight or obese[6].

Given the worldwide importance of these diseases, much research is currently in progress seeking answers to unresolved questions about their physiopathology, the pathways involved and new therapies to treat these conditions. Although mainly mammalian models, such as rabbits, rats and mice, are used for these studies[7-10], other models that have been gaining ground in the field of inflammatory diseases do exist[11-13]. The zebrafish is a small tropical freshwater fish primarily used as a vertebrate model in developmental biology because of its characteristic high fecundity, short *ex vivo* development time, ease of observation in the embryonic and larval states, relative ease of genetic manipulation, and low cost of production[14]. Additionally, the zebrafish genome is fully mapped (http://www.sanger.ac.uk/resources/zebrafish/), having approximately 70% of orthologs with the human genome[15]. Zebrafish have specific technical advantages as model for the *in vivo* analysis and knock-down technology[12,16]. They have anatomical features commonly found in mammals, including a central, autonomic and enteric nervous system, a multi-chambered heart, a liver, an intestinal system, a pancreas, and a kidney responsible for the production of hematopoietic cells, as well as immunological maturation sites such as the thymus and the spleen[13]. Zebrafish have a functional innate immune system at 48 h post-fertilization (hpf) and a mature adaptive system approximately 4-6 wk post-fertilization (wpf)[17], with many of the same immune cells, cytokines and chemokines known in humans[18]. Furthermore, in the last decade, zebrafish have become a model for different human diseases and a tool for drug screening[11,13,19]. All of these characteristics make the zebrafish an excellent model for the study of inflammatory pathologies.

In this review, we discuss and summarize the current larval and adult zebrafish models of Western diseases with an inflammatory component, including IBD and obesity.

IBD MODELS

In humans, the IBD are a group of chronic inflammatory conditions of the small intestine and colon, appearing as a result of deregulated interactions between the immune system and the commensal microbiota, triggered by a genetic predisposition of the affected individual and external factors[20-22]. In mice, there is a wide range of genetic, spontaneous and chemical models[23-25] that have been used in an attempt to find answers to different about IBD issues. Beginning some years ago, an increasing variety of zebrafish IBD chemical models have arisen, which are based on models previously tested in mice.

The zebrafish intestine has been described by many authors[26-28] as very similar in anatomy and architecture to the mammalian small intestine. It is a compartmentalized tubular structure with three intestinal segments defined by histological morphology of the epithelial folds and the distribution of different cell types. It has a mucosal layer of simple folded epithelium formed by columnar absorptive enterocytes, goblet cells and enteroendocrine cells; it lacks Paneth cells and a lamina propria beneath the epithelium. The mucosa is directly surrounded by circular and longitudinal smooth muscle layers, and small groups of enteric nerves can be observed between the two muscular layers with the nerve fibers innervating the connective tissue beneath the epithelium. Because of these simple anatomical characteristics, the zebrafish has proven to be an excellent model to study intestinal inflammation.

Genetic susceptibility

Nowadays, there's little evidence of genetic spontaneous colitis models in zebrafish as exist in mice [*e.g.,* NEMO KO, STAT3 KO in myeloid cells, interleukin (IL)-10 KO][29-31], however, there have been discovered many zebrafish genes related to genetic susceptibility in human IBD. The loss of *myd88*[32,33], an adaptor molecule, central in innate immune signaling[34], induce predisposition to bacterial infections and compromised expression of immune transcription factors (*nfκb, ap-1*) and molecules (*il-1β, mmp9*), proving to be an important molecule in the development of the inflammatory process in zebrafish. NOD proteins are intracellular pattern recognition receptors involved in innate immune response and have been associated with genetic vulnerability to IBD[35].

In zebrafish, *nod1* and *nod2* genes are expressed in intestinal epithelial cells (IECs) and neutrophils. In a model of infection with *Salmonella enterica,* morpholino (MO) knock-down (KD) of *nod1* and *nod2* have decreased survival after infection, and *nod1* KD also had a decreased expression of dual oxidase (*duox*), which is responsible for the synthesis of reactive oxygen species and has role in neutrophils migration, since its depletion slows down the repopulation of the caudal hematopoietic tissue[36]. In mammals, the cytokine IL-22, which is produced by T-helper cells and innate lymphocytes, has important functions in host defense at mucosal surfaces and in tissue repair[37]. In zebrafish, IL-22 expression was detected predominantly in the myeloid innate linage during early developmental stages, and proved to have participation in host-microbe interaction since its knock-down present high susceptibility to bacterial infections and an increased pro-inflammatory cytokine expression. IL-22 is increased in patients with Crohn's disease (CD) but is decreased in ulcerative colitis (UC) patients[38,39], thus would be interesting investigate the role of this cytokine during zebrafish intestinal inflammation. Furthermore, IL-10 and IL-23 expression have been related to an upregulated response to LPS and bacterial infection in zebrafish[40,41], and both have proven roles in IBD, since the *il10*$^{-/-}$ mice develops a spontaneous colitis[31] and IL-23 is essential for T cell–mediated colitis[42].

