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Role of Antimicrobial Peptides in Eosinophilic Esophagitis

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“*Per aspera sic itur ad astra*”
(L.A.Seneca)

A mio fratello Andrea

Ringraziamenti

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ABSTRACT

Background: Eosinophilic Esophagitis (EoE) is a Th2 mediated disease characterized by patchy eosinophilic esophageal infiltration. In other Th2 mediated diseases such as Atopic Dermatitis (AD), there is a significantly reduced expression of the antimicrobial peptides and proteins (AMPs), a family of small cationic peptides that protect their hosts against infectious microorganisms. AMPs have never been characterized before in EoE. In the present work we hypothesize that similarly to AD AMPS may be dysregulated in EoE.

Aim: We investigated the presence and expression levels of selected AMPs in esophageal biopsies of children with active EoE (n=5), with EoE in remission (n=5) and in healthy controls (n=5).

Methods: Biopsies were analyzed by Real-Time quantitative PCR (RT-qPCR) for human Beta-Defensin-(hBD) 1 and 2, cathelicidin, elafin and psoriasin and by immunohistochemestry hBD1, elafin and psoriasin. Statistical analysis was performed using Kruskall-Wallis test or One-Way ANOVA followed by Bonferroni post-hoc test. Values of $P<0.05$ were considered statistically significant.

Results: Table 1 summarizes principal findings.

AMP	RealTime qPCR		IHC		
	Active EoE	*Not Active EoE*	*Active EoE*	*Not Active EoE*	*Active + Not Active EoE*
hBD1	⬆ vs control (1.87 ± 3.56)	⬇ vs control (0.54 ± 0.54)		⬇vs active ✵	N/A
hBD2	⬇ vs control (0.15 ± 0.14)	⬇ vs control (0.22 ± 0.14)	N/A	N/A	N/A
Cathelici-din	=vs control (1.19 ± 1.12)	=vs control (1.32 ± 1.45)	N/A	N/A	N/A
Elafin	⬇ vs control (0.41± 0.38)	⬇ vs control (0.52 ± 0.42)	⬇ vs control	⬇ vs control	⬇vs control ✵
Psoriasin	=vs control (1.87 ± 2.14)	=vs control (1.45 ± 2.16)	⬇vs control ✵	⬇vs control ✵	⬇vs control ✵

Table 1: summary of presented data. Red boxes indicate upregulation, green boxes indicate down-regulation. Numbers between parenthesis indicate fold expression. ✵ indicate statistically significant data, p<0.05. N/A not assessed

Conclusions: Although this is a small population, we were able to demonstrate that a possible dysregulation of AMPs expression is present in children with EoE. Larger studies will be required to confirm the above findings.

BACKGROUND

Eosinophilic esophagitis

Definition

In the last decades it has been observed an increase of patients with esophageal eosinophilia, who were initially thought to be affected by gastro-esophageal reflux disease (GERD). Due to the poor response to common therapies for GERD, such as protonic pomp inhibitors, a new disease was delineated, subsequently known as Eosinophilic Esophagitis (EoE).

EoE, as defined during the First International Gastrointestinal Eosinophil Research Symposium in 2007, is a "a clinico-pathologic condition characterized by oesophageal symptoms and a dense oesophageal eosinophilia, both of which persist despite prolonged treatment with proton pump inhibitors, whereas eosinophilic inflammation is absent in the other sections of the digestive tract" [1]. However, very recently, a new conceptual definition of EoE has been delineated by a task force of 33 physicians with recognized expertise on this disease. A number of recent basic, translational and clinical studies have, indeed, highlighted the increasing role of genetics and the existence of different phenotypes of EoE, rather defining a "spectrum" than a single disease. In this way, EoE should be defined as "*a chronic, immune/antigen-mediated esophageal disease characterized clinically by symptoms related to esophageal dysfunction and histologically by eosinophil-predominant inflammation*." [2].

Epidemiology

EoE has been reported from every part of the world except Africa, an observation that may be consistent with the hygiene hypothesis of allergic disease's distribution. It seems to be more predominant in men and in white population [3-5], clusters into families and it is associated with allergic sensitization [6] and other atopic diseases [7, 8].

In the last decade an increase in the incidence of EoE has been noted, and, according to different epidemiological studies, the prevalence of this disease seems to be comparable to that of inflammatory bowel diseases but less than celiac dis-

ease [9, 10]. Over a 16-year observation period, between 1989 and 2004, *Straumann et al* found an increasing prevalence from 2 to 23 per 100,000 persons in a Swiss population that included children and adults [11]. A population based study from the United States reported an increasing incidence from 0.35 to 9.45 per 100,000 persons from 1991-1995 to 2001-2005 [12]. Another study from the United States demonstrated a 35- fold increase between 1994 and 2003 [7] and an Australian study [13] an 18-fold increase in the prevalence of EoE. However, there are some pediatric studies suggesting that the incidence of EoE remains stable, at least in pediatric age. From 2000 to 2003, in Hamilton county (Ohio, United States), *Noel et al* [14] reported an annual incidence of EoE of 1 case per 10,000 in children and a prevalence of 4.3 cases per 10,000. Recently, *DeBrosse et al* [15] did not find any changes in the incidence of pediatric EoE in an observation period from 1982 to 1999, even after correction for a 40-fold increase in the number of performed endoscopies. For these contrasting results, further prospective and population-based studies, on children and adults, are needed to confirm these epidemiological observations.

Pathophysiology

In normal conditions, esophageal mucosa is devoid of eosinophils, which can however be found in duodenal mucosa and in small intestine; therefore attractants and degranulation triggers for eosinophils must play a central role in the pathogenesis of EoE. The pathophysiology of EoE is due to multifactorial causes including environmental and allergic triggers, genetic predisposition and, probably, a defect of the innate immune system (figure 1).

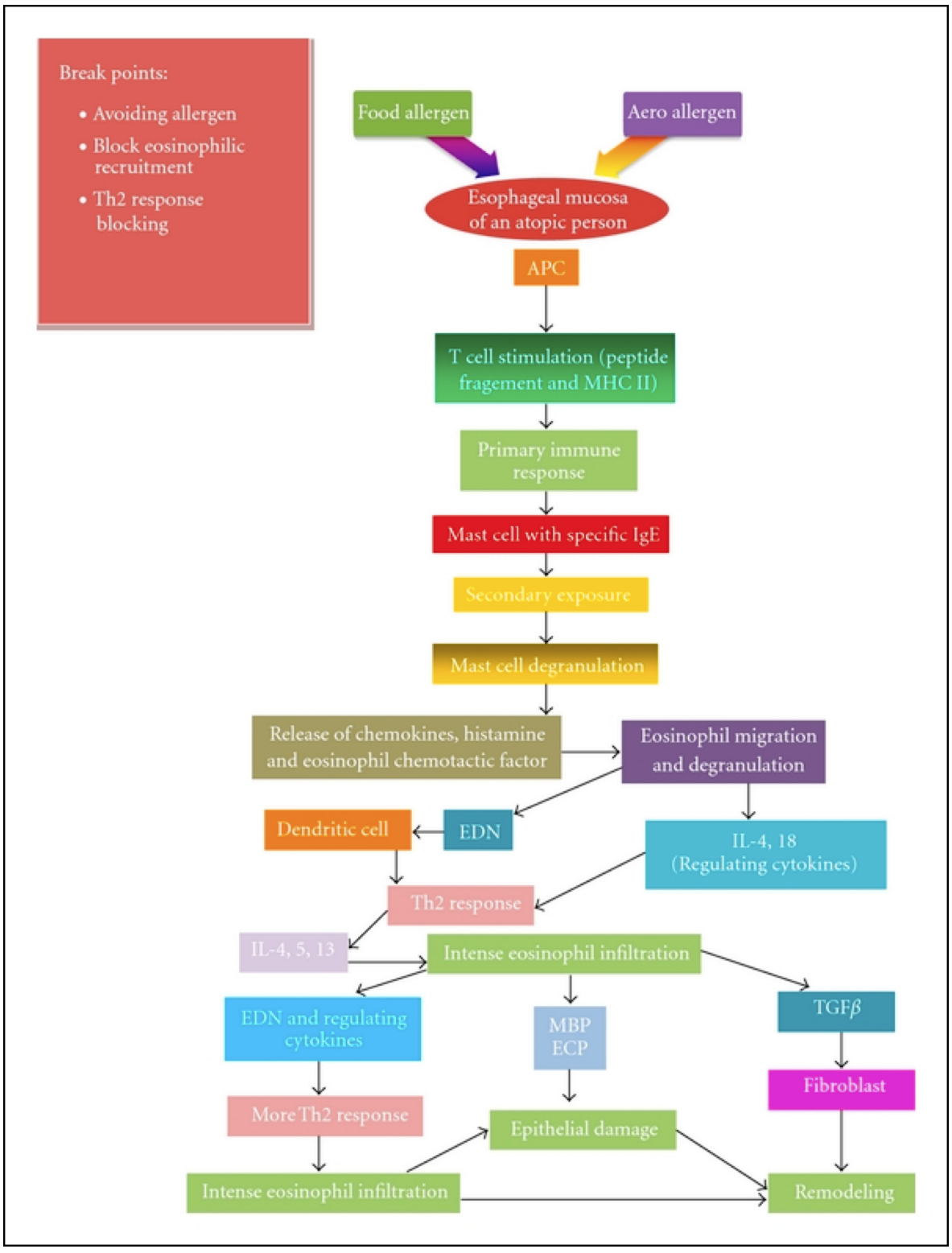

Figure 1: pathophysiology of EoE. APC: antigen presenting cells, ECP: eosinophil cationic protein, EDN: eosinophil derived neurotoxin, MBP: major basic protein, TGFβ: transforming growth factor β. (From *Shahzad* et al. [16])

In fact, recent studies have highlighted that EoE is thought to be a Th2-mediated disease, in the context of genetically predisposed individuals and an inciting environmental trigger [17]. A very interesting review deeply analyzed the possible role for immune system cells and cytokines in the pathogenesis of EoE [18]. This re-

view concludes that "*the causative events that lead to EoE in humans remain unknown*".

Eosinophils are recruited and activated into inflammatory sites and are regulated by Th2-skewed cytokines such as IL-4, IL-5, IL-13, IL-14 and TNF which are produced by activated Th2 and mast cells [19]. In EoE, esophageal mast cells and Th2 become activated by antigen-presenting cells (APC) processing food or air allergens (figure 2).

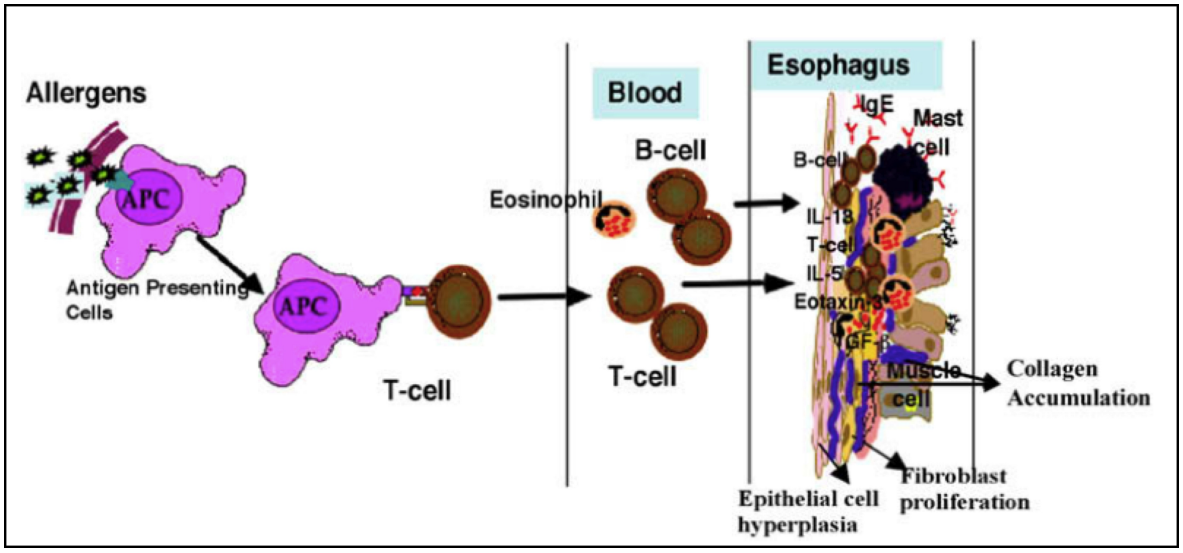

Figure 2: APC process and present the allergens to the T cells, which home to the esophagus by way of blood circulation and, on activation, release eosinophil-specific cytokines (IL-5 and IL-13), which induce chemokines (eotaxin -1, -2, and -3) in the esophageal epithelium that attract eosinophils into the esophagus. The activated eosinophil and mast cells are a rich source of TGF-β, which may play a critical role in the disease pathogenesis, including esophageal remodeling (from *Mishra A.* [20]).

After their activation, eosinophils degranulate and up-regulate their cytokines production. Eosinophils release four major cationic proteins contained in pre-formed granules (eosinophil peroxidase, eosinophil cationic protein, eosinophil-derived neurotoxin and major basic protein) and produce a wide range of cytokines that potentiate the inflammatory response (IL-1, IL-3, IL-4, IL-5, IL-6, IL-8, GMCSF, TGFα/β, TNFα and eotaxin). The chronic eosinophilic inflammation leads to the deposition of fibrous tissue in the sub-epithelial layers, a process called 'esophageal remodeling' that represents a major long-term sequela of untreated persistent EoE [21, 22]. The connective tissue deposition in the sub-epithelial layer may, in the long-term, lead to the alteration of the esophageal function and to luminal narrow-

ing, resulting in some of the typical findings of eosinophilic esophagitis, such as strictures and rings, that lead to the typical clinical presentation (figure 3, below).

The role of allergy in the pathogenesis of EoE is suggested by several findings: most patients are atopic (food and inhalants sensitization), demonstrate full disease remission after starting an elemental diet and recrudescence after reintroductions of food allergens [23, 24], and respond to steroid therapy (as in other atopic diseases, as atopic dermatitis and asthma).

In a large pediatric cohort, one study showed that two-thirds of children with EoE had concomitant atopy, of which 231 had asthma (37%), 243 had allergic rhinitis (39%) and 78 had atopic dermatitis (13%) [25]. Other studies have reported a higher prevalence of atopy with food and inhalants sensitizations in pediatric or adult patients with EoE, higher than the general population [5, 8, 26]. Moreover, aero- and food allergens also play a role in the pathogenesis of EoE, as demonstrated by the model by *Seema* et *Aceves* (figure 3) [27].

