**Conserved function of zebrafish (*Danio rerio*) Gdf15 as a sepsis
tolerance mediator**

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Abstract

GDF15 is frequently detected in patients suffering from various diseases, especially those associated with pro-inflammatory processes and/or metabolic disorders. Accordingly, sepsis, whose major complications are related to metabolic alterations and systemic inflammation, significantly increases the secretion of GDF15. Indeed, this cytokine could be considered a marker of sepsis severity. However, until the last several years, the involvement of GDF15 in these disorders had not been widely characterized. In mice, GDF15 was recently described as a pivotal inducer of sepsis tolerance by mediating metabolic alterations that reduce tissue damage.

In this work we describe a zebrafish *gdf15* gene. We found that *gdf15* follows an expression pattern similar to that observed in mammals, being highly expressed in the liver and kidney and induced after pro-inflammatory stimuli. Moreover, larvae overexpressing *gdf15* were more resistant to bacterial and viral challenges without affecting the pathogen load. Consequently, Gdf15 also protected zebrafish larvae against LPS-induced mortality. As in mice, zebrafish Gdf15 seems to induce sepsis tolerance by altering the metabolic parameters of the individuals.

Keywords: Gdf15, zebrafish, sepsis, tolerance, LPS, metabolism

37 1. Introduction

38 Growth differentiation factor-15 (GDF15), also known as macrophage inhibitory
 39 cytokine-1 (MIC1), is a member of the transforming growth factor (TGF)- β superfamily
 40 (Bootcov et al., 1997). This cytokine is widely associated with a variety of diseases,
 41 metabolic disorders, and stress responses, as well as obesity and tissue injury (Breit et
 42 al., 2011; Unsicker et al., 2013; Adela and Banerjee, 2015). For that reason, GDF15 is
 43 considered a general prognostic marker of several diseases (Corre et al., 2013). In
 44 general terms, GDF15 is linked to pro-inflammatory conditions, and most studies
 45 concluded that GDF15 plays a protective anti-inflammatory role, decreasing the tissue
 46 damage associated with inflammation (Kempf et al., 2011; Abulizi et al., 2017; Luan et
 47 al., 2019). Indeed, a recent publication reported that murine GDF15 is involved in
 48 survival after bacterial and viral challenges, not by affecting pathogen virulence and/or
 49 propagation but by increasing tolerance to inflammatory damage (Luan et al., 2019).
 50 Moreover, the authors observed that GDF15 was essential for protecting animals from
 51 lipopolysaccharide (LPS)-induced sepsis (Luan et al., 2019).

52 The term sepsis (from the Greek *sipsi*, meaning “make rotten”) was first used
 53 approximately 2,700 years ago in Homer’s poems and by Hippocrates (460–370 BC) to
 54 define the putrefaction process of organic matter (Funk et al., 2009). Since then, the
 55 meaning of sepsis remained practically unaltered until the 19th century, when the “germ
 56 theory” of disease was conceived and the relationship between sepsis and harmful
 57 microorganisms was established (Gül et al., 2017). Nevertheless, the inclusion of the
 58 host responses in the term sepsis was finally achieved in the 20th century based on
 59 different experiments demonstrating the importance of the host processes and that
 60 people succumbed to sepsis even when the pathogenic microorganism was successfully
 61 eradicated or after the administration of bacterial endotoxins (Funk et al., 2009). These
 62 advances confirmed that the host response to the microorganism drives the pathogenesis
 63 of sepsis. Currently, sepsis is defined as “life-threatening organ dysfunction caused by a
 64 dysregulated host response to infection” (Singer et al., 2016), although an active
 65 infection is not necessary to induce sepsis. Indeed, the bacterial endotoxin LPS is
 66 widely used to study sepsis (Opal, 2010).

GDF15 has been described as a predictor of mortality in critical patients with sepsis because its serum levels are significantly associated with organ failure and disease severity (Buendgens et al., 2017). However, the involvement of GDF15 in this syndrome has not been demonstrated and remains uncertain. Interestingly, it was recently published that GDF15-deficient mice are protected against abdominal polymicrobial sepsis induced by caecal ligation and puncture (CLP) due to the CXCL5-mediated recruitment of neutrophils into the peritoneum, which allows better bacterial control (Santos et al., 2019). These results are in contrast with those obtained by Luan et al., who observed an unaltered bacterial burden after CLP or no variations in the influenza titre when GDF15 was neutralized with specific antibodies or when recombinant GDF15 was administered (Luan et al., 2019). Moreover, in this last work, the authors found a protective effect of GDF15 against LPS, bacterial and viral infections by reducing sepsis-associated damage (Luan et al., 2019). The authors concluded that GDF15 coordinates tolerance to inflammatory damage by regulating triglyceride export from the liver via adrenergic signalling and without altering the plasma levels of pro-inflammatory or anti-viral cytokines, such as IL-12, TNF- α and IFN- α (Luan et al., 2019). GDF15, when interacting with its receptor GFRAL, which is exclusively expressed in the hindbrain (Hsu et al., 2017; Mullican et al., 2017; Yang et al., 2017), plays a physiological role in energy balance by influencing such parameters as food intake, body weight, insulin levels, glucose tolerance, lipid and oxidative metabolism (Chrysovergis et al., 2014; Lee et al., 2017; Tsai et al., 2018; Patel et al., 2019).

Although fish are generally more resistant to LPS-induced sepsis than mammals (Berczi et al., 1966; Novoa et al., 2009) and zebrafish are able to develop tolerance to LPS when exposed to a previous sublethal dose (Novoa et al. 2009; Dios et al., 2014), the mechanisms activated in zebrafish during LPS-induced inflammation are highly comparable to those observed in mammals (Dios et al., 2014; Forn-Cuní et al., 2017; Philip et al., 2017). For this purpose, zebrafish could serve as a useful model for the development of strategies and drug screening aimed at protecting against sepsis.

Due to the importance of zebrafish in biomedical research, we attempted to identify and characterize the *gdf15* gene in this species and to study basic aspects of its expression by examining the effect of *gdf15* overexpression on survival against bacterial and viral infections, as well as LPS-induced sepsis. We found that zebrafish *gdf15*

follows a similar expression pattern to that observed for mammals and that it seems to be involved in sepsis tolerance without significantly affecting pathogen progression. Although zebrafish *gdf15* slightly reduced the expression of some pro-inflammatory mediators, the main modulations induced by this cytokine are related to metabolic processes, suggesting a function similar to that observed in mice by Luan et al. (2019). The description of a *gdf15* gene in zebrafish, which was not previously identified in this species, and our results concerning the evolutionary conservation of this gene may facilitate further research in the sepsis and tolerance field.

2. Materials and Methods

2.1. Sequence retrieval and characterization of zebrafish *gdf15*

The zebrafish *gdf15* cDNA sequence was obtained by blast search against the last version of the zebrafish genome (GRCz11) via Ensembl (https://www.ensembl.org/Danio_rerio/Tools/Blast) (Zerbino et al., 2018) and using the human Gdf15 sequence as entry because it was not identified in the zebrafish genome. Synteny conservation between zebrafish and mammals (*Homo sapiens* and *Mus musculus*) was investigated using Genomicus v98.01 (Muffato et al., 2010).