Endoplasmic reticulum (ER) stress is a defense mechanism triggered by a variety of conditions that disturb folding of proteins in the ER. To alleviate this stress the unfolded protein response (UPR) is activated, restoring ER homeostasis, promoting cell survival and adaptation. Modifications in genes that are centrally involved in the UPR appears as a risk factor for both forms of IBD, UC and CD[43]. In zebrafish, two mutants present ER stress in IECs, the *sec13*^{sq198} and *cdipt*^{hi559}, with defects in intestinal development in the first one and alteration of the villi, disorganization in the proliferation of IECs, apoptosis of goblet cells, abnormal mucosecretion, bacterial overgrowth and leucocyte infiltration, in the second one, characteristics resembling the IBDs[44,45]. All these examples, demonstrates conserved genes and pathway in zebrafish, that makes it an interesting model for the research of new IBD related genes.

Chemically induced adult models

Disruption of the intestinal epithelial barrier and exposition to luminal bacterial antigens into the mucosa is one of the key characteristic of mammalian IBD[21], and is the main accomplishment of most of the chemicals used to induce colitis, closely resembling morphological, histopathological and symptomatical features of human IBD. The first chemical model of intestinal inflammation studied in adult zebrafish[46] was based on the mouse oxazolone model of UC[47]. A concentration of 0.2% Oxazolone/50% ethanol was intrarectally injected inducing an inflammation characterized by the intestinal infiltration of granulocytes, eosinophils, macrophages and lymphocytes, as well as changes in the intestinal architecture such as bowel-wall thickening, loss of intestinal folds, and depletion of goblet cells. An increase of *tnfα*, *il1β*, and *il10* transcripts was also observed and was reversed by the use of antibiotics prior the induction of the colitis. A marked influence of the microbiota was evidenced by an enhanced susceptibility to inflammation due to an increase in the bacterial load when fishes where kept in stand-alone/static tanks than in continuous flow-tanks. Treatment with vancomycin, an antibiotic active against gram-positive bacteria, resulted in the reduction of the enterocolitis score and the infiltration of neutrophils, as well as an outgrowth of the *Fusobacteria* phylum; while treatment with colistin, targeting gram-negative bacteria, did not affect the total enterocolitis score but reduced eosinophilic and lymphocytic infiltration and increase in Proteobacteria group. This indicates that the oxazolone model in zebrafish can be used as a complementary model for the study of experimental UC.

A second model of intestinal inflammation in adult zebrafish used 2,4,6-trinitrobenzenesulfonic acid (TNBS) in a 30% ethanol solution injected intrarectally[48]. In mice, this model is widely used to study IBD because of clinical and histopathological findings resembling those seen in CD[49,50]. Using a wide range of TNBS concentrations, the authors observed a reduction of fish survival in a dose-dependent way with a recovery in survival rate when fishes were treated with vancomycin. A histological analysis showed disruption of the epithelial integrity with presence of ulcerations, swelling, thickening and the detachment of villi. No changes in goblet cells were observed in TNBS treated fishes. Inflammatory events peaked at 6 h post-induction (hpi), with an increase in infiltrated neutrophils in Tg(*mpx:eGFP)* animals and a significant increase in mRNA expression of *il1β, il8* and *il10,* in TNBS-exposed fishes compared with controls. These results are similar to a murine TNBS model in which an increase in neutrophilic infiltration in damaged tissue associated with a high myeloperoxidase activity[51] and an increase in TNFα levels[10] can be observed. Melanine-Concentrating Hormone (MCH) is a conserved neuropeptide involved in appetite regulation that recently has been related with intestinal inflammation[52]. The *mch2* gene isoform, equivalent to mammalian *mch*[53], was upregulated in the intestine in TNBS-exposed zebrafish; however the *mch1* isoform did not show any change. The MCH receptor MCHR1b was also upregulated, while MCHR2 was downregulated after TNBS treatment. This downregulation of MCHR2 is different than the expression of MCHR2 in humans, which was shown to increase during intestinal inflammation. Further studies are necessary to discover the contribution of MCH to intestinal inflammation.

