Universitat Autònoma de Barcelona

# MECANISMOS CITOTÓXICOS Y HUMORALES IMPLICADOS EN LA ETIOPATOGÉNESIS DE LA ESOFAGITIS EOSINOFÍLICA

**MARINA FORTEA GUILLAMÓN**

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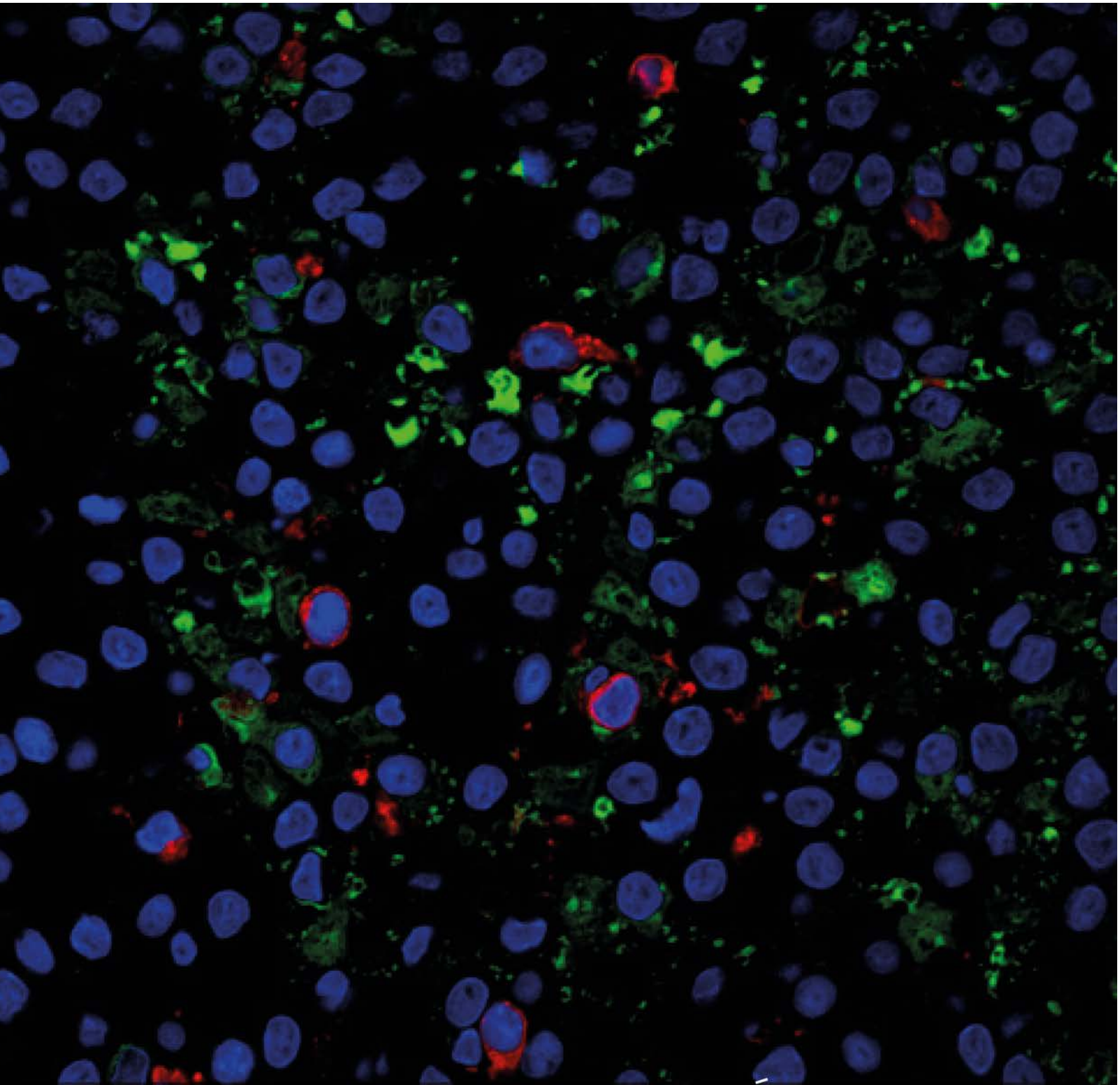

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TESIS DOCTORAL

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Universitat Autònoma de Barcelona

# "MECANISMOS CITOTÓXICOS Y HUMORALES IMPLICADOS EN LA ETIOPATOGÉNESIS DE LA ESOFAGITIS EOSINOFÍLICA"


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Tesis presentada para obtener el título de Doctor

Programa de doctorado de Bioquímica, Biología Molecular y Biomedicina

Departamento de Bioquímica i Biología Molecular

Universitat Autònoma de Barcelona

Barcelona, enero 2020

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HACEN CONSTAR

Que la memoria titulada "Mecanismos citotóxicos y humorales implicados en la etiopatogénesis de la Esofagitis Eosinofílica" presentada por Marina Fortea Guillamón para optar al grado de Doctor, se ha realizado bajo su dirección, y al considerarla concluida, autorizan su presentación para ser juzgada por el tribunal correspondiente.

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Dra. María Vicario Pérez

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La imagen de la portada corresponde a una micrografía tomada por microscopía confocal del epitelio esofágico de un paciente de EEo en la que se muestra marcaje de IgG4 (verde) y mastocito (rojo)

La imagen de la contraportada corresponde a una micrografía tomada por microscopía electrónica de la lamina propia del esófago de un paciente de EEo en la que se muestra el contacto entre una célula plasmática y un mastocito en proceso de degranulación, y que forma parte de los resultados de esta tesis.

"La educación no es aprender hechos,

sino entrenar la mente a pensar"

***Albert Einstein***

Mi sincero agradecimiento para todas las personas con las que he compartido con pasión las dificultades de este viaje fascinante y a todas aquellas personas de las que he tenido la oportunidad de aprender y crecer, tanto científica como personalmente.

A mi álter-ego, gracias por ser mi mentora, por no ser jefa, ser líder, por enseñarme que el factor "grupo" es lo más importante, que no siempre iba a ser fácil pero que si éramos una piña todo saldría adelante. Por enseñarme el amor incondicional a la ciencia. Y por ser rubia, por supuesto.

A Truman, por ser tan tú y tan único. Por esa actitud en la vida que te permitió crear el show, que menudo show y menudo lujo haber podido formarte parte de el.

A la directora del show en la sombra, que más que pitbull te quedas en labrador. Un placer tus puñales, sobretodo los afilados. Porque sumas y ayudas a crecer.

A Anita, Bea, Danila, Cristina Martínez, Marc y Amanda porque sin vuestra ayuda esto habría sido más que imposible. Por ser el mejor equipo de post-docs imaginable. Vuestro conocimiento, actitud y apoyo. Por escucharme y por estar ahí, siempre.

A Bruno, Elo y César por acogerme cuando entré, César con sus chistes y sus debates, Elo con su filosofía de vida y Bruno con su manera de cultivar mentes. Nunca podrá agradecer suficiente toda tu ayuda prestada y porque gracias a ti creo haber crecido mucho como científica. Se os echa de menos.

A todos los miembros del show, actuales y temporales. A Mercé y Elba porque habéis sido muchísimo para mí, sobretodo en esta recta final. Y los que nos queda señoras. A Noe, por ser mi compañera de poyata y de viajes. A Esther por ser la mejor estudiante que podía pedir, porque parte de esta tesis es tuya también, y me hace muy feliz. A Cris, Dori, Irene, María, Elisa (calabaza), Dani, Raquel, Helena por haber formado parte de esta etapa de mi vida, porque de todos he aprendido y todos me habéis regalado momentos épicos en este viaje.

A los vecinos. A Alba por apuntarte a todos mis bombardeos y porque me llevo una rubia de por vida. A mi Encarnita bonita, Fran, Marta, Guillaume, David porque sin vosotros hubiera sido todo un poco más aburrido.

A los infec-nanos, a Michele por inspirarme en los agradecimientos, a Leeena ppppor el macccccadrawwwer, a Anne por su castillo, a Marta por su inocencia y a Mur por ser científica, pero también diseñadora, informática y correctora, por tu ayuda y tu apoyo, sin ti esta desastresis me hubiera costado mil veces más, gracias.

A mi familia, que nunca fallan. Sobretodo a mis padres, que, aunque todo el mundo lo piense de los suyos, los míos si que son los mejores. Esta tesis es en gran medida vuestra, por animarme a todo.

*A mis padres,*

# RESUMEN

# RESUMEN

La esofagitis eosinofílica (EEo) es una enfermedad inflamatoria crónica del esófago caracterizada por disfunción esofágica asociada, principalmente, a la presencia de eosinófilos intraepiteliales. La EoE provoca un deterioro considerable en la calidad de vida de los pacientes, tanto niños como adultos, y los estudios epidemiológicos indican un aumento en su prevalencia en los últimos años. Aunque los mecanismos etiopatogénicos no están del todo definidos, se considera una enfermedad alérgica, dada la respuesta clínica e histológica a la eliminación de ciertos alérgenos y al tipo de infiltrado inflamatorio en el tejido. Actualmente su diagnóstico se basa en la presencia de 15 o más eosinófilos en el epitelio esofágico tras descartar la presencia de otras enfermedades que cursan con infiltrado eosinofílico esofágico.

Pese al creciente interés en esta entidad, los mecanismos que desencadenan la respuesta inflamatoria no están del todo dilucidados. Así pues, el objetivo de este trabajo ha sido analizar los integrantes de la respuesta inmunitaria, con especial interés en mediadores de respuestas citotóxicas y humorales, y su contribución al desarrollo de la EEo. Para ello se han desarrollado tres estudios; en el primero se ha caracterizado el perfil inmunitario en el esófago en sujetos sanos; en el segunde ha o analizado la función de los linfocitos CD8 y su modulación tras el tratamiento con inhibidores de la bomba de protones o con excusión dietética, así como los posibles mecanismos en un modelo celular in vitro; y en el tercero se ha evaluado la respuesta humoral en el tejido y su modulación tras la dieta de exclusión.

En el primer estudio se realizó la caracterización fenotípica de linfocitos T, linfocitos B, macrófagos y mastocitos. Se demostró que el esófago está ampliamente infiltrado por estas células sin observarse diferencias en las distintas regiones esofágicas. En cuanto a los estratos histológicos, se identificó que los más densamente poblados para todos los tipos celulares eran la papila vascular y la lamina propia, seguido del epitelio, en el caso de los linfocitos, y de la capa muscular en el caso de macrófagos y mastocitos.

El segundo estudio reveló un mayor infiltrado de células CD8+, caracterizadas por el marcador de activación CD2, en pacientes con EEo que revirtió de forma más eficaz en pacientes tratados con dieta de exclusión que en pacientes tratados con inhibidores de la bomba de protones. Además, se identificó un aumento de expresión de varios genes relacionados con la actividad de las células CD8 como enzimas citotóxicos (granzima A, granzima B, granulisina…) interleuquinas proinflamatorias (IL-10, IL-12B, IL-15) y catepsinas relacionadas con actividad peptidasa. En el modelo in vitro, se utilizó la línea celular Het-1A como modelo experimental, pero ciertas limitaciones no permitieron su desarrollo completo, mientras que la exposición de biopsias esofágicas ex-vivo parece ser una metodología más prometedora para el estudio de mecanismos citotóxicos en el epitelio esofágico.

El tercer estudio identificó un aumento de inmunoglobulinas (Ig) IgE, IgG total e IgG4 específicas frente a alérgenos, expresadas en mastocitos y células plasmáticas en el esófago de pacientes con EEo. La dieta de exclusión revirtió la producción de estas inmunoglobulinas en paralelo a la mejoría clínica.

Esta tesis contribuye a reforzar el potencial inmunológico del esófago y revela la coexistencia de mecanismos activos citotóxicos y humorales en la EEo que son modulados mediante el tratamiento. La presencia de inmunoglobulinas especificas frente a alérgenos en el esófago representa una nueva vía para el diseño de dietas eficaces en pacientes con EEo y suponen una aproximación a la medicina personalizada en esta enfermedad.

# RESUM

La esofagitis eosinofílica (EEo) és una malaltia inflamatòria crònica de l'esòfag caracteritzada per disfunció esofàgica associada, principalment, a la presència d'eosinòfils intraepitelials. La EEo provoca un deteriorament considerable en la qualitat de vida dels pacients, tan en nens com en adults, i els estudis epidemiològics indiquen un augment en la seva prevalença en els darrers anys. Tot i que els mecanismes etiopatogènics no estan del tot esclarits, es considera una malaltia al·lèrgica, donada la resposta clínica i histològica dels pacients a l'eliminació de certs al·lergògens i al tipus d'infiltrat inflamatori en el teixit. Actualment el seu diagnòstic es basa en la presència de 15 o més eosinòfils per camp de gran augment en l'epiteli esofàgic, després de descartar la presència d'altres malalties que cursen amb infiltrat eosinofílic esofàgic.