The *gdf15* full-length open reading frame (ORF) was amplified by PCR using specific primers (**Supplementary Table S2**), cloned in the plasmid pcDNA 3.1/V5-His TOPO TA Expression Kit (Invitrogen) and sequenced using an automated ABI 3730 DNA Analyzer (Applied Biosystems, Inc. Foster City, CA, USA) with the primers T7 and BGH. The predicted amino acid sequence was obtained with the ExPASy Translate Tool (Gasteiger et al., 2003). The presence of the signal peptide was analysed with the SignalP 5.0 Server (<http://www.cbs.dtu.dk/services/SignalP/>) (Almagro Armenteros et al., 2019) and the presence of specific domains and signature family sequences with PROSITE (<https://prosite.expasy.org/>) (Sigrist et al., 2013). The mature protein was predicted based on the RXXR cleavage site on Gdf15 genes from mammals. Molecular weight was calculated with the Compute pI/Mw tool from ExPASy (Gasteiger et al., 2003). The three-dimensional structure was predicted with the I-TASSER (Iterative Threading ASSEmbly Refinement) server (Zhang, 2008) by selecting the model with the best C-score. The template modelling score (TM-score), a measure of structural

similarity between two proteins, was also used to identify structural analogues with known crystal architecture in the Protein Data Bank (PDB; <http://www.rcsb.org/pdb/>).

For phylogenetic analysis, GDF15 amino acid sequences from other vertebrate species were aligned using the ClustalW server (Thompson et al, 1994), and the phylogenetic tree was constructed with Mega 6.0 software (Tamura et al., 2013). The neighbour-joining algorithm (Saitou and Nei, 1987) was used as the clustering method, the distance matrix was computed using the Poisson correction method, and complete deletion of the positions containing alignment gaps and missing data was conducted. Statistical confidence of the inferred phylogenetic relationships was assessed by performing 10,000 bootstrap replicates. Sequence identity and similarity scores were calculated with the software MatGAT (Campanella et al., 2003) using the BLOSUM50 matrix. The GenBank accession numbers of the sequences are listed in **Supplementary Table S1**.

2.2. Animals, virus and bacterium

Wild-type zebrafish were obtained from the facilities at the Instituto de Investigaciones Marinas (Vigo, Spain), where zebrafish are maintained following established protocols (Westerfield, 2000; Nusslein-Volhard and Dahm, 2002). Zebrafish were euthanized using a tricaine methanesulfonate (MS-222) overdose (500 mg/l). Fish care and challenge experiments were conducted according to the guidelines of the CSIC National Committee on Bioethics under approval number ES360570202001/16/FUN01/PAT.05/tipoE/BNG.

The rhabdovirus Spring Viremia of Carp virus (SVCV isolate 56/70) was propagated on ZF4 cells (ATCC CRL-2050) and titred in 96-well plates. The TCID₅₀/ml was calculated according to the Reed and Muench method (Reed and Muench, 1938).

The *Aeromonas hydrophila* strain AH-1 (Yu et al., 2004) was grown on tryptic soy agar (TSA) plates overnight at 28°C and resuspended in phosphate-buffered saline (PBS), and its final concentration was determined by analysing the number of colony forming units (CFUs) using 10-fold serial dilutions of the bacterial suspension seeded on TSA plates.

2.3. Zebrafish *gdf15* constitutive expression

The expression of *gdf15* was analysed in different tissues, cell types and developmental stages of zebrafish. In all these cases, RNA was isolated and retrotranscribed, and *gdf15* expression was determined as mentioned below.

2.3.1. Tissue expression profile

A total of 20 adult zebrafish were euthanized, and different tissues were sampled: spleen, kidney, gills, intestine, liver, heart and brain. Tissues from 5 animals were separately pooled, yielding a total of 4 pools of 5 fish per organ.

2.3.2. Expression in different immune cell types

To determine the magnitude of the expression of *gdf15* in different immune cell types, a total leukocyte suspension from kidney (using 10 adult zebrafish) was prepared by passing the tissues through a 100-µm nylon, and the cells were diluted in Leibovitz's L-15 medium (Gibco) supplemented with Primocin (InvivoGen) and 2% foetal bovine serum (FBS; Gibco). Cells were centrifuged at 400 xg (4 °C) for 10 min and diluted in L-15 medium supplemented with Primocin (InvivoGen) and 10% FBS for analysis by flow cytometry based on forward and side scatter on a FACSCalibur flow cytometer (Beckton Dickinson) equipped for cell sorting (Traver et al., 2003). A total of 300,000 events were sorted in phosphate-buffered saline (PBS; Gibco) from the regions corresponding to myeloid (R2), lymphoid (R3) and precursor (R4) populations, and they were pelleted by centrifugation at 400 xg for 5 min at 4 °C and processed for gene expression. The correct distribution of the cell populations was previously corroborated by using specific cell markers (Pereiro et al., 2015).

2.3.3. Expression during zebrafish ontogeny

To determine the expression level of *gdf15* during zebrafish ontogeny, zebrafish larvae were sampled at the following days postfertilization (dpf): 1, 3, 5, 8, 11, 14, 17, 20, 23, 26. Approximately 10–15 animals (from 3-14 dpf) or 6-8 (17-26 dpf) were pooled to obtain a total of 3 biological replicates per sampling point.

2.4. *In vitro* stimulation of zebrafish kidney cells with recombinant interleukin-4 (IL4) or interferon-gamma (Ifng)

Kidney was extracted from 24 adult zebrafish, and the tissue from 6 fish was pooled, obtaining 4 pooled biological replicates. The kidneys were homogenized

through a 40-µm mesh, and the mixed kidneys were adjusted to a concentration of approximately 10^6 cells/ml in L-15 medium + Primocin + 2% FBS. The cells were seeded into 24-well plates at 0.4 ml per well, and for each pool, six wells were obtained. One well from each pool (4 wells) was stimulated with zebrafish recombinant Il4 (Kingfisher Biotech, Ref. RP1034Z; 0.5 µg/ml), either alone or in combination with LPS (50 µg/ml), with zebrafish recombinant Ifng1.2 (Kingfisher Biotech, Ref. RP1045Z; 0.5 µg/ml), either alone or in combination with LPS, with LPS alone; finally, 4 wells remained unstimulated and served as a control. Cells were maintained at 28 °C, and after 9 h, they were collected for RNA isolation. This sampling point was selected based on previous publications reporting the *in vitro* stimulation of fish macrophages/head kidney cells with recombinant Ifng, which mainly ranged from 4 to 12 h (revised in Pereiro et al., 2019).