The oxazolone and TNBS adult zebrafish enterocolitis models are comparable with the respective murine models in some aspects and provide a complementary tool for the study of IBD. However, because these models have been recently developed, studies that delineate all their inflammatory and pathologic features in order to

enhance their use are scarce in the literature, and more studies are necessary to accomplish this goal.

Chemically induced larval models

Contrary to adult models, the larval model lacks a functional adaptive immune system[17,54]; therefore it allows the observation of the isolated participation of innate immunity in inflammatory intestinal conditions. Additionally, the use of a larval model permits exploitation of transgenic lines to visualize *in vivo* changes in digestive organs and immune cells, such as gutGFP[55], Tg(*mpx:EGFP*)[114], Tg(*ptprc:DsRed*)[sd3][56], MO microinjections[57] and the CRISPR/Cas9 system[58], along with mutagenesis screening to discover novel candidate genes involved in diseases, among other applications.

The most used colitis model in zebrafish larva was developed by two different research groups[59,60] using TNBS in concentrations between 50-75 μg/mL diluted directly in the swimming water of embryos at 3 dpf to 6-8 dpf, higher concentrations resulted in less than 50% survival 3 d post-exposure[60]. Different histological characteristics were observed depending on the time and concentration of the TNBS exposition. Several changes were shown for a treatment of 5 d and 75 μg/mL TNBS including an expansion of the intestinal lumen, a smoothing of the epithelial line, a loss of villi and epithelial clefts, and an increase in the number of goblet cell throughout the mid and posterior intestine[59], with first changes appearing at 6 dpf[61]. Whereas, a 3 d exposition of 50 μg/mL TNBS does not induce any change in intestinal cell morphology or increase in goblet cell number[60]. We think that discordance on these characteristics in different research groups could also be influenced by the variability of the microbiota in the different facilities[62], nevertheless, more studies need to be conducted to test this hypothesis. Subcellular changes in TNBS-exposed larvae included the accumulation of lysosomes in the apical region of the epithelial cells and the loss of tight and gap junctions between IECs[59], as observed in human IBD[63,64]. A well preserved microvilli were present in both TNBS and controls larvae, suggesting a direct action of the TNBS on the physiology of the IECs and not an erosive action. TNBS-exposed fishes showed increases expression of *il1β*, *tnfα*, *il8*, *il12a*, *infg1-2*, *il10* at 6 dpf in the intestinal tissue[60] (Morales Fénero CI *et al*, unpublished data). Interestingly, some of these molecules are Th1 type cytokines, and though the larvae lacks circulating lymphocytes, they are probably produced by epithelial and/or infiltrating myeloid cells[65,66], as observed in the intestine of Tg(*mpx:EGFP*; *ifabp:RFP*)[60] and Tg(*lys:DSRED*) TNBS-exposed larvae (Morales Fénero CI *et al*, unpublished data). Moreover, an increase in TNFα expression in the intestinal lumen was directly related to the TNBS dose and could be reverted with a prednisolone treatment[59,61]. Decrease in RNA expression of ileal fatty acid binding protein *(fabp6)*[67] and increased intestinal lipids accumulation visualized by Nile red (NR) lipophilic stain, reflect alterations in fatty acid metabolism in TNBS-treated fishes, as well as, loss in the endocytic function in the mid-intestinal region[68]. Furthermore, a slight disruption of the intestinal vasculature was evidenced by a reduction of intestinal capillary branches and a decrease in the expression of vascular endothelial growth factor, in colitis induced larvae.

The microbiota is a source of pathogen-associated molecular patterns against which the immune cells can react and is a principal factor in IBD pathology[22]. Broad-spectrum antibiotics increased the low survival rate obtained with high concentrations of TNBS, and even recover the low survival rate of TNBS-exposed *myd88* morphants to control levels[60]. In addition, TNBS exposition in larva induced a lesser diversity of bacteria, with an increase in the phylum Proteobacteria (*Hydrocarboniphaga daqingensis*, *Limnobacter sp.*, *Citrobacter freundii*, *Comamonas sp.*, *Salmonella sp.)* and a decrease in the phylum Firmicutes (*Lactococcus plantarum* and *Streptococcus sp.*) compared with the control group[61]. The same bacteria phyla have been previously observed altered in human IBD[69,70]. Finally, treatment with 5-aminosalicylic acid co-administered with TNBS, prevented the disease alterations induced by the hapten, decreased the expression of *il1β, tnfα, ccl20* and *il8,* and inhibited the increase of myeloid cells in (*lys:EGFP)* transgenic larva, as well as reduced the recruitment of leucocytes to the intestine and skin in Tg(*mpx:EGFP*) larvae[59,60]. Similar results were obtained with prednisolone which was also effective against the generation of disease changes, including reduced expression of *il1β, tnfα* and *il8*[60], a decrease in TNFα and reduction of the number of goblet cells to normal levels[59]. Treatment with NOS inhibitors, rescued the *in vivo* and histological disease phenotype, while treatment with the immunomodulatory drugs thalidomide and parthenolide failed to rescue the changes induced by TNBS despite a decrease in the expression of TNFα in the intestinal tissue. These results indicate that the zebrafish can be an excellent pharmacological tool for drug screening in the search for new treatments for inflammatory diseases.