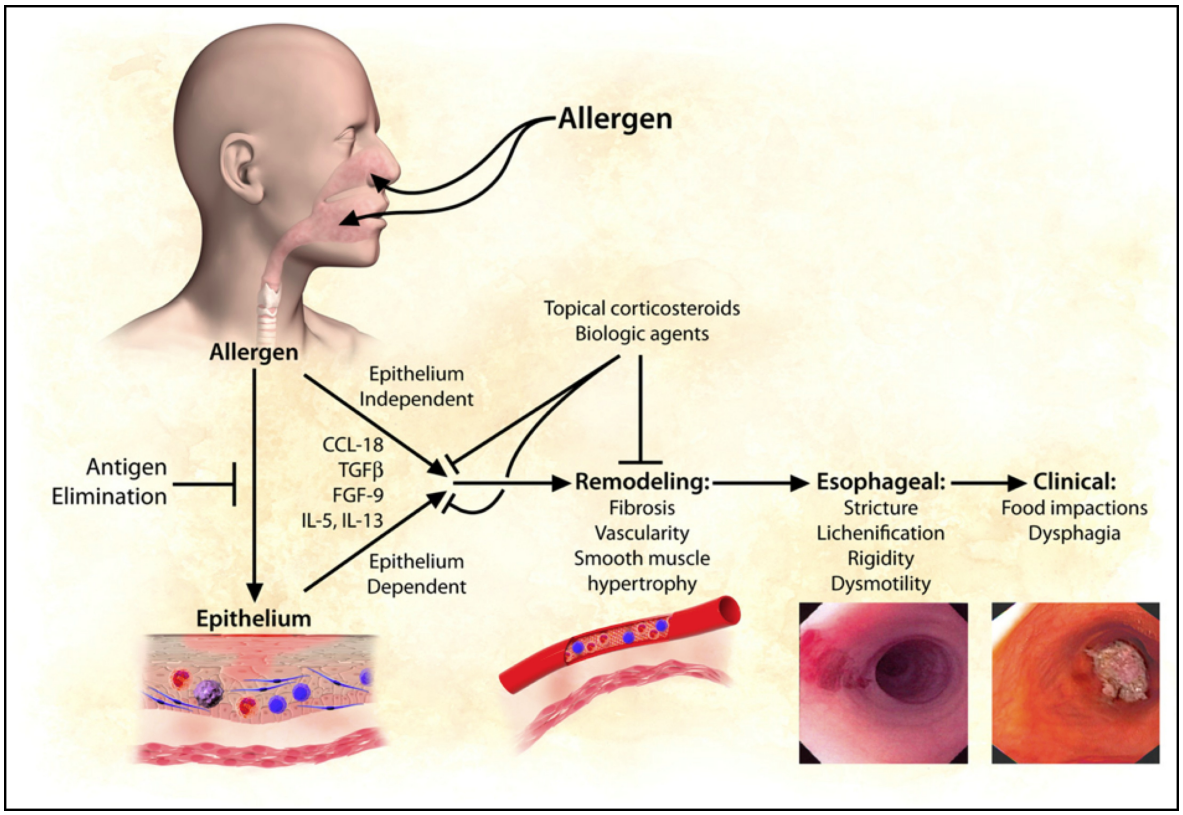

Figure 3: Potential pathogenesis and effects of tissue remodeling in patients with EoE. Aero and Food allergen drives epithelial inflammation and production of chemokines, interleukins, and growth factors, causing the remodeling. This remodeling, in turn, causes the abnormalities visible on endoscopy and esophageal dysfunction, determining clinical symptoms and further complications. Topical steroids, biologic agents, and antigen elimination might be able to alter the course to remodeling. FGF-9, Fibroblast growth factor 9. (From *Seema S. Aceves*. [27])

Genetics

Genetics surely plays an important role in the pathogenesis of EoE, as highlighted in a recent review by *Brown-Whitehorn* and *Spergel* [28].

EoE shows a strong familial association with nearly 10% of parents of EoE patients having a history of esophageal strictures and about 8% having biopsy-proven EoE [29].

Blanchard et al [30] have demonstrated, using a genome-wide microarray, a dysregulation of 1% of the expressed genes in patients with EoE compared to healthy controls (Figure 4). The highest up-regulated gene was eotaxin-3, a specific chemo-attractive cytokine responsible for the accumulation and adhesion of eosinophils. The role of eotaxin-3 in EoE pathogenesis is also confirmed by the observation that eotaxin receptor-deficient mice are protected from experimental EoE.

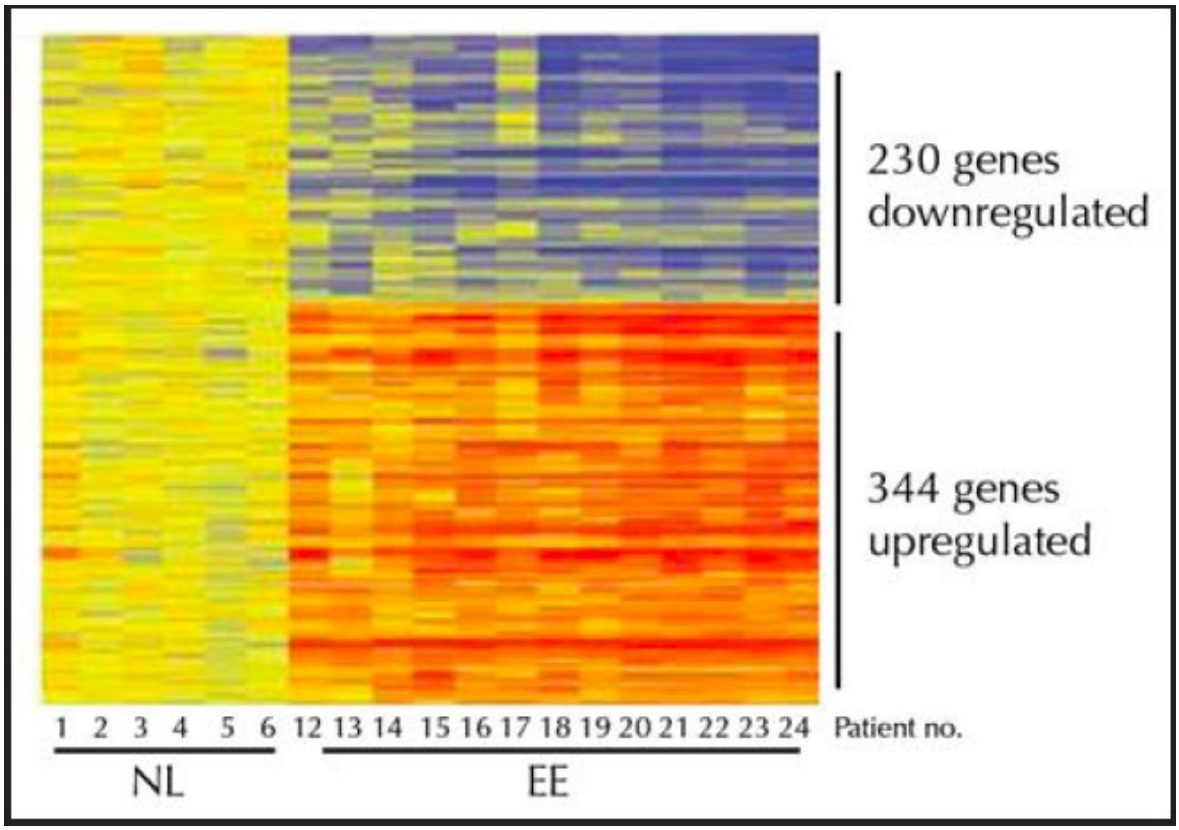

Figure 4: microarray of mRNA from normal (NL) human esophageal mucosa and from eosinophilic esophagitis (EE). Down-regulated genes are shown in blue and up-regulated genes in red (From: *Blanchard* et al [30]).

Rothenberg et al [31] have reported an association of EoE with variants at chromosome 5q22 encompassing TSLP (thymic stromal lymphopoietin) and WDR36

(WD repeat containing protein 36) genes. TSLP is an epithelial-derived cytokine that activates professional antigen-presenting cells, such as dendritic cells, which initiate Th2-type allergic responses, highlighted in EoE patients [32]. TSLP has also been found to be associated with atopic dermatitis [33] and asthma [34] compared with healthy controls.

A recent study by *Sherrill et al* [35] has showed a significant association between specific TSLP variants (found with the genetic analysis of single nucleotide polymorphism (SNP) of TSLP and TSLP receptor) and atopic disease. Moreover, results from this study have suggested a unique potential mechanism for the induction of EoE. Food allergens can trigger the TLR-3 (toll like receptor) receptor, inducing TSLP and finally causing the activation of the Th2 pathway and subsequently leading to the eosinophilic esophageal inflammation. However, more studies are needed to better understand the genetics of EoE.

Diagnosis

Diagnosing EoE presents some clinical and histological issues.

Clinical symptoms (table 1) complained by patients are not exclusively found in this disease, and even histological finding (table 2) are, sometimes, non completely reliable.

Table 1 Clinical presentation of eosinophilic esophagitis

Gastrointestinal symptoms	Other symptoms
Dysphagia	Chest pain
Food impaction	Rhinitis
Nausea and vomiting	Asthma
Heartburn	Allergies
Abdominal pain	Atopic dermatitis
Feeding disorders (pediatric)	Hoarseness
Failure to thrive (pediatric)	Croup, cough
	Sleep disordered breathing

Table 1: Clinical picture of EoE. (From: *Gupte* and *Draganov*. [36])

Table 2 Clinical signs in eosinophilic esophagitis

Endoscopic features	Histologic features
Diminished vascular pattern	Thick epithelium with eosinophilia
Mucosal furrows	Abnormally long papillae
Thick mucosa	Fibrotic lamina propria
Exudates	Microabscesses
Strictures	Extracellular Eosinophilic granules
Rings	Increased extracellular major basic protein (MBP)
Laryngeal edema, vocal cord nodules, laryngeal ventricular obliteration	

Table 2: Clinical signs in EoE. (From: *Gupte* and *Draganov*. [36])

Different guidelines for diagnosis of EoE have been published; although, most of them could be summarized in these following (table 3):

Table 1. Diagnostic guidelines for eosinophilic oesophagitis

Clinical manifestations	Symptoms of oesophageal dysfunction
Histologic manifestations	≥15 eosinophils in one hpf
Exclusion criteria	Lack of response to high-dose PPIs or normal pH monitoring of the distal oesophagus for GERD exclusion Exclusion of other conditions that cause oesophageal eosinophilia

hpf, high-power field; PPI, proton pump inhibitor; GERD, gastro-oesophageal reflux disease.

Table 3: Diagnostic guidelines for EoE. (From: *Schoepfer AM*, et al. [37])

Clinical presentation

In children typical symptoms are: refuse of feeding, food impaction, dysphagia, vomiting, abdominal pain and heartburn; some children could also present with failure to thrive (Table 1). Although these symptoms could suggest other diagnoses, such as GERD, and different endoscopic findings have been identified, none of them are strictly pathognomonic for EoE. Moreover, although esophageal eosinophilia is a primary histologic feature of EoE, this finding could be present in other diseases, such as GERD [38], Crohn's disease and ulcerative colitis [39], drug associated esophagitis, and hypereosinophilic syndrome [40].

Histologic findings

The first records of histologic findings associated with EoE were reported in the 1970s [41, 42], although one of the most characteristic feature of EoE, circular rings, was already reported twenty years before, in 1953 [43]. Histological features (figure 5, 6) associated with EoE are: i) at least 15 intraepithelial eosinophils/HPF at peak count; ii) presence of eosinophils micro-abscesses iii) superficial layering of eosinophils iiii) basal zone hyperplasia. Moreover, due to the high rate of allergic diseases in EoE patients, a complete allergy evaluation is recommended.

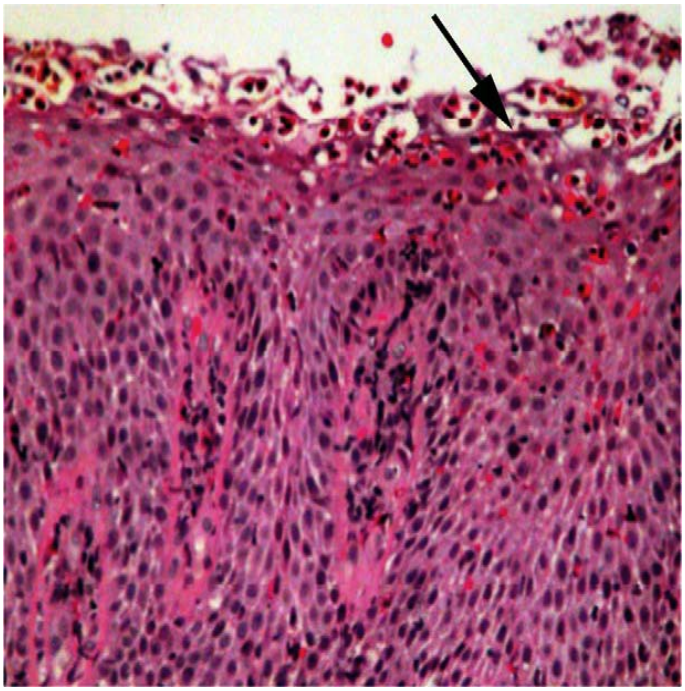

Figure 5: Microscopic appearance of EoE: biopsy specimens have characteristic mucosal eosinophilia and epithelial proliferation. Eosinophils layer on the luminal surface and form micro-abscesses (arrow). Microscopic examination would also note numerous degranulating eosinophils. *(*From: *Noel* and *Tipnis* [44])

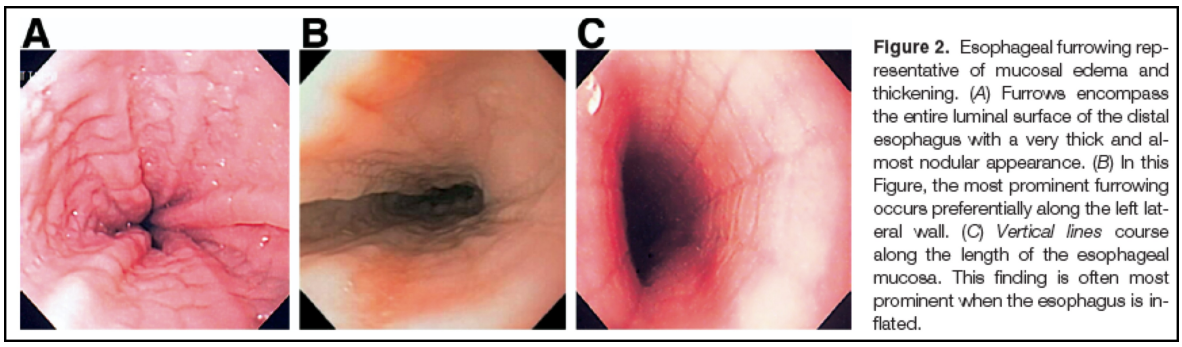

Figure 6: Pictures of esophageal furrowing. (From: *Furuta* et al. [1])

Differential diagnosis and management of EoE

The differential diagnosis of EoE, such as GERD, eosinophilic gastroenteritis, Crohn's disease, connective tissue diseases, hyper-eosinophilic syndromes, should be ruled out. Treatment usually comprises isolated or a combination of approaches which include: medical (local and systemic steroids, acid suppression drugs, leukotriene receptor antagonists and mast cell stabilizers, biological drugs), surgical (esophageal dilatation) and/or dietary (food elimination).

