Immunohistochemical expression of E–cadherin in different tissues of the teleost fish *Scophthalmus maximus*

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Abstract

E–cadherin is an evolutionary conserved protein, whose main role as the principal component of adherens junctions is supporting epithelial cell–cell adhesion. It is an essential molecule for the maintenance of the epithelial barrier function and the analysis of its immunohistochemical expression is a valuable resource in morphopathological, ontogenetic and pathogenesis studies in mammals. As well, there is an increasing understanding of the importance of E-cadherin in the physiology of the immune system

and the development of the immune response. Mucosal health is a primary issue in aquaculture research; nevertheless, there is a lack of immunohistochemical studies of cell junction proteins in fish species. In this work, an immunohistochemical technique was optimized in Bouin- and formalin-fixed paraffin-embedded tissues of turbot *Scophthalmus maximus*, employing a commercial antibody raised against human E-cadherin. The specificity of the antibody in recognizing the molecule in this teleost species was tested by western blot and mass spectrometry-based proteomic analyses. The assays showed a good specificity and indicated that the antibody recognizes the well conserved cytoplasmic domain of the protein. Immunohistochemistry showed the localisation of E-cadherin at cell-cell contact in the epithelia of the different organs, between the hepatocytes and the pancreatic acinar cells, as well as in the reticulo-epithelial stroma of the thymus. Also, the immunoreaction was observed in the cells constituting the melano-macrophage centres in the spleen and kidney. No immunostaining was detected, as expected, only in the heart and brain. No significant difference was noticed between the two fixative used for collecting the tissues samples. This is the first description of E-cadherin immunohistochemical expression in several tissues of a teleost. The immunohistochemical technique represents a useful tool to be used in the different areas of fish health research.

Keyword: *Immunohistochemistry*; *cadherin-1*; *cell junctions*; *epithelial barriers*; *mucosal health*

1. Introduction

Cell–cell adhesion is a fundamental structural feature of multicellular organisms, mediated by a set of specialized membrane structures termed intercellular junctions. Particularly, the ability of epithelial cells to organize into monolayered sheets is a prerequisite for multicellularity, thereby providing tissue integrity, barrier function, and tissue polarity in metazoan organisms (Bruser and Bogdan, 2017). Over the course of morphological evolution, metazoan animals have diversified the architecture of their cell–cell junctions, which includes tight junctions, adherens junctions (AJ), and desmosomes. Among them, AJs are the only detected throughout the metazoan phyla, whereas other junctional types show restricted phylogenetic distributions (Oda and Takeichi, 2011). Thus, AJs could be considered the universal adhesion machinery for the generation and maintenance of multicellular animal bodies. E-cadherin, a calcium–dependent transmembrane protein, is the structural and functional core of AJs, mediating *trans*–homophilic interactions between neighbouring cells. Its adhesive functions are mediated by the extracellular cadherin repeat domains, while the highly conserved intracellular domains form a complex with catenins linking E–cadherin to the actin cytoskeleton, being so involved in junctional maintenance, dynamics, and plasticity of epithelial tissues (Bruser and Bogdan, 2017; Oda and Takeichi, 2011; van Roy and Berx, 2008).

In teleost, the mucosal surfaces (skin, gills and gastrointestinal tract) constitute the first line of defence against pathogens invasion and carry out multiple physiological processes, such as osmoregulation, waste excretion and nutrient adsorption. For fish, even more than for terrestrial species, mucosal barriers and their health are of primary importance to face the continue interactions with the aquatic microbiota (Peatman and Beck, 2015). Almost all of the most urgent areas of aquaculture research require a deeper understanding of

mucosal barriers, from pathology issues to vaccine delivery, nutrition, nutraceutics and microflora modulation (Peatman et al., 2015). The importance of E–cadherin as diagnostic marker and the main role it plays in human diseases, from several cancer types to different skin and gastrointestinal conditions, have been widely reported (Baniak et al., 2016; Christou et al., 2017; Maretzky et al., 2008; Singhai et al., 2011; Sugihara, 2016; von Zeidler et al., 2014; Wu et al., 2007; Zbar et al., 2004). In fish pathology, E–cadherin has recently been identified as a key molecule for resistance to infectious pancreatic necrosis (IPN) in Atlantic salmon (Moen et al., 2015), and another work on the same species reported modulated intestinal E–cadherin gene expression in response to an experimental dietary treatment affecting intestinal fluid permeability (Hu et al., 2016).

The investigation of E–cadherin immunohistochemical expression is a primary focus in morphological studies, including ontogenetic and morphopathological characterization of the different organs, as well as an irreplaceable complement for studies based on gene expression analysis (Bondow et al., 2012; Fuertes et al., 2013; Gassler et al., 2001; Kuwahara et al., 2001; Sakamoto et al., 2008; Schneider et al., 2010). However, no comprehensive study of E–cadherin immunolocalisation in teleost tissues has been addressed and there is still a scarce knowledge of the physiological distribution of this protein and its change under pathological conditions. In this study, an immunohistochemical technique based on a commercial antibody was optimized in Bouin- and formalin-fixed paraffin–embedded tissues from turbot (*Scophthalmus maximus*), an economically–important marine species, and the distribution of E–cadherin in different tissues of healthy specimens analysed.