2.5. *In vivo* stimulation of adult zebrafish

Adult zebrafish were intraperitoneally (i.p.) inoculated with 10 µl of an SVCV suspension (3×10^6 TCID₅₀/ml), and the corresponding controls were injected with the same volume of viral medium (MEM + 2% FBS + penicillin/streptomycin). The same experiment was conducted using polyinosinic:polycytidylic acid (Poly I:C) (1 mg/ml in PBS; Sigma-P1530) or lipopolysaccharide (LPS) from *Escherichia coli* serotype 0111:B4 (1 mg/ml in PBS; Sigma-L2630), and the corresponding controls were injected with PBS. Kidney samples were taken at 3, 6 and 24 h poststimulation, and 4 biological replicates (4 fish/replicate) per time point were obtained. The samples were stored at -80 °C until RNA isolation.

2.6. *In vivo* stimulation or injury in zebrafish larvae

To determine whether different pro-inflammatory stimuli are able to induce the expression of *gdf15* in larvae, we conducted different experiments using zebrafish embryos and larvae. In all experiments, RNA was isolated and retrotranscribed, and *gdf15* expression was determined as described below.

2.6.1. LPS stimulation by bath

Approximately 90 larvae with an age of 3 dpf were placed in 6-well plates (10 larvae/well) in a volume of 6 ml of water and exposed to 50 µg/ml LPS from *E. coli*, whereas another 90 larvae remained untreated. The larvae were maintained at 28 °C. At

30 min, 6 h and 24 h posttreatment, larvae were sampled (3 pools, 10 larvae/pool) and maintained at -80 °C until RNA isolation.

2.6.2. Tail fin injury

A group of 60 larvae (7 dpf) were anaesthetized and placed on a Petri dish filled with 1% agarose, and the tails were amputated at a position immediately caudal to the notochord using a 1-mm sapphire blade under a Nikon SMZ800 stereomicroscope. Another 60 larvae were not amputated and served as controls. After 1.5, 5 and 17 h, four biological replicates (5 larvae/replicate) were taken and stored until RNA isolation.

2.6.3. Overexpression of *il6*

Because IL-6 has been described as an inducer of Gdf15 expression (Tsui et al., 2012) and both are considered acute phase proteins, we investigated whether zebrafish Il6 has the ability to increase the expression of *gdf15*. To this end, we used an expression plasmid encoding zebrafish *il6* (pcDNA3.1-*il6*) previously synthesized (Bello-Perez et al., 2017). The recombinant plasmid pcDNA3.1-*il6* or the corresponding control empty plasmid (pcDNA3.1) was microinjected into one-cell stage zebrafish embryos with a glass microneedle using a Narishige MN-151 micromanipulator and a Narishige IM-30 microinjector. Zebrafish embryos were microinjected with 150 pg/egg (final volume of 2 nl, diluted in PBS) of the recombinant or empty plasmid and maintained in darkness at 28 °C. Three days after plasmid injection (3 dpf larvae), four biological replicates of approximately 10 larvae were collected from each treatment. Samples were processed for qPCR detection of *gdf15* expression.

2.7. Effect of nutritional alterations on *gdf15* expression

Because mammalian GDF15 is involved in the regulation of food intake, energy expenditure and body weight (Hsu et al., 2017), we wanted to confirm if this function is potentially conserved in zebrafish by analysing the expression of *gdf15* under fasting conditions or fatty acid supplementation.

2.7.1. Food deprivation in adult zebrafish

Thirty adult zebrafish were divided into two tanks (15 fish/tank). One of the tanks was fed as usual and the other was food deprived for a period of 3 days. Liver, kidney, intestine and brain were sampled, and 5 biological replicates (3 fish/replicate) were obtained for *gdf15* expression analysis.

2.7.2. Stimulation of zebrafish larvae with fatty acids

Three different non-esterified fatty acids (NEFAs) were used: arachidonic acid (ARA), oleic acid (OA) and palmitic acid (PA). These NEFAs were purchased from Sigma-Aldrich (Ref. A3611, O1008 and P0500, respectively) and dissolved in pre-heated 0.1N NaOH at 60°C. A 10 mM stock solution was prepared for each NEFA by conjugating them with a pre-warmed fatty acid-free BSA solution (Sigma-Aldrich, Ref. A7030).

Zebrafish larvae (3 dpf) were placed in 6-well plates (10 larvae/well) in a volume of 6 ml of water and maintained at 28 °C in the presence of 1 mM ARA, OA or PA for 24 h or in control medium containing 0.1N NaOH and BSA. After this period, larvae were rinsed three times with zebrafish water and maintained in the absence of NEFAs for an additional period of 24 h. Five pools (5-6 larvae) were sampled for *gdf15* expression analysis.

2.8. Production of an expression plasmid encoding zebrafish *gdf15*

The full-length cDNA sequence of zebrafish *gdf15* was amplified by PCR (primers in **Supplementary Table S2**), and the PCR product was cloned using the pcDNA 3.1/V5-His TOPO TA Expression Kit (Invitrogen), but the epitope V5 and the polyhistidine (6xHis) tag were not included because the reverse primers contained the stop codon of *gdf15*. One Shot TOP10F' competent *E. coli* (Invitrogen) were transformed to generate the plasmid construct (pcDNA 3.1-*gdf15*). Plasmid purifications were conducted using the PureLink HiPure Plasmid Midiprep Kit (Invitrogen) following the manufacturer's instructions.

2.9. Overexpression of *gdf15* in zebrafish embryos and its effect on *A. hydrophila* and SVCV-associated mortality or LPS-induced sepsis

The pcDNA3.1-*gdf15* plasmid and the corresponding control plasmid (pcDNA3.1) were microinjected into one-cell stage zebrafish embryos as mentioned above for the pcDNA3.1-*il6* plasmid. Embryos were microinjected with 112.5 pg/egg of plasmid (final volume of 1.5 nl, diluted in PBS) and maintained in darkness at 28 °C. Three days after plasmid injections (3 dpf), the larvae were placed onto 6-well plates (10 larvae/well; volume 6 ml of water), and the following bath challenges were conducted:

- Infection with *A. hydrophila* (1.4×10^{10} CFU/ml) for 24 h. The corresponding untreated controls were included.
- Infection with SVCV (3.16×10^7 TCID₅₀/ml) for 24 h. The control wells were treated with the same volume of cell culture medium for 24 h.
- Treatment with a lethal dose (50 µg/ml) of LPS from *Pseudomonas aeruginosa* (Sigma–L9143) and the corresponding untreated controls.

For survival analysis, four wells per condition (pcDNA3.1 ± stimuli, pcDNA3.1-*gdf15* ± stimuli) were analysed, mortality was assessed during the next 6 days for the infections and 3 days for LPS treatment, and Kaplan-Meier survival curves were constructed. These experiments were replicated twice.