An additional murine version of UC model uses Dextran Sulfate Sodium (DSS) in the drinking water in an acute protocol of 5-7 d[9,71] or with the induction of chronic inflammation interspersed with periods of DSS-water and periods of recuperation with normal water[72,73]. In another attempt to use the zebrafish as a model for intestinal diseases, researchers exposed larvae at 3 dpf to 0.5% DSS for 3 d, generating a phenotype of marked mucus production with no changes observed in the number of goblet cells in the mid-intestine and the esophagus[74]. Nevertheless, exposure to DSS did not affect the expression levels of the *muc* gene, an ortholog of the human MUC5 gene family that is expressed in the esophagus. However, other "*muc* genes", such as *muc2.1,* which is highly expressed in the gut can also be analyzed for changes in this model[75]. An analysis of *Tg (kita:GAL4, UAS:EGFP)* larvae that labels fin fold mucus producing cells revealed a slight increase in the number of positive

cells when the larvae were treated with DSS, compared with controls[76]. Suggesting that intestinal goblet cells and not esophagus goblet cells, are altered and that they are responsible for the production of excess mucus. An augmented bacterial load and an increased number of intestine-infiltrating neutrophils was observed in *Tg(mpx: EGFP)^{i114}* DSS-exposed larvae compared with controls. This was reversed by antibiotic and dexamethasone treatment, however, depletion of the microbiota prevented the appearance of the DSS-induced mucosecretory phenotype while dexamethasone did not have the same effect. Exposition to DSS also increased the levels of *ccl20, il1β, il23, il8, mmp9* and *tnfα,* and decreased the proliferating cell nuclear antigen *(pcna)* gene. Curiously, using DSS at lower concentrations (0.25%) caused a loss of the inflammatory characteristics but a persistence of the mucosecretory phenotype, which was protective against TNBS induced colitis, and could be suppressed with a retinoic acid treatment, resulting in a worse survival rate and increased neutrophil infiltration.

Based on the premise that the use of non-steroidal anti-inflammatory drugs (NSAIDs), could lead to the impairment of mucosal barrier function[77,78], researchers used the NSAID glafenine as an inducer of intestinal inflammation[79]. An overnight treatment of 12 μmol/L glafenine in 5 dpf larvae produced an obstruction of cells and debris in the lumen of the mid-intestine and posterior intestine of the zebrafish, which consisted of dead IECs. Analysis of transversal sections showed intestinal shedding and an obstructed lumen with hypertrophic and hyperplasic IECs, with apical-pyknotic nuclei, sings of cellular damage and apoptosis, which were confirmed by an increase in activated caspase-3 positive cells. The visualization of glafenine-treated fish with transmission electron microscopy showed apoptotic IECs, cellular debris and microvesiculation of IECs, as well as, a pitted ER and organelles enveloped by membranes, which are signs of ER stress[80]. This characteristics were reverted by treatment with the μ-opioid receptor agonist (D-Arg2,Lys4) dermorphin-(1,4)-amide (DALDA), which decreased the formation of debris and apoptotic cell obstruction, improved the survival of glafenine-exposed larvae, decreased the expression of caspase-3, and increased the number of proliferative 5-ethynyl-2′-deoxyuridine (EdU) positive cells. Furthermore, DALDA-treated larvae showed decreased ER stress in IECs and a conserved epithelial architecture, as well as, upregulation of the UPR mediators spliced-xbp1 *(s-xbp1)* and activating transcription factor 6 (*atf6*), which were normal in glafenine-exposed animals. For other side, *atf6*-MO suppressed the rescue mediated by DALDA. This is anomalous with the function of the mammalian ATF6, which has apoptotic effects[81]. A lack of study on immune parameters such as innate cells infiltration or the quantification of cytokines and chemokines, leaves us with little knowledge about this model, which could be an excellent model to study of ER stress during intestinal inflammation. Modifications in genes that are centrally involved in the UPR appear to be a risk factor for both forms of IBD, UC and CD[43-45].