2. Materials and methods

2.1. Fish and sampling procedures

For this study, 10 adult turbot (2,127 ± 182.2 g mean weight) were employed. Fish were euthanized by overexposure to tricaine methane sulfonate (MS222, Sigma–Aldrich, Denmark) and necropsied. For histological examination and immunohistochemistry, tissues samples from kidney, spleen, thymus, digestive tract, liver, pancreas, heart, gills, brain and skin were collected. Samples were fixed in Bouin's fluid at 4 ºC or in formalin during 24 hours and then paraffin–embedded. Histological analysis was performed on H&E and toluidine blue–stained sections. For western blot analysis, samples (1 g) of anterior and posterior intestine were collected and extensively washed with ice cold PBS containing 1 mM of phenylmethylsulfonyl fluoride (PMSF) before storage at –80ºC.

All experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Santiago de Compostela (Spain).

2.2. Western blotting and E-cadherin sequences analysis

Western blot analysis was performed to assess the specificity of the primary antibody on protein extracts from anterior and posterior intestine of turbot. Protein extracts from human skin and intestine (ileum) were used as positive control. Tissues were homogenized in ice–cold homogenization buffer (50 mM Tris–HCl, 150 mM NaCl, 5mM EDTA, 1 mM dithiothreitol, 1 mM PMSF, 1 mM benzamidine hydrochloride hydrate, 5 µg/mL pepstatin A, 5 µg/mL aprotinin, 5 µg/mL leupeptin, 1% triton X–100, 0,5 % sodium deoxycholate, 0,1 % sodium dodecyl sulfate pH 7.4). Homogenates were centrifuged at 35,000 *g* for 15 minutes at 4 ºC, and supernatants filtered through gauze and frozen in aliquots at –80º C until required. Protein concentrations were determined by Bradford method according to manufacturer's instruction (Bio–Rad, Hercules, CA, USA).

Samples of protein extracts were mixed with 1/4 vol of 5X SDS sample buffer (250 mM Tris–HCl, 8 % SDS, 40% glycerol, 20% β–mercaptoethanol, pH 6.8) and denatured by heating at 37 ºC for 20 min. Approximately 30 µg of total protein per tissue were resolved by SDS–PAGE using 7,5% polyacrylamide slabs–gel and proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon–P; Millipore, Bedford, MA, USA) by applying a 275 mA current for 3 h at 4 ºC. Sodium dodecyl sulphate (SDS) was added (0,025%) to the transference buffer in order to facilitate the transfer of high molecular weight proteins. After blocking for 2 h and 30 min at room temperature with 5% non–fat dry milk in TTBS (20 mM Tris–HCl buffer, pH 7.5, 0.15 M NaCl, 0.1% Tween 20), membranes were incubated overnight at 4º C with the anti–human E–cadherin primary antibody (mouse monoclonal antibody, clone NCH–38, M3612, Dako, Denmark) diluted 1:500 with TTBS. The blots were then incubated for 1 h at room temperature with anti–mouse secondary antibody conjugated to horseradish peroxidase (Sigma–Aldrich, St Louis, MO, USA) diluted 1:50,000 with TTBS and, finally, developed with the chemiluminiscent HRP substrate (Millipore) and exposed to X–ray film (Curix RP2 Plus; Agfa–Gevaert, Mortsel, Belgium) for a few seconds. Membranes were washed 5 times for 10 min with TTBS between subsequent steps. Negative control was performed by substituting primary antibody with TTBS. In order to estimate the apparent molecular weight of detectable bands, a sample of molecular weight standards (Unstained Broad Range SDS-PAGE Standards, Bio-Rad) was also applied on the gel. The membranes were finally stained with a Ponceau-S solution to mark the positions of the molecular weight standards.

Additionally, for all tested samples SDS–PAGE and gel staining with Coomassie Brilliant Blue R (Sigma–Aldrich) were carried out.

138 The amino acid sequence of turbot E–cadherin was inferred using ExPASy Translate tool
139 (http://web.expasy.org/translate/) from the nucleotide sequence (GenBank accession
140 number: MG137250) identified in a previous study (Robledo et al., 2014), and its
141 theoretical molecular weight estimated by ExPASy Compute pI/Mw tool
142 (http://web.expasy.org/compute_pi/). For comparison purposes, the amino acid sequence
143 alignments were performed by using the BLASTP 2.6.1 online program (Altschul et al.,
144 2005; Altschul et al., 1997).