To study pathogen proliferation by qPCR, larvae were sampled at 24 h postinfection (5 biological replicates composed of 5-7 larvae each). For the detection of *A. hydrophila*, a pair of primers against the 16S ribosomal RNA gene was used. SVCV replication was analysed by amplification of the SVCV nucleoprotein (N) gene. Samples were also taken for qPCR analysis of different genes related to inflammation and metabolism at 2 h posttreatment with LPS and the corresponding controls, constituting 4 biological replicates (5-7 larvae/replicate). Primers are listed in **Supplementary Table S2**.

To determine whether *gdf15* overexpression influences the larval development, the embryos microinjected at one-cell stage with pcDNA3.1 or pcDNA3.1-*gdf15* were photographed at 3 and 6 dpf using a Multi-Zoom AZ100 microscope equipped with a DS-Ri1 digital camera (Nikon, Melville, NY). The total length was measured using the NIS-Elements software (Nikon).

2.10. RNA isolation, cDNA synthesis and quantitative PCR (qPCR) analyses

RNA for all the samples was isolated using a Maxwell 16 LEV simplyRNA Tissue kit (Promega) with an automated Maxwell 16 Instrument in accordance with the instructions provided by the manufacturer. The quantity of RNA was measured in a NanoDrop ND-1000 (NanoDrop Technologies), and depending on the experiment, different quantities of RNA were retrotranscribed (0.025-0.5 µg RNA). cDNA synthesis of the samples was performed with an NZY First-Strand cDNA Synthesis kit (NZYTech) following the manufacturer's instructions.

Specific qPCR primers were designed using Primer 3 software (Rozen and Skaletsky, 2000), and their amplification efficiency was calculated with the threshold cycle (CT) slope method (Pfaffl, 2001). Primer sequences are listed in **Supplementary Table S2**. Individual qPCR reactions were carried out in a 25- μ l reaction volume that contained 12.5 μ l of SYBR GREEN PCR Master Mix (Applied Biosystems), 10.5 μ l of ultrapure water, 0.5 μ l of each specific primer (10 μ M) and 1 μ l of cDNA template (two-fold diluted or not depending on the experiment); reactions were performed in MicroAmp optical 96-well reaction plates (Applied Biosystems) using technical triplicates in a 7300 Real-Time PCR System thermocycler (Applied Biosystems). qPCR conditions consisted of an initial denaturation step (95°C, 10 min), which was followed by 40 cycles of a denaturation step (95°C, 15 s) and one hybridization-elongation step (60°C, 1 min). The relative expression levels of the different genes were normalized following the Pfaffl method (Pfaffl, 2001); *18S ribosomal RNA (18S)* was used as a reference gene. Fold-change units were calculated by dividing the normalized expression values for the different treatments by the normalized expression values of the corresponding controls.

2.11. Metabolite measurements

For the metabolite measurement experiments, the same approach was used. The pcDNA3.1-*gdf15* plasmid and the corresponding control plasmid (pcDNA3.1) were microinjected into one-cell stage zebrafish embryos, and three days after plasmid injections, zebrafish larvae were sampled (8-9 biological replicates composed of 5-7 larvae each). Samples used to assess larvae metabolite levels were homogenized immediately by mechanical disruption using a potter linked to a motor pestle in 7.5 vol. of ice-cold 6% perchloric acid and neutralized (using 1 M potassium bicarbonate). The homogenate was centrifuged (16,000 xg) for 4 min, and the supernatant was used to assay tissue metabolites. Triglyceride, cholesterol, glucose and lactate levels were determined enzymatically using commercial kits (Spinreact, Barcelona, Spain) adapted to a microplate format.

2.12. Statistical analyses

Survival data were analysed with Kaplan–Meier survival curves, and statistically significant differences were determined with a log-rank (Mantel-Cox) test. Expression results were represented graphically as the means $\pm$ standard error (SE) of the biological

replicates. To determine significant differences, data were analysed with Student's t-test or one-way ANOVA with Tukey's post hoc test. Significant differences are defined as *** ($0.0001 < p < 0.001$), ** ($0.001 < p < 0.01$) or * ($0.01 < p < 0.05$).

3. Results

3.1. Characterization of the zebrafish *gdf15* and the encoded protein

The zebrafish *gdf15* gene was located on chromosome 2, and it partially conserved synteny with mammalian Gdf15 genes (**Figure 1A**). The predicted ORF was cloned and sequenced to confirm the sequence and submitted to GenBank under accession number MN857471. It was constituted by 1,050 bp encoding a 350 aa pre-pro-Gdf15 (39.6 kDa), which showed a signal peptide of 18 aa, the canonical RXXR furin-like cleavage site, the transforming growth factor-beta (TGF- β)-like domain (aa 237-350) and the characteristic TGF- β family signature (aa 269-284) (**Figure 1B**). After enzymatic processing, a mature protein of 113 aa should be produced with a molecular weight of 12.7 kDa. The predicted cleavage site RXXR is well conserved across vertebrates (**Supplementary Figure S1**), and the mature protein shows 9 well-conserved Cys positions (**Supplementary Figure S1**); however, zebrafish Gdf15 has only 7 conserved Cys residues on its mature protein and common horse *Equus caballus* 6 Cys. Nevertheless, the 3-D structure of the mature zebrafish Gdf15 was highly similar to that observed for other members of the TGF- β family (**Supplementary Figure S2**), and a growth factor receptor-like binding site was predicted for this protein (**Supplementary Figure S2**).

Phylogenetic analysis of the pre-pro-Gdf15 proteins from different vertebrate species revealed three main clusters, with one containing the mammalian species and another containing the other vertebrates (**Figure 1C**). This second cluster also showed differentiation into two main branches, that is, one for the teleost species and another for birds, reptiles and amphibians (**Figure 1C**). An identity/similarity matrix was constructed with the mature predicted protein (**Supplementary Table S3**). The lowest values of identity for zebrafish Gdf15 were obtained with the mammalian sequences (ranging from 33.6 to 42.2%), whereas for other fish, the values were higher than 68%.

3.2. Constitutive expression of *gdf15*

378 Zebrafish *gdf15* gene expression was observed in all the tested tissues from
379 healthy adult zebrafish, but as occurs in mammals, higher expression was detected in
380 the liver and kidney (**Figure 2A**). When different immune kidney cell populations were
381 identified and sorted (myeloid, lymphoid and precursor lineages) and the expression of
382 *gdf15* was analysed, we found that the myeloid and precursor populations showed
383 similar expression, but the lymphoid lineage showed very low expression of *gdf15*
384 (**Figure 2B**). Moreover, through ontogeny development, *gdf15* progressively tended to
385 increase its general expression in the whole organism (**Figure 2C**).

386 **3.3. Effect of recombinant Il4 and Ifng on the expression of *gdf15* in kidney cells**

387 We analysed the expression of *gdf15* in a primary culture of kidney cells after
388 stimulation with recombinant Il4 or Ifng in combination or not with LPS. Zebrafish Il4
389 significantly increased the expression of *gdf15*, but this induction was not observed
390 when Il4 was combined with LPS (**Figure 3**). On the other hand, Ifng ± LPS or LPS
391 alone did not affect the transcription of *gdf15* after 9 h of *in vitro* stimulation (**Figure**
392 **3**).