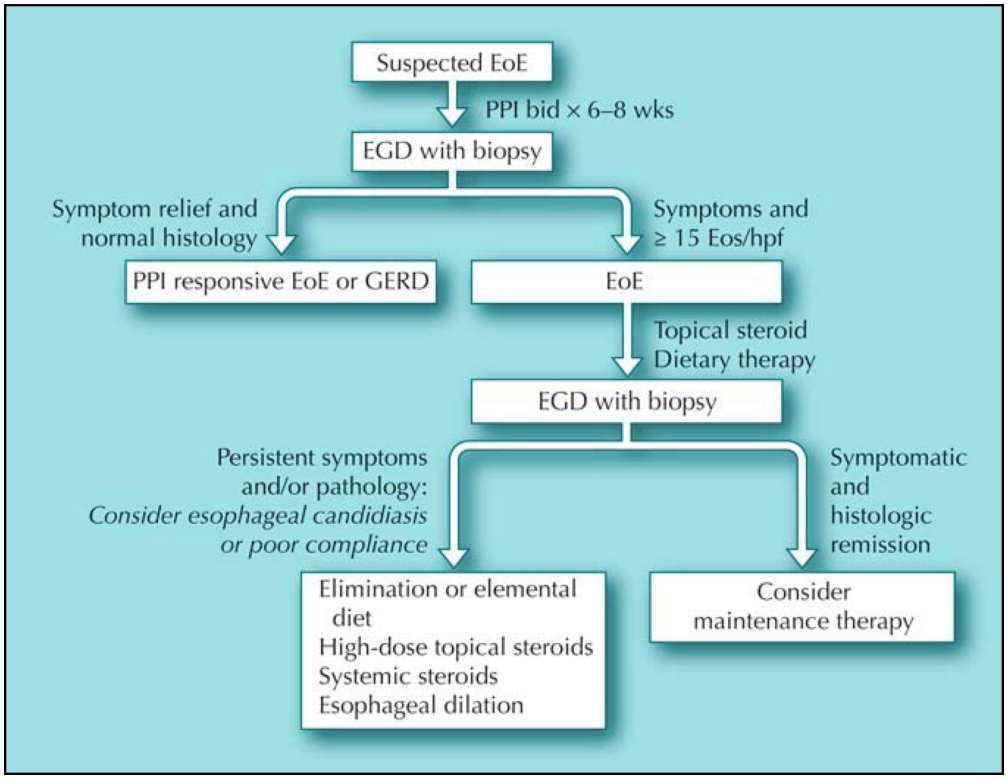

Figure 7: Algorithm for EoE management and treatment. (From: Garrean and Hirano. [45]

The first step (figure 7) in the treatment of EoE is an empiric 6- to 8- week trial of *acid suppression therapy*, as suggested by the First International Gastrointestinal

Eosinophilic Research Symposium in 2007. Due to the possible concomitant GERD, without the acid suppression therapy, endoscopy cannot confirm EoE diagnosis.

Systemic corticosteroids, although highly effective in improving symptoms and esophageal histology, should be reserved for patients with severe symptoms. A short-term course could be considered when hospitalization is required or in case of development of esophageal stricture. *Topical swallowed corticosteroids* is a mainstay of EoE treatment in adults and in children, as some studies have already demonstrated [46-48]. Topical steroids have less side effects than those seen with systemic corticosteroids; however, disease recurrence generally occurs when treatment is suspended.

Anti IL-5 therapy is currently under investigation in two pediatric studies, based on the efficacy observed in a open-label phase TT study in four adult patients [49]. Although new recent discoveries and innovative therapeutical approaches, some questions still remain unresolved, among them, the understanding of the etiology and pathogenesis of EoE which could better address diagnostic tests and treatment choices.

Antimicrobial peptides and proteins

The human immune system is composed of an innate and an adaptive system, to protect itself from exogenous and endogenous insults. The innate immune system has two major components: cells (e.g. leukocytes) and humoral mediators (e.g. cytokines, antimicrobial substances). Antimicrobial substances comprise microbicidal chemicals (e.g., hydrogen peroxide, nitric oxide, etc.) and a wide variety of host gene-encoded antimicrobial peptides and proteins [50] .

Antimicrobial peptides (AMPs) are a family of small cationic peptides that belongs to the innate branch of the immune system [51]. These peptides, which are present in several species, protect their hosts against infectious microorganisms and have a role in the molecular cascade of some inflammatory diseases.

Different AMPs have been described in humans, that could be classified upon their molecular structure into [52, 53]:

1) linear α-helical peptides free of cysteine residues (e.g. human cathelicidin);

2) peptides with a β-sheet globular structure stabilized by 3 intramolecular disulfide bonds (e.g. human defensins);

3) peptides with unusual bias in certain aminoacids, such ad histidine, glycine, proline or tryptophan (e.g. bovine indolicin).

The most documented and known AMPs are defensins, although some others have been studied, as cathelicidin and psoriasin in skin diseases. AMPs have been studied mostly in inflammatory bowel diseases (table 4) [54], in skin (table 5) and respiratory diseases [55].

Antimicrobial peptide	Chromosomal location	Molecular mass (kDa)	Secretory stimuli	Distribution in gastrointestinal tract	Biological function	Changes in inflammatory bowel disease
hBD-1	8p23.1	3.5–4.5	Constitutive in epithelial cells, IFN-γ and LPS in monocytes	Ubiquitous in epithelial cells of small and large intestine, monocytes, monocyte-derived dendritic cells	Antimicrobial, chemotactic	Reduction in colonic IBD
hBD-2, 3, 4	8p23.1	3.5–4.5	LPS, flagellin mediated by NF-κB and AP-1	Epithelial cells, monocytes	- Antimicrobial, chemoattractant for macrophages and monocytes, - hBD-2: mast cells and neutrophils	- Attenuated induction observed in colonic CD - Reduced copy numbers for hBD-2 in colonic CD
HD-5 and HD-6	8p23.1	3.5–4.5	NOD2 activation (MDP, LPS) TLR	Granules of ileal Paneth cells (also metaplastic Paneth cells in other areas of intestinal tract)	Antimicrobial, induction of IL-8	- Reduction in ileal CD, more pronounced in patients with NOD2 mutation - HD-5 and HD-6 expression due to metaplastic Paneth cells in UC and CD colon
Cathelicidin ("LL-37")	3p21.3	18	Butyrate, vitamin D, bile acids, MDP	Epithelial cells, leukocytes	Antimicrobial, chemotactic	- Attenuated induction in colonic CD - Ileal CD and UC show regular induction
Elafin	20q13.12	9.8	IL-1, TNF-α	Epithelial cells, leukocytes	Antiprotease with antimicrobial and chemotactic properties	Attenuated induction in colonic CD
Secretory phospholipase A2	16p13.1–p12	14	LPS	Epithelial and inflammatory cells, Paneth cell granules	- Acute phase protein involved in eicosanoide metabolism - Small intestinal mucosal defense	?
Lysozyme	12q15	16.5	?	Gastric, pyloric and duodenal glands, small intestine, macrophages and monocytes, not in colonic tissue	Antimicrobial against Gram-positive bacteria, chemotactic	- Small intestine: no changes observed - Increased colonic expression due to metaplastic Paneth cells
BPI (bactericidal/ permeability-increasing protein)	20q11.23	50	LPS	Epithelial cells, neutrophils	Antimicrobial, binds LPS-compounds	No changes observed, regular induction in IBD

Table 4: AMPs in inflammatory bowel diseases (from *Jager* S et al. [54])

Peptide	Cellular source	Susceptible organisms[1]	Comments
dermcidin	eccrine sweat glands	broad-spectrum	-principal sweat antimicrobial peptide; -not inducible by injury or inflammation
psoriasin	keratinocytes, sebocytes	Gram-negatives *E. coli*	-most abundant antimicrobial peptide in healthy skin; -induced by *E. coli* flagellin
RNase 7	keratinocytes	broad-spectrum *Enterococcus faecium*	-antimicrobial activity independent of RNase activity
RNase 5/angiogenin	keratinocytes	*Candida albicans*	-also plays an important role in blood vessel formation; -importance of RNase activity not clear
cathelicidin (LL-37)	keratinocytes, sebocytes	Gram-positives Gram-negatives	-induced by injury or inflammation; -also plays a role in wound healing
hBD-1	keratinocytes, sebocytes	Gram-negatives	-constitutively produced at low amounts
hBD-2	keratinocytes, sebocytes	Gram-negatives	-induced by injury or inflammation
hBD-3	keratinocytes	broad-spectrum	-induced by injury or inflammation
hBD-4	keratinocytes	Gram-positives Gram-negatives	-inducible in primary keratinocytes in vitro; -the actual concentration and function in skin is unknown
SLPI	keratinocytes	broad-spectrum	-upregulated during inflammation; -also functions as an inhibitor of neutrophil elastase and cathepsin G; -plays a role in wound healing probably dependent on its antiprotease activity
elafin	keratinocytes	broad-spectrum	-upregulated during inflammation; -also functions as an inhibitor of neutrophil elastase and proteinase 3
adrenomedullin	keratinocytes, hair follicles, eccrine and apocrine sweat glands, sebocytes	Gram-positives Gram-negatives	-pluripotent peptide also involved in wound healing and various other processes
MIP-3α/CCL20	Keratinocytes	broad-spectrum	-belongs to a group of chemokines with antimicrobial activity collectively called kinocidins
lysozyme	keratinocytes, sebocytes, hair bulb cells	Gram-positives (Gram-negatives)	-degrades the bacterial cell wall by its muramidase activity; -lyses bacterial membranes in a non-enzymatic manner

Table 5: AMPs in the skin (from *Wiesner* J and *Vilcinskas* A. [56])

Role and functions of AMPs

Several reviews have analyzed different types and roles of AMPs [53, 57-59]. AMPs are virtually present in the whole human body, especially in the skin and in mucosal epithelia, which are tissues in direct contact with the outside environment. It has been demonstrated that AMPs have a double role in humans: they have antimicrobial functions and act as immuno-modulatory molecules in several diseases, such as inflammatory bowel diseases (IBDs), atherosclerosis, cystic fibrosis and psoriasis (figure 8).

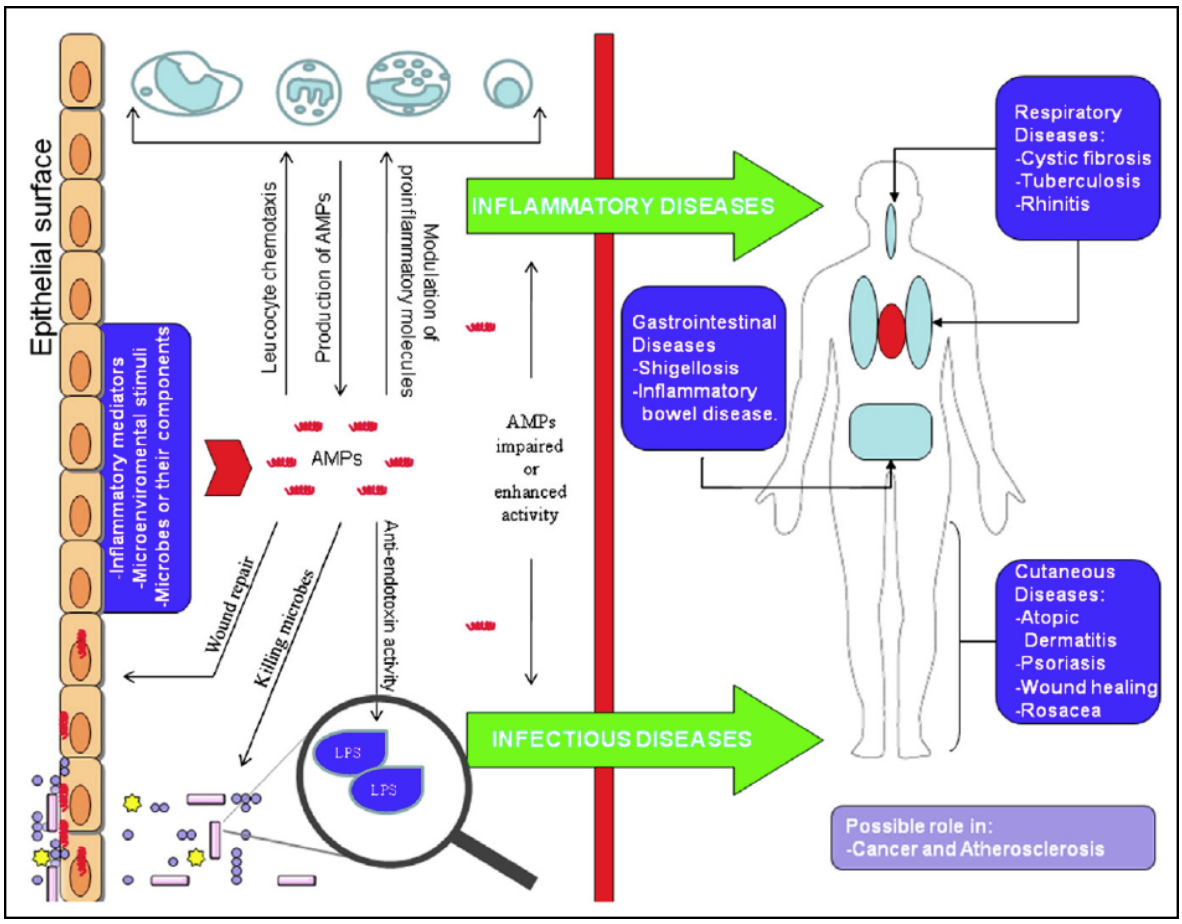

Figure 8: Functions of AMPs in inflammatory diseases. Various cell types are activated by microbes and inflammatory mediators, causing the production and release of AMPs. These peptides show different functions including antimicrobial activity and modulation of the inflammatory response. However, an imbalance in the activity of AMPs leads to the development of infectious or inflammatory diseases. Abbreviations: AMPs, antimicrobial peptides; LPS, lipopolysaccharides. (from *Guaní-Guerra E* et al. [57])

Currently, the exact mechanism of action of each AMPs is not completely known. Different models (figure 9) have been proposed but it is unknown which of the possible mechanisms is closest to reality [60].

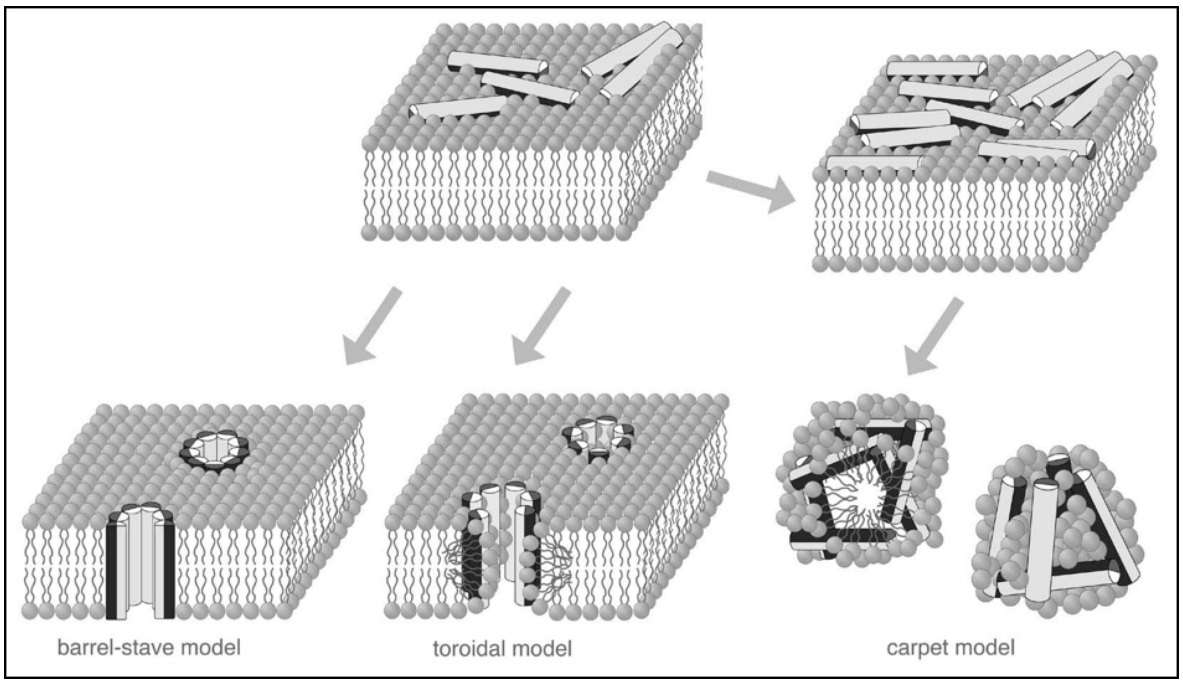

Figure 9: Schematic presentation of the three major models explaining how cationic amphipathic AMPs insert into lipid bilayers or lead to membrane disruption. Hydrophilic and lipophilic parts of the AMPs are indicated in light grey and black respectively. (from *Wiesner J and Vilcinskas*. [56])

For the purpose of this thesis, only a selection of human AMPs will be analyzed in depth: beta-defensins (hBD), cathelicidin, elafin and psoriasin.