The aforementioned models of intestinal inflammation, summarized in Table 1, encompass the current options to analyze different aspects of IBD, including the possibility of study in conditions of isolated innate immune system. Other tools, as the generation of gnotobiotic zebrafish[82,83] make this good model system for the study of the microbiota that is central to the pathology of IBD.

OBESITY AND METABOLIC SYNDROME

Another western-lifestyle disease that has worldwide impact and involves chronic inflammation is obesity and the associated metabolic syndrome. Subjects related to lipid metabolism in zebrafish are relatively new but have begun to gain ground in the study of adipogenesis, metabolic alterations and obesity.

Zebrafish, as other teleosts, are poikilothermic animals and they only have white adipose tissue (WAT) and lack brown fat, which is more characteristic of homoeothermic organisms. The first visceral adipocytes appear to form in proximity to the pancreas after exogenous feeding is initiated, and they increase in number and distribution as the zebrafish grow, with the participation of the markers of adipocyte lineage peroxisome-proliferator activated receptor γ (*pparγ*) and fatty acid binding protein 11a (*fabp11a*)[84]. Lipid absorption in zebrafish is very similar to the process in mammals. Bile is synthesized in the liver, stored in the gall bladder and brought to the intestine through the bile duct, where it emulsifies lipids that are broken down by luminal lipases and absorbed like fatty acids and triacylglycerols. Lipids are transported in the plasma as unbound fatty acids or bound to carrier proteins, as triacylglycerols (TAG)-rich chylomicrons, and then they are delivered to the liver and stored in visceral, intramuscular and subcutaneous reservoirs, mainly as TAG[85-87]. Currently, a variety of techniques exists that allows visualization of the lipids in zebrafish without sacrificing the animal, including the fluorescent compounds NR and BODIPY® - conjugated lipids and Oil Red O, which is suitable for fixed fish and is an excellent tool for the study of lipid metabolism[86].

Genetic models of obesity

Energy homeostasis in the zebrafish is conserved and regulated by peripheral signals like PYY, GLP-1, ghrelin, adiponectin, leptin and insulin[88-93], originated in the gastrointestinal tract and adipose tissue, processed in the brain by the central melanocortin system (CMS), as in mammals[86,94,95]. The CMS circuits include the pro-opiomelacortin (*POMC*) gene, melanocortin peptides and its receptors (MC1R-MC5R), as well as the melanocortin antagonist agouti-related protein (AgRP)[96,97]. AgRP mRNA is upregulated by fasting in humans, mice and zebrafish[97-100], and its overexpression has been related to obesity in mice[101]. Song *et al*[102] created a transgenic animal that overexpressed the *AgRP* gene under the control of the *β-actin* promoter. AgRP transgenic animals were demonstrated to gain more weight, present

Table 1 Zebrafish models of inflammatory bowel diseases

Model	Age	Induction	Characteristics
Oxazolone	Adult	Intrarectal administration of 0.2% oxazolone in 50% ethanol. Stand-alone tanks	Epithelial damage; infiltration of neutrophils and eosinophils in intestine; depletion of goblet cells; upregulation of IL1β, TNFα and IL10[46]
TNBS	Adult	Intrarectal administration of TNBS (160 mmol/L) in 30% ethanol. Stand-alone tanks	Dose-dependent fish survival; disruption of the epithelial integrity; ulcerations; swelling, thickening and detachment of villi; no changes in goblet cells; upregulation of IL1β, IL8 and IL10[48]
	Larva (3-8 dpf)	50-75 μg/mL TNBS in swimming water (E3 medium)	Dose-dependent survival; expansion of intestinal lumen; loss of villi; increased number of goblet cell; upregulation of IL1β, TNFα, IL8, and MMP9; increased TNFα expression in lumen; infiltrate of myeloid cells[59,60]
DSS	Larva (3-6 dpf)	0.5% DSS in swimming water (E3 medium)	Mucosecretory phenotype; neutrophilic infiltration microbiota - dependent; upregulation of CCl20, IL1β, IL23, IL8, MMP9 and PCNAIL; increased proliferating cells[76]
Glafenine	Larva (5 dpf)	25 μmol/L glafenine for 12 h in in swimming water (E3 medium)	Apoptosis in intestinal epithelial cells; ER stress in IECs[79]