145 *2.3. In–gel digestion and protein identification by nano–HPLC–ESI–IT–MS/MS*

146 Based on western blot results, the labelled bands of 110 kDa y 38 kDa obtained in turbot
147 were manually excised from SDS–PAGE Coomassie-stained gels. Proteins were in–gel
148 reduced, alkylated and digested with trypsin as previously described (Jensen et al., 1999).
149 Briefly, protein bands were three times washed with water and dehydrated with
150 acetonitrile. Gel plugs were then dehydrated in a vacuum centrifuge and finally
151 rehydrated with a 0.5 µM solution of sequencing grade bovine trypsin (Promega,
152 Madison, WI) in 25 mM ammonium bicarbonate buffer, pH 8.0, for at least 40 min on
153 ice. After the rehydration step, samples were digested overnight at 37 °C and peptides
154 were then cleaned–up by using ZipTip C18 according to manufacturer's instructions. The
155 peptide mixture was dried in vacuum centrifugation, and dissolved again in 15 µL of 1%
156 formic acid prior to mass analysis.

157 Trypsin–digested proteins were analysed using a Dionex UltiMate 3000 RSLCnano
158 system (Thermo Fisher Scientific) coupled to a LTQ Velos–Pro mass spectrometer
159 (Thermo Fisher Scientific). The separation of the peptides was done on an Acclaim
160 PepMap100 Nano Trap Column, C18, 5 µm, 100 Å, 100 µm × 1 cm (Thermo Fisher
161 Scientific) coupled to an RP column Acclaim PepMap RSLC 75 µm × 150 mm, C18, 2

µm, 100 Å (Thermo Fisher Scientific). Mobile phases A and B were respectively 0.1% formic acid in water and in 100% acetonitrile. A 90 min linear gradient from 5% to 35% B, at a flow rate of 300 nL/min, was used. For ionization, a spray voltage of 2.10 kV and a capillary temperature of 200 °C were used. Peptide detection was performed by using survey scans from 350 to 1600 Da (2 µscans), followed by MS/MS scans (2 µscans) of the six more intense peaks using an isolation width of 1 Da and a normalised collision energy of 35%. Singly charged ions were excluded from MS/MS fragmentation and a dynamic exclusion enabled, with repeat count set to 2 and exclusion duration of 30 s. Protein identification was performed using the database searching function of the PEAKS 7 software (Ma et al., 2003; Zhang et al., 2012) (Bioinformatics Solutions Inc, Waterloo, Ontario, Canada), to compare experimental MS/MS spectra against reference MS/MS spectra from the UniProtKB/TrEMBL database (release 2017_04; 84827567 sequence entries), which also included their respective decoy sequences. The following limitations were used for the searches: tryptic cleavage, up to 2 missed cleavage sites, and tolerances ±1.0 Da for precursor ions and ±0.8 Da for MS/MS fragments ions. The FDR was kept below 1% and only proteins with at least one unique peptide were considered. When protein identification failed, the PEAKS algorithm was used to perform *de novo* sequencing for each input spectrum. The same parameters (mass error tolerance and PTMs) specified for database search are also used for *de novo* sequencing. Average Local confidence (ALC) for each *de novo* sequence was set at ≥60%.

2.4. Immunohistochemistry

Thin sections (3 µm thick) were obtained from all the sampled organs. The sections were placed on slides treated with silane to improve section adherence and dried overnight at 37 ºC. After deparaffination with xylene (two 5–min washes) and hydration through a graded ethanol series, slides were incubated with a peroxidase–blocking solution (Dako,

Denmark) for 30 minutes to quench endogenous peroxidase activity. Antigen retrieval was performed using high pH antigen retrieval buffer (Dako, Denmark), following the manufacturer instruction. The optimal working dilution for the monoclonal antibody anti–human E–cadherin was determined to be 1:50 with an incubation time of 2 h at room temperature. Then, slides were incubated during 30 min with horseradish peroxidase (HRP)–labelled polymer conjugated to rabbit secondary antibody and peroxidase reaction was developed using a diaminobenzidine–positive chromogen (EnVision+ System–HRP kit, K4007, Dako). All incubations were performed in a humid chamber at room temperature, and the sections were washed three times for 5 min in 0.1 M phosphate buffered saline containing 0.05% Tween–20 between all subsequent steps. Sections were finally counterstained with haematoxylin, dehydrated and coverslipped with DePeX mounting medium for microscopic observation. Tissue sections from formalin-fixed paraffin-embedded human intestine were used as positive controls. In sections included as negative controls, primary antibody was replaced with either PBS or antibody diluent.

3. Results

3.1. Histological analysis

The histological evaluation of the sampled tissues did not show any significant pathological alteration or presence of pathogenic agents.

3.2. Western blotting

In human tissues, an expected signal corresponding to a molecular weight of approximately 97 kDa was detected in both the skin and intestinal extracts. Furthermore, an additional intensely labelled band of about 38 kDa was observed for the skin. In turbot, the band corresponding to the mature form of E–cadherin reached approximately 110

kDa, identical for the anterior and posterior intestine. This result was consistent with the theoretical molecular weight indicated by ExPASy Compute pI/Mw tool for the protein. As well, additional 38–kDa labelled bands were observed in both intestinal regions (Fig 1).