Tot i el creixent interès d'aquesta entitat clínica, els mecanismes que desencadenen la resposta inflamatòria no estan del tot dilucidats. Així doncs, l'objectiu d'aquest treball ha estat analitzar els integrants de la resposta immunitària, amb especial interès en mediadors de respostes citotòxiques i humorals, i la seva contribució al desenvolupament de la EEo. Per a això s'han desenvolupat tres estudis. En el primer s'ha caracteritzat el perfil de l'infiltrat immunològic en l'esòfag en subjectes sans; en el segon s'ha analitzat la funció dels limfòcits CD8 en la EEo i la seva modulació després del tractament amb inhibidors de la bomba de protons o amb exclusió dietètica, així com els possibles mecanismes en un model cel·lular in vitro; finalment, en el tercer estudi, s'ha avaluat la resposta humoral en el teixit i la seva modulació després de la dieta d'exclusió.

En el primer estudi es va realitzar la caracterització fenotípica de limfòcits T, limfòcits B, macròfags, mastòcits i eosinòfils. Es va demostrar que excepte d'eosinòfils, totes les capes de l'esòfag estan àmpliament infiltrades per aquestes cèl·lules sense observar-se diferències en les diferents regions esofàgics. Pel que fa als estrats histològics, es va identificar que els estrats més densament poblats per a tots els tipus

cel·lulars son la papil·la vascular i la lamina pròpia, seguit de l'epiteli en el cas dels limfòcits i de la capa muscular en el cas de macròfags i mastòcits.

El segon estudi va revelar un major infiltrat de limfòcits CD8+, caracteritzats pel marcador d'activació CD2, en pacients amb EEo que va revertir de forma més eficaç en pacients tractats amb dieta d'exclusió que en pacients tractats amb inhibidors de la bomba de protons. A més, es va identificar un augment d'expressió de diversos gens relacionats amb l'activitat de les cèl·lules CD8, com ara enzims citotòxics (granzima A, granzima B, granulisina...), interleuquines pro-inflamatòries (IL-10, IL-12B, IL-15) i catepsines relacionades amb activitat peptidasa. En el model in-vitro, la línia cel·lular Het-1A es va fer servir per a l'estudi d'aquesta malaltia, tot i que les limitacions van impedir el desenvolupament del model, mentre que el cultiu de biòpsies esofàgiques *ex-vivo* sembla ser un mètode més prometedor per a l'estudi de mecanismes citotòxics en l'epiteli esofàgic.

El tercer estudi va identificar un augment d'immunoglobulines (Ig) IgE, IgG i IgG4 totals i IgE i IgG4 específiques enfront d'al·lergògens, expressades en mastòcits i cèl·lules plasmàtiques en l'esòfag de pacients amb EEo. La dieta d'exclusió va revertir la producció d'aquestes immunoglobulines en paral·lel a la millora clínica.

Aquesta tesi contribueix a reforçar el potencial immunològic de l'esòfag i revela la coexistència de mecanismes actius tan citotòxics com humorals en la EEo que són modulats amb el tractament. La presència d'immunoglobulines especifiques enfront d'al·lergògens en l'esòfag representa una nova via per al disseny de dietes eficaces en pacients amb EEo i suposen una aproximació a la medicina personalitzada en aquesta malaltia.

# SUMMARY

Eosinophilic esophagitis (EoE) is a chronic inflammatory disease of the esophagus characterized by esophageal dysfunction and is associated with a dense infiltrate of intraepithelial eosinophils. EoE causes a considerable deterioration in the quality of life of patients, both children and adults, and epidemiological studies indicate a prevalence increase in the last years. Although the etiopathogenic mechanisms are not fully defined, it is considered an allergic disease, given the clinical and histological response to the elimination of certain allergens and the type of inflammatory infiltrate in the tissue. Currently, its diagnosis is based on the presence of 15 or more eosinophils per high power field in the esophageal epithelium after excluding the presence of other diseases with esophageal eosinophilic infiltrate.

Despite the growing interest in this entity, the mechanisms that trigger the inflammatory response are not entirely elucidated. Thus, the objective of this work was to analyze the participants of the immune response, with special interest in mechanisms leading cytotoxic and humoral responses, and their contribution to the development of EoE. For this, three studies were developed. In the first one, the cellular immune profile of the esophagus was characterized in health. In the second study, the function of CD8 lymphocytes and their modulation after treatment with proton pump inhibitors or with dietary food avoidance, were analyzed, as well as the possible mechanisms in an in vitro cell model. In the third study, the humoral response in the tissue and its modulation after the exclusion diet was evaluated.

In the first part, the phenotypic characterization of T lymphocytes, B lymphocytes, macrophages, mast cells and eosinophils was performed. It showed that except for eosinophils, the all esophageal layers are widely infiltrated by these cells without observing differences in the esophageal regions. Regarding the histological strata, it was identified that the most densely populated, for all cell types, were the vascular papilla and the lamina propria, followed by the epithelium in the case of lymphocytes and the muscular layer in the case of macrophages and mast cells.

The second study revealed a greater infiltration of CD8+ lymphocytes, featured by the activation marker CD2, in patients with EoE, which reversed more effectively in patients treated with an exclusion diet than in patients treated with proton pump inhibitors. In addition, an increase in the expression of several genes related to the activity of CD8 cells was identified as cytotoxic enzymes (granzyme A, granzyme B, granulysin...), proinflammatory interleukins (IL-10, IL-12B, IL-15) and cathepsins related to peptidase activity. In the in vitro model, the Het-1A cell line was used as a model for the study of this disease, however some limitations did not allow to fully developing it. Additionally, ex-vivo esophageal biopsy culture seems to be a more promising approach for the study of cytotoxic mechanisms in the esophageal epithelium.

The third study identified an increase in total immunogloblins (Ig) IgE, IgG and IgG4 and specific IgE and against allergens, expressed in mast cells and plasma cells in the esophagus of patients with EoE. The exclusion diet reversed the production of these immunoglobulins in parallel to clinical improvement.

This thesis contributes to reinforcing the immunological potential of the esophagus and reveals the coexistence of cytotoxic and humoral active mechanisms in the EoE that are modulated by treatment. The presence of specific immunoglobulins against allergens in the esophagus represents a new way for the design of effective diets in patients with EoE and represents an approach to personalized medicine in this disease.

# ABREVIATURAS

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ALI, *air-liquid-interface,* interfaz aire-líquido

APC, *antigen presenting cell*, célula presentadora de antígeno

AUC, *area under the curve*, area bajo la curva

BC, *B cell*, Célula B

BCL-6, *B cell lymphoblastoma protein 6*, proteina 6 de linfoblastoma de células B

CAPN, *calpain*, calpaina

CCL, *C-C motif chemokine ligand*, ligando de quimiocinas C-C

CD, *cluster of differentiation*, cúmulo de diferenciación

cga, campo de gran aumento

CLDN, *claudin*, claudina

CPA, célula presentadora de antígeno

CTS, *cathepsines*, catepsinas

DC, *dendritic cell*, célula dendrítica

DISC, *death inducing signalling complex*, complejo señalizador de inducción de muerte

DSG1, *desmoglein 1*, desmogleina 1

E, *epithelium*, epitelio

EDN, *eosinophil-derived neurotoxin*, neurotoxina derivada del eosinófilo

EE, *erosive esophagitis*, esofagitis erosiva

EEI, enfermedad inflamatoria intestinal

EEo, esofagitis eosinofílica

EoE, *eosinophilic esophagitis*, esofagitis eosinofílica

EVOM, *electrodes of the epithelial voltohmmeter*, electrodos para el voltohmetro epitelial

FADD, *Fas associated dead domain*, dominio de muerte asociado a FAS

FAS, *Fas cell surface death receptor*, receptor superficial de muerte celular fas

FDR, *false discovery rate*, tasa de descubrimientos falsos

GALT, *gut associated lymphoid tissue*, tejido linfoide asociado al intestino

GERD, *gastroesophageal reflux disease*, enfermedad por reflujo gastroesofágico

GZM, *granzyme*, granzima

HIER, *heat-induced epitope retrieval*, desenmascaramiento enzimático inducido por calor

hpf, *high power field*, campo de gran aumento

IBP, inhibidor de la bomba de protones

ICAM, *intercellular adhesion molecule*, molécula de adhesion celular

IELs, *intraepithelial lymphocytes*, linfocitos intraepiteliales

IFN-γ, *interferon gamm*a, interferon gamma

Ig, *immunoglobulin*, immunoglobulina

IL, *interleukin*, interleuquina

iNKT, *invariant NKT*, NKT invariante

JAM, *junctional adhesion molecules*, moléculas de adhesion celular

LE, *lymphocytic esophagitis*, esofagitis linfocítica

LP, *lamina propria*, lamina propia

LPLs, *lamina propria lymphocytes*, linfocitos de la lamina propia

LTc, *T cytotoxic lymphocytes*, linfocito T citotóxico

LTh, *T helper lymphocytes*, linfocitos T cooperadores

MBP, *major basic protein*, proteina básica principal

MC, *mast cell*, mastocito

MC, *circular musculature*, musculatura circular

ME, *musuclaris externa*, muscular externa

MHCI, *major histocompatibility complex class I*, comlejo principal de histocompatibilidad clase I

MHCII, *major histocompatibility complex class II*, comlejo principal de histocompatibilidad clase II

ML, *longitudinal musculature*, musculatura longitudinal

MM, *muscularis mucosae*, muscularis mucosa

MUC5B, *mucin 5B*, mucina 5B

NK, *natural killer*, célula asesina

NKT, *natural killer T cell*, célula T asesina

OCLN, *occludin*, ocludina

PAMPs, *pathogen-associated molecular patterns*, patrones moleculares asociados a patógenos

PBMC, *peripheral blood mononuclear cells*, células mononucleares de sangre periférica

PC, *plasma cell*, célula plasmática

POSTN, *periostin*, periostina

PPI, *proton pump inhibitors*, inhibidor de la bomba de protones

PRF1, *perforin 1*, perforina 1

PRR, *pattern recognition receptors*, receptores de reconocimiento de patrón

ROS, *reactive oxigen species*, especies reactivas de oxígeno

SC, *stratum conreum*, estrato córneo

SFED, *six-food elimination diet*, dieta de eliminación de seis alimentos

SG, *stratum germinativum*, estrato germinativo

SME, *esophageal submucosa*, submucosa esofágica

SNP, *single nucleotide polymorphism*, polimorfismo de base única

SPINK, *serine peptidase inhibitor kazal type*, inhibidor de la serina peptidasa tipo kazal

SS, *stratum spinosum*, estrato espinoso

STAT, *signal transducer and activator of transriptión*, transductores de señal y activadores de la transcripción

SYNPO, *synaptopodin*, synaptopodina

TEER, *transepithelial electrical resistance*, resistencia eléctrica transepitelial

TFH, *T folicular helper lymphocyte*, linfocito T cooperador folicular

TGF-β, *transforming growth factor beta*, factor de crecimiento transformante beta

Th1, *T helper type 1 lymphocyte*, linfocito T cooperador tipo 1

Th17, *T helper type 17 lymphocyte*, linfocito T cooperador tipo 17

Th2, *T helper type 2 lymphocyte*, linfocito T cooperador tipo 2

Th3, *T helper type 3 lymphocyte*, linfocito T cooperador tipo 3

TIA, *T-cell-restricted intracellular antigen*, antígenos intracelulares restringidos a células T

TNF-α, *tumor necrosis factor alpha*, factor de necrosis tumoral alfa

Tr1, *T regulator type 1 lymphocyte*, linfocito T regulador tipo 1

Treg, *T regulator lymphocyte*, linfocito T regulador

TSLP, *thymic stromal lymphopoietin*, linfopoietina tímica del estroma

VEGF, *vascular endothelial growth factor*, factor de crecimiento endotelial vascular

VP, *vascular papillae*, papila vascular

ZO, *zonula occludens*

# ÍNDICE

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# INTRODUCCIÓN

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## 1 FUNCIÓN Y ANATOMÍA DEL ESÓFAGO

El esófago es un órgano tubular con una longitud promedio de 25 cm y un diámetro de 1,5 cm, cuya función principal es transportar del bolo alimenticio desde la hipofaringe al estómago (**Figura 1**). Anatómicamente, el extremo superior comienza en la faringe, a la altura del cartílago cricoides, y desciende por el centro del mediastino posterior hasta el extremo inferior que termina en la cavidad abdominal por debajo del hiato esofágico del diafragma. El esófago se une al estómago en la unión esófago-gástrica, en el cardias o esfínter esofágico.[1]

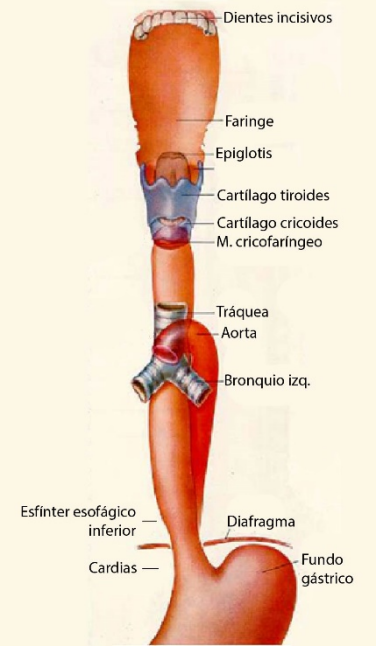


**Figura 1 | Esófago y órganos adjuntos.** Adaptado de Netter *et al.*,[2]

### 1.1 ESTRUCTURA DEL ESÓFAGO

El esófago está formado por una serie de capas de tejido consecutivas, cuya estructura, desde la luz esofágica, es la siguiente3,4 (**Figura 2**):

- Epitelio: está formado por un epitelio epidermoide plano pluri-estratificado no queratinizado, cuyas células se aplanan al aproximarse al lumen esofágico.