393 **3.4. Expression of *gdf15* increases with pro-inflammatory stimuli**

394 When adult zebrafish were i.p. injected with SVCV, LPS or Poly I:C, and the
395 expression of *gdf15* was analysed in kidney samples at 3, 6 and 24 h postchallenge, we
396 observed that all stimuli significantly increased the expression of *gdf15* (**Figure 4A**).
397 The higher expression level was obtained with SVCV after 24 h, which is probably
398 related to a time-increasing viral titre. The stimulation of zebrafish larvae with LPS by
399 bath also significantly induced the expression of *gdf15* (**Figure 4B**), showing that
400 animals without fully developed immunity are also able to increase the expression of
401 this gene after an inflammatory stimulus. Because it has also been shown that Gdf15
402 expression increases after tissue injury in mammals (Emmerson et al., 2018), we also
403 amputated the tail fin of 7 dpf larvae and analysed the expression of *gdf15* (**Figure 4C**).
404 In this case, we did not observe significant modulation of the transcription level of this
405 gene. However, the overexpression of the cytokine *il6* significantly increased the
406 mRNA level of *gdf15* (**Figure 4D**).

407 **3.5. Nutritional alterations influence the expression of *gdf15***

Adult zebrafish subjected to a 72 h fasting period showed inhibition of the *gdf15* expression in liver, kidney and intestine, whereas it was not affected in brain (**Figure 5A**). On the other hand, when zebrafish larvae were stimulated for 24 h with three different NEFAs, we observed a higher expression of *gdf15* compared to the control larvae (**Figure 5B**). The higher gene overexpression was observed for ARA, followed by OA and PA.

3.6. Gdf15 protects zebrafish larvae against LPS-induced sepsis and infectious diseases

The correct replication of the plasmid pcDNA3.1-*gdf15* was confirmed by qPCR in zebrafish larvae (3 dpf) inoculated at the one-cell stage with the expression plasmid (**Supplementary Figure S3**). The overexpression of *gdf15* did not seem to influence the larval development, as no significant differences in total length were observed between the embryos microinjected with pcDNA3.1 or pcDNA3.1-*gdf15* at 3 and 6 dpf (**Supplementary Figure S4**).

At 3 dpf, larvae previously inoculated with the control empty plasmid (pcDNA3.1) or the expression plasmid (pcDNA3.1-*gdf15*) were exposed to an *A. hydrophila* and SVCV bath challenge. Gdf15 induced enormous protection against the bacteria *A. hydrophila*, increasing survival from 17.5 to 92.9% (**Figure 6A**). The overexpression of *gdf15* also induced statistically significant protection against SVCV, increasing survival by 20% (**Figure 6B**). To determine whether Gdf15 could mediate antibacterial or antiviral mechanisms, we also analysed the bacterial content and viral titre at 24 hpi in larvae overexpressing *gdf15* or not. No significant differences were observed in *A. hydrophila* (**Figure 6C**) or SVCV qPCR detection (**Figure 6D**). Because zebrafish Gdf15 seemed to protect against inflammatory damage, we also exposed larvae to a highly lethal dose of LPS. Whereas those animals inoculated with the empty plasmid showed a survival rate of 3.3%, this value increased to 46.7% in those larvae overexpressing *gdf15* (**Figure 6D**).

3.7. Gene modulations induced by *gdf15* overexpression and LPS treatment

Based on the protective role of Gdf15 against sepsis-associated mortalities, we wanted to analyse different processes potentially involved in the induction of tolerance. For that, we analysed by qPCR genes belonging to the following main groups: pro-inflammatory cytokines, hormone genes involved in metabolism,

glycolysis/glycogenolysis, fatty acid synthesis, cholesterol metabolism, lipolysis and lipid transport (**Table 1**).

Zebrafish larvae overexpressing *gdf15* did not show significant differences in the expression of *il1b* and *il6* compared to larvae previously inoculated with the control plasmid, but LPS challenge did not affect the expression of these genes (**Figure 7A**). On the other hand, at 2 h post-stimulation with LPS, a slight but significant increase in the transcription of *tnfa* was observed, but the overexpression of *gdf15* was able to reduce the mRNA level of this pro-inflammatory cytokine (**Figure 7A**). With regard to the expression of metabolic hormones, *ghrl* and *insa* genes were inhibited by LPS stimulation, but *insb* was only downregulated by LPS in larvae overexpressing *gdf15* (**Figure 7B**). Hypersecretion of Gdf15 significantly inhibited the expression of two key genes in glycolysis, *pfkma* and *pkmb* (**Figure 7C**). The synthesis of triglycerides and cholesterol was also affected by LPS and *gdf15* overexpression; in general terms, both LPS and Gdf15 inhibited the expression of *fasn*, *acc*, *srebf2* and *hmgcr* (**Figure 8A, B**). Interestingly, *srebf1* was only inhibited by Gdf15 independent of LPS stimulation (**Figure 8A**). Genes involved in lipolysis were also affected by both stimuli; LPS significantly inhibited the expression of *lipeb*, but *mgll* was expressed at lower levels in larvae overexpressing *gdf15* (**Figure 8C**). However, the gene most inhibited by Gdf15 was the fatty acid cell importer *cd36* (**Figure 8D**), whereas the genes encoding apolipoproteins involved in triglyceride export from the liver were not significantly modulated (**Figure 8D**).

3.8. Effect of Gdf15 on the content of metabolites

The measurement of triglycerides, cholesterol, glucose and lactate in the whole larvae revealed that the overexpression of *gdf15* did not affect the total amount of triglycerides (**Figure 9A**), but a higher concentration of cholesterol was observed (**Figure 9B**). On the other hand, those larvae inoculated with pcDNA3.1-*gdf15* showed a lower concentration of glucose (**Figure 9C**), whereas lactate was not affected (**Figure 9D**).

4. Discussion

GDF15 has been widely associated with a variety of disease states, including cancer, diabetes, cardiovascular complications, liver injury, renal failure, chronic inflammatory diseases, obesity, and ageing (Breit et al., 2011). However, knowledge about the role of this molecule during disease-associated inflammation remained obscure until the simultaneous identification by three independent research groups of its specific receptor, GFRAL, which is exclusively expressed in neurons located in the hindbrain (Hsu et al., 2017; Mullican et al., 2017; Yang et al., 2017). For that reason, all those investigations conducted with recombinant GDF15 *in vitro* should now be carefully reconsidered, especially because it was shown that batches of recombinant GDF15 were contaminated with TGF- β (Olsen et al., 2017). Therefore, it seems clear that GDF15 exerts its functions via the central nervous system (CNS). In zebrafish, the potential Gdf15 receptor Gfral has been also identified in the zebrafish genome (chromosome 13; ENSDARG00000089709). However, the interaction of both molecules needs to be further studied. Interestingly, a recent work conducted in goldfish (*Carassius auratus*) revealed that *gfral* was expressed in different tissues, reaching its higher mRNA level in gallbladder (Blanco et al., 2020). This indicates a certain differentiation in the expression pattern of the GDF15 receptor between fish and mammals, which could suggest additional functions for Gdf15 in fish.