The alignment performed by BLASTP 2.6.1 showed that turbot and human E–cadherin (Uniprot code P12830) amino acid sequences exhibited more than 53% of identity (Fig 2 A). Furthermore, the alignment of the human 38 kDa C–terminal fragment of E–cadherin, known as CTF1 (Marambaud et al., 2002; Maretzky et al., 2005), with the correspondent last 182 amino acids of turbot protein resulted in more than 75% of identity (Fig 2 B).

3.3. Validation of western blot results by MS–proteomics analysis

Proteomics data supported the presence of E–cadherin in both 110 kDa and 38 kDa bands (Table 1) by finding a unique peptide sequence which matched with the E–cadherin protein sequence of *Fundulus heteroclitus*. That fish sequence, which is currently available in the UniProtKB/TrEMBL database, exhibited more than 45% of identity with the human E–cadherin (Uniprot code P12830), and more than 57% with turbot's, after their sequence alignment. The further alignment of that unique peptide sequence with the E–cadherin protein sequence of *S. maximus* resulted in 100% of identity (Fig 2 B). Moreover, in the 110 kDa band, *de novo* PEAKS data analysis found an additional peptide sequence (NQGLSQDNTVQTK) in the sample which also exhibited 100% of identity with the E–cadherin protein sequence of turbot.

3.4. Immunohistochemistry

The expected negative or positive reactions were observed in the negative or positive controls, respectively. E–cadherin was detected in all the studied tissues of turbot, with the exception of the brain and heart, in both formalin- and Bouin-fixed samples. As

234 expected, the immunostaining was mostly observed in the epithelia, located at the cell–
235 cell junction area with a basolateral position. The epidermis and the epithelium covering
236 the gill *lamellae* showed this kind of immunoreactivity (Fig 3 A, B), as well as the lining
237 epithelium of the digestive tract (Fig 3 C) from the oesophagus to the anus. In the stomach
238 the gastric pits were also labelled, but not the entire epithelium of the gastric glands (Fig
239 3 D). As well, in the kidney the expression of E–cadherin was observed in the epithelia
240 of the tubules, ducts and Bowman's capsule (Fig 4 A). In this organ and in the spleen, the
241 melano–macrophage centres (MMCs) also showed immunoreactivity, which appeared
242 located at the contact area between the cells constituting the centres (Fig 4 B). E–cadherin
243 immunostaining was also noticed in the opercular epithelium that separates the thymus
244 from the gill chamber, as well as drawing a reticular pattern in the thymus parenchyma,
245 with a stronger intensity in the inner part of the organ (Fig 4 C). Finally, E–cadherin was
246 immunolocalised at the intercellular junction between the hepatocytes in the liver and the
247 acinar cells in the pancreas, as well as in the epithelium of the biliar and pancreatic ducts.
248 On contrary, no immunostaining was observed in the endocrine components of the
249 pancreas, the islets of Langerhans (Fig 5).

250 ## 4. Discussion

251 To our knowledge, this is the first work addressing the immunolocalisation of E–cadherin
252 in different tissues of a teleost fish. The commercial antibody employed was thoroughly
253 evaluated to test its specificity in recognizing the protein in turbot by western blot and
254 MS–based proteomic analyses.

255 The results suggest that the monoclonal antibody recognizes the C–terminal region of the
256 protein, corresponding to the cytoplasmic domain (CD). In fact, the CD of E–cadherin
257 remained quite conserved from placozoa to man (Hulpiau and van Roy, 2011) and

cleavage fragments of human E–cadherin CD have been reported. Particularly, the intramembrane protease complex gamma secretase, through the action of a presenilin–1(PS1), is responsible for the cleavage of the 38 kDa fragment known as CTF1. Upon formation of cell–cell contacts, PS1 is recruited to sites of cell–cell adhesion, where it forms complexes with E–cadherin and b–catenin at the cell surface (van Roy and Berx, 2008). There is a lack of information about cleavage sites and fragments of E–cadherin in teleost, however, the observation of 38 kDa labelled bands in turbot tissues indicate the possibility that they might be similar to those described in mammals. As well, a high similarity between the amino acid sequences of the C–terminal region of turbot and human E–cadherin was observed, which would support the cross–reactivity of the antibody employed.

The immunohistochemical expression observed in turbot was full consistent with that described in mammalian tissues, being E–cadherin immunolocalised at cell–cell contacts in the lining epithelia covering the body surfaces exposed to the external environment (skin, gills, gastrointestinal tract), as well as in the epithelia of the biliar and pancreatic ducts and the collecting duct system of the kidney. In this last organ, also the simple squamous epithelium of the Bowman's capsule was labelled, as previously seen in human kidney (Tsuchiya et al., 2006). Membranous labelling of pancreas acinar cells and of hepatocytes was also observed, in accordance with what described in mammals (Lim et al., 2007; Tsuchiya et al., 2006). Besides, immunostaining of hepatocytes was also reported in the cell line RTL–W1, originated from a primary culture of rainbow trout normal liver (Malhao et al., 2013).