Lo constituye una capa de 500 a 800 µm de grosor medio y contiene prolongaciones o invaginaciones de la *lámina propria* llamadas papilas vasculares que proporcionan irrigación al epitelio y cuya altura es inferior a los 2/3 del total del grosor del epitelio. El epitelio esofágico se divide en 3 capas funcionalmente distintas, la capa basal o *stratum germinativum*, la capa intermedia, *stratum spinosum* o *prickle layer* y la capa superficial o *stratum corneum*.

La capa basal del epitelio contiene varias capas de células cúbicas y/o rectangulares con núcleos oscuros que carecen de glucógeno en el citoplasma. En esta capa se sitúan melanocitos y células neuroendocrinas, además de células linfoides. En la zona basal, sobre todo en las regiones cercanas a las papilas, se encuentran las células madre encargadas de generar las células del epitelio esofágico que se diferenciaran y migraran hacia las capas superiores. La capa intermedia contiene células de mayor tamaño y ricas en glucógeno que se van diferenciando, aplanando y cuyo tamaño del núcleo va disminuyendo conforme ascienden en el epitelio hacia la luz esofágica. En esta capa, las células presentan una elevada expresión de uniones estrechas, así como de digitaciones citoplasmáticas en los espacios intracelulares uniéndose mediante desmosomas a las células colindantes.

La capa superficial es muy estrecha y sus células contienen una elevada cantidad de tonofilamentos. Está diseñada para actuar como barrera mecánica entre la mucosa y el contenido luminal y frente a la penetración del ácido. Esta función barrera se lleva a cabo gracias a las estructuras intercelulares de unión.[5] Además, contiene una fina capa exterior de moco procedente de las glándulas de la submucosa que facilita el paso del bolo alimenticio.

- *Lámina propria*: Está compuesta por fibras de colágeno y fibroblastos que están incluidos en una matriz de glucosaminoglucanos. Esta capa presenta vasos sanguíneos y la mayoría de tipos de células inmunitarias como mastocitos, linfocitos, macrófagos y células plasmáticas.

- Muscular de la mucosa: Esta capa es de grosor variable, desde unas pocas fibras en la unión de la faringe hasta llegar a ser especialmente gruesa en el extremo inferior donde se acerca a la unión esofágico-gástrica. Está compuesta por fibras de músculo liso organizadas aleatoriamente en las zonas esofágicas proximales mientras que en las distales se organizan en capas longitudinales y circulares.[6]

- Submucosa: Está constituida por tejido conectivo laxo y contiene glándulas mucosas que segregan mucinas a la luz esofágica[7] cuya función es facilitar el transporte del bolo y proteger al epitelio frente al ácido. Distribuidos alrededor de estas glándulas abundan diseminados linfocitos, células plasmáticas y eosinófilos, que, en la región submucosa cercana a la unión esófago-gástrica se organizan en numerosos cúmulos de células linfoides formando pequeños folículos. La submucosa contiene numerosos vasos sanguíneos (plexo de Heller), vasos linfáticos y el plexo submucoso o plexo de Meissner, cuya función principal es el control de la secreción.

- Muscular: Esta capa está compuesta por fibras musculares, tanto estriadas como lisas, organizadas en capas circulares internas y longitudinales externas. Entre ambas capas se sitúa el plexo mientérico o plexo de Auerbach, que coordina los movimientos peristálticos propios del órgano. En el tercio superior las capas están formadas casi totalmente por músculo estriado, pero en el tercio medio se produce una transición gradual a musculatura lisa, de manera que las capas musculares del tercio inferior están formadas totalmente por músculo liso, continuando con las capas de músculo liso del estómago.

- Capa adventicia: Es la capa más alejada de la luz esofágica, a continuación de la capa muscular, y está formada por tejido conjuntivo laxo rico en adipocitos, pequeños vasos sanguíneos, canales linfáticos y fibras nerviosas.

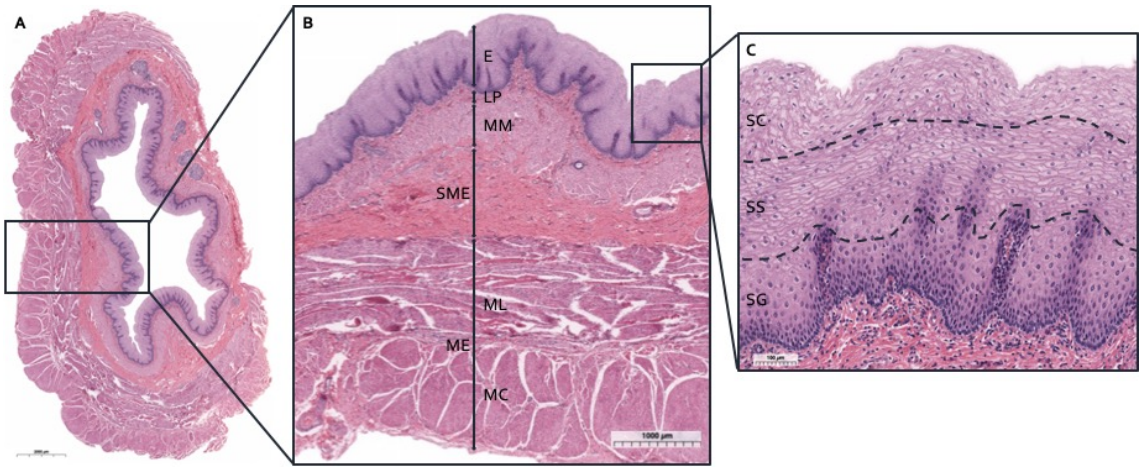


**Figura 2** | **Corte transversal del esófago medio** (Tinción con hematoxilina-eosina)[8].

A. Visión histológica general del esófago humano
B. Sección histológica transmural del esófago humano. E: epitelio, LP: *lamina propria*, MM: *muscularis mucosae* SME: submucosa esofágica, ME: *muscularis externa*, ML: musculatura longitudinal, MC: musculatura circular.
C. Sección histológica del epitelio esofágico humano. SC: stratum corneum, SS: stratum spinosum, SG: stratum germinativum

## 1.2 FUNCIÓN ESOFÁGICA

El esófago lleva a cabo las siguientes funciones:

### 1.2.1 FUNCIÓN MOTORA

La función principal del esófago es el transporte del bolo alimenticio hacia el estómago una vez finalizada la fase bucofaríngea de la deglución; constituyendo la tercera fase o la fase esofágica de la deglución. Cada región esofágica desempeña una función motora particular, esencial para el proceso de digestión del alimento:

a) **Esfínter esofágico superior:** es una zona de alta presión de unos 2-4 cm entre la faringe y el esófago constituido por músculo estriado. Su función principal es deglutir e impedir el paso de aire hacia el tubo digestivo durante la inspiración y el reflujo del bolo y/o gástrico a la faringe.[9]

b) **Cuerpo esofágico**, realiza tres tipos de movimiento peristáltico:
   - Peristaltismo primario: Estas contracciones u ondas peristálticas son la continuación de las originadas en la faringe. Las fibras musculares localizadas en la zona superior del bolo se contraen y las inferiores se

relajan impulsando el bolo hacía el esfínter esofágico inferior esfínter esofágico inferior.[10,11]

- Peristaltismo secundario: Esta contracción se inicia cuando la onda peristáltica primaria no consigue transportar el bolo por completo. La distención causada por un bolo residual o por reflujo del contenido gástrico activa los receptores sensitivos del esófago.
- Peristaltismo terciario: Esta onda peristáltica se inicia en el cuerpo esofágico en presencia o ausencia del bolo. La presión se eleva simultáneamente en transductores del cuerpo esofágico produciendo una onda peristáltica en el músculo liso de forma anómala y asociada al dolor.

c) **Esfínter esofágico inferior:** tras la deglución, cuando las ondas peristálticas esofágicas alcanzan el estómago, se produce una relajación del EEI, cuya presión se reduce a niveles similares a los del *fundus* gástrico, lo que permite el paso del bolo al estómago. Esta relajación dura entre 5 y 10 segundos y se produce una contracción para impedir el reflujo del contenido gástrico al esófago.[12]

### 1.2.2 FUNCIÓN BARRERA O DEFENSIVA

El esófago es un órgano complejo e inmunológicamente activo. Los diferentes mecanismos defensivos que tienen lugar en este órgano actúan como barrera protectora frente a sustancias deglutidas potencialmente nocivas, tales como sustancias microbianas o proteínas de la dieta, e incluso sustancias propias del organismo como la exposición al ácido del estómago. Los diferentes mecanismos defensivos se pueden estructurar en tres grupos: mecanismos físicos, químicos e inmunológicos.

#### 1.2.2.1 MECANISMOS FÍSICOS:

Dado que este órgano carece de funciones de absorción, las células epiteliales son las responsables de restringir la entrada de antígenos desde el lumen al organismo

y constituyen una importante barrera. Para ello existen varias proteínas implicadas en la formación de estructuras intercelulares de unión localizadas a lo largo de la membrana celular, esenciales para la integridad de la barrera epitelial.[13,14] Entre las distintas estructuras de unión el epitelio expresa: uniones estrechas o *tight junctions,* formadas por proteínas transmembrana como ocludina y claudinas, y las proteínas JAM (*Junctional Adhesion Molecules)* y no transmembrana como ZO (*Zonula Occludens*); uniones adherentes formadas por complejos de unión como e-cadherinas y desmosomas formado por proteínas desmogleinas.[5,15,16]

Se han identificado alteraciones de la barrera esofágica asociadas a la modulación de proteínas de uniones estrechas en enfermedades como el reflujo gastro-esofágico.[17] Estas alteraciones en las uniones epiteliales se han descrito en otras regiones del aparato digestivo, concretamente en enfermedades asociadas a disfunción de la barrera intestinal, como en el síndrome del intestino irritable, en el que dichas alteraciones se asocian a un aumento del espacio intercelular.[18]

#### 1.2.2.2 MECANISMOS BIOQUÍMICOS:

El esófago tiene la capacidad de responder ante estímulos externos con el objetivo de mantener la homeostasis interna. Para ello, cuenta con un gran repertorio de proteasas e inhibidores de proteasas a lo largo de las distintas capas del epitelio y de las regiones subyacentes, tanto en compartimentos celulares como extracelulares.[19] El epitelio desempeña una función barrera esencial, manteniendo no sólo la estructura física, sino también un correcto balance entre la producción de proteasas y sus correspondientes inhibidores, dado que la hidrólisis llevada a cabo por las proteasas es irreversible. El desequilibrio de esta homeostasis, además, sirve como sensor ante la entrada de antígenos o ante daño del propio epitelio, lo cual promueve respuestas inmunitarias y, en función del estímulo y del tipo de lesión, inicia la regeneración epitelial.[20]

El epitelio esofágico, dada la proximidad al estómago, cuenta con mecanismos para evitar el daño por ácido. La eliminación del ácido luminal se lleva a cabo mediante el

paso rápido del bolo alimentario gracias a la gravedad y a la peristalsis, además de la secreción de bicarbonato y de moco. El esófago también cuenta con cierta resistencia epitelial que le permite tener contacto con el contenido luminal ácido sin producir daños en su estructura. La resistencia epitelial se puede dividir en 3 componentes dependientes e interconectados entre sí: el pre-epitelial, el epitelial y el post-epitelial.

El componente pre-epitelial está compuesto principalmente por la capa de moco, agua y los iones de bicarbonato, cuya función es generar un gradiente de pH entre la luz esofágica y la superficie del epitelio para evitar el contacto directo del medio ácido de la luz con el epitelio. La función principal del moco es limitar la entrada de moléculas grandes, aun así, el moco esofágico no es tan eficaz como el del resto del tracto digestivo dado que su composición es principalmente de mucinas de tipo soluble, como Muc5B, incapaces de formar una red visco-elástica protectora. Aunque la concentración de iones de bicarbonato existentes en el moco en condiciones fisiológicas en el esófago no tiene la misma capacidad tamponadora que el moco de resto del tracto gastrointestinal, es suficiente para mantener el pH ente 5 y 7, condiciones en las que la pepsina permanece inactiva.

La defensa epitelial se basa en la capacidad esofágica de generar potencial eléctrico diferencial y mantener el pH en valores estables. La acidificación de los espacios intracelulares se resuelve acidificando el citosol mediante bombas de $Cl^-/HCO_3^-$ independientes de $Na^+$. Si, en consecuencia, disminuye mucho el pH en el interior de las células se previene con el efecto tamponador de $HCO3^-$, capaz de tamponar hasta 39 mmol de $H^+$. En caso de que no sea suficiente se compensa mediante bombas de $Na^+/H^+$ y, si hay suficiente $HCO3^-$ extracelular, con bombas $Cl^-/HCO3^-$, en este caso dependientes de $Na^+$.

La defensa post-epitelial se lleva a cabo por el torrente sanguíneo, mediante el aporte de nutrientes y oxígeno para la reparación celular y la eliminación de los deshechos del metabolismo como CO2 y ácido. Además, las células epiteliales también aportan HCO3- al espacio intercelular en caso de acidificación.