TNBS: 2,4,6-trinitrobenzenesulfonic acid; DSS: Dextran sulfate sodium.

increased total triglycerides, present larger visceral adipocytes and increased linear growth, compared with wild type (WT) animals. However, though they could not demonstrate the direct action of the zebrafish AgRP protein as a competitive antagonist of the melanocortin receptors, the authors showed that the positive response to α-melanocyte stimulating hormone (α-MSH) by zebrafish MC3R, MC4R and MC5bR transfected cells could be antagonized with mouse-AgRP. Further research on this pathway revealed an *in vivo* interaction of MC4R with two forms of melanocortin receptor accessory protein 2 (MRAP2) in zebrafish[103]. In cell culture, MRAP2a binds to MC4R and reduces its ability to bind to its ligand αMSH, and *in vivo,* MRAP2a is expressed during larval stages and stimulates growth by blocking the action of MC4R. The MC4R antagonist AgRP is also highly expressed in larvae and collaborates with MARP2 to maintain MC4R in a stable inactive state. On the other hand, MRAP2b is highly expressed in the adult zebrafish brain and it causes a moderate increase in the expression of MC4R in transfected cells, and even increase MC4R affinity to its ligand αMSH, suggesting that MRAP2b is the homologous isoform of the mammalian MRAP2. Given the chronic inflammatory state in obese mammals, it would be interesting to analyze the inflammatory state resulting from genetic modifications of this pathway, in order to find a genetic relationship to inflammation.

An interesting transgenic of exogenous human constitutively active Akt1 (*myrAkt1*) expressed in the skin protein Keratin-4 (*krt4*) presented a severe obese phenotype in adult zebrafish[104]. Tg(*krt4:Hsa.myrAkt1*)^{cy18} animals exhibit hypertrophic and hyperplastic growth of the epidermis during the larval stages, caused by the upregulation of the activated Akt1 downstream targets glycogen synthase kinase 3 alpha/beta (GSK3α/β), mammalian target of rapamycin (mTOR) and 70-kDa S6 protein kinase (70S6K). The adult Tg(*krt4:Hsa.myrAkt1*)^{cy18} had an increased body weight but not body length and also an augmented conditional factor, equivalent to human BMI, compared to WT siblings. Analysis with Oil Red O revealed pronounced lipid accumulation in the entire body that primarily arose from an excess of triglycerides with normal cholesterol accumulation. Sagittal sections of the entire body of obese transgenic adult zebrafish showed adipocyte hyperplasia rather than hypertrophy, as well as ectopic adipocytes in the muscles of the dorsal body and the gill arch that also infiltrated and replaced bone and skeletal muscle cells. This seemed to be triggered by upregulation of the ectopic expression of *myAkt1* in liver, muscle and bone and activation of the mTOR pathway in adipose tissue. Exploring the mRNA expression in tail samples of Tg (*krt4:Hsa.myrAkt1*) cy18, Rasouli *et al*[105] found downregulation of the transcripts of myogenic factor 5 (*myf5*), myogenic factor 6 (*myf6*) myogenic differentiation 1 (*myod1*) and myosin light polypeptide 2 (*mylz2*), which are myogenic regulatory factors and structural proteins. Factors participating of skeletogenesis as runt-related transcription factor 2 (*runx2)* and collagen type Ⅱ alpha-1a (*col2α1a*) were also down-regulated, while genes related to lipid metabolism such as *pparγ* and CCAAT/enhancer binding protein α (*cebpα*) were intensely upregulated, in addition to fatty acid-binding proteins (*fabp11a* and *fabp11b*), sterol regulatory element binding transcription factor 1 (*srebf1*), lipoprotein lipase (*lpl*) and stearoyl-CoA desaturase (*scd*). Analysis of the inflammatory state of this transgenic animal revealed high expression of adiponectin (*adipoql* and *adipoql2*), of the adiponectin receptors *adipor1a* and *adipor1b*, the leptin receptor (*lepr*), and *lipin1*, known as adipocytokines. Inflammatory molecules such as *tnfα, il1β, mmp2* and *mmp9* were also upregulated, and although no differences in the number of whole body neutrophils were found, neutrophil aggregation could be seen in the obese animals' tails. It would be interesting to see if these neutrophils aggregate in WAT. Other characteristics of this transgenic animal were a "sedentary" swimming behavior because muscles were replaced by fat, a lower survival rate compared with WT, and reduced glucose clearance after feeding, suggesting impaired glucose tolerance in these animals.