HUMAN β-DEFENSINS

The most described and represented human AMPs are defensins, which are characterized by a triple-stranded β-hairpin structure, six conserved disulfide-linked cysteine residues and a positive net charge. Based on the cysteine pairing, it is possible to divide them in two subfamilies: the *alpha(α)-defensins* and the *beta(β)-defensins* (figure 10).

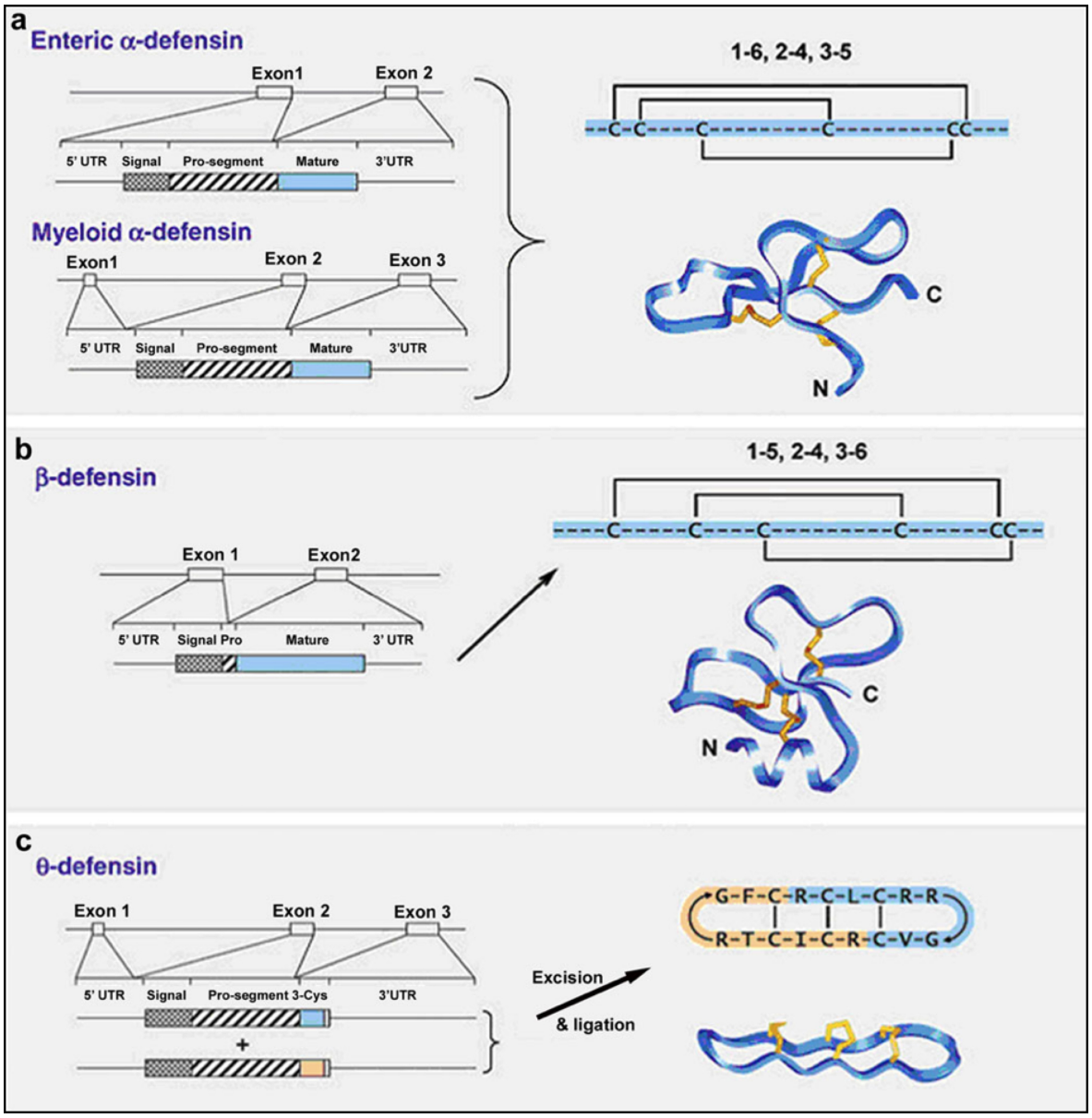

Figure 10: Structure of α-defensins (A), β-defensins (B) and θ-defensins (C). (from Hazlett L, Wu M. [61])

In humans, six *α-defensins* have been identified: HNP (human neutrophil peptide) -1 to HNP-4 in the azurophilic granules of neutrophils, and HD (human defensin) -5 and -6 mainly secreted from the Paneth cells in the small intestine. Several *β-*

defensins, too, have been characterized (table 6) and are mainly produced by epithelial cells; hBD (human beta defensin) -1 to -4 are those better known and characterized. All *α*- and the *β-defensins* have broad antimicrobial activities (viruses, bacteria, fungi) and some chemoattractant properties. A third class of defensins (*θ-defensins*) have been described, but not in humans.

Defensin	Tissue distribution	Cell source	Synthesis and regulation
HBD1	Oral and nasal mucosa, lungs, plasma, salivary glands, small and large bowel, stomach, skin, eyes, mammary glands, urogenital tract and kidneys	Epithelial cells*, monocytes, macrophages, monocyte-derived dendritic cells and keratinocytes	Constitutive or inducible in response to interferon-γ, lipopolysaccharide and peptidoglycan
HBD2 and HBD3	Oral and nasal mucosa, lungs, plasma, salivary glands, small and large bowel, stomach, skin, eyes, mammary glands, urogenital tract and kidneys	Epithelial cells*, monocytes, macrophages, monocyte-derived dendritic cells and keratinocytes	Inducible in response to viruses, bacteria, lipopolysaccharide, peptidoglycan, lipoproteins, cytokines (IL-1β, TNF) and growth factors
HBD4	Gastric antrum and testes	Epithelial cells*	Constitutive or inducible in response to PMA and bacteria
HD5 and HD6	Salivary glands, small bowel, inflamed large bowel, stomach, eye, female genital tract (HD5 only), breast milk and inflamed urethral lumen	Intestinal paneth cells* and vaginal epithelial cells (HD5 only)	Constitutive or inducible, such as by sexually transmitted infection

Table 6: Tissue distribution, cell source, synthesis and regulation of β-defensins. (from *Klotman ME, Chang TL*. [62])

Genetics and structure

The genes encoding human *β-defensins* are located on the chromosome 8:
hBD1 (DEFB1) [63]; hBD2 (DEFB4) [64]; hBD3 (DEFB103) [65]; hBD4 (DEFB104) [66, 67] ; hBD5-9 (DEFB05-DEFB9) [66, 68-70].

Source and expression

The primary source of hBD1-4 is epithelial cells; however, these β-defensins are also expressed in monocytes and macrophages [62]. Whereas the highest levels of hBD1-3 were detected in skin, sweat glands, lung, respiratory, and urogenital tracts [71-77], hBD4 and several recently discovered β-defensins (hBD5-9, hBD18-21, hBD23 and hBD25-29) are mainly present in placenta, testis, and epididymis [66, 67, 69, 70, 78, 79].

While in the primary epithelial cells a basic level of hBD1 is supplied by the constitutive expression with slight modulation during the inflammation [72, 74, 80-82], expression of hBD2-4 is clearly inducible [80-82]. The synthesis pattern of human epithelial defensins 2 through 4 involves multiple, distinct signaling pathways [83]. In general, inducible up-regulation of the hBD2-3 expression was observed in re-

sponse to bacterial and viral infections or such microbial components as lipoproteins, peptidoglycan, lipopolysaccharides (LPSs), lipoteichoic acid (LTA), proinflammatory cytokines (IL-1α, IL-1β, TNF-α), growth factors (TGF-α, IGFI), and some chemical agents like 1,25-dihydroxyvitamin D3 or phorbol 12-myristate 13-acetate [65, 67, 71, 80, 82, 84-87].

Functions and role in humans

The first **hBD1** was identified in 1995 and purified from the plasmafiltrates of patients with renal disease, from haemofiltrates [88], while its mRNA was found to be predominantly expressed in epithelia of the urogenital tract [72]. The previously reported genomic hBD-1 sequence does not contain transcription factor regulatory elements for NF-kB and AP-1 [89], making it likely that HBD-1 is constitutively produced and is not transcriptionally regulated by inflammatory agents [72, 73, 90-93] but could also be augmented by inflammatory stimuli. However, expression of hBD1 could be induced and upregulated by LPS, heat-inactivated Pseudomonas and INFγ.

hBD2 was originally isolated in 1997 from psoriasic skin lesions [71]. The most prevalent expression of hBD2 is observed in the skin and the gastrointestinal and respiratory tracts; however, substantial amounts of this defensin are present throughout the entire epithelia. In contrast to hBD1, the hBD-2 gene expression is inducible by various proinflammatory agents such as TNF-α, IL-1 β and Gram-negative bacteria and to a lesser extent by Gram-positive bacteria and yeasts [71, 76], thus it may represent the human equivalent of bovine TAP and LAP. Indeed the 5'-flanking region of hBD-2 has been demonstrated to contain consensus binding sequences for NF-kB [94]. Therefore, hBD2 can be considered as the first described human inducible defensin. This psoriatic-scale derived hBD2 shows preferential antimicrobial activity against Gram negative bacteria (*E.Coli*, *Pseudomonas*) and less activity against *Candida Albicans* and only bacteriostatic activity against *St. Aureus*. It is interesting to note that hBD2 ability to inhibit bacteria growth dimin-

ishes when salt concentration is increased, suggesting that hBD2 will be unable to kill bacteria in serum or on the skin surface covered with evaporated sweat. hBD2 immunoreactivity is localized to the uppermost layers of the epidermis and/or stratum corneum. On a subcellular level, hBD2 is stored in lamellar bodies of stimulated KC of the spinous layer of the epidermis, suggesting that hBD2 is released with the lipid-like contents of lamellar bodies. Inter-individual and site-specific differences in intensity of immunostaining were observed, and the pattern of peptide localization was seen to be rather focal, similar to psoriasin staining, suggesting that hBD2 is locally induced. Stimulators of hBD2 expression include IL-1a (++), IL-1b (++), TNFa, INFg, PMA, isoleucine, vitamin D3, LPS and some Gram negative bacteria.

hBD3 was nearly simultaneously purified from psoriasic scales. hBD3 is a broad spectrum antimicrobial peptide, active against Gram negative and Gram positive bacteria, fungi, including MRSA (multi resistant Staphilococcus Aureus) and VRE (vancomycin resistant *Enterococcus*). hBD3 mRNA is expressed throughout the epithelia of many organs and in some non-epithelial tissues. Transcripts were found in skin, tonsils, gingival KC, esophagus, trachea, placenta, adult heart, skeletal muscle and fetal thymus. Similar to hBD2, expression of hBD3 is induced in keratinocytes and the respiratory epithelium by TNFα (+), IL-1β, INFγ (++), various bacteria and yeast. In contrast to hBD2, upregulation of hBD3 expression in keratinocytes was observed in the presence of TGFα and IGF1. hBD3 seems to be less widely expressed than other human β-defensins such as hBD1 and hBD2. *Harder et al* investigated the tissue distribution of hBD3 mRNA expression from various body sites by real-time RT-PCR and found low or no HBD3 mRNA expression in most of the analyzed organs including the respiratory, gastrointestinal, and genitourinary tracts [84]. Despite the low hBD3 expression in biopsies from gastrointestinal tract, purified epithelial cells of normal small and large intestine were found to express high level of HBD3 mRNA [95].

CATHELICIDIN

Cathelicidins are linear alpha-helical peptides and represent another major group of mammalian AMPs [96, 97]. Cathelicidins have been found in nearly all investigated mammals [98]; about 35 cathelicidin members have been identified in various mammalian species but in humans there is only one cathelicidin, called LL-37 (or hCAP18) [99].

Genetics and structure

The gene encoding LL-37 is localized to chromosome 3 and contains four exons [100]. Cathelicidins are synthesized as pre-pro-peptides. Generally, the cathelin pro-peptide must be removed from the C-terminal peptide to unleash the microbicidal activity [101]. All cathelicidins (figure 11) contain an N-terminal putative signal peptide (pre-region), a conserved pro-region cathelin-like domain (hence the name cathelicidin), and a carboxy-terminal microbicidal domain.

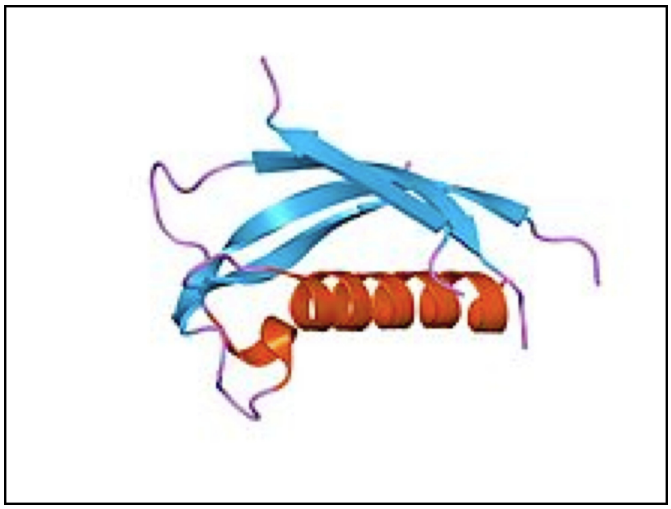

Figure 11: Schematic structure of human cathelicidin. The promoter region of the gene contains putative binding sites for many transcription factors such as nuclear factor NF-*k*B, IL-6, acute-phase response factor, activator protein 4 and CCAAT/enhancer-binding protein (C/EBP). (From Bals and Wilson [101].

Source and expression

Initially, LL-37 was isolated from myelocytes and metamyelocytes and localized into specific neutrophils granules [102]. Thereafter LL-37 was demonstrated in very different cells and tissue types (table 7).

Cell and tissue types	
Leukocytes	Developing lung
Myelocytes and metamyelocytes	Bronchoalveolar lavage fluid
Bone Marrow	Salivary glands
Breast milk	Saliva
Skin of Newborns	Gigiva
Squamous epithelia	Colon epithelium
Nail	Colo mucosa
Sweat	Testis
Wound and blister fluid	Epididymis epithelium
Ocular surface epithelia	Spermatozoa
Synovial membranes	Seminal plasma
Nasal mucosa	Vernix caseosa
Lung epithelia	Amniotic fluid

Table 7: cell and tissue types where LL-37 has been found (adapted from *Ulrich* et al. [103])

Several reports of up- and down-regulation of LL-37 have been described (table 8). Up-regulation seems to be more common than down-regulation, indicating that these AMPs assists the immune system in fighting disease.