Due to the recent description of murine GDF15 as a potential sepsis tolerance mediator (Luan et al., 2019), we wanted to identify this molecule in the model species zebrafish, which is widely used in biomedical research, and to investigate its similarities with its mammalian counterpart. As occurs in other vertebrates, only one copy of *gdf15* seems to exist in zebrafish, and the gene synteny between zebrafish and mammals is partially well conserved, which is usually indicative of conserved function (Ghiurcuta and Moret, 2014). The zebrafish Gdf15 also conserved different characteristics, such as the presence of a signal peptide, the TGF- β -like domain, and the canonical RXXR cleavage site. Therefore, it is predicted that the zebrafish mature Gdf15 is a 12.7-kDa protein, which is a similar molecular weight to that of mammals (Bootcov et al., 1997). Whereas the Gdf15 proteins from other vertebrates possess 9 well-conserved Cys residues, zebrafish has only 7, but this does not seem to affect its 3-D structure. Although it is predicted that GDF15 is active as a dimer (Bootcov et al., 1997), the absence of specific antibodies did not allow us to corroborate this dimeric structure in zebrafish.

The study of the *gdf15* gene expression profiles revealed that the tissue distribution was notably similar to that of mammals, with the kidney and liver being the main Gdf15 producers (Luan et al., 2019; Zhao et al., 2019). Because GDF15 was first identified in macrophages and named macrophage inhibitory cytokine 1 (MIC1) (Bootcov et al., 1997), we also analysed *gdf15* transcription in different immune cell populations isolated from the kidney. As expected, the myeloid population showed high expression of this gene compared to lymphoid cells. GDF15 is secreted by macrophages and other cell types under pro-inflammatory stimuli (Bootcov et al., 1997; Zimmers et al., 2006). We confirmed that the overexpression of the *il6* gene in zebrafish larvae significantly increased the mRNA level of *gdf15*, suggesting a conserved induction mechanism. Moreover, it has been shown that GDF15 is suppressed in M1-like macrophages and has been implicated in polarization towards an M2-like phenotype (Lee et al., 2017; Jung et al., 2018). Because IFN- γ induces macrophage polarization towards an M1 phenotype and IL-4 towards an M2 phenotype (Sica and Mantovani, 2012; Piccolo et al., 2017), which seems to be conserved in teleosts (Wiegertjes et al., 2016; Pereiro et al., 2019), we stimulated zebrafish kidney cells *in vitro* with recombinant zebrafish Ifng and Il4 in combination or not with LPS. Significant modulations were only observed for Il4 administered alone, indicating that Gdf15 is probably also an M2 marker in fish and that LPS (known to induce macrophage polarization towards the M1 phenotype) counteracts this effect. Contrary to that observed for kidney cells, *in vivo* LPS stimulation of adult zebrafish and larvae significantly increased the transcription of *gdf15* in kidney and whole larvae, respectively, indicating that the induction of a pro-inflammatory state increases the synthesis of this cytokine. According to that observed for LPS i.p. injection in adults, stimulation with Poly I:C or infection with SVCV also increased the expression of *gdf15* in the kidney. Moreover, when we overexpressed *gdf15* in zebrafish embryos, we found that higher levels of this cytokine were able to protect larvae from LPS septic shock, significantly increasing their survival. Protection was also achieved for the larvae infected with the bacterium *A. hydrophila* and, to a lesser extent with the virus SVCV without affecting the bacterial and viral titres. These results are in agreement with those obtained by Luan et al. (2019) in mice, suggesting that zebrafish Gdf15 is also a sepsis tolerance mediator.

As in mammals, zebrafish Gdf15 seems to be involved in metabolism. Although in general terms mammalian GDF15 was not found to be regulated by fasting (Patel et al., 2019), goldfish *gdf15* was upregulated in foregut but inhibited in liver after 3 and 7 days of food deprivation, whereas it was not affected in brain (Blanco et al., 2020). Our results revealed inhibition of *gdf15* in liver, kidney and intestine but, as it was observed in goldfish (Blanco et al., 2020), no modulations were found in brain. On the other hand, when zebrafish larvae were exposed to NEFAs, an increase in the transcription of *gdf15* was observed. Fatty acid supplementation also raised the levels of GDF15 both in mammals (Lee et al., 2006; Patel et al., 2019) and goldfish (Blanco et al., 2020).

To test whether the tolerance induced by Gdf15 in zebrafish is also based on metabolic alterations, as occurs in mammals, we analysed the regulation of different pro-inflammatory and metabolic genes in zebrafish larvae (3 dpf). Although Gdf15 was able to slightly reduce the expression level of the pro-inflammatory cytokine *tnfa*, this does not appear to be sufficient to induce such levels of protection. It is known that mammalian GDF15 can be induced by TNF- α (Bootcov et al., 1997) and that GDF15 knockout mice produce higher levels of TNF- α (Abulizi et al., 2017; Chung et al., 2017a); therefore, negative feedback from GDF15 to TNF- α could be established to control inflammation. However, Luan et al. (2019) did not find differences in plasma TNF- α when mice were treated with a GDF15-neutralizing antibody, suggesting that inflammation reduction is not involved in GDF15-induced tolerance. On the other hand, numerous genes encoding key metabolic proteins were modulated by both LPS and/or *gdf15* overexpression. LPS inhibited the expression of *ghrl* and *insa*. It has been previously reported in mammals that LPS can reduce the plasma levels of ghrelin (Basa et al, 2003; Wang et al., 2006; Stengel et al., 2010), and interestingly, ghrelin has been proposed as a promising anti-inflammatory therapeutic agent during sepsis in rats (Chang et al., 2003; Wu et al., 2007; Zhou et al., 2017). The same occurs with insulin administration; because hyperglycaemia is a common feature of sepsis and is related to the severity of the illness, insulin therapy has been shown to ameliorate sepsis outcome (Andersen et al., 2004; Van Cromphaut et al., 2008). Zebrafish possess two insulin genes (*insa* and *insb*), and while *insa* expression increases with development, *insb* mRNA levels decrease during embryonic development, and its expression is mainly restricted to proliferating blastomeres and, at later stages, to the head and pancreas, suggesting that *insb* could act as a pro-growth neurotrophic factor during development

(Papasani et al., 2006). Interestingly, *insb* larval expression was only affected by costimulation with Gdf15 and LPS, suggesting that this hormone could be pivotal in the sepsis tolerance induced by Gdf15. However, further research is needed to elucidate this question.