#### 1.2.2.3 MECANISMOS INMUNOLÓGICOS

A pesar de que el esófago no cuenta con un sistema de defensa inmunológico tan bien estructurado como el resto del tracto gastrointestinal existe cierto paralelismo entre el tejido linfático del esófago y la organización del tejido linfoide asociado al intestino (GALT; *gut associated lymphoid tissue*) (**Figura 3**).

El esófago contiene dos plexos capilares que constituyen el sistema linfático, formados por canales de 20-30 μm de diámetro[21], que se originan en la mucosa y en la capa muscular, a partir de los cuales se desprenden troncos colectores, de 100-200 μm de grosor, formando el plexo linfático peri-esofágico que desemboca en los ganglios para-esofágicos, escalonados a lo largo del esófago.[22] A pesar de carecer de una estructura organizada como el GALT, estos plexos capilares y nódulos linfáticos localizados en el esófago proporcionan los elementos necesarios para llevar a cabo la respuesta inmunitaria inductora mientras que los linfocitos distribuidos por el epitelio y por la *lamina propria* forman la respuesta efectora.

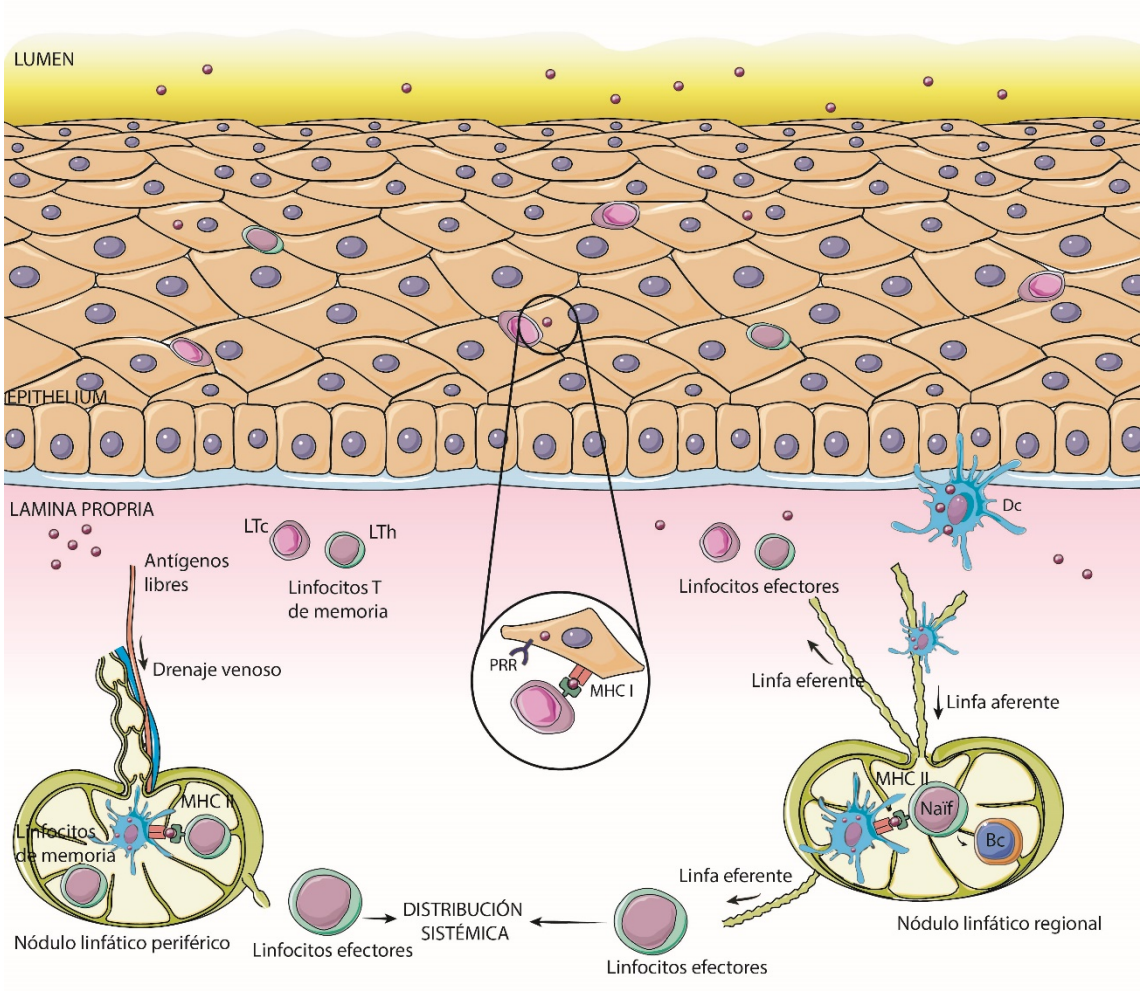


**Figura 3 | Esquema resumen de la respuesta inductiva y efectora en el esófago.** Dc: célula dendrítica; LTc: linfocito T citotóxico, LTh: linfocito T cooperador o *helper*, BC: linfocito B, MHC I: complejo principal de histocompatibilidad (*major histocompatibility complex*) tipo I; MHC II: complejo principal de histocompatibilidad tipo II; PRR: receptores de reconocimiento de patrón (*Pattern Recognition Receptor*). (Figura propia)

## RESPUESTA INDUCTORA

La respuesta inductora se lleva a cabo en los nódulos linfáticos, punto clave para el inicio de la respuesta inmunitaria ya que es el lugar donde se va a producir el contacto entre los linfocitos y los antígenos, ya sean libres o mediante células presentadoras de antígeno (CPA). Los nódulos linfáticos son órganos encapsulados que se ubican en la periferia del esófago (nódulos periféricos) o en el tejido esofágico (nódulos regionales), y constan de tres regiones anatómicas: la corteza, el paracortex y la médula. La corteza contiene folículos primarios y secundarios ricos en linfocitos B y células dendríticas. La paracorteza (paracórtex) contiene una elevada cantidad de

linfocitos T y células dendríticas. La médula, la región más profunda del nódulo, está compuesta principalmente por linfocitos B, linfocitos T y células plasmáticas.[23,24]

La respuesta inductora que se origina frente a la entrada de antígenos extraños precisa de la coordinación perfecta de todas las células que participan en sus diferentes fases. La entrada de antígenos se produce por la vía transcelular o por la vía paracelular. Estos antígenos pueden ser presentados por las células epiteliales mediante moléculas del complejo principal de histocompatibilidad (*major histocompatibility complex*, MHC) de tipo I, o viajar hasta los nódulos linfáticos de forma libre o capturada por medio de células dendríticas, siendo posteriormente presentados a linfocitos T vírgenes o *naïve* mediante MHC de tipo II.

Además de la respuesta inmunitaria específica, el huésped cuenta con receptores de reconocimiento de patrones (*pattern recognition receptor,* PRR) tanto en las células inmunitarias como en las células epiteliales. Estos receptores reconocen patrones moleculares asociados a patógenos (*pathogen-associated molecular patterns*, PAMPs)[25], moléculas presentes en microorganismos, lo que permite al sistema inmunitario detectar y responder a su entrada.

En el córtex las células dendríticas y los macrófagos reconocen y procesan los antígenos que llegan al nódulo linfático desde los plexos capilares. Una vez maduras, migran al paracórtex donde actúan como CPA a linfocitos *naïve* T cooperadores o *helper* (Th), mediante el MHC de tipo II y a células citotóxicas, mediante el MHC de tipo I. Esta presentación genera la activación de linfocitos T efectores, iniciando la respuesta inmunitaria adaptativa.[26] Por un lado, los linfocitos T efectores migran desde los nódulos hasta el tejido, mientras otros linfocitos se quedan en el nódulo como linfocitos de memoria o se desplazarán hasta los centros germinales para promover la diferenciación de célula B a célula plasmática. Los linfocitos asesinos naturales, o *natural killer* (NK), con actividad principalmente citotóxica, también participan en respuestas inmunitarias en el esófago.[27]

Los linfocitos T $CD4^+$ se diferencian según las citoquinas presentes en el medio (**Figura 4**), como consecuencia del reconocimiento antigénico por parte de las CPA[28],

en T-*helper* tipo 1 (Th1), T-*helper* tipo 2 (Th2), T-*helper* 17 (Th17), T-*helper* foliculares (TFH), células T-reguladoras (Treg), T reguladoras tipo 1 (Tr1) o T-*helper* tipo 3 (Th3) (**Figura 4**). La expresión de citoquinas como la interleuquina 12 (*interleukin*, IL) y el interferón gamma (*interferon gamma*, IFN-γ) favorecen el desarrollo de los linfocitos Th1. Por otra parte, la secreción de la IL-12 promueve la activación de las células NK lo que conlleva la producción de IFN-γ y el factor de necrosis tumoral alfa (*tumor necrosis factor alpha,* TNF-α) por parte de los linfocitos Th1.[29] Por otra parte, la expresión de IL-4 por parte de CPA inicia la diferenciación de las células $CD4^+$ hacia Th2, linfocitos que serán productores de IL-4, IL-5 e IL-13. Los linfocitos Th17 se generan tras la producción de factor de crecimiento transformante beta (*tumor growth factor beta,* TGF-β), IL-23 e IL-6 por parte de las CPA, generalmente en respuesta a una infección extracelular.[30] La diferenciación de las células TFH requieren señales de activación tanto por parte de CPA (IL-6 e IL-21)[31], como de células B, (Bcl6)[32]. Los linfocitos Treg se generan en ausencia relativa de patógenos y tras la unión de TGF-β a FoxP3, un factor regulador de la transcripción, en ausencia de otras interleuquinas[28], estas células pueden producir mensajeros solubles con función inmunosupresora como el TGF-β, IL-10 y adenosina.[33] Los linfocitos Tr1 se producen mediante (y son productores de) IL-10, principalmente, y los Th3 también producen IL-10 tras la detección de TGF-β. Estos dos tipos de linfocitos reguladores son especialmente importantes para el desarrollo de tolerancia a antígenos orales de la dieta y a la microbiota.

Los linfocitos T $CD8^+$ (LTc) se diferencian (**Figura 4**) a células efectoras citotóxicas mediante la expresión de IL-2, IL-12, IL-15 e IL-21 así como mediante el contacto con linfocitos T *helper*[26]. La IL-15, producida por células dendríticas, monocitos, macrófagos, células epiteliales y células B y T, además de tener un papel importante en la supervivencia y en la proliferación de los LTc, es clave en el desarrollo, diferenciación y supervivencia de las células NK y NKT invariantes (también linfocitos del sistema inmunitario innato).

Por otra parte, la diferenciación de las células B (**Figura 4**) requiere la presencia en el medio de TGF-β, IL-4, IL-5, IL-6 e IL-10, citoquinas producidas por las células dendríticas y también IL-21 producida por las células TFH.[34]

Una vez activados, los linfocitos T y B y las células plasmáticas migrarán principalmente a la médula y a los tejidos donde se haya inducido la respuesta inmunitaria, mientras células de memoria y algunas células plasmáticas quedarán residentes en los ganglios periféricos.[24]

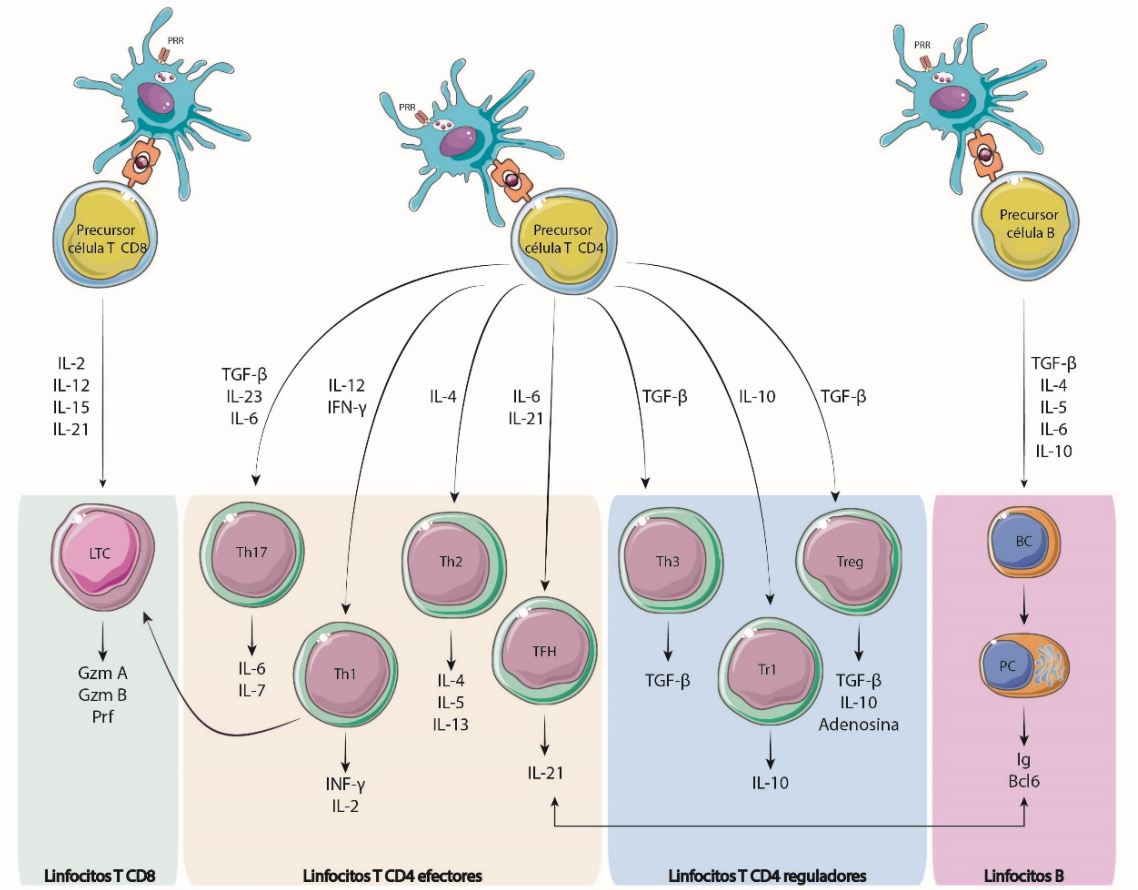


**Figura 4 | Clasificación de linfocitos B y T activos** y las citoquinas correspondientes para su diferenciación. IL: interleuquina; Gzm: granzima; Prf: perforina; TGF-β: factor de crecimiento tumoral beta (*tumor growth factor beta*), IFN-γ: interferon gamma; Ig: inmunoglobulina; BCL-6: proteina 6 de linfoblastoma de células B (*B Cell lymphoblastoma protein 6*); PRR: receptores de reconocimiento de patrón (*pattern recognition receptor*); LTC: linfocito T citotóxico, Th17: linfocito T *helper* 17; Th1: linfocito T *helper* tipo 1; Th2: linfocito T *helper* tipo 2, TFH: linfocito T *helper* folicular; Th3: linfocito T *helper* tipo 3; Tr1: linfocito T regulador tipo 1; Treg: linfocitos T reguladores; BC: linfocito B; PC: célula plasmática.