Up-regulation	Keratinocytes in inflammatory disorders (psoriasis, lupus erythematosus, contact dermatitis); keratinocytes in condyloma acuminatum and verruca vulgaris; cholesteatoma; gastric epithelia, *H.pylori* infection; inflamed synovial membranes; chronic nasal inflammatory disease; bronchoalveolar lavage fluid in cystic fibrosis and sarcoidosis; tracheal aspirated of newborns; breast cancer.
Down-regulation	Atopic dermatitis; chronic ulcer epithelium; enteric infections; neutrophils, acute myeloid leukemia.
Absent	Kostmann's disease.

Table 8: up- and down-regulation of cathelicidin (adapted from *Ulrich* et al. [103]

LL-37 is up-regulated in skin in response to cutaneous infection or injury and in cutaneous inflammatory disorders, such as psoriasis. In contrast, low expression of LL-37 has been detected in patients with atopic dermatitis. This apparent deficiency of LL-37 could suggest an explanation for the increased susceptibility of patients with atopic dermatitis to skin infection, compared to patients with psoriasis, who are not more prone to skin infections than to healthy subjects.

Functions and role in humans

Cathelicidins have different functions (figure 12), similar to the other members of AMPs.

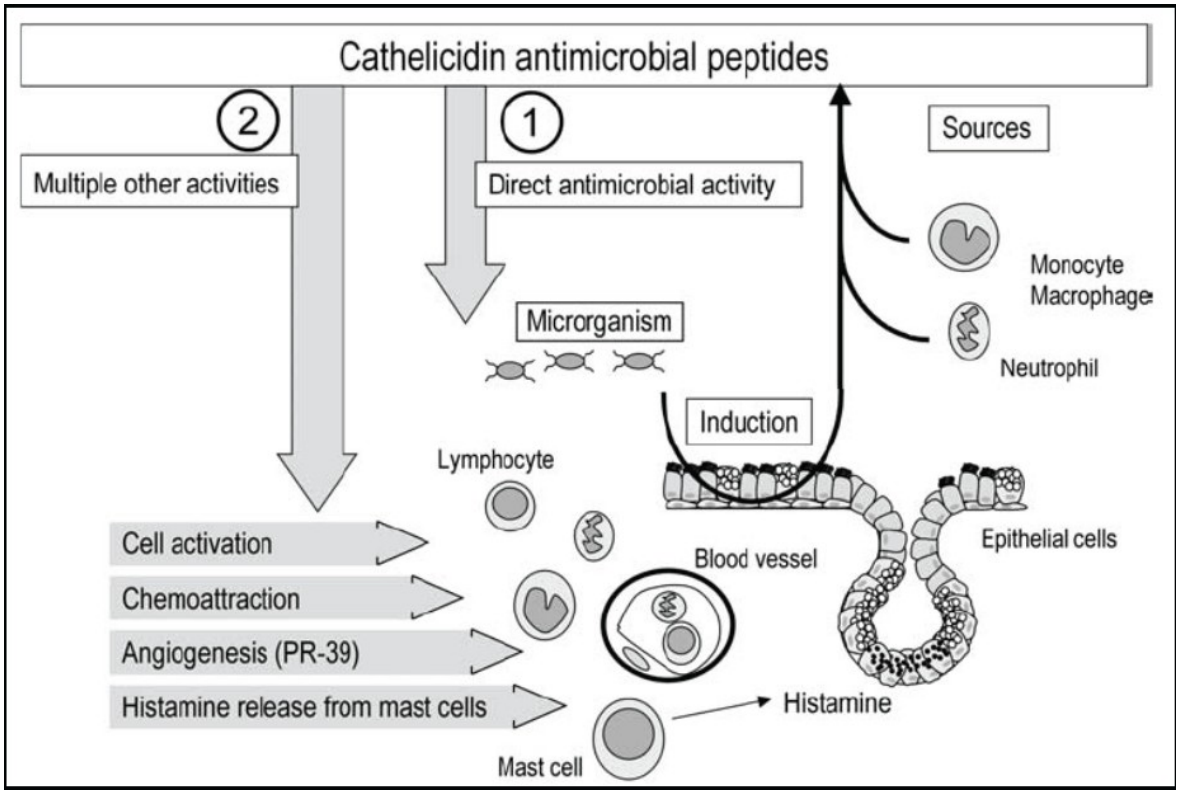

Figure 12: biological functions of cathelicidin antimicrobial peptides. Cathelicidins are secreted by several cell types during infection and inflammation. Cathelicidins have direct antimicrobial activity and regulate cellular responses including cell proliferation, cell migration of inflammatory cells, release of cytokines and angiogenesis. (From *Bals R, Wilson JM.* [101]

It has been demonstrated that LL-37 has specific functions and interacts with several other inflammatory molecules at very different concentrations (figure 13).

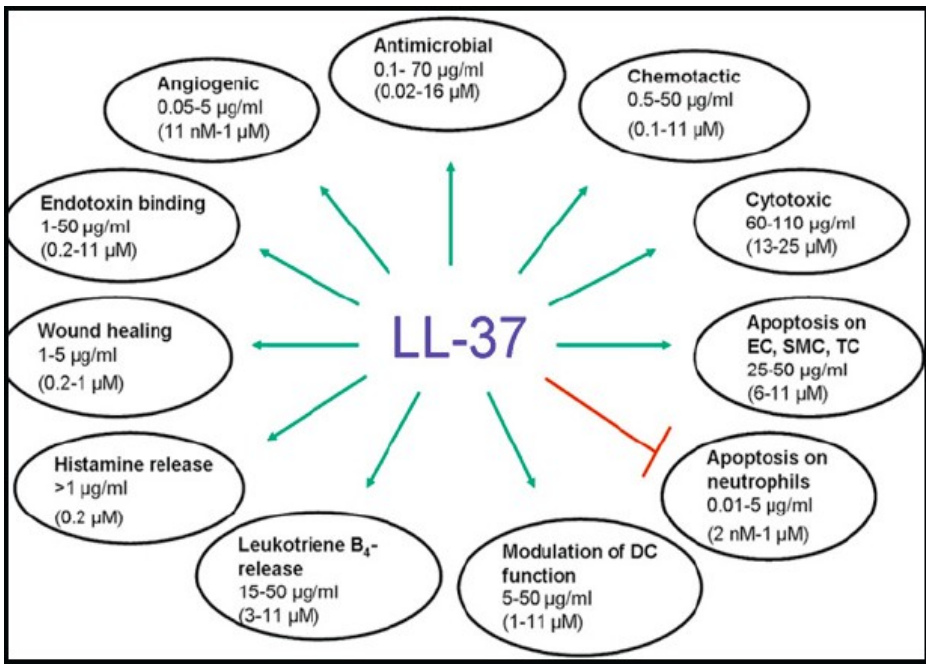

Figure 13: concentrations of LL-37 required to different activities and functions.

Cathelicidin has a broad spectrum of antimicrobial activity against gram positive and gram negative bacteria, and even against yeasts. It is noteworthy that in patients with Kostmann's disease there is an absence of LL-37 in the granulocytes and saliva and, unless treated with G-CSF (granulocytes colony stimulation factor), these patients usually die from bacterial infections within their first year of life [104]. This clearly highlights the role of cathelicidin in the regulation of the immune system in fighting infectious diseases.

Cathelicidin has a chemotactic activity on several cells of the immune systems, i.e. leukocytes [105], and increased levels of this AMP are regularly found in inflamed or infected tissues. Mast cells are also under the influence of LL-37: it has been demonstrated that LL-37 could induce degranulation [106] and migration [107] of mast cells.

Some recent studies have demonstrated increased levels of LL-37 in human skin after a wound had been inflicted [108]. The role of AMPs in wound healing has also been studied for other AMPs.

ELAFIN

Elafin was first isolated from sputum secretions of patients with chronic obstructive pulmonary disease and cystic fibrosis [109, 110]. This protein, together with the secretory-leukocytes-proteinase-inhibitor (SLPI), belongs to the family of “alarm antiproteases”.

These anti-proteases have been classified as either “systemic” or “alarm” [111]. Alarm anti-proteases are synthesized and secreted by cells local to the site of inflammation in response to the release of inflammatory cytokines (as IL-1) [112]. Therefore, anti-proteases could be crucial to prevent and contrast tissue injury from excessive release of proteolytic enzymes and inflammatory molecules by inflammatory cells.

Genetics and structure

Characterization of elafin was complete 20 years after its discovery [113]. Elafin is composed of two regions (figure 14): a globular C-terminus WAP/four-disulphide core domains and a flexible NH_2 domain referred to as “cementoin,” or “trappin” domain (hence one of the acronyms), which provides a substrate for the enzyme transglutaminase [115, 116]. This enzyme allows elafin to be cross-linked into polymers or with extracellular matrix components [116].

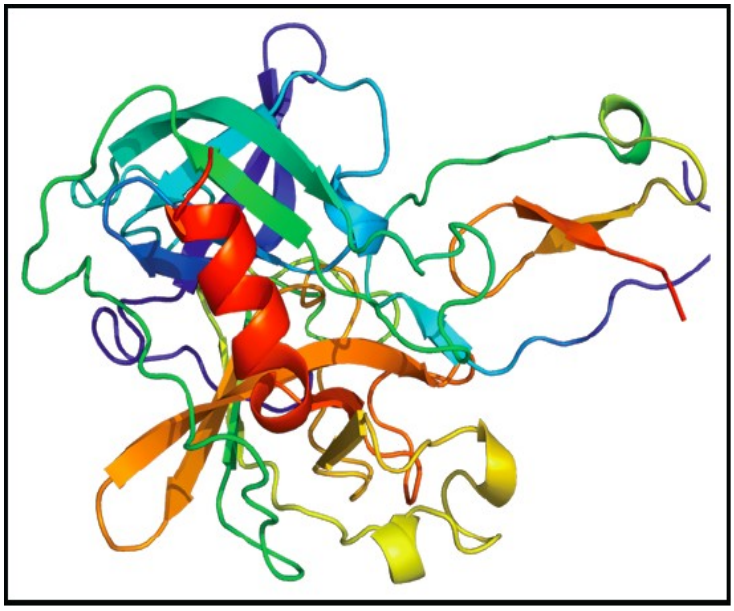

Figure 14: structure of elafin

Source and expression

Elafin is mainly produced by the body mucosae and by the skin. It was initially purified from highly inflamed mucosal secretions where neutrophils recruitment is prominent.

In the skin, elafin expression is constitutive in the squamous epithelium as a reflection of its immuno-modularity properties [117]. It has been observed that in some inflammatory diseases, as psoriasis, the expression of elafin is increased, correlating with the degree of neutrophils influx [118].

Functions and role in humans

It has been shown that elafin possesses a wide repertoire of activities, including antimicrobial [119-123] and immuno-modulator properties (figure 15).

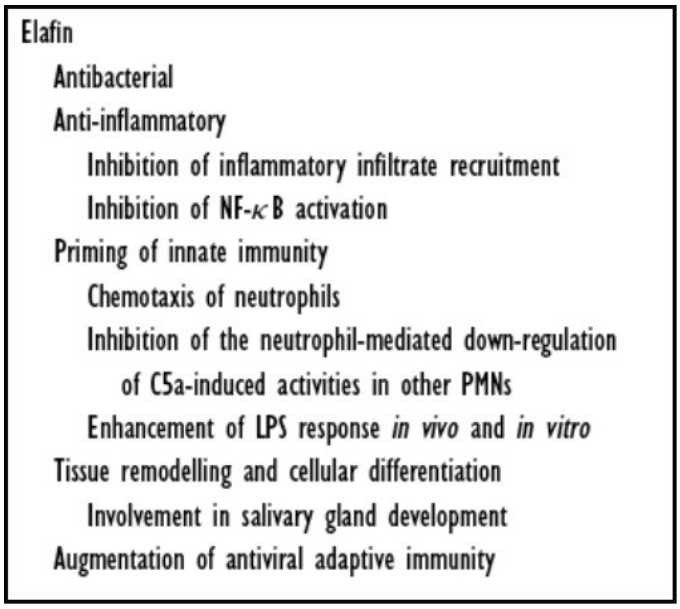

Figure 15: antimicrobial and antiinflammatory functions of elafin

The biochemical mechanisms of these properties have not been fully clarified but it has been speculated that the cationic nature of elafin allows it to disruptively interact with the anionic cell membrane.

It has been found that there is an increase of anti-proteases levels in some diseases where there is also an increase of protease activity, such as psoriasis and emphysema [125, 126]. Moreover, an increase in the level of these alarm anti-pro-

teases has also been found in diseases where no proteases activity is relevant, such as ischemic heart disease and lung cancer.

It has been recently demonstrated that the over-expression of elafin seems to prevent intestinal inflammation in the mouse model of colitis [127]. Furthermore, a recent study on humans has shown an increasing of elafin staining in inflamed tissue of ulcerative colitis and Crohn's disease compared to healthy controls (figure 16). This study has also shown a higher expression of elafin in Crohn's disease compared to Ulcerative Colitis. This observation, coupled with an imbalance between elastase and this antiprotease activity, could explain the more aggressive tissue destruction observed in Crohn's disease.

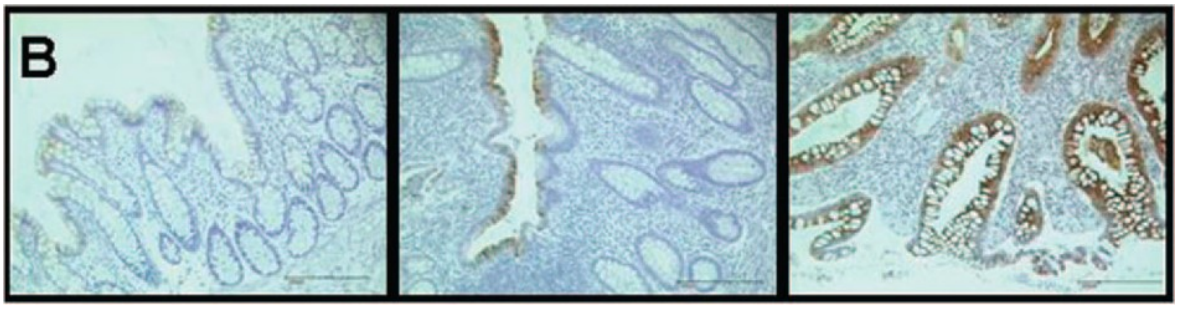

Figure 16: Representative immunohistochemical staining of elafin in controls (left), Crohn's disease (middle), and ulcerative colitis (right). From *Schmid M* et al. [128].

PSORIASIN

Psoriasin (also known as S100A7) is a member of the S100 gene family and was first isolated in 1991 from keratinocytes of psoriatic epidermis [129].

Genetics and structure

The psoriasin gene is located to chromosome 1q21.2-q22, within a cluster of genes that include at least 12 of the S100 gene family termed the “epidermal differentiation complex” (EDC) [130, 131].

Source and expression

In skin, psoriasin is focally expressed and released from keratinocytes, particularly in areas where high bacterial colonization is well documented, such as the uppermost parts of hair follicles and nose skin. Dry areas of the skin, such as lower leg skin, show instead a rather “patchy” staining for psoriasin. It is interesting to note that, apart from keratinocytes, sebocytes, the lipid-secreting cells of sebaceous glands, also show immuno-reactive psoriasin, suggesting that psoriasin is possibly also secreted together with lipids; probably, psoriasin is stored in the lipid layer of healthy skin [132].

Psoriasin has been found in other different tissues and organs beside skin, such as tongue, ear and eye. Recently it has been demonstrated that psoriasin is secreted by neoplastic keratinocytes in bladder carcinoma, in neoplastic breast ductal epithelium and in bronchial epithelium of patients affected by cystic fibrosis. Interestingly, it has been found that psoriasin protects the embryo from infections, since this molecule was found both in the vernix caseosa and in amniotic fluid [133].