Diet induced obesity models

In addition to the genetic mutations that could lead to

an obesogenic phenotype several diet induced obesity (DIO) models also exist, that use different combinations of high fat food to generate the phenotype. Oka *et al*[106] designed a DIO model in zebrafish by overfeeding adults for 8 wk with freshly hatched nauplii *Artemia* (brine shrimp), which are part of the normal food in zebrafish facilities. The DIO animals exhibited an increased BMI (calculated as the body weight divided by the square of the body length), increased plasma triglycerides and hepatosteatosis. These parameters were improved by a calorie restricted diet following overfeeding. A comparative transcriptome analysis between visceral adipose tissue of DIO zebrafish, DIO mice, DIO rats and obese humans revealed common pathophysiological pathways. Genes related to blood coagulation and lipid metabolism were significantly dysregulated in the four obese groups, including apolipoprotein H (*apoh*), *il6* and *il1β* as regulatory molecules appearing in the coagulation cascade, as well as *srebf1*, peroxisome proliferator activated receptor alpha (*pparα*) and gamma (*pparγ),* nuclear receptor subfamily 1 group H member 3 (*nr1h3*) and leptin (*lep*), which are regulatory molecules involved in lipid metabolism in obese zebrafish, rats, mice and humans. These results indicates that immune molecules occur in obesity pathways in both zebrafish and mammals. Further research with this DIO model tested the anti-obesity effects of different vegetables in zebrafish, including regular and Campari tomatoes, pumpkins, eggplants, and others[107]. Campari tomatoes have significant lipid-lowering proprieties because they suppressed the increase of body weight and plasma triglycerides in DIO zebrafish, reduced lipid accumulation in the liver, and increased the genes involved in fatty acid oxidation such as proliferator-activated receptor gamma co-activator 1α (*pparγc1α*) and peroxisome proliferator-activated receptor αb (*ppar-αb*). Additionally, the same group tested the anti-adipogenesis proprieties of green tea extract (GTE) in the same DIO model[108]. GTE treatment decreased the volume of visceral but not subcutaneous WAT and increased the liver expression of acyl-coenzyme A oxidase 1, palmitoyl (*acox1*), acyl-coenzyme A dehydrogenase (*acadam*) and *pparα*, which are part of β-oxidation and lipid catabolism. Also decreased the expression of suppressor of cytokine signaling 3b (*socs3*) in visceral fat, which inhibits leptin signaling. Another approach using GTE as a treatment in adult zebrafish used a standard chow supplemented with gluten, α-potato starch, corn oil and lard in order to create four diets for DIO models with different fat contents. The results indicated no differences in fat accumulation between the groups of high-fat (HF) or of low-fat (LF). As in the previous model, GTE decreased body weight and fat volume in animals on a HF diet, and increased the activity of the enzyme 3-hydroxyacyl-coenzyme A dehydrogenase in liver and skeletal muscle, which is part of the β-oxidation pathway, demonstrating the utility of this model to test different natural anti-obesogenic compounds.

Another recent DIO model based on overfeeding adult zebrafish was generated by feeding them two times the standard fish chow than the controls[109]. DIO animals showed increased total weight, showed liver steatosis, as well as the overexpression of *tac4*, *col4a3*, *col4a5*, lysyl oxidases and genes involved in retinoid metabolism. A liver transcriptomic analysis after inoculation with LPS showed that immune system genes responded to LPS stimulation, including Toll-like receptors, ubiquitin mediated proteolysis, RIG-I-like receptor signaling pathway, MAPK and Jak–STAT signaling pathway in control lean animals. No alterations were observed in obese animals, and there was also no difference between obese animals and uninjected obese controls. Studying the differences between obese and non-obese zebrafish in other organs during LPS-stimulation or in other infection models could be of great interest, because obese animals are in a basal inflammatory state.