RESPUESTA EFECTORA

El epitelio esofágico, sobre todo la capa basal, es capaz de responder a señales pro-inflamatorias así como de iniciar y potenciar una respuesta inflamatoria al actuar como células presentadoras de antígeno.[35–37]

La respuesta efectora se lleva a cabo por el tejido linfoide difuso. Está formada por linfocitos dispersos a lo largo de la capa epitelial llamados linfocitos intraepiteliales (*intraepithelial lymphocytes,* IELs) y de la *lamina propria* (*lamina propria lymphocytes,* LPLs).

A lo largo de la *lamina propria* existe un elevado número tanto de linfocitos T $CD4^+$ como de linfocitos T CD8 residentes, una fracción de los cuales, principalmente de linfocitos $CD8^+$, migra hacia el epitelio.[38]

### Respuesta mediada por linfocitos T helper

La actividad de los linfocitos $CD4^+$ es fundamental en el control de infecciones; los linfocitos Th1 potencian respuestas contra virus y patógenos intracelulares mediante la producción de IFN-γ y TNF-α. El INF-γ aumenta la producción de especies reactivas del oxígeno y del nitrógeno en macrófagos, lo que aumenta su actividad antimicrobiana. El TNF-α también promueve la activación de macrófagos y aumenta su habilidad para fagocitar patógenos. Los linfocitos Th2 desarrollan la respuesta humoral y son clave en el control de infecciones por parásitos. Estos linfocitos facilitan la activación y a la diferenciación de los linfocitos B mediante la producción de IL-4, IL-5 e IL-13 y están involucrados en el reclutamiento de eosinófilos y mastocitos a los tejidos.

Los linfocitos Th17 participan en la respuesta ante patógenos extracelulares mediante la producción de IL-17A e IL-22.[39,40] La interleucina IL-17A juega un papel importante en la defensa de patógenos en las barreras epiteliales y mucosas. Esta interleucina activa el epitelio para que inicie la producción de quimioquinas responsables del reclutamiento de neutrófilos y macrófagos[41] y, juntamente con la

IL-22, participa en funciones de prevención de infecciones bacterianas mediante la producción de péptidos antimicrobianos y la activación de células epiteliales para atraer granulocitos.[41,42]

Respuesta mediada por linfocitos T citotóxicos

Los linfocitos $CD8^+$ efectores, difusos por el epitelio y la *lamina propria* son responsables de la defensa ante patógenos intracelulares, tanto virus como bacterias, lisando las células infectadas. La citolisis de las células infectadas se lleva a cabo tanto por células NK como por linfocitos T mediante la liberación de gránulos citoplasmáticos citotóxicos (**Figura 5**). La apoptosis llevada a cabo por las células T puede dividirse en dos vías, la vía apoptótica intrínseca y la vía apoptótica extrínseca. La vía apoptótica intrínseca esta regulada por la familia de la proteína 2 del linfoma de células B (BCL-2; *B cell lymphoblastome protein 2*) que mantienen el equilibrio entre la supervivencia y la muerte celular.[43] Estas proteínas regulan la integridad de la membrana mitocondrial y su potencial. La vía apoptótica extrínseca se desencadena mediante receptores de muerte transmembrana como FAS/CD95 o TNFR (receptor de TNF), ente otros, responsables de iniciar una cascada de reclutamiento tanto de proteínas adaptadoras (FADD (dominio de muerte de FAS; *FAS death domain*) o DISC (complejo señalizador de inducción de muerte; *death-inducing signaling complex*)) como de caspasas iniciadoras (caspasa 8 y caspasa 10) que escindirán caspasas efectoras (caspasa 3, 6 y 7)[44] y promoverán la activación de mecanismos de muerte celular.

La función efectora de los linfocitos citotóxicos se lleva a cabo mediante la exocitosis de diversas proteínas como proteínas formadoras de poro (perforina 1, PRF-1), proteasas (granzimas), péptidos antimicrobianos (granulisinas) o ligandos de muerte (FAS) así como varias hidrolasas lisosómicas incluyendo las cistein-catepsinas C, H y L.[45–48] Esta degranulación se ejecuta rápidamente tras la exposición de las células T a células somáticas infectadas o disfuncionales, que desencadenan la movilización de

gránulos preformados que contienen perforina, granzimas o granulisina así como ligandos de muerte.[49–51]

La entrada de granzimas y de péptidos microbianos en el citosol de la célula diana se produce gracias a la formación de poros por parte de la proteína perforina 1.[52–54] Las granzimas A y B son las serin-proteasas más abundantes de las 6 descritas en humanos (A, B, C, H, K y M).[55,56] La muerte celular mediada por granzima A es independiente de la activación de vías apoptóticas asociadas a BCL-2 o a la escisión caspasas[57]; actualmente se cree que esta respuesta se basa en la generación de especies reactivas de oxígeno (ROS *Reactive Oxigen Species*).[58] Además se ha demostrado que son capaces de regular la producción de citoquinas pro-inflamatorias como la IL1b.[59] Sin embargo, la citolisis mediada por granzima B se induce por la vía apoptótica intrínseca.[60,61] La actividad citolítica mediada por el resto de granzimas es todavía desconocida.

Es importante destacar el papel de una amplia familia de cistein-proteasas con elevada actividad proteolítica reguladas por citoquinas pro-inflamatorias, denominadas catepsinas (CTS). Existen varios tipos de CTS descritas, entre las cuales la CTS B, C, G, K, L1, L2, S y W están implicadas en el desarrollo o activación de los linfocitos T citotóxicos. La CTSC actúa como coordinador central de la activación de muchas serin proteasas en células inflamatorias y en la activación de granzima A y B. La CTSG desarrolla la función de eliminar patógenos y romper tejidos en órganos infamados. La CTSL2 se encarga de procesar la cadena invariante en la selección tímica de linfocitos. La CTSS promueve la degradación de proteínas antigénicas a péptidos para la presentación mediante MHC de tipo II. La CTSW es responsable de la regulación de la actividad citolítica de las células T.[62,63]

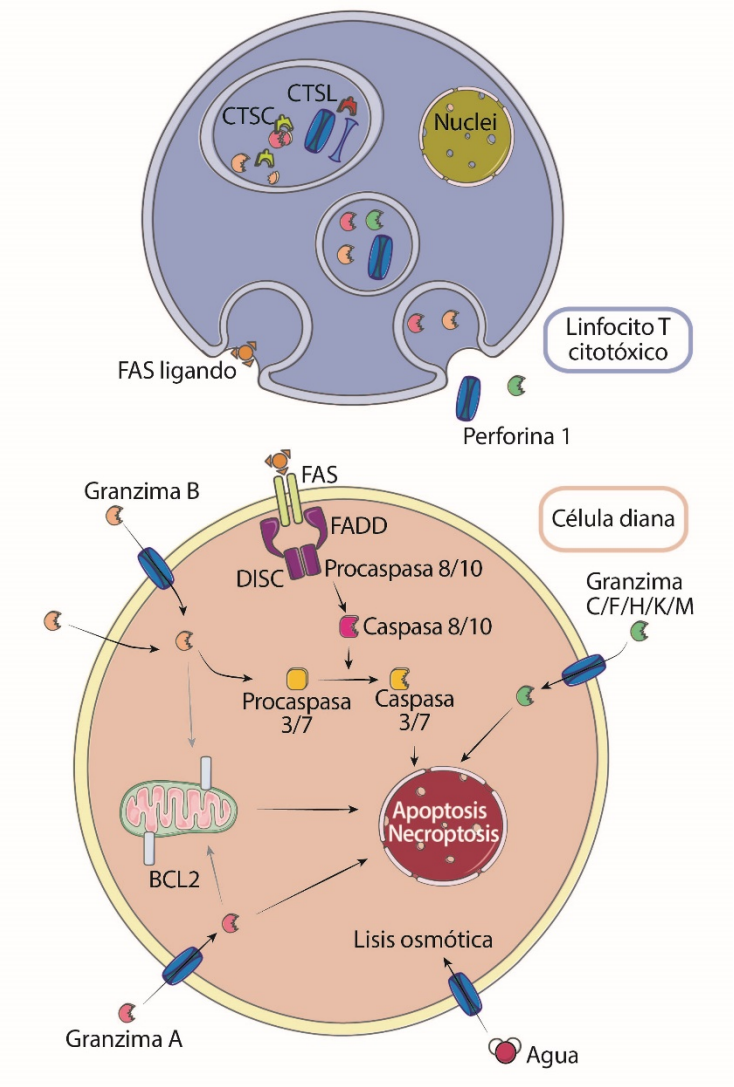


**Figura 5 | Mecanismos de muerte celular mediada por célula T.** Las células T pueden lisar las células diana mediante la exocitosis de gránulos, como granzimas y perforina, y la expresión de ligandos de muerte, como FAS. FADD: Dominio de muerte asociado a FAS, FAS associated *dead domain*. DISC: complejo de señalización de inducción de muerte, *death-inducing signaling complex.* BCL-2: célula B de linfoma 2, *B-cell lymphoma 2*. CTSC: catepsina C. CTSL: catepsina L.

En la *lamina propria* residen también linfocitos T $CD4^+$ y $CD8^+$ de memoria, los cuales, si vuelven a encontrarse con el antígeno para el que fueron activados, iniciaran una expansión clonal respondiendo de manera rápida y efectiva a la esta nueva exposición.

RESPUESTA ALÉRGICA Y REACCIONES DE HIPERSENSIBILIDAD

Las reacciones de hiperactividad se dan como respuestas inmunitarias desencadenadas por antígenos no relacionados con agentes infecciosos, generalmente ambientales, como polen, alimentos y fármacos.[28]

La alergia es el tipo de hipersensibilidad más común y corresponde a la hipersensibilidad e tipo I mediada por IgE en respuesta a antígenos solubles desencadenando la activación de células cebadas principalmente mastocitos y, en menor grado basófilos. La respuesta tipo II y III están mediadas por IgG y puede responder a distintas clases de antígeno, involucrando mecanismos de complemento y mecanismos efectores fagocíticos. Las respuestas tipo II se dirigen contra antígenos de la superficie celular o de matriz mientras que las de tipo III contra antígenos solubles.