Functions and role in humans

The skin is in direct contact with the environment and produces different AMPs with a broad spectrum of activity against several bacteria. *Glaser et al* [136] have demonstrated that, although different bacterial strains survive on fingertips [137],

E.Coli is rapidly killed. These authors have identified psoriasin (S100A7) as the principal *E.Coli*-killing AMP and the expression of this protein on the skin could explain why skin regions after exposed to high concentrations of E.Coli (as anogenital skin) are not usually infected from these bacteria.

Moreover, this observation could explain why patients with psoriasis, an inflammatory skin disease characterized by inflammatory lesions and hyper-proliferation of epidermis, could suffer from skin infections but rarely from *E.Coli* [138].

Patients with atopic dermatitis frequently have *Staphylococcus Aureus* super-infections and colonization of the skin, but not from *E.Coli*. The same group of authors have demonstrated that, although a deficiency of some AMPs is detectable, there is an enhanced epidermal psoriasin expression in atopic dermatitis [140]. This could indicate that the antimicrobial response in this skin disease is not generally impaired, but greatly differs according to the type of AMP produced by the skin.

RATIONALE AND AIM OF THE STUDY

Rationale of the study

The possible link between eosinophilic esophagitis (EoE) and other atopic diseases has already been investigated and extensively reviewed by *Jyonouchi et al* [141]. In particular, this link seems to be stronger between asthma/atopic dermatitis (AD) and EoE. In those diseases the remodeling of the involved tissues (basement membrane in the lung, basal layer of esophagus and epithelial layer in the skin) is due to the deposition of collagen with consequent fibrosis [142-144]. The Th2-skewed environment enhances the production and activation of eosinophils, creating favorable conditions to the development of atopy. In addition, the homing signals for Th2 cells and eosinophils are increased both in EoE and atopic diseases [30, 145-148]. These findings suggest that EoE, even when no allergic sensitization is proven, belongs to the Th2-driven diseases.

In particular, EoE shares some clinical and histological characteristics with AD, such as the macroscopic (i.e. they both are a “patchy” disease) and microscopic appareance (i.e. both of them are characterized by eosinophilic infiltration), the good response to topical steroids treatment and allergen avoidance, the difficult-to-find allergic causative sensitization.

Regarding etiopathogenesis, a hypothetical common mechanism between EoE and AD could be a dysregulation of the innate immune system. Several Authors have demonstrated a genetically determined epithelial barrier dysfunction in AD due to abnormalities in filaggrin function [149-151], while other studies have suggested that an alteration of epithelial barrier functions could be the basis for the development of atopic sensitization, atopic dermatitis and, possibly, asthma in patients with eczema [152]. In line with this observation, filaggrin mutations seem to have a role in the pathogenesis of asthma [153] and filaggrin expression seems to be down-regulated also in EoE, as recently demonstrated [154], although a previous study [155] failed to stain filaggrin in normal esophageal biopsies.

Another crucial component of the innate immune system is constituted by antimicrobial peptides and proteins (AMPs). In lesional AD skin it has been shown that some AMPs, such as cathelicidin and the β-defensins, may show defective functions [156, 157] and their expression is significantly reduced if compared to psoriasis, another chronic skin disease [158].

To underscore the importance of our investigation, while we were conducting this study, a study group from Colorado [159] has addressed the same hypothesis, although testing a more restricted panel of AMPs on a population that included only active EoE patients and controls.

These observations could re-enforce the possible link between EoE and AD.

Aim of the study

In this study we wanted to investigate the presence and expression levels of selected AMPs in esophageal biopsies (hBD-1, hBD-2, cathelicidin, elafin, and psoriasin) of children with active EoE (5 children), with EoE in remission (5 children) and in healthy controls (5 children).

METHODS

Patients and tissue selection

A total of 15 children (age range 0-18 years), undergoing EGD between January 2008 and January 2011, were studied.

Children were randomly and anonymously selected from a larger cohort of children, referred to the Children's Hospital of Philadelphia to undergo esophageal biopsies. These children were prior to the randomization divided into three groups prior to the randomization:

- children with positive esophageal biopsies (more than 20 eosinophils/hpf) and a clinical history compatible with EoE ("active" group);
- children with negative esophageal biopsies and a clinical history compatible with EoE ("not active" group);
- children with negative esophageal biopsies and no clinical history compatible with EoE ("negative" group).

From each group, 5 children were randomly selected. Biopsies were marked only with the archive's number and retrieved from Pathology Core archive.

The study was approved by the local ethical committee and a written consensus was signed by the parents and by the child (if older than 7 years old).

Immunohistochemistry (IHC)

Due to the limited number of slides obtained by the paraffinated biopsies, we chose to stain the slides with the most known hBD1, elafin and psoriasin. IHC for elafin, psoriasin and hBD1 was performed as follows (from [160]):

- deparaffinated and alcohol-fixed tissue sections from esophageal biopsies of children who underwent EGD were boiled for 30 minutes in buffered saline (pH 6.0), for antigen retrieval;
- after blocking of endogenous peroxidase, slides were incubated overnight with the primary antibody directed against elafin (Santa Cruz Biotechnology, Santa Cruz, California, USA; sc-20637, diluted 1 : 200), psoriasin (Biocarta, San Diego, California, USA, diluted 1 : 200) and hBD1 (AlphaDiagnostic International, San Antonio, Texas, USA, diluted 1 : 1.000);

- immunostaining was visualized using a detection kit as outlined by the supplier (Dako; K-5007, horseradish peroxidase-labeled secondary antibody, detection with 3'-diaminobenzidine tetrahydrochloride);
- sections were counterstained with hematoxylin.

Evaluation of immunostaining

To quantify AMPs expression we performed an image analysis study employing Image J software (NIH; http://rsbweb.nih.gov/ij/). Briefly, digital images were acquired with a dedicated microscope set-up (*Leica DMD 180*), RGB (red-green-blue) channels were split, blue channel (the one containing the largest information on immunostaining positivity) was thresholded and positive pixels were quantified. The green channel was employed to threshold the whole image section. The volume fraction occupied by the positive signal was quantified as a ratio between positive area/total area.

Real Time quantitative PCR

RNA preparation and reverse transcription

Frozen biopsies were disrupted mechanically in 1 mL of Trizol (*Gibco BRL,* Eggenstein, Germany) with an Ultra-Turrax (Branson, Danbury, Connecticut, USA)until complete fragmentation. Total RNA was extracted according to the supplier's protocol. RNA quality was determined by electrophoresis and quantified by photometry. Subsequently, 2 µg RNA was reverse transcribed with oligo dT-primers and 200U reverse transcriptase (RT) (*Superscript; Gibco BRL*), according to routine procedure.

Real-time PCR analysis

Real-time PCR analyses were performed using single-stranded cDNA from biopsies with specific oligonucleotide primer pairs in a thermocycler equipped with a fluorescence detection monitor (SD-7000, Applied Biosystem).

1 uL cDNA (corresponding to 10 ng of RNA) served as a template in a 20 uL reaction containing 10 uL of Power SYBR Green Master Mix (Roche Diagnostic), 0.5 uL of each primer (forward and reverse, 10 uM stock, ready to use) and 8 uL of RNA free water. All amplifications were carried out in triplicate. The temperature profile was 95°C for 10 seconds, 62°C for 5 seconds and 72°C for 2 seconds.

Primers sequences and amplicon size

Table 1 Oligonucleotide sequences used in real-time PCR analysis

Gene	Sense	Antisense	PCR product size
HBD1	5′ ATA CTT CAA AAG CAA TTT TCC TTT AT 3′	5′ TTG TCT GAG ATG GCC TCA GGT GGT AAC 3′	253 bp
HBD2	5′ ATC AGC CAT CAG GGT CTT GT 3′	5′ GAG ACC ACA GGT GCC AAT TT 3′	172 bp
HBD3	5′ TGA AGC CTA GCA GCT ATG AGG ATC 3′	5′ CCG CCT CTG ACT CTG CAA TAA 3′	206 bp
HBD4	5′ ATT CCT GAT GCC TCT TCC AG 3′	5′ CAT GGC TTT TTG CAG CAT TT 3′	156 bp
HD5	5′ GCC ATC CTT GCT GCC ATT C 3′	5′ AGA TTT CAC ACA CCC CGG AGA 3′	241 bp
HD6	5′ CCTCACCATCCTCACTGCTGTTC 3′	5′ TCAGCAGCAGAATGCCCAGAGTC 3′	269 bp
Elafin	5′ CGT GGT GGT GTT CCT CAT C 3′	5′ TTC AAG CAG CGG TTA GGG 3′	258 bp
Psoriasin	5′-AGA CGT GAT GAC AAG ATT GAC-3′	5′-TGT CCT TTT TCT CAA AGA CGT C-3′	234 bp
BPI	5′ GCA CCT GTT CCT GAT GGG 3′	5′ AGC ACA AAT GGA AAT TTC TTG 3′	255 bp
LL-37	5′ TCG GAT GCT AAC CTC TAC CG 3′	5′ GGG TCA CTG TCC CCA TAC AC 3′	190 bp
GAPDH	5′ CCA GCC GAG CCA CAT CGC TC 3′	5′ ATG AGC CCC AGC CTT CTC CAT 3′	360 bp
Il-8	5′ATG ACT TCC AAG CTG GCC GTG GC 3′	5′ TCT CAG CCC TCT TCA AAA ACT TC 3′	292 bp

BP, bactericidal/permeability-increasing protein.

From: Hosakaa Y et al. [160]

RT-qPCR data analysis

Analysis of relative gene expression data was carried out by employing the $2^{-\Delta\Delta Ct}$ method, as previously described [161].

Statistics

All statistical analyses and graphs were carried out using Prism 4.0 software (La Jolla, California, USA). For comparison of nonparametric quantitative RT-qPCR data we used the Kruskall-Wallis test. To compare differences among groups of IHC data we employed One-Way ANOVA followed by Bonferroni post-hoc test. Values of $P<0.05$ were considered statistically significant.

RESULTS

hBD1

Real-Time quantitative PCR

In order to verify whether the expression of hBD1 was dysregulated in patients with active and not active EoE, we performed a Real-Time quantitative PCR (RT-qPCR) experiment comparing EoE patients with healthy controls.

As shown in figure 1, when we compared the relative amount of hBD1 normalized to the internal reference (GAPDH) and relative to a calibrator (healthy controls) with the $2^{-\Delta\Delta Ct}$ method, we observed that patients with active EoE showed a trend toward increased expression of this gene with respect to both not active EoE and healthy controls. Furthermore not active EoE patients showed a trend toward a reduced expression compared to healthy controls. However, no statistically significant difference was observed. Moreover, the relative amount of target gene quantified by the $2^{-\Delta\Delta Ct}$ method showed that active EoE patients had 1.87 ± 3.56 fold the hBD1 expressed by healthy controls, and not active EoE patients had 0.54 ± 0.54 fold the hBD1 expressed by healthy controls.

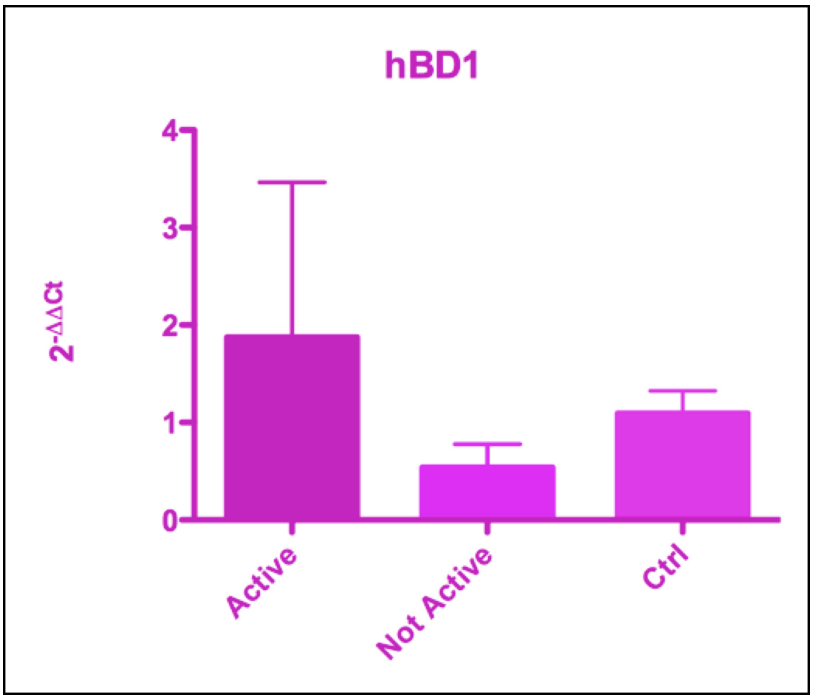

Figure 1: hBD1 expression evaluated by RT-qPCR; histograms represent mean and error bars indicate standard deviation

Immunohistochemistry (IHC)

Moving from the consideration that RT-qPCR data were suggestive but non conclusive of differential expression of the tested gene between active/not active EoE and healthy controls, we decided to quantify the protein expression by IHC and morphometry. As shown in figure 2, hBD1 could be detected in all cases; however, differences in protein expression between the three groups could be observed. To quantify these differences, a morphometric analysis was performed and the volume fraction occupied by the cells positive to this protein was computed. Figure 3 summarizes the results obtained from the statistical analysis. Interestingly, active EoE patients showed a statistically significant increased expression compared to not active EoE patients.

Figure 2: hBD1 expression in esophageal biopsies of active EoE, not active EoE and healthy controls.

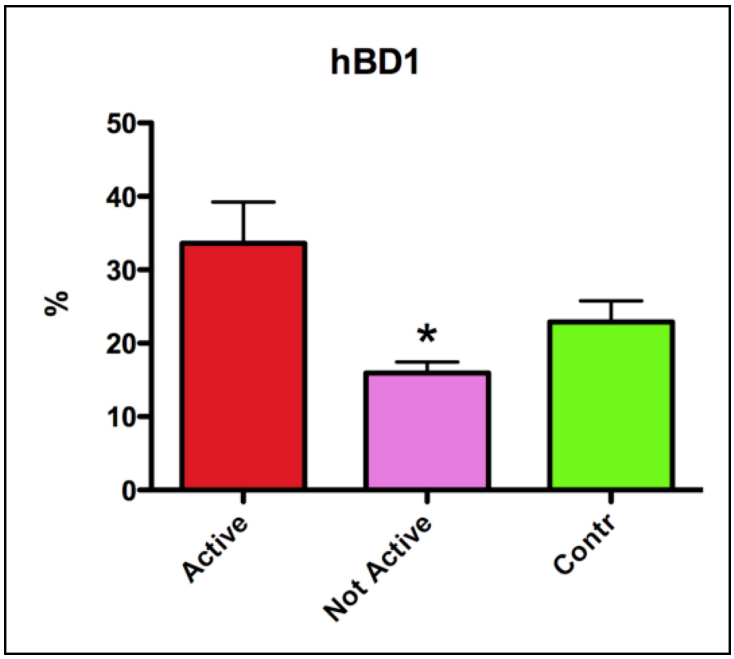

Figure 3: hBD1 expression evaluated by IHC; histograms represent mean and error bars indicate standard deviation.
p<0.05 versus active EoE patients.

Altogether these results indicate that EoE patients are characterized by altered expression of hBD1.

hBD2

Real-Time quantitative PCR

In order to verify whether the expression of hBD2 was altered in patients with active and not active EoE, we performed a RT-qPCR experiment, comparing EoE patients with healthy controls.