The most controversial results were the lack of immunostaining of the gastric glands and islets of Langerhans in the pancreas, where the expression of E–cadherin was described in human by Tsuchiya et al. (2006), who performed an immunohistochemical study on

283 different healthy human tissues. However, the same authors also observed in both cases
284 immunoreactivity to another member of cadherin family, N–cadherin. Particularly, in the
285 stomach, N–cadherin was detected in parietal cells, which are specialized in secreting
286 hydrochloric acid (Tsuchiya et al., 2006). In teleost, on the other hand, only one cell type
287 is observed in gastric glands, secreting both pepsinogen and hydrochloric acid (Grosell et
288 al., 2010). The presence of other cadherins other than E-cadherin in these locations cannot
289 be discarded and the development of molecular markers is needed for a better
290 characterization of the cadherin family in teleost. Concerning the lack of immunostaining
291 for E–cadherin in turbot heart and brain, this is in accordance with that described in
292 human, where N–cadherin was in turn detected in the intercalated discs of cardiac muscle
293 and in the central nervous system (Tsuchiya et al., 2006).

294 E–cadherin was also immunolocalised, as expected, at cell–cell contacts in the epithelium
295 of the gill chamber separating the thymus from the aquatic milieu. In the thymus, the
296 immunostaining corresponded to the stromal tissue, constituted by a framework of
297 reticulo–epithelial cells that support the parenchyma (Vigliano et al., 2011). This staining
298 was more intense in the inner zone of the organ. Zonation of the thymus has been reported
299 in turbot, consisting in an outer part mainly composed by thymocytes and the inner
300 thymus, where mostly lymphoblasts and macrophages are present (Padrós and Crespo,
301 1996). This might resemble the cortical and medullary portions of human thymus, where
302 a strongest staining intensity of E–cadherin was found in the medulla and has been related
303 with mechanisms involved in T cells development and proliferation (Kutlesa et al., 2002).
304 Further research has to be carried out to investigate the functions of fish E–cadherin others
305 than junctional maintenance, but, based on the emerging evidences in mammals (Van den
306 Bossche and Van Ginderachter, 2013), it is plausible a broader implication in the
307 functioning of teleost immune system.

In the same way, immunohistochemical localisation of E–cadherin was observed in the MMCs in kidney and spleen. These are nodular accumulations of closely packed pigment–containing cells, mostly macrophages, which play significant roles in physiological processes, such as destruction, detoxification or recycling of endogenous and exogenous materials, and in the immune response, being proposed as the evolutionary precursors of mammalian germinal centres (Agius and Roberts, 2003; Steinel and Bolnick, 2017). MMCs in spleen and kidney increase in size and/or number in response to infection, vaccination, environmental and physiological changes, as well as they have been seen developing elsewhere in fish body, often in association with chronic inflammation (Agius and Roberts, 2003; Bermúdez et al., 2010). A comprehensive understanding of MMCs functions and of the kinetics of the cells that join, leave or form the centres needs further and systematic investigations (Steinel and Bolnick, 2017), but this is the first evidence of E–cadherin involvement in the interaction between the cells constituting MMCs in a teleost.

In conclusion, the immunohistochemical technique optimized with a commercial antibody showed a good specificity and allowed the study of E–cadherin distribution in several tissues of turbot. The recognition of the CD of the protein, well evolutionary conserved, points toward a successful employment of this technique in other fish species. This is a useful tool for the investigation of mucosal barriers and their physiological and pathological changes, for ontogeny studies, as well as to explore the occurrence and significance of cell–cell adhesion in the functioning of the immune system.

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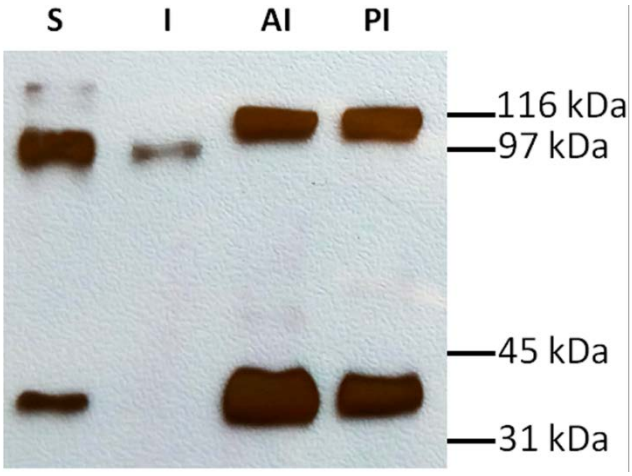
Table 1. NanoHPLC-ESI-IT-MS/MS analysis of the 110 kDa and 38 kDa labeled bands found by western blot with an anti-E-cadherin antibody in *Scophthalmus maximus*.