Las reacciones de hipersensibilidad tipo IV se clasifican en subgrupos. El subtipo IVa se lleva a cabo por linfocitos Th1 que responden a antígenos solubles mediando la activación de macrófagos y originando una respuesta inflamatoria. La respuesta IVb está mediada por linfocitos Th2 quienes activan, sobre todo, eosinófilos, mastocitos y promueven la producción de IgE. El subtipo IVc esta mediado por células citotóxicas al reconocer antígenos propios, encargadas de lisar las células que presentan dicho antígeno. Por último, el subtipo IVd corresponde a respuestas inflamatorias mediadas por neutrófilos tras la exposición a antígenos externos.[64]

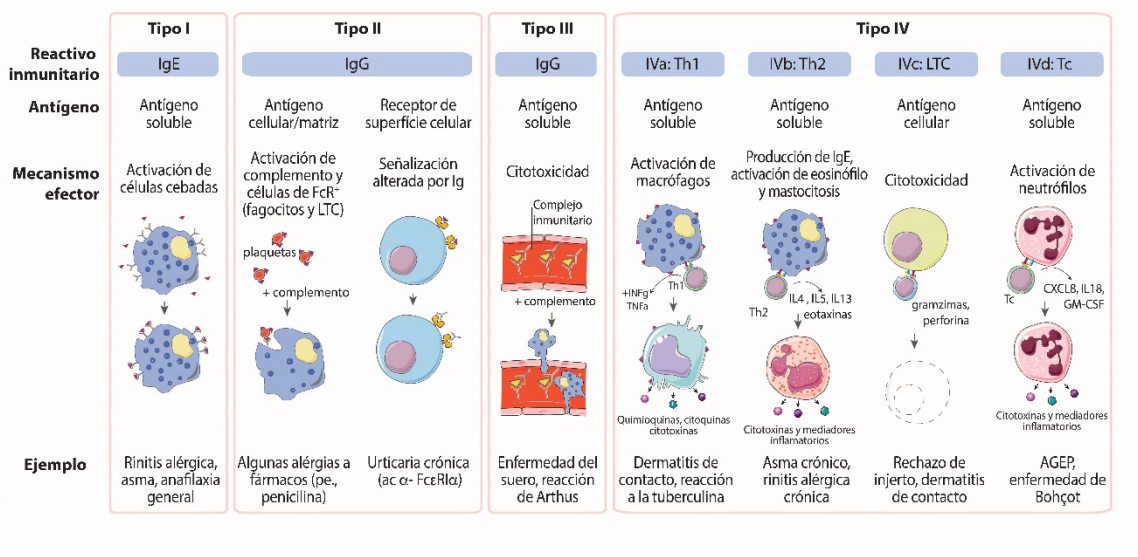


**Figura 6 | Respuestas de hipersensibilidad.** Adaptado de Janeway 2008[28].

# 2 ESOFAGITIS EOSINOFÍLICA

La esofagitis eosinofílica (EEo) es una enfermedad inflamatoria crónica del esófago mediada por antígenos, histológicamente caracterizada por la presencia de 15 o más eosinófilos por campo de gran aumento en el epitelio esofágico y clínicamente caracterizada por la presencia de síntomas de disfunción esofágica.[65–67] Esta enfermedad tiene un impacto significativo en la calidad de vida tanto de niños como de adultos.[68]

A diferencia del resto del tracto gastrointestinal, el epitelio esofágico carece de eosinófilos, de manera que la presencia de estos denota patología. Además de la EEo, otras enfermedades también se manifiestan con presencia de eosinófilos en el esófago, como la enfermedad por reflujo gastroesofágico (ERGE), infecciones parasitarias o fúngicas, o la enfermedad inflamatoria intestinal (EII), por lo que es necesario un análisis detallado para conocer la causa de la eosinofilia esofágica y establecer el diagnóstico adecuado.

## 2.1 EPIDEMIOLOGIA

La EEo es más común en varones (ratio de 2-3:1) de origen caucásico. La edad media de la población pediátrica afectada se encuentra entre 5,5 y 9,6 años[69] mientras que en adulto el rango de mayor afectación se encuentra entre los 30 y los 50 años.[70]

La EEo es una enfermedad emergente ya que su reconocimiento como entidad propia se produjo hace solo 25 años.[71] De todas formas, la incidencia y la prevalencia de la EEo ha ido aumentando en el tiempo de forma significativa. En un reciente meta-análisis realizado por Navarro P *et al.*,[72] la incidencia en la población pediátrica es de 7,7 por 100.000 habitantes/año y en la población adulta es de 6,6 individuos por 100.000 habitantes/año. En este último meta-análisis[72] (**Tabla 1**), se observa una prevalencia anual global de 34,2 por cada 100.000 habitantes en países desarrollados con una prevalencia más alta en población adulta comparada con la población pediátrica (42,2; 95%IC, 31,1-55 vs 34; 95%IC: 22,3-49,2, respectivamente). En el sub-

análisis según áreas geográficas, se describe una prevalencia más alta en Norte America en comparación con Europa (41 vs 29, respectivamente), aunque estas diferencias no alcanzan significación estadística (p=0,571).

**Tabla 1 | Estimaciones e intervalos de confianza (95%) de la prevalencia de EEo** basado en estudios epidemiológicos en niños y adultos. Adaptado de Navarro et al.,[72]

| Prevalencia | Total/100,000 | I2(%) | n | Adultos/100,000 | I2(%) | n | Niños/100,000 | I2(%) | n |
|---|---|---|---|---|---|---|---|---|---|
| **Total** | 34.2 (23.1-47.5) | 99.9 | 24 | 42.2 (31.1-55) | 99.9 | 9 | | 99.8 | 14 |
| **Subgrupos según área geográfica** | | | | | | | | | |
| **N. América*** | 41 (25.7-59.9) | 99.9 | 13 | 31.9 (21.5-44.3) | 100 | 7 | 38.3 (23.7-56.4) | 99.9 | 9 |
| **Europa** | 29 (19.9-39.8) | 99.6 | 9 | 95.8 (68.4-127.8) | - | 2 | 41 (3.2-121.4) | 92 | 3 |
| **Subgrupos según criterios diagnósticos** | | | | | | | | | |
| **Antes consenso 2007** | 15.4 (10.4-21.2) | 99.8 | 9 | - | - | - | - | - | - |
| **Después consenso 2007** | 30.1 (19.6-42.9) | - | 3 | - | - | - | - | - | - |
| **Después guías 2017 y AGREE 2018** | 63.2 (34.6-100.3) | 97.1 | 4 | - | - | - | - | - | - |
| **Subgrupos según el riesgo de seso del estudio** | | | | | | | | | |
| **Alto riesgo** | 32 (16.6-52.4) | 100 | 10 | 34.2 (21.4-49.9) | 100 | 7 | 35 (21.3-52.2) | 99.9 | 6 |
| **Bajo riesgo** | 38.3 (17.7-66.8) | 99.6 | 11 | 95.8 (68.4-127.8) | - | 2 | 34.9 (11.3-71.6) | 99.7 | 8 |

* EEUU y Canadá

## 2.2 SÍNTOMAS Y CARACTERÍSTICAS CLÍNICAS

Las manifestaciones clínicas difieren en función de la edad del paciente (Tabla 2). En la población pediátrica predomina la intolerancia alimentaria, vómitos, pérdida de peso, dolor abdominal y problemas para desarrollar patrones de alimentación, mientras que adolescentes y adultos presentan principalmente disfagia e impactación alimentaria. Estos síntomas pueden estar acompañados por pirosis, dolor torácico o epigástrico y dificultad de deglución.[73,74]

**Tabla 2 | Tabla de manifestaciones clínicas de EEo.** Adaptado de Carr y Watson 2011[75]

| | Bebés | Niños | Adolescentes y adultos |
|---|---|---|---|
| **Síntomas** | - Aversión / intolerancia a la alimentación<br>- Vómitos<br>- Rechazo de alimentos<br>- Asfixia con las comidas<br>- Problemas en e desarrollo | - Disfagia<br>- Asfixia / arcadas con texturas ásperas<br>- Impactaciones<br>- Dolor torácico / abdominal<br>- Vómitos / regurgitación | - Disfagia<br>- Impactaciones<br>- Evitación de alimentos<br>- Acidez intratable |
| **Condiciones asociadas** | - Alergia alimentaria<br>- Dermatitis atópica | - Asma<br>- Rinitis alérgica<br>- Alergia alimentaria | - Historia de atopia<br>- Asma<br>- Rinitis alérgica |

La EEo es una enfermedad crónica que no presenta periodos espontáneos de remisión, aun así, algunos estudios sugieren que ciertos pacientes sufren variaciones estacionales en los síntomas, consistente con la idea de que la enfermedad tenga una etiología relacionada con la exposición a aero-alérgenos.

Los hallazgos endoscópicos no son determinantes a la hora de diagnosticar la enfermedad dado que aproximadamente un 20 % de los pacientes presentan aspecto y calibre normales del esófago[76] (**Figura 7**). Las características morfológicas no-patognomónicas incluyen estenosis esofágica, anillos esofágicos, surcos longitudinales, pápulas blanquecinas y ulceraciones.[65,77]

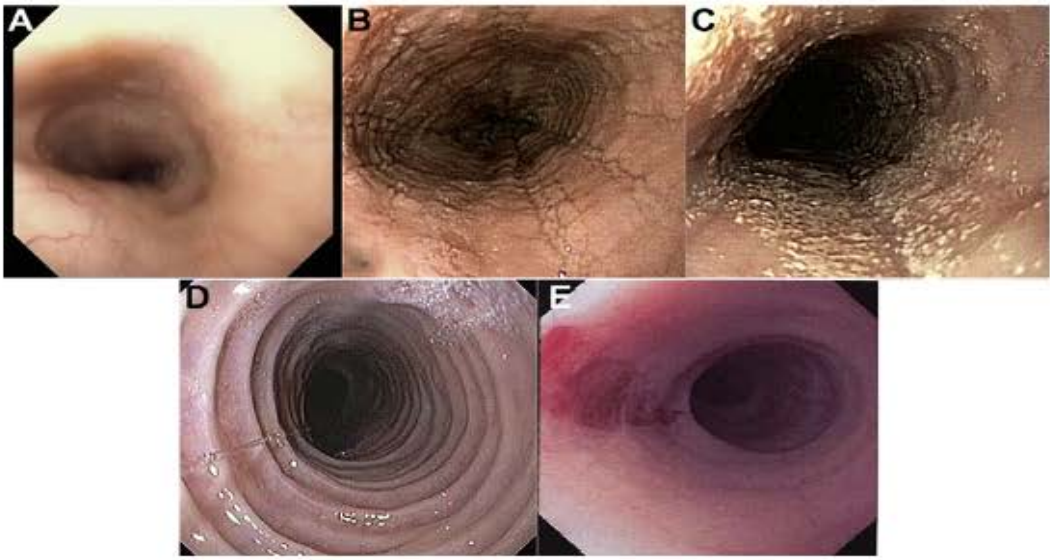


**Figura 7 | Hallazgos endoscópicos de pacientes con EEo.** Esófago normal B. Surcos longitudinales C. Placas de mucosa blancas D. Anillos esofágicos E. Estenosis esofágica.[65]

## 2.3 DIAGNÓSTICO

El diagnóstico de la EEo es complejo y debe ser realizado mediante la combinación de criterios clínicos e histológicos, así como la exploración de los diferentes factores de riesgo que aumentan la sospecha de la enfermedad. Es importante valorar la presencia de otras comorbilidades atópicas como asma, dermatitis o alergias alimentarias, así como la existencia previa de dicha enfermedad en la familia.

La detección de hallazgos endoscópicos característicos de la enfermedad durante la realización de gastroscopia aumentan la sospecha de su diagnóstico y pueden ser cuantificados mediante el criterio de puntuación endoscópico validado (eosinophilic esophagitis Endoscopic Reference Score, EREFS).[78] La toma de biopsias y su estudio posterior son determinantes para el diagnóstico de la EEo. Se deben tomar múltiples biopsias esofágicas, dado que histológicamente se manifiesta como una enfermedad parcheada, además de biopsias gástricas y duodenales para excluir otras enfermedades al descartar eosinofília gastrointestinal.[65,66,79]

Una vez realizado el análisis histológico, y tras confirmar el infiltrado de al menos 15 eosinófilos por campo de gran aumento, se realiza un diagnóstico diferencial de otras enfermedades asociadas con eosinofília esofágica como son: gastritis eosinofílica, gastroenteritis, colitis, ERGE, acalasia, trastornos motores esofágicos, síndrome hipereosinofílico, enfermedad de Crohn, infecciones fúngicas o virales, enfermedades de tejidos conectivos, enfermedades autoinmunes, pénfigo, hipersensibilidad a fármacos, enfermedad de injerto contra huésped o enfermedades mendelianas [66]. En particular, hay que subrayar la relación entre ERGE y EEo dada su complejidad y bidireccionalidad, pudiendo coexistir las dos entidades en el mismo paciente. De hecho, la persistencia del infiltrado eosinofílico tras el tratamiento durante 8 semanas con inhibidores de la bomba de protones (IBPs) ha sido recientemente eliminado de los criterios diagnósticos de la EEo. Los IBPs dejan de tener una utilidad diagnóstica para convertirse en una alternativa de tratamiento de primer orden en la EEo, a través de un mecanismo anti-inflamatorio directo, independiente de su acción inhibidora de la secreción ácido en el estómago.