Although we cannot discern between serum and cellular metabolites in this work, the total content of glucose in the whole larvae was lower in those individuals overexpressing *gdf15*, which could be contributing to the higher survival of these larvae. It has been previously shown in a murine model that glucose supplementation has a detrimental effect in bacterial sepsis, although it was protective against influenza infection, and in both cases, the effects mediated by glucose were independent of pathogen load and magnitude of inflammation but were linked to cellular adaptation to different inflammatory states (Wang et al., 2016). Therefore, the lower glucose content of the larvae overexpressing *gdf15* could also favour tolerance to sepsis. It has been reported that GDF15 improved glucose tolerance in mice (Macia et al., 2012); therefore, glucose utilization by cells could reduce the total glucose content. However, contrary to expectations, some pivotal genes involved in glycolysis were inhibited by Gdf15.

It has been previously shown that GDF15 favours fatty acid β -oxidation (FAO) (Chung et al., 2017b; Zhang et al., 2018). Unexpectedly, the lipolysis-related genes analysed in this work tended to be inhibited by Gdf15 but also by LPS challenge. This inhibition of lipolysis and FAO after LPS stimulation has been suggested to be a protective strategy of the host for increasing the level of fatty acids and triglycerides available for host defence and tissue repair (Feingold et al., 2008). Indeed, the gene most inhibited by Gdf15 in zebrafish larvae was *cd36*, which is a cell surface protein involved in the import of fatty acids into the cells. Therefore, lower *cd36* expression could be indicative of lower FAO. Indeed, CD36-deficient mice showed improved protection from antibiotic-treated CLP-induced sepsis (Leelahavanichkul et al., 2012). However, when key genes involved in the synthesis of fatty acids and cholesterol were analysed, we also found that both LPS and Gdf15 reduced their expression. This reduction could also be a tolerance strategy, since lipogenesis inhibition has been demonstrated to reduce inflammation and organ injury during CLP-induced sepsis in mice (Idrovo et al., 2016). This gene inhibition mediated by Gdf15 was especially remarkable for the lipogenic transcription factor *Srebf1*. The deficiency of this protein in mice increased the resistance to an endotoxic shock induced by CLP (Im et al., 2011).

However, although the genes involved in lipid synthesis were downregulated, the total cholesterol content was higher in the larvae overproducing Gdf15. Serum cholesterol content has been inversely related to sepsis severity outcome, indicating that high cholesterol levels favour survival (Abraham et al., 2005; Shor et al., 2007; Cirstea et al., 2017; Trinder et al., 2019). Moreover, cholesterol metabolism has been linked to LPS-induced sepsis and tolerance in zebrafish larvae (Dios et al., 2014). GDF15^{-/-} mice exhibited a 19% lower cholesterol concentration in plasma compared to WT mice (Bonaterra et al., 2012), indicating the potential involvement of this cytokine in cholesterol metabolism.

5. Conclusions

In this work, for the first time, we identified and described a zebrafish Gdf15 molecule. We found that the expression pattern of Gdf15 is relatively well conserved compared to mammals, being induced by pro-inflammatory stimuli. Moreover, the use of an expression plasmid encoding Gdf15 allowed us to suggest that this molecule is also involved in sepsis tolerance in teleost. Nevertheless, additional functional studies are necessary to fully confirm this. As in mammals (Luan et al., 2019), the potential mechanisms induced by Gdf15 to mediate tolerance are probably related to metabolic regulation. In this case, the use of a whole organism prevented us from separately analysing the metabolic modulations and/or metabolite concentrations in different body compartments, but glucose reduction and cholesterol abundance are probably involved in Gdf15-induced tolerance in zebrafish. However, the use of whole organisms is one of the main advantages of zebrafish due to the transparency of the larvae, and therefore, this approach is an attractive model to monitor different diseases, treatments and biological processes. To this end, the identification of a Gdf15 molecule in zebrafish could facilitate further investigations in the biomedical field and provide a highly useful tool for sepsis research. Although more specific analyses could elucidate the mode of action of Gdf15 in zebrafish, all these results seem to suggest the conserved function of GDF15 as a metabolism regulator and its promising therapeutic applications in treating sepsis.

Declaration of competing interest

The authors have declared that no competing interests exist.

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947 Tables

948 **Table 1. Pro-inflammatory and metabolic genes analysed by qPCR**

	Gene name	Gene symbol
Pro-inflammatory cytokines	<i>interleukin 1 beta</i>	<i>il1b</i>
	<i>tumor necrosis factor alpha</i>	<i>tnfa</i>
Metabolic hormones	<i>ghrelin</i>	<i>ghrl</i>
	<i>leptin a</i>	<i>lepa</i>
	<i>insulin a</i>	<i>insa</i>
	<i>insulin b</i>	<i>insb</i>
Glycolysis/glycogenolysis	<i>glucose-6-phosphate isomerase b</i>	<i>gpib</i>
	<i>phosphofructokinase, muscle a</i>	<i>pfkma</i>
	<i>phosphofructokinase, muscle b</i>	<i>pfkmb</i>
	<i>lactate dehydrogenase a4</i>	<i>ldha</i>
	<i>pyruvate kinase M1/2b</i>	<i>pkmb</i>
	<i>glycogen phosphorylase, muscle a</i>	<i>pygma</i>
Fatty acid synthesis	<i>sterol regulatory element binding transcription factor 1</i>	<i>srebf1</i>
	<i>acetyl-CoA carboxylase alpha</i>	<i>acc</i>
	<i>fatty acid synthase</i>	<i>fasn</i>
Cholesterol metabolism	<i>sterol regulatory element binding transcription factor 2</i>	<i>screbf2</i>
	<i>3-hydroxy-3-methylglutaryl-CoA reductase a</i>	<i>hmgcra</i>
	<i>cholesterol-25-hydroxylase b</i>	<i>ch25hb</i>
Lipolysis	<i>lipoprotein lipase</i>	<i>lpl</i>
	<i>lipase, hormone-sensitive</i>	<i>lipeb</i>

	<i>monoglyceride lipase</i>	<i>mgll</i>
Lipid transport	<i>cluster of differentiation 36</i> <i>apolipoprotein ea</i> <i>apolipoprotein c1</i>	<i>cd36</i> <i>apoea</i> <i>apoc1</i>

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953 **Figure Legends**

954 **Figure 1. Zebrafish growth differentiation factor-15 features.** A) Synteny
955 conservation of the zebrafish *gdf15* gene compared to mammals. B) Nucleotide and
956 amino acid sequences of zebrafish *gdf15*. The predicted signal peptide is highlighted in
957 blue, and the TGF- β -like domain is highlighted in red. The canonical RXXR furin-like
958 cleavage site is boxed with a continuous line, and the TGF- β family signature is boxed
959 with a dashed line. C) Phylogenetic analysis of vertebrate Gdf15 amino acid sequences.

960 **Figure 2. Constitutive expression of zebrafish *gdf15*.** Gene expression was analysed
961 in A) different tissues from healthy adult zebrafish, B) sorted kidney cell populations,
962 C) and during ontogeny development.