Band ID	Presence of E-cadherin	Protein description	Uniprot Code	-10lgP	#Peptides	#Unique	#Sequences	SC (%)	MWt (kDa)
110 kDa Band	YES	Cadherin-1 OS= *Fundulus heteroclitus* PE=4 SV=1	A0A146XRX5	47,63	1	1	R.PANPDELGNFIDDNLK.A	2	83.6
38 kDa Band	YES	Cadherin-1 OS= *Fundulus heteroclitus* PE=4 SV=1	A0A146XRX5	37,07	1	1	R.PANPDELGNFIDDNLK.A	2	83.6
110 kDa Band	YES	E–cadherin protein sequence of *S. maximus*	-	-	1	1	D.NQGLSQDNTVQTK.V*	1.3	110

Different parameters supporting the successful protein identification of E-cadherin by MS are indicated: PEAKS protein score (–10lgP), number of matching total and unique peptides (FDR < 1%), peptide sequences, % sequence coverage (SC) and theoretical protein mass (MWt).

*Obtained by *de novo* sequencing using PEAKS algorithm and alignment with E–cadherin protein sequence of *S. maximus* using the BLASTP 2.6.1 (Score: 28.9 bits(63); Expect: 1^{e-06}; Method: Compositional matrix adjust; Identities: 13/13(100%); Gaps: 0/13(0%)).



463

464 **Fig 1. Western blot analysis of E-cadherin expression in protein extracts from**
465 **human and turbot (*Scophthalmus maximus*) tissues.** S = human skin; I = human ileum;
466 AI = turbot anterior intestine; PI = turbot posterior intestine. At the right, the position of
467 the molecular weight standards is indicated.

A

Score	Expect	Method	Identities	Positives	Gaps
713 bits (1841)	0.0	Compositional matrix adjust.	416/791(53%)	524/791(66%)	23/791(2%)

B

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HUMAN   701  VEAGLQIPAILGILGGILALLILILLLLL-FLRRRAVVKEPLLPPEDDTRDNVYYYDEEGG  760
             +  G  +P ILGILGGIL LL+L+LLLLL   RR    KEPLL  +DD RDN+YYYDEEGG
TURBOT  816  IAGGTSLPMILGILGGILLLLMLVLLLLLFARRRGGEKKEPLLQ-DDDIRDNIYYYDEEGG  875

HUMAN   761  GEEDQDFDLSQLHRGLDARPEVTRNDVAPTLMSVPRYLPRPANPDEIGNFIDENLKAADTD  821
             GE+DQD+DLS LHRGLD RP+V RNDV P  M  P+Y PRPANPDEIGNFID+NLKAAD D
TURBOT  876  GEDDQDYDLSVLHRGLDNRPDVFRNDVLPNFMPAPQYRPRPANPDEIGNFIDDNLKAADND  936

HUMAN   822  PTAPPYDSLLVFDYEGSGSEAASLSSLNSSESDKDQDYDYLNEWGNRFKKLADMYGGGEDD  882
             PTAPPYDSLLVFDYEG GS+A SLSSLNSS S  DQDYD L +WG RFKKLADMYGGGEDD
TURBOT  937  PTAPPYDSLLVFDYEGGGSDAGSLSSLNSSSS-GDQDYDCLGDWGPRFKKLADMYGGGEDD  996
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182 AA C-TERMINAL FRAGMENT (CTF1)

Identities	Positives
136/182(75%)	149/182(82%)

468

469 **Fig 2. Comparisons between human and turbot E-cadherin amino acid sequences.**
470 A) Output of BLASTp alignment of human (P12830) and turbot (*Scophthalmus maximus*)
471 E-cadherin. B) Comparison between the amino acid sequences of the C-terminal fragment

known in human as CTF1 and its counterpart in turbot. The amino acids highlighted in red corresponds to the unique peptide sequence found by nano–LC–MS/MS analysis of the excised bands from Western blot in turbot tissues that matched with the E–cadherin sequence of the teleost *Fundulus heteroclitus*. Conservative amino acid changes are shown by a "+" sign between the aligned residues.