Though adult models of obesity seems to be more popular, larvae obesity models are also promising. The zebrafish obesogenic test[110] was created for the *in vivo* study of the effect of diet composition, chemical pollutants, and/or drugs on white adipocyte tissue. Larvae of a standard length of 7.5-9 mm were fed with a three-day protocol, in which the first day started with a high-fat diet (HFD) based on hard-boiled chicken egg-yolks *ad libitum* for the entire day, followed by starvation the next day, and by exposure to different obesogenic/non-obesogenic compounds the third day. After the feeding period, HFD animals showed an increase in NR staining in blood vessels, which were reduced after the fasting period. This phenomenon was not observed in control animals fed with a standard diet (SD). When studying the interaction of the initial diet with different compounds, the researchers found that exposure to rosiglitazone, a PPARγ agonist used in type Ⅱ diabetes treatment, increased the lipids deposits in both SD and HFD animals, and this effect was inhibited by T0070907, a PPARγ antagonist. A similar result was observed with tributyltin, a renowned environmental obesogen that binds to PPARγ and retinoid X receptor, for which both SD and HDF larvae exhibited an increase in adipose deposits. However, additive effects between any of the two chemical and HFD were not observed. Although this model showed an increase in blood vessel lipids for a HFD in a short period of time, this result does not reflect obesity as a chronic disease, because differences in the accumulation of lipids in the visceral WAT between SD and HFD animals or increase in weight and size of HFD larvae were not observed. Perhaps a longer exposure period to the HFD would affect these parameters.

Progatzky *et al*[111], showed that the exposure to a HF diet or a high-cholesterol diet (HCD) in zebrafish larvae induced an inflammatory response in hours, with infiltration of myeloid cells in the intestine, dependent on inflammasome activation by IECs. They demonstrated that the inflammation was directly induced by cholesterol binding to the Niemann-Pick C1-like receptor, with the participation of the apoptosis-associated speck-like protein containing a CARD (ASC) and activation of

Table 2 Zebrafish models of obesity

Model	Age	Induction	Characteristics
Genetic models			
AgRP overexpression	All stages	AgRP expressed under the control of β-actin promoter	Weight gain and linear growth; increased BMI; visceral adipose accumulation; increased triglycerides; larger visceral adipocytes[102]
Tg(krt4:Hsa.myrAkt1)cy18	All stages	Expression of constitutively active human AKt1	Weight gain; increased BMI; triglycerides accumulation; adipocyte hyperplasia; ectopic adipose tissue; increased expression of adiponectin, adiponectin receptors, leptin receptor; increased inflammatory molecules TNFα, IL1β, MMP2 and MMP9[104]
DIO models			
Artemia overfeeding	Adult	Overfeeding with nauplii artemia for 8 wk	Increased BMI; high plasma triglycerides; hepatosteatosis[106]
Chow overfeeding	Adult	Overfeeding with standard fish chow for 8 mo	Weight gain; hepatosteatosis[109]
Zebrafish obesogenic test (OZ)	Larva	High-fat diet based in hard-boiled chicken egg-yolk ad libitum during one day	Increase in blood vessel lipids in a short time[110]
HCD	Larva	HCD, cholesterol mixed in fish standard dry food for 6 h. Extended HCD for 10 d	Infiltration of myeloid cells in intestine dependent of the inflammasome, microbiota and NFκB activation; extended feeding leads to visceral fat accumulation, liver steatosis, intestine inflammation, impaired peristalsis[111]

HCD: High cholesterol diet; BMI: Body mass index; TNF: Tumor necrosis factor; IL: Interleukin; MMP: Matrix metalloprotease.

caspase-1, which is part of the inflammasome complex[112] that produces high levels of active IL-1β. Furthermore, this inflammation was dependent on the microbiota and NFκB activation. Finally, extended feeding with a HCD produced the accumulation of visceral fat, liver steatosis, sustained inflammation in the intestine, and impaired peristalsis. This study verified a direct link between inflammation and high-fat diets, specifically the activation of the inflammasome complex by cholesterol in the intestine, and opened a new window to the study of innate inflammation in the context of obesity and its influence in other chronic inflammatory diseases.

By last, a study analyzing two flame retardants, tetrabromobisphenol-A and tetrachlorobisphenol-A, as possible obesogens using zebrafish larvae[113] showed lipid accumulation in larval stage and late-onset weight gain in juvenile animals, which was most likely caused by the compounds' activity as a PPARγ agonist. This method could be interesting for the analysis of the inflammatory state under such conditions, using these substances as agonists of PPARγ.

The zebrafish models related to obesity maybe are not so well known as mice models, nonetheless, the examples presented here (Table 2) are evidence of the conserved signals that control lipids metabolism and the flexibility of the zebrafish as model of metabolic diseases.

CONCLUSION

The models presented in this review exhibit the utility of zebrafish as a model of diseases and demonstrate that this animal as an intermediate between models involving simpler invertebrates and more complex higher mammals and can be used as an alternative or a complement to pre-clinical and drug screening studies that involve conserved metabolic and inflammatory pathways. Furthermore, the characteristics of zebrafish such as physiological homology, rapid development and a low cost of production, make this animal a great option for research on new therapies for inflammatory diseases.

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