963 **Figure 3. Expression of *gdf15* in zebrafish kidney cells after *in vitro* stimulation**
964 **with the recombinant cytokines Il4 and Ifng alone or in combination with LPS.**
965 Only Il4 alone was able to significantly induce the expression of *gdf15* in kidney cells.
966 The expression level of *gdf15* was normalized to the expression of the *18S ribosomal*
967 *RNA* gene and expressed as the fold change with respect to the levels detected in the
968 control group (unstimulated cells). The graph represents the mean + SE of 4
969 independent biological replicates. Significant differences are displayed as *** (0.0001 <
970 $p < 0.001$), ** (0.001 < $p < 0.01$) or * (0.01 < $p < 0.05$).

971 **Figure 4. Modulation of zebrafish *gdf15* expression under different pro-**
972 **inflammatory stimuli.** A) Expression of *gdf15* in the kidneys of adult zebrafish i.p.
973 injected with SVCV, LPS or Poly I:C. B) *gdf15* gene modulation in zebrafish larvae
974 stimulated by bath with a sublethal dose of LPS. C) Expression of *gdf15* in whole larvae
975 after tail fin injury. D) Modulation of *gdf15* transcription in zebrafish larvae

overexpressing *il6*. In all cases, the expression of *gdf15* was normalized to the expression of the *18S ribosomal RNA* gene and expressed as the fold change with respect to the levels detected in the corresponding control group. The graphs represent the means + SE of the biological replicates. Statistically significant differences are displayed as *** ($0.0001 < p < 0.001$), ** ($0.001 < p < 0.01$) or * ($0.01 < p < 0.05$).

Figure 5. Nutritional alterations modulate the expression of *gdf15*. A) Expression of *gdf15* in liver, kidney, intestine and brain from adult zebrafish under fasting conditions compared to those receiving a normal diet. B) Expression of *gdf15* in zebrafish larvae exposed by bath to three different NEFAs. The expression of *gdf15* was normalized to the expression of the *18S ribosomal RNA* gene and expressed as the fold change with respect to the levels detected in the control groups. The graphs represent the means $\pm$ SE of 5 independent biological replicates. Significant differences are displayed as *** ($0.0001 < p < 0.001$), ** ($0.001 < p < 0.01$) or * ($0.01 < p < 0.05$).

Figure 6. Survival of zebrafish larvae overexpressing *gdf15* after different immune challenges. Kaplan-Meier survival curves of zebrafish larvae infected by bath with A) *A. hydrophila* or B) SVCV. The pathogen load was analysed by qPCR at 24 h postinfection with *A. hydrophila* or SVCV C) by using *A. hydrophila*-specific primers or D) by analysing the expression of the SVCV nucleoprotein (N) gene. The expression levels of *A. hydrophila* 16S and N-SVCV were normalized to the expression of the *18S ribosomal RNA* gene. The graphs represent the means $\pm$ SE of 5 independent biological replicates. E) Kaplan-Meier survival curves of zebrafish larvae stimulated by bath with a highly lethal dose of LPS. In all graphs, significant differences are displayed as *** ($0.0001 < p < 0.001$), ** ($0.001 < p < 0.01$) or * ($0.01 < p < 0.05$).

Figure 7. Expression of different immune and metabolic genes in zebrafish larvae inoculated with pcDNA3.1-empty or pcDNA3.1-*gdf15* in the absence or presence of LPS bath stimulation for 2 h. A) Expression of pro-inflammatory cytokines. B) Expression of metabolic hormones. C) Expression of genes involved in glycolysis. In all cases, the expression of the different genes was normalized to the expression of the *18S ribosomal RNA* gene and expressed as the fold change with respect to the levels detected in the control group (embryos injected with pcDNA3.1-empty and unstimulated). The graphs represent the means + SE of 4 biological replicates. Statistically significant differences are displayed as *** ($0.0001 < p < 0.001$), ** ($0.001 < p < 0.01$) or * ($0.01 < p < 0.05$).

Figure 8. Expression of different metabolic genes in zebrafish larvae inoculated with pcDNA3.1-empty or pcDNA3.1-*gdf15* in the absence or presence of LPS bath stimulation for 2 h. A) Expression of key genes involved in the synthesis of fatty acids and B) cholesterol metabolism. C) Modulation of genes involved in lipolysis. D) Expression of genes involved in the transport of lipids. In all cases, the expression of the different genes was normalized to the expression of the *18S ribosomal RNA* gene and expressed as the fold change with respect to the levels detected in the control group (embryos injected with pcDNA3.1-empty and unstimulated). The graphs represent the means + SE of 4 biological replicates. Statistically significant differences are displayed as *** (0.0001 < p < 0.001), ** (0.001 < p < 0.01) or * (0.01 < p < 0.05).

Figure 9. Quantification of different metabolites in zebrafish larvae overexpressing or not overexpressing *gdf15*: A) triglycerides, B) cholesterol, C) glucose and D) lactate. The graphs represent the means ± SE of 8-9 pools of larvae. Statistically significant differences are displayed as *** (0.0001 < p < 0.001), ** (0.001 < p < 0.01) or * (0.01 < p < 0.05).

Supplementary material

Supplementary Table S1. GenBank accession number of the GDF15 amino acid sequences used in this work.

Supplementary Table S2. Primer pairs used in this work.

Supplementary Table S3. Identity/similarity matrix showing the identity (upper right) and similarity (lower left) of the GDF15 mature protein in different vertebrates.

Supplementary Figure S1. Alignment of pre-pro-GDF15 proteins from different vertebrate species. The canonical RXXR furin-like cleavage site is highlighted in yellow. Although the region prior to the mature protein is not highly conserved among vertebrates, the mature proteins show a high degree of identity. Indeed, the Cys residues of the functional proteins are well conserved across vertebrates.

Supplementary Figure S2. Predicted three-dimensional structure of zebrafish Gdf15 mature protein. A) Comparison of the predicted structure of zebrafish Gdf15 (colour cartoon view) with human TGF-β2 (purple ribbon diagram). TM-scores

observed after I-TASSER submission revealed that human TGF- β 2 is the preferred crystal structure analogue for *D. rerio* Gdf15 (TM-score 0.869). **B)** Deduced binding site of zebrafish Gdf15 to a growth factor receptor-like.

Supplementary Figure S3. Detection of *gdf15* expression in zebrafish larvae (3 dpf) microinjected with pcDNA3.1 or pcDNA3.1-*gdf15* in the one-cell embryo stage. The data are represented as the mean of 4 independent biological replicates + SE. Significant differences are displayed as *** ($0.0001 < p < 0.001$), ** ($0.001 < p < 0.01$) or * ($0.01 < p < 0.05$).

Supplementary Figure S4. Effect of *gdf15* overexpression on larval development. Zebrafish embryos were microinjected with pcDNA3.1 or pcDNA3.1-*gdf15*. The total length of the larvae was measured at 3 and 6 dpf. The data are represented as the mean of 25-30 larvae + SE. No significant differences were observed between both groups. Scale bar: 1 mm.

Figure 1

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