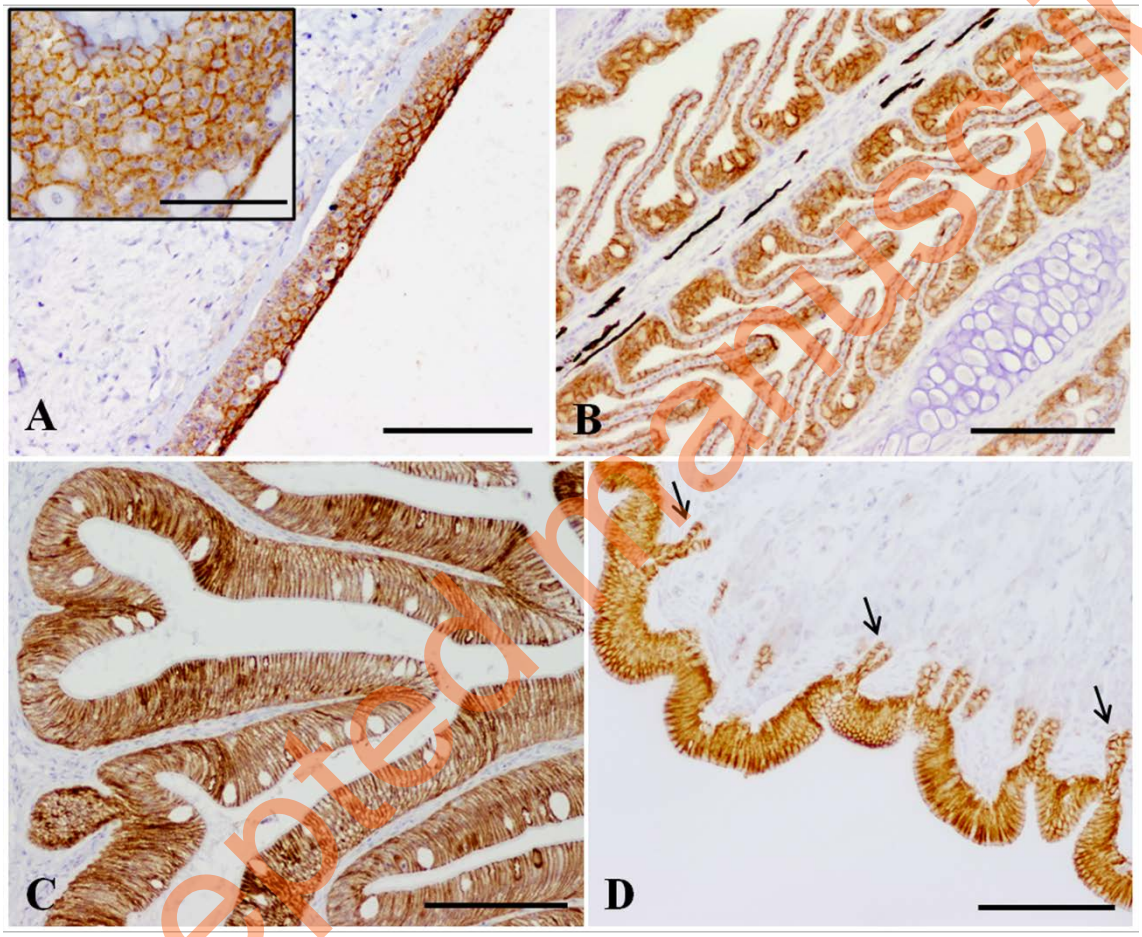

Fig 3. E-cadherin immunolocalisation at cell-cell contacts in the lining epithelia of turbot skin (A), gills (B), intestine (C) and stomach (D). Note the immunoreaction of the gastric pits (3D, arrows), while this is not present in the underlying gastric glands. Bars = 100 µm (Inset 3A= 50 µm).