Actualmente, a pesar de varias propuestas testadas, no existen marcadores no invasivos disponibles para el diagnostico o la monitorización de la enfermedad. No obstante, se esta trabajando en el desarrollo de herramientas mínimamente invasivas que presentan resultados muy esperanzadores como por ejemplo el *String test* o la cito-esponja **(Figura 8**). El *String test* consta de un hilo de 90 cm de longitud unido a una cápsula de gelatina en la que se pueden analizar proteínas derivadas de los eosinófilos puesto que el hilo se impregna de secreciones esofágicas mientras que la

cito-esponja es una esponja plegada en una cápsula que se expande al llegar al estómago y al extraerla realiza un barrido de las paredes esofágicas donde quedan adheridos restos de tejido que pueden analizarse posteriormente, tras la inclusión del dispositivo en parafina.[80,81]

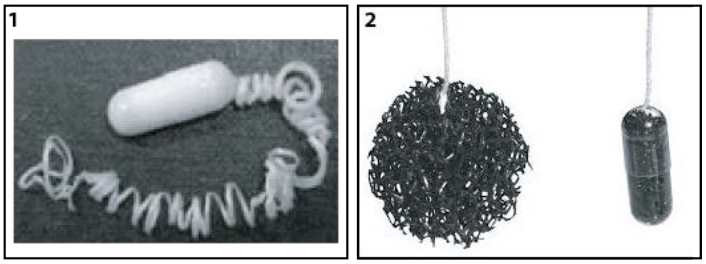


**Figura 8 | Herramientas mínimamente invasivas testadas en EEo**. 1. *String test* 2. Citoesponja

## 2.4 TRATAMIENTO

Actualmente se aplica el siguiente algoritmo[67] para el tratamiento en pacientes con EEo. El orden de tratamiento se decide según la experiencia del centro y mediante consenso entre el médico y el paciente. El tratamiento se considerará efectivo no sólo por la mejora de la sintomatología sino por la resolución de la inflamación esofágica. Los síntomas clínicos no correlacionan con la actividad histológica de la EEo y, por lo tanto, la valoración histológica mediante realización de gastroscopia con biopsias sigue siendo necesaria (declaración nº18 de la guía europea de EEo).[67]

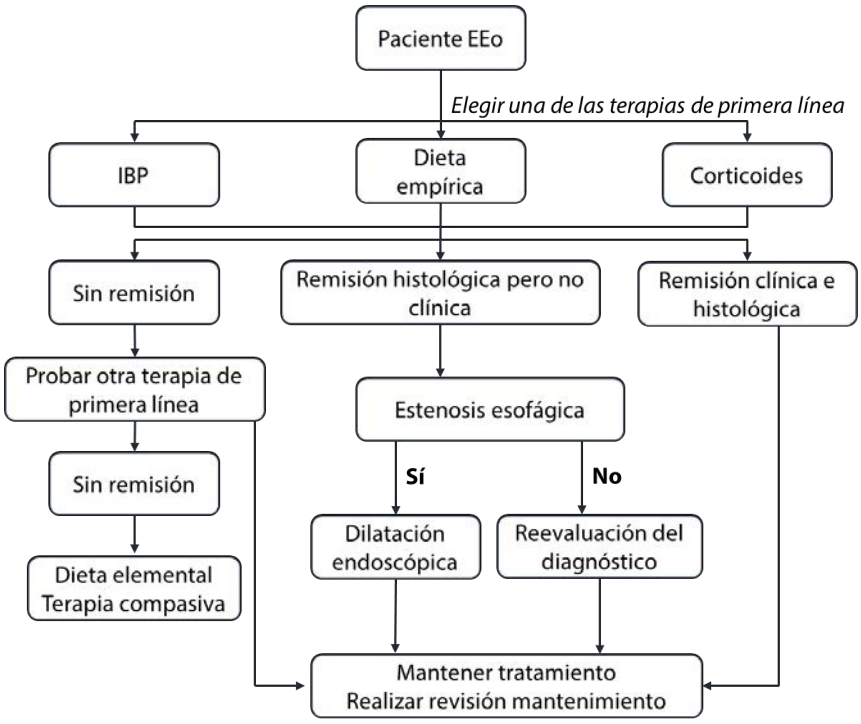


**Figura 9 Algoritmo de tratamiento de la EEo.** Adaptado de Lucendo 2017 [67].

### 2.4.1 TRATAMIENTO DIETÉTICO

El tratamiento dietético consistente en evitar la exposición a alimentos según su potencial alergénico. Es una alternativa efectiva en el 50-70 % de los pacientes con EEo[82,83] y reduce tanto el infiltrado inflamatorio, la hiperplasia epitelial como la fibrosis subepitelial en paralelo a la mejoría clínica de los pacientes.

Existen varios modelos dietéticos:

- Dieta elemental: consiste en la administración de una fórmula elemental amino-acídica durante un periodo de tiempo concreto [77]. A pesar de resultar muy eficaz, llegando a un 90,8 % de respuesta en pacientes pediátricos[67,84] (Kelly *et al.*, 1995; Lucendo *et al.*, 2017), su uso especialmente a largo plazo resulta poco factible (80 % de los niños pueden precisar sonda naso-gástrica).
- Dieta de exclusión guiada por test de alergia: consiste en la eliminación de alimentos según tests de alergia en la piel o en parches. Esta dieta ha obtenido resultados poco satisfactorios, obteniéndose en adultos una respuesta de alrededor del 24 %[85,86], mientras en niños la respuesta varía entre un 55 y un 77 %.[87,88]

- Dieta de exclusión de 2-4 o 6 alimentos: consiste en la eliminación de 2, 4 o 6 alimentos de la lista de 8 alimentos potencialmente alergénicos, responsables del 90 % de las reacciones alérgicas.[89] En pacientes adultos bajo dieta de 2 alimentos se retiran la leche y el gluten y se logran unas tasas de remisión del 43 %.[89] En la dieta de 4 alimentos se retira a los pacientes legumbres y huevo además del gluten y la leche. En estos casos la remisión es del 60 %.[89] Por último, se puede realizar una dieta de exclusión de 6 alimentos dónde se retirarán el pescado y el marisco y los frutos secos además de los alimentos excluidos en la dieta de 4 alimentos, esta dieta ha resultado ser efectiva para el 79% de los pacientes.[83,89–91]

### 2.4.2 TRATAMIENTO FARMACOLÓGICO

Los tratamientos farmacológicos utilizados actualmente son los siguientes:

- Glucocorticoides sistémicos: como la prednisona o metilprednisolona, que han resultado ser un tratamiento efectivo en el 60 % de los pacientes en ambas poblaciones. Aun así, los resultados son muy variables según tipo de glucocorticoides, dosis y tiempo de mantenimiento.[67,77] Dado los efectos adversos, este tratamiento ha quedado restringido a situaciones y pacientes excepcionales y de emergencia.

- Glucocorticoides tópicos: propionato de fluticasona o budesonida en formula viscosa, es el tratamiento más utilizado por su alta eficacia tanto en la inducción como en el mantenimiento de la remisión de la enfermedad. Este tratamiento tiene una eficacia media del 75 %.[67] La eficacia de respuesta es variables según el tipo de administración (inhalada, viscosa, efervescente…), la dosis y el tiempo de mantenimiento, llegando en algunas cohortes a un 94 % de remisión.[92]

- Inhibidores de la bomba de protones: su uso ha tenido una evolución en los últimos años. Aunque hasta 2007, la falta de respuesta al tratamiento con IBP se consideraba una herramienta diagnóstica para la EEo y, entre 2011 y 2015, se definió como una entidad distinta en aquellos pacientes que respondían a

IBP (EEo respondedora a IBP), a partir de 2017 se consideró a los IBP como un tratamiento de primera línea. Este tratamiento consigue ser eficaz en el 50,5 % de los pacientes[93] y se utiliza tanto en la inducción de la remisión como en el mantenimiento de los pacientes con EEo.

- Anticuerpos monoclonales: AcMo anti-IL-5 o anti-IL-13 han sido testados, aunque sin demostrar ser suficientemente efectivos en la inducción de la remisión histológica. A pesar de ello, en ocasiones, se usan como terapia compasiva.[94,95]

### 2.4.3 TRATAMIENTO ENDOSCÓPICO

El tratamiento endoscópico se aplica de forma urgente durante los episodios de impactación alimentaria a pacientes con estenosis esofágica, con un calibre inferior a 10 mm. Este tratamiento es paliativo y temporal y, dado el riesgo de perforación, se realiza como tratamiento complementario a los otros tratamientos.

## 2.5 HISTOPATOLOGÍA

El estudio histológico en esta enfermedad es fundamental para establecer el diagnóstico, cuyo criterio está definido por consenso en la presencia de un pico mínimo de 15 eosinófilos por campo de gran aumento (cga) en el epitelio esofágico.[79]

Empíricamente, se determinó la sensibilidad que ofrecían 1, 2, 3 y 6 biopsias del esófago distal y medio, siendo del 73 %, 84 %, 97 % y 100 % respectivamente,[96], por ello se recomienda la toma de al menos 3 biopsias por individuo para diagnosticar la enfermedad, de al menos 2 zonas distintas. Existen otras alteraciones histológicas, aparte del infiltrado eosinofílico, que contribuyen al diagnóstico de la EEo tales como la presencia de hiperplasia en la capa basal y un ensanchamiento de las papilas[97,98] mucho más pronunciado que en otras enfermedades esofágicas.[99] En las biopsias de pacientes con EEo pueden existir eosinófilos en la superficie y microabscesos

eosinofílicos[100] (**Figura 10**). Además de los eosinófilos, el epitelio contiene otras estirpes celulares en mayor número que en tejidos sanos como por ejemplo células dendríticas, mastocitos, linfocitos T, linfocitos B y células unidas a IgE e IgG.[101–105]

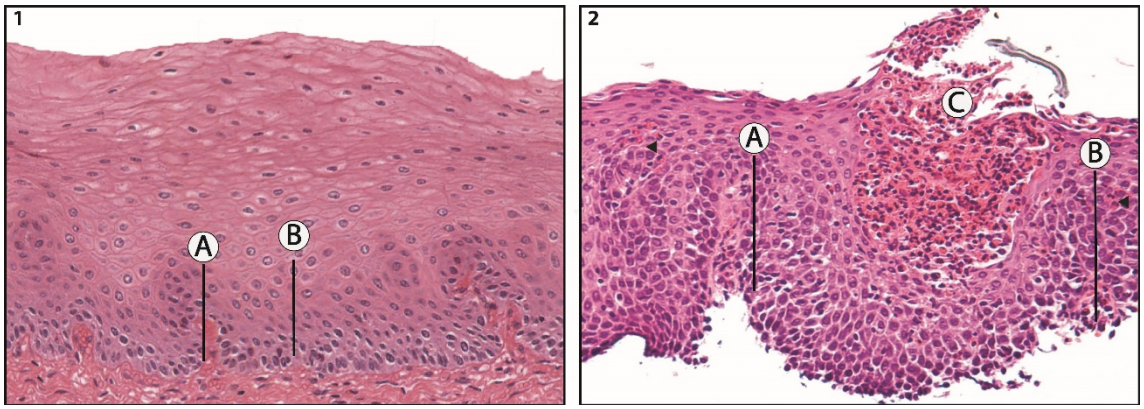


**Figura 10 | Tinción de hematoxilina-eosina en biopsias esofágicas.** 1. Muestra de sujeto control 2. Muestra de paciente con esofagitis eosinofílica. A. Elongamiento de papilas vasculares. B. Hiperplasia de la capa basal. C; Edema, ▶Infiltrado eosinofílico.

## 2.6 PATOGÉNESIS

Aunque aún no se conocen con exactitud los mecanismos etiopatogénicos de la EEo, ésta se considera una enfermedad alérgica, dada su asociación con la atopia, el tipo de infiltrado celular presente en el tejido, los estudios en modelos animales y la mejoría clínica y la remisión histológica tras el tratamiento con dietas de exclusión. En 1995 Kelly *et al.*[84] aportaron evidencias sobre el origen immunoalérgico en la EEo, resolviendo la inflamación eosinofílica de pacientes pediátricos diagnosticados con ERGE que no respondían al tratamiento de IBP mediante una dieta elemental aminoacídica durante 8 semanas. Sin embargo, se desconoce la causa exacta de eosinofilia exclusiva en el esófago, por qué unos pacientes responden mejor a un tratamiento frente a otro y si esta respuesta diferencial responde a un origen alérgico o a otro factor no descrito en la actualidad.

### 2.6.1 EPITELIO ESOFÁGICO

En la hipótesis más aceptada actualmente sobre la patogénesis de la EEo, la respuesta inmunitaria se desencadena a partir de la entrada de antígenos alimentarios o aero-alérgenos a través del epitelio esofágico. Esta entrada esta favorecida por un aumento de los espacios intracelulares del epitelio esofágico tras la desregulación de proteínas de adhesión como E-cadherina, desmogleina-1 (DSG1), involucrina y filagrina. También se ha observado una expresión alterada de proteínas de unión como Claudina (CLDN)-1, CLDN-4, CLDN-7, Ocludina (OCLN) y Zónula-occludens (ZO)-1 y 3. Además, el ambiente pro-inflamatorio facilita la alteración de proteínas del citoesqueleto como la synaptopodina (SYNPO).[16,106,107]

Recientemente, se ha apuntado a la desregulación de ciertas proteasas como causa de la alteración ya descrita de proteínas de unión y adhesión, como por ejemplo Calpaina 14 (CAPN14) encargada de regular el citoesqueleto y que se encuentra sobre-expresada en la enfermedad.[108] Se han descrito alteraciones no solo en las proteasas sino también en sus inhibidores, tales como SPINK 5 y SPINK7 que se encuentran prácticamente ausentes en pacientes con EEo.[109]

Para reforzar el papel del epitelio en esta enfermedad, es importante destacar su capacidad de expresar el complejo principal de histocompatibilidad tipo II (MHCII) en procesos de inflamación y comportarse como CPA no profesionales. El epitelio es también el principal productor de la linfopoyetina tímica del estroma (*thymic stromal lymphopoietin,* TSLP), una citoquina esencial para la activación de células presentadoras de antígeno, maduración de células T y polarización a células Th2[110], implicada en la fisiopatología de la EEo.

### 2.6.2 RESPUESTA INMUNITARIA

En la hipótesis convencional de esta enfermedad (**Figura 11**) la entrada de antígenos desencadena una respuesta inmunitaria del tipo Th2. Estos linfocitos Th2, reclutados por parte de las células dendríticas, inician la producción de IL-5 e IL-13[111]. La IL-5 es necesaria para la formación de eosinófilos desde la médula ósea y la atracción hacia

el tejido. Por otro lado, la IL-13 lleva a cabo dos funciones; la disrupción de la barrera epitelial mediante la disminución de producción de filagrina y el aumento de expresión de CAPN14 y promueve la activación epitelial lo que inicia la producción de TSLP, CCL11, CCL24, pero sobre todo CCL26, principal factor quemotáctico de los eosinófilos, por parte del epitelio esofágico.[112,113]

Los eosinófilos infiltrados en el esófago activan varios tipos celulares al liberar el contenido de sus gránulos. Mediante la producción de la proteína básica principal (*major basic protein*, MBP) que principalmente activa el epitelio esofágico, aumentando la producción de CCL26, y a los mastocitos que, a su vez, liberan diversos mediadores como TGF-β que favorecen la dismotilidad y, si persiste, la fibrosis subepitelial. Mediante la producción de neurotoxina derivada del eosinófilo (*eosinophil-derived neurotoxin*, EDN) los eosinófilos activan las células dendríticas que promueven la respuesta tipo Th2. Asimismo, se inician procesos de angiogénesis en los vasos esofágicos mediante la producción del factor de crecimiento endotelial vascular (*vascular endotelial growth factor*, VEGF), del TNF-α y de la molécula de adhesión celular 1 (*intercellular adhesion molecule 1*, ICAM1), facilitando la infiltración de células inmunitarias en el epitelio esofágico desde el torrente circulatorio. Además, los eosinófilos, mediante la producción de TGF-β, activan a los fibroblastos que, juntamente con la actividad mastocitaria, favorecen la hipertrofia muscular y la fibrosis.

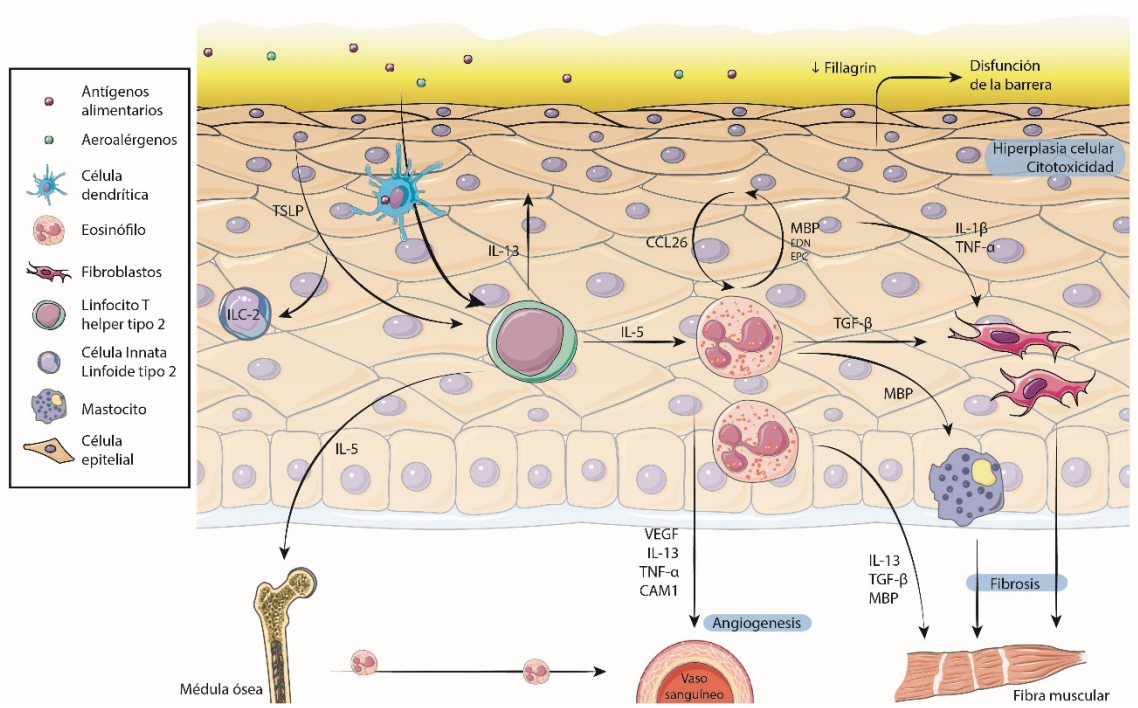


**Figura 11 | Hipotesis actual de la fisiopatología de la EEo.** El epitelio esofágico y las respuestas inmunitarias que inician y perpetuan la respuesta inflamatoria, así como los cambios estructurales presentes en el epitelio esofágico. La entrada de alérgenos y aeroalérgenos a través del epitelio esofágico es presentado por las células dendríticas a linfocitos T helper tipo 2, que inician la producción de IL-5 e IL-13. La IL-5 es responsable del reclutamiento de eosinófilos desde la médula ósea hasta el tejido mientras la IL-13 activa el epitelio esofágico. El epitelio esofágico activo produce linfopoyetina tímica del estroma (TSLP, thymical stromal lymphopoietin) que activa varios tipos linfocitarios, como LTh o ILC-2. Además, el epitelio produce CCL26, principal factor quemotáctico de eosinófilos, lo que aumenta su infiltrado. Los eosinófilos liberan el contenido citotóxico de sus gránulos perpetuando un feedback positivo en la activación del epitelio y la producción de CCL26 que reclutará más eosinófilos. Los eosinófilos además mediante la producción de sustancias proinflamatorias producirán angiogénesis lo que aumentará todavía más su infiltración en el tejido. Por otro lado, tanto el epitelio como los eosinófilos reclutaran fibroblastos y mastocitos responsables de la fibrosis muscular. TSLP: linfopoietina tímica del estroma, *thymical stromal lymphopoietin*; MBP: proteina basica principal, *major basic protein*; EDN: neurotoxina derivada del eosinófilo, *eosinophil-derived neurotoxin*; ECP: proteina catiónica del eosinófilo; *eosinophil cationic protein*; IL: interleuquina; TNF: Factor de necrosis tumoral alfa, *tumor necrosis factor alpha*; CCL26: quimioquina ligando 26 (tipo C-C), *C-C motif chemokine 26*; TGFb: factor tumoral beta, *tumor growth factor beta,* VEGF: factor de crecimiento vascular endotelial, *vascular endothelial growth factor, CAM1:* molecula de adhesión celular 1, *cell adhesión molecule 1*.

A pesar de ello, distintas hipótesis alternativas han sido propuestas para el desarrollo de la inflamación esofágica en la EEo (**Figura 12**):

#### 2.6.2.1 INMUNOGLOBULINAS

Las inmunoglobulinas (Ig) han sido una de las propuestas alternativas dada su asociación con las enfermedades de origen alérgico. Ya en 2010, Vicario *et al.*[104] demostraron un aumento tanto de linfocitos B tanto en el epitelio como en la *lamina propria* de pacientes con EEo además de un aumento de los transcritos germinales y en la producción local de IgE [104]. Puesto a que la mayoría de pacientes con EEo presentan test cutáneos alérgicos positivos, es común observar en algunos pacientes un aumento de la concentración de IgE específica en sangre.[114] Sin embargo, hasta la fecha, no se ha observado un aumento de expresión de dicha Ig en el tejido[105] y los tratamientos con anticuerpos monoclonales anti-IgE no son concluyentes ni homogéneos.[105,115]

A partir de la identificación del aumento de los transcritos germinales de IgG4[104], posteriormente, esta Ig cobró importancia tras observarse un aumento de 45 veces en su concentración en el tejido esofágico y el aumento de la IgG4 específica a distintos alimentos aunque una cohorte poco delimitada.[105] Sin embargo, el tratamiento a ratones con anti-IgG no fue efectivo en la reducción de eosinófilos.[116] A pesar de no haberse determinado aún el papel de las Igs en la EEo, tanto IgE como IgG4 tienen la capacidad de activar otras células pro-inflamatorias como los mastocitos, productores de IL-5 e IL-13, principales citoquinas involucradas en el desarrollo de la inflamación esofágica en la EEo.

#### 2.6.2.2 LINFOCITOS T $CD8^+$

Aunque la respuesta inmunitaria descrita en el tejido es mayoritariamente de tipo Th2, llevada a cambio por linfocitos $CD4^+$ y mastocitos, el aumento del infiltrado de linfocitos T en el epitelio esofágico corresponde principalmente a la población

$CD8^{+}$[103], células efectoras tanto de la inmunidad innata (NK) como de la adaptativa (linfocitos T).

Esta población $CD8^{+}$ en pacientes con EEo se ha descrito positiva para granzima B y TIA1 (antígenos intracelulares restringidos células T, *T-cell-restricted intracellular antigen* TIA1) lo que indicaría que poseen un perfil citotóxico.[117] Mediante el uso de modelos murinos, se ha observado que la deficiencia de células T garantiza protección ante el desarrollo de EEo.[118] Además, estudios previos observaron que los ratones deficientes en células $CD4^{+}$ estaban más protegidos ante el desarrollo de EEo (aunque mantenían un infiltrado patológico), pero que los ratones deficientes en células $CD8^{+}$ desarrollaban EEo con el mismo infiltrado patológico, lo que podría sugerir un papel protector de estas células. También en modelos murinos, se ha observado que los ratones deficientes en CD1d están protegidos frente a la inducción de EEo.[119] En el mismo grupo se observó que la administración de PBS57, un activador de iNKT, aumentaba el infiltrado de eosinófilo en el esófago de los ratones, revertía tras la administración de un inhibidor (anti-CD1d).[116]

En cuanto a los estudios en humanos, se ha observado que los iNKT son activados mediante esfingolípidos presentes en la leche, un alimento desencadenante de EEo en el 50% de los casos.[120]

De la misma manera que las Ig, las células $CD8^{+}$ también son capaces de reproducir el infiltrado inflamatorio presente en el esófago de pacientes con EEo. Tras reconocer antígenos presentados por las células epiteliales vía MHC II, se inicia la lisis celular de dichas células. Esta citotoxicidad epitelial desencadena el aumento de expresión de CCL26 y TSLP lo que estimula el reclutamiento de eosinófilos y linfocitos T, respectivamente, y se inicia la inflamación esofágica característica en la EEo (**Figura 12**).

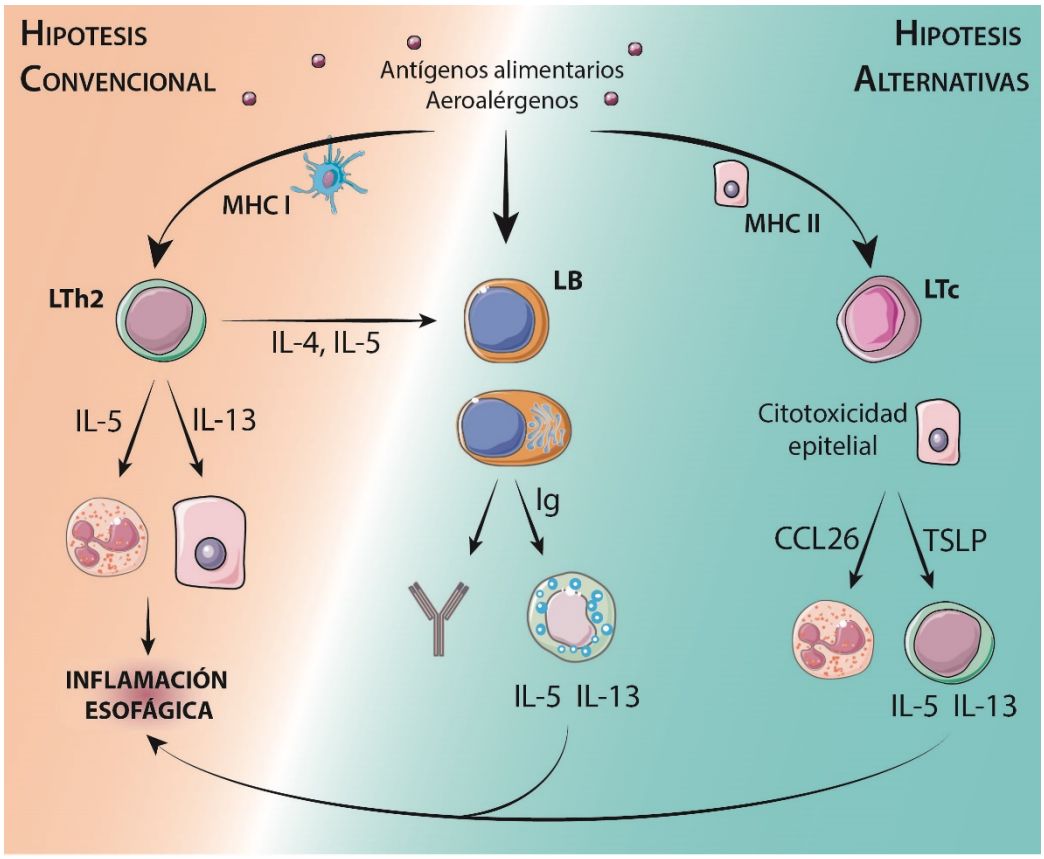


**Figura 12 | Hipotesis convencional y nuevas hipotesis alternativas del desencadenamiento de la fisiopatología en EEo.** MHC: complejo mayor de histocompatibilidad