As shown in figure 4, when we compared the relative amount of hBD2 normalized to the internal reference (GAPDH) and relative to a calibrator (healthy controls) with the $2^{-\Delta\Delta Ct}$ method, we observed that patients with active EoE showed a trend toward reduced expression of this gene with respect to both non active EoE and healthy controls. However this difference failed to reach statistical significance. Quantitatively, the relative amount of target gene quantified by the $2^{-\Delta\Delta Ct}$ method showed that active EoE patients had 0.15 ± 0.14 fold the hBD2 expressed by healthy controls, and non active EoE patients had 0.22 ± 0.14 fold the hBD2 expressed by healthy controls.

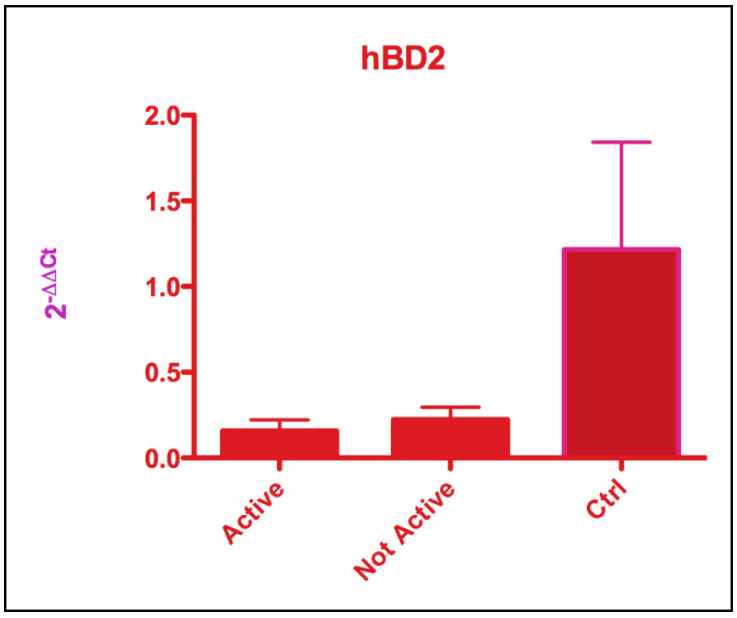

Figure 4: hBD2 expression evaluated by RT-qPCR ; histograms represent mean and error bars indicate standard deviation.

Cathelicidin (LL-37)

Real-Time quantitative PCR

In order to verify whether the expression of cathelicidin was dysregulated in patients with active and non active EoE, we performed a RTqPCR experiment, comparing EoE patients with healthy controls.

As shown in figure 5, when we compared the relative amount of cathelicidin normalized to the internal reference (GAPDH) and relative to a calibrator (healthy controls) with the $2^{-\Delta\Delta Ct}$ method, we observed that neither patients with active EoE nor those with not active EoE showed levels of cathelicidin different from healthy controls.

Quantitatively, the relative amount of target gene quantified by the $2^{-\Delta\Delta Ct}$ method showed that active EoE patients had 1.19 ± 1.12 fold the cathelicidin expressed by healthy controls, and not active EoE patients had 1.32 ± 1.45 fold the cathelicidin expressed by healthy controls.

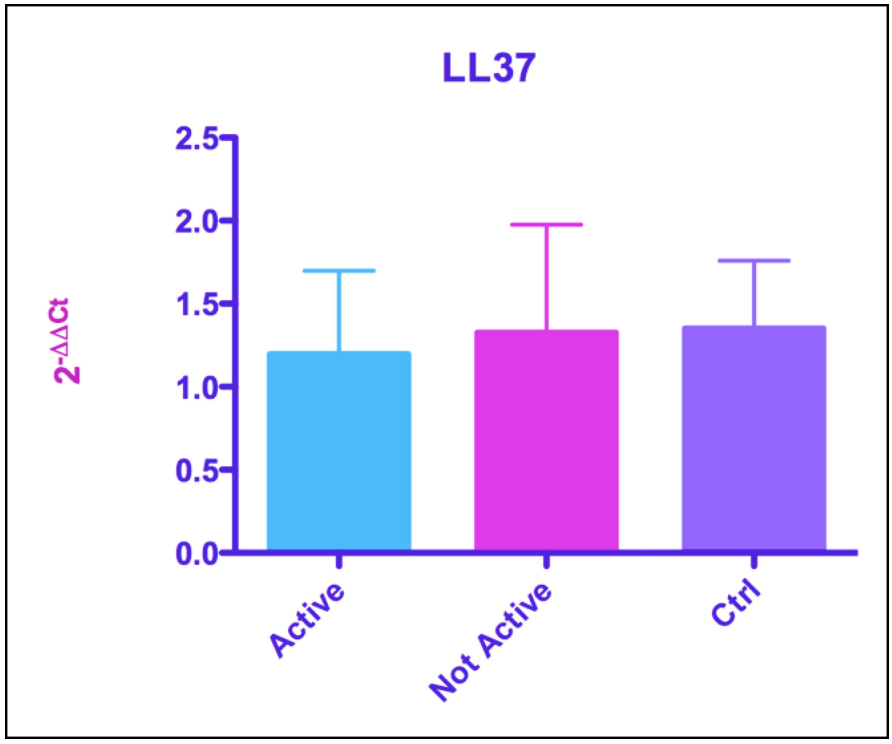

Figure 5: cathelicidin expression evaluated by RT-qPCR; histograms represent average and error bars indicate standard deviation.

Elafin

Real-Time quantitative PCR

In order to verify whether the expression of elafin was dysregulated in patients with active and not active EoE, we performed a RT-qPCR experiment, comparing EoE patients with healthy controls.

As shown in figure 6, when we compared the relative amount of elafin normalized to the internal reference (GAPDH) and relative to a calibrator (healthy controls) with the $2^{-\Delta\Delta Ct}$ method, we observed that patients with EoE showed a trend toward reduced expression of this gene with respect to healthy controls. However this difference failed to reach statistical significance.

The amount of target gene quantified by the $2^{-\Delta\Delta Ct}$ method showed that active EoE patients had 0.41± 0.38 fold the elafin expressed by healthy controls, and not active EoE patients had 0.52 ± 0.42 fold the elafin expressed by healthy controls.

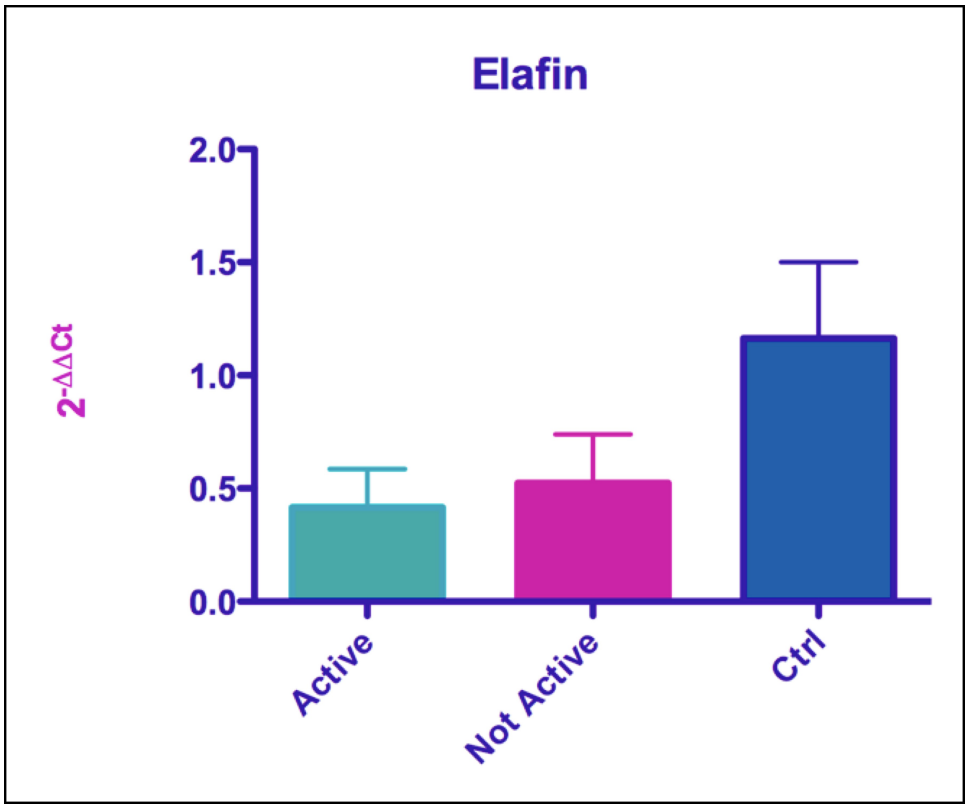

Figure 6: elafin expression evaluated by RT-qPCR; histograms represent average and error bars indicate standard deviation.

Immunohistochemistry

Moving from the consideration that RT-qPCR data were suggestive but non conclusive of differential expression of the tested gene between active/not active EoE and healthy controls, we decided to quantify the protein expression by IHC and morphometry. As shown in figure 7, elafin was detected in all cases; in addition, differences in protein expression between the three groups could be observed. To quantify these differences, a morphometric analysis was performed and the volume fraction occupied by the cells positive to this protein was computed. Figure 8 and Figure 9 summarize the results obtained from the statistical analysis. Although active EoE and not active EoE patients, taken as separate groups, failed to reach a statistically significant difference, the combined group of children suffering from EoE showed, irrespectively from the active or not active status of the disease, a statistically significant decreased expression of Elafin, compared to healthy controls.

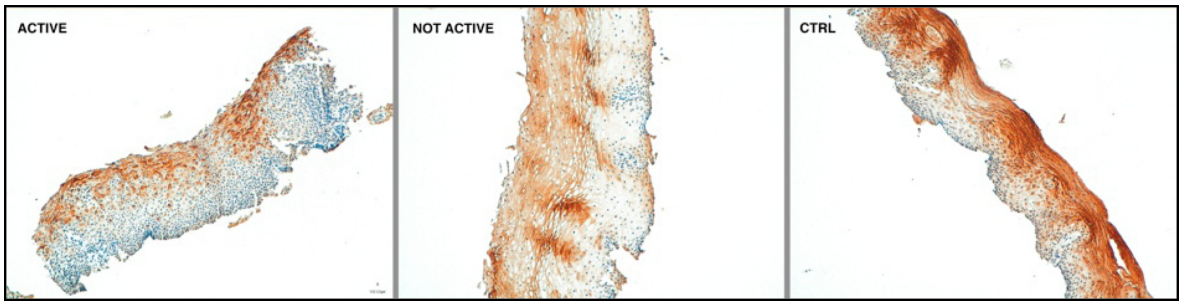

Figure 7: Elafin expression in esophageal biopsies of active EoE, not active EoE and healthy controls.

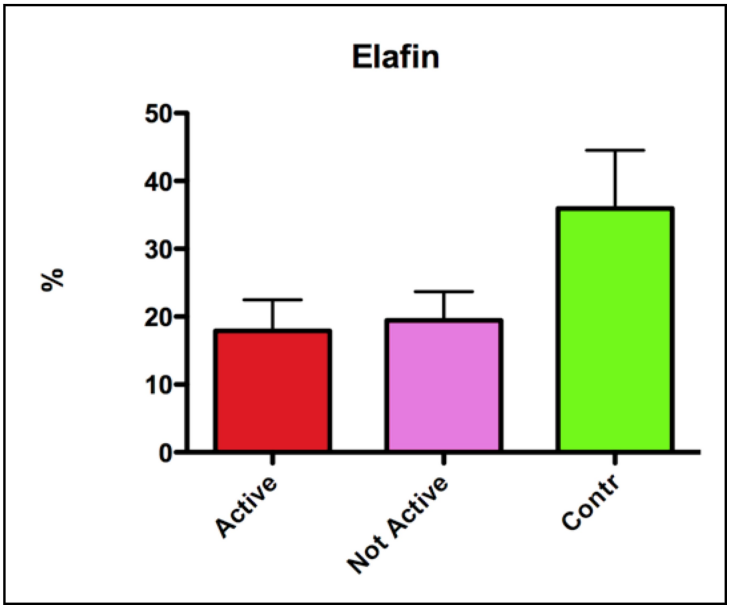

Figure 8: Elafin expression evaluated by IHC; histograms represent mean and error bars indicate standard deviation.

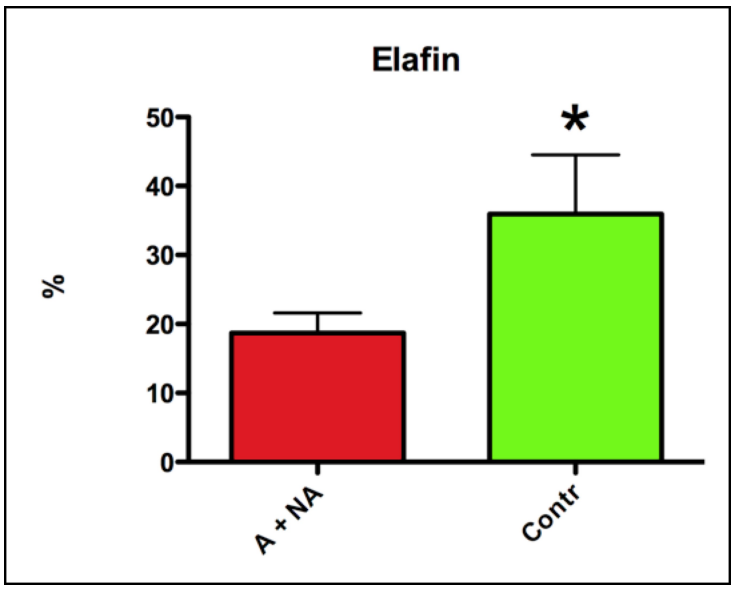

Figure 9: Elafin expression evaluated by IHC; histograms represent mean and error bars indicate standard deviation.
$p<0.05$ versus active+not active EoE patients.

Altogether these results indicate that the expression of Elafin is significantly reduced in EoE patients, irrespectively of the disease activity, strongly suggesting a constitutive down-regulation of this protein.

Psoriasin

Real-Time quantitative PCR

In order to verify an abnormal expression of psoriasin in patients with active and not active EoE, we performed a RT-qPCR experiment, comparing EoE patients with healthy controls.

As shown in figure 10, when we compared the relative amount of psoriasin normalized to the internal reference (GAPDH) and relative to a calibrator (healthy controls) with the $2^{-\Delta\Delta Ct}$ method, we observed that patients with active EoE showed a trend toward increased expression of this gene with respect to both not active EoE and healthy controls even that this difference is not statistically significant. The not active EoE patients showed a similar expression compared to healthy controls. The amount of target gene quantified by the $2^{-\Delta\Delta Ct}$ method showed that active EoE patients had 1.87 ± 2.14 fold the psoriasin expressed by healthy controls, and not active EoE patients had 1.45 ± 2.16 fold the psoriasin expressed by healthy controls.

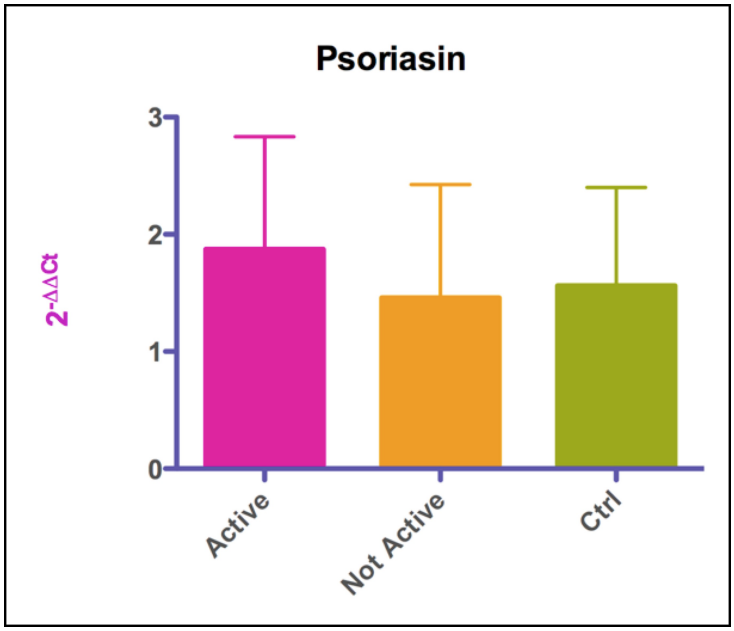

Figure 10: psoriasin expression evaluated by RT-qPCR; histograms represent average and error bars indicate standard deviation.

Immunohistochemistry

Moving from the consideration that RT-qPCR data were suggestive but not conclusive of differential expression of the tested gene between active/not active EoE and healthy controls, we decided to quantify the protein expression by IHC and

morphometry. As shown in figure 11, psoriasin was detected in all cases; and in all times, differences in protein expression between the three groups could be observed. To quantify these differences, a morphometric analysis was performed and the volume fraction occupied by the cells positive to this protein was computed. Figure 12 and Figure 13 summarize the results obtained from the statistical analysis. Interestingly, both active EoE and not active EoE patients showed a statistically significant difference compared to healthy controls.

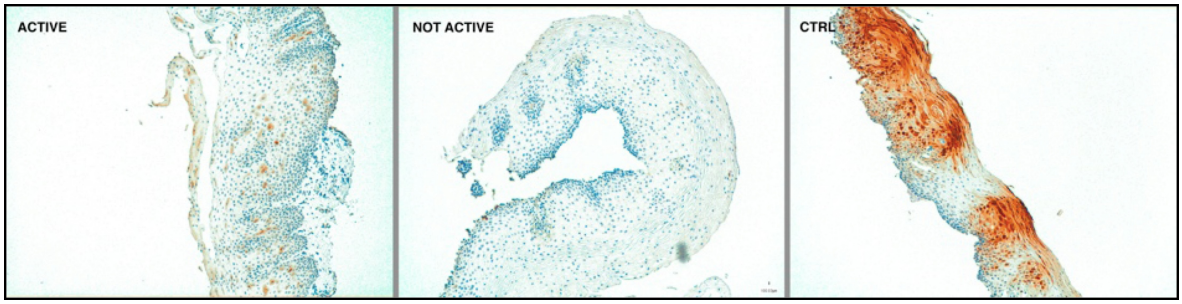

Figure 11: Psoriasin expression in esophageal biopsies of active EoE, not active EoE and healthy controls.

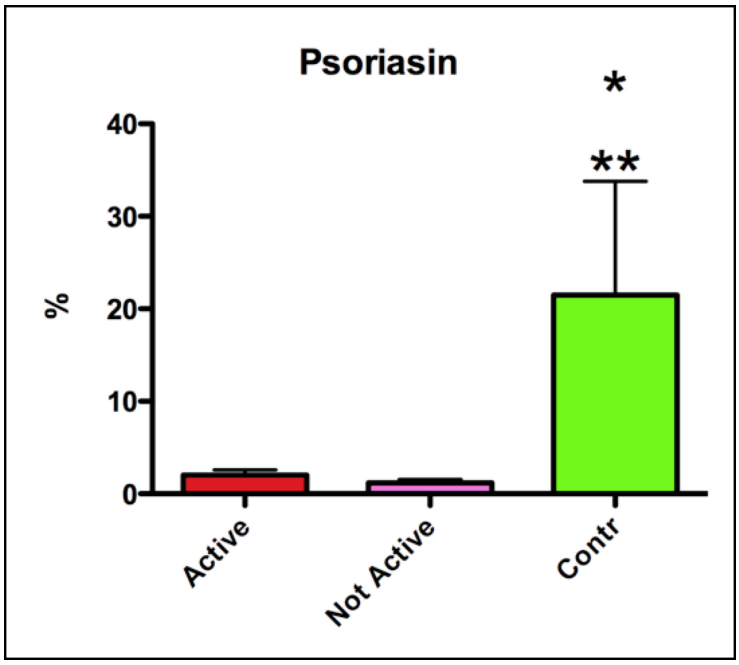

Figure 12: Psoriasin expression evaluated by IHC; histograms represent mean and error bars indicate standard deviation.

* p<0.05 versus active.

**p<0.05 versus not active EoE patients.

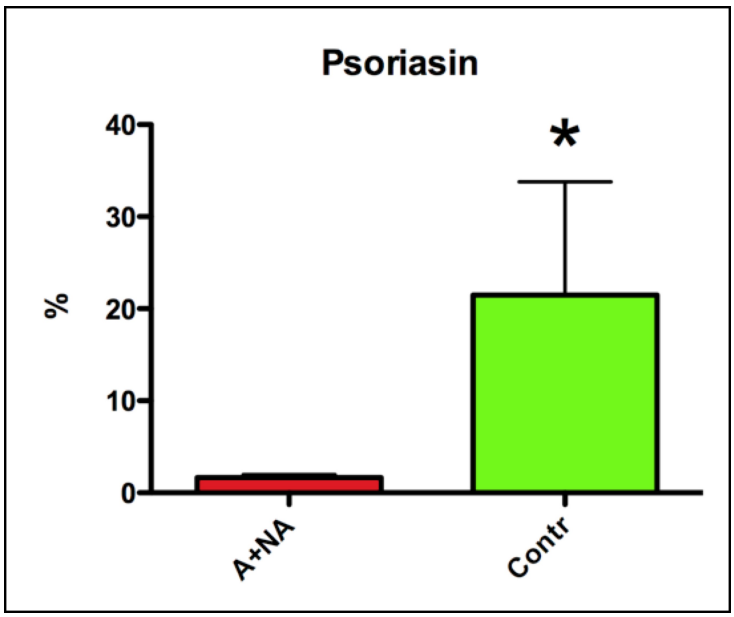

Figure 13: Psoriasin expression evaluated by IHC; histograms represent mean and error bars indicate standard deviation.

* $p<0.05$ versus active+not active EoE.

Altogether these results indicate that the expression of psoriasin is reduced in EoE patients, independently of the disease status, suggesting a constitutive downregulation of this protein.

Summary of the principal findings

Table 1 summarizes the principal findings of RealTime qPCR and immunohistochemistry analysis. It is apparent at a glance that there is an alteration of all tested AMPs.

AMP	RealTime qPCR		IHC		
	Active EoE	*Not Active EoE*	*Active EoE*	*Not Active EoE*	*Active + Not Active EoE*
hBD1	⬆ vs control (1.87 ± 3.56)	⬇ vs control (0.54 ± 0.54)		⬇vs active ❋	N/A
hBD2	⬇ vs control (0.15 ± 0.14)	⬇ vs control (0.22 ± 0.14)	N/A	N/A	N/A
Cathelicidin	=vs control (1.19 ± 1.12)	=vs control (1.32 ± 1.45)	N/A	N/A	N/A
Elafin	⬇ vs control (0.41± 0.38)	⬇ vs control (0.52 ± 0.42)	⬇ vs control	⬇ vs control	⬇vs control ❋
Psoriasin	=vs control (1.87 ± 2.14)	=vs control (1.45 ± 2.16)	⬇vs control ❋	⬇vs control ❋	⬇vs control ❋

Table 1: summary of presented data. Red boxes indicate upregulation, green boxes indicate down-regulation. Numbers between parenthesis indicate fold expression. ❋ indicate statistically significant data, p<0.05. N/A not assessed

DISCUSSION

The present work deals with a clinically relevant and original issue to deepen our knowledge on the pathophysiology of EoE and to improve both the diagnostic and therapeutic options for patients affected by this chronic invalidating disease.

This study was based on the consideration that EoE seems to share some clinical characteristics with AD. These two diseases, although localized to different districts with distinct embryological origins, have a similar macroscopic appearance, both being a “patchy” disease characterized by eosinophilic infiltration, and show a good clinical and histological response to topical steroid treatment and widened allergens’ avoidance. Regarding etiology, both diseases are characterized by a difficulty in finding a specific allergic causative sensitization. Indeed EoE is, at least partially, a Th2-driven disease and a pathogenetic role for Th2-cytokines, as IL-5 and IL-13, has been observed in EoE models [6, 21, 162, 163]. In AD a Th1/Th2 imbalance has also been demonstrated [164], and a defect in innate immunity has been detected [165]. The possible link between EoE, AD and other atopic diseases has been recently addressed by *Brown-Whitehorn and Spergel* [28]. In this review it is discussed how EoE shares clinical and histological characteristics with other atopic diseases, as AD, asthma, allergic rhinitis. However, a clear relationship between EoE and AD has yet to be proven.

The etiopathogenesis of EoE is an open research field and different studies have tried to analyze the role of defects in both innate and adaptive immune systems. Beside in well-known immunodeficiencies, alterations of the innate immune system have been found in other human diseases, such as in inflammatory bowel diseases [95, 127] and in atopic dermatitis [165]. It has been suggested that a defect in innate immunity and, specifically, in some AMPs could contribute to the increased susceptibility of patients with AD for viral skin infections, as eczema herpeticum [166], although controversies are still open in this regard [167].

However, it seems to be clear that a dysregulation of AMPs is present in AD (figure 1).

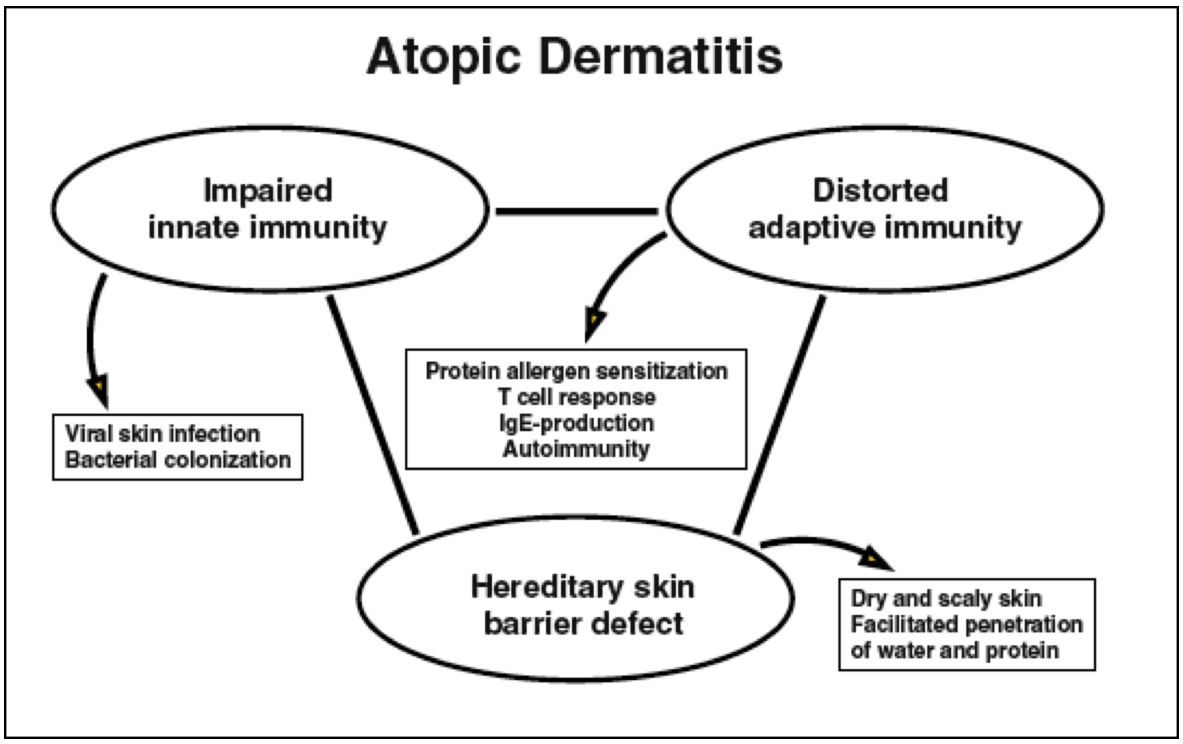

Figure 1: Immunopathogenesis of AD. (from Wollenberg et al. [156])

Therefore, we tested the hypothesis that, in analogy with AD, EoE patients could have a dysregulation of AMPs, too. For this purpose, a total of fifteen children were enrolled in this study; of these, five were affected by EoE (histology-proven) in active state, five were in remission state and five healthy children were tested as controls. We decided to test not only active EoE, but not active EoE patients too, since EoE is a progressive chronic disease, even when in a remission state. In this way, we aimed to evaluate whether there was an alteration of AMPs depending not only on active lesions but also on chronic inflammation, since AMPs comprise proteins and peptides whose expression is both constitutive and inducible by inflammatory cytokines and stimuli.

In our study population, we evaluated the expression of hBD1, hBD2, cathelicidin, elafin and psoriasin genes by RT-qPCR, observing an altered expression in the tested AMPs. Specifically, we documented a reduced expression of hBD2, cathelicidin and elafin in EoE patients (both active and not active) compared to controls; regarding hBD1, we found a slight increased expression in active EoE compared to controls (not statistically significant) and a reduced expression in not active EoE compared to controls. Last, we documented a slight increased expression of psori-

asin in active EoE with respect to controls, while not active EoE showed almost equal expression levels compared to controls. However, all these results failed to reach statistical significance.

Although RTqPCR is nearly the most sensitive and accurate method for gene expression quantification, these findings do not necessarily correspond to protein expression levels. Therefore, in order to verify whether these trends resulted in a similar altered expression of AMPs proteins, we analyzed the esophageal biopsies by IHC. We were able to confirm RT-qPCR data for hBD1 and elafin, while psoriasin showed reduced expression levels both in active and not active EoE patients compared to controls.

The possibility to recruit both EoE patients with active and not active disease gave us the opportunity to verify the behavior of inducible AMPs in response to inflammation. In this regard, the behavior of elafin and psoriasin is paradigmatic, since both active and not active EoE patients showed protein levels below controls. Importantly, a reduced induction of elafin [128] and psoriasin [168] has also been described in patients suffering from Crohn's disease (CD). Although this is not an atopic disease, CD is characterized by inflammation and dysregulation of AMPs. The importance of the topic addressed by this thesis is testified by the fact that, while we were in the process of conducting the analysis, a study group in Colorado (from the Section of Pediatric Gastroenterology Hepatology and Nutrition, Children's Hospital Colorado, CO) has reported in an international conference results that are in line with our observation. In particular, they observed a statistically significant reduced expression of hBD1-2-3 in EoE patients compared to healthy controls [159]. With respect to this latter study, however, the work of this thesis has broadened the observation to other AMPs (cathelicidin, elafin and psoriasin) and has added the information on non active EoE patients.

Limits of the study

This is an exploratory study whose aim was to investigate possible alterations of AMPs in EoE. For this reason the study population was limited thus reducing the

statistical power, especially for RT-qPCR analysis. Nonetheless, we were able to find important and novel statistically significant alterations that suggest that this phenomenon deserves further investigations on a wider cohort.

In addition, our study has shown an association between EoE and AMP alterations but it did not investigate whether these latter have a causative role in the etiopathogenesis of this disease. To address this issue, experimental studies both *in vitro* and *in vivo* are advocated.

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