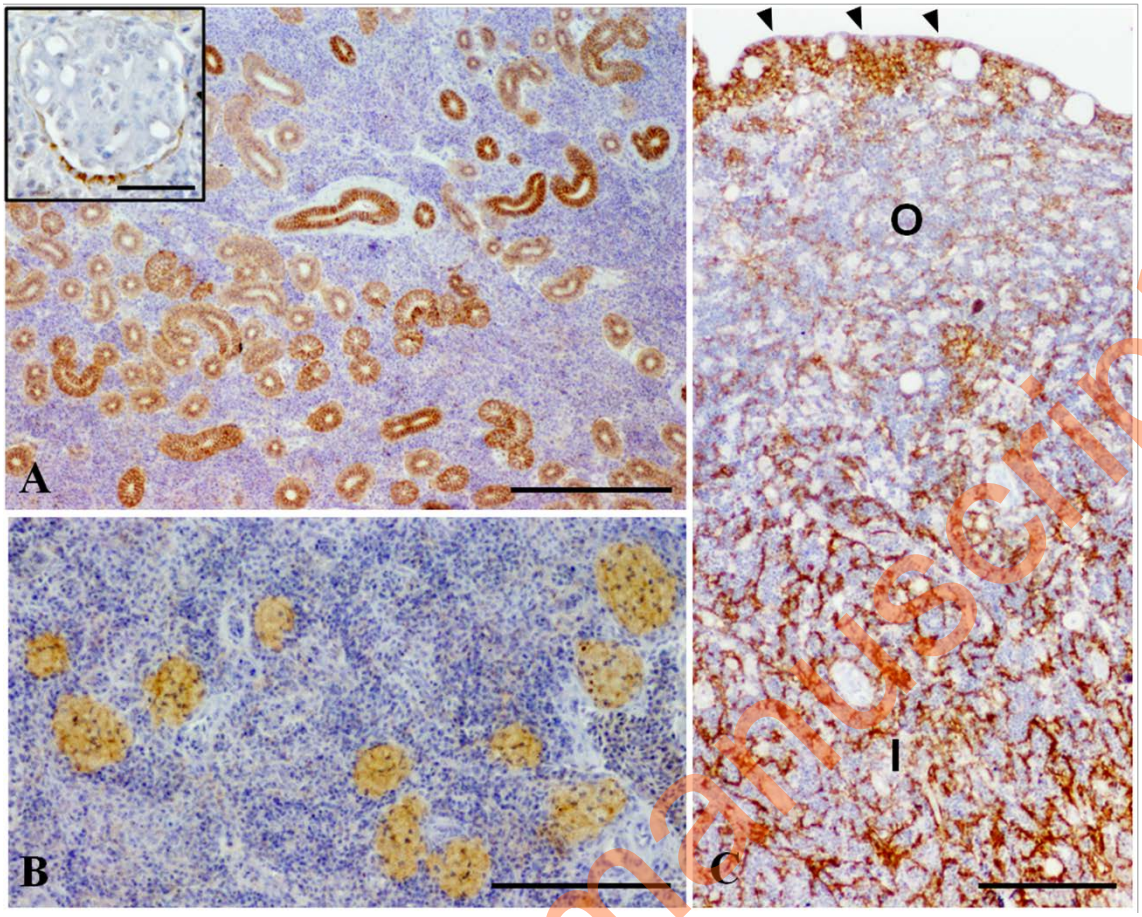

Fig 4. E-cadherin immunohistochemical expression in turbot (*Scophthalmus maximus*) kidney (A), spleen (B) and thymus (C). A) The immunoreaction was observed in the epithelia of the renal ducts and tubules, as well as in the epithelium of the Bowman's capsule (inset). Bar = 200 µm; Inset's bar = 25 µm. B) Immunostaining of the splenic melano-macrophage centres. Bar = 100 µm. C) Immunolocalisation of E-cadherin in thymus stroma, showing a stronger staining intensity in the inner (I) than in the outer (O) part of the organ. Note also the immunoreaction of the epithelium separating the thymus from the gill chamber (arrowheads). Bar = 100 µm.

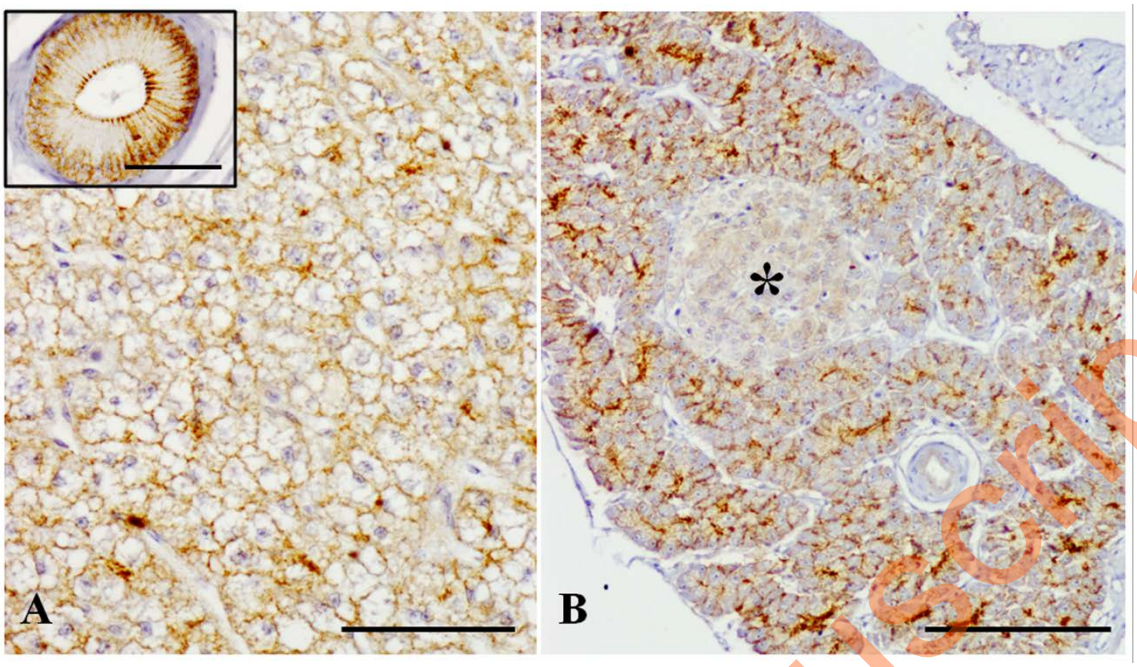

Fig 5. E-cadherin immunohistochemistry in turbot (*Scophthalmus maximus*) liver (A) and pancreas (B). A) Membranous staining pattern of the hepatocytes and in the epithelium of a biliar duct (Inset). Bars = 50 µm. B) Immunostaining of E-cadherin at cell-cell contacts between acinar cells, while this is not noticed in a pancreatic islet of Langerhans (star). Bar = 100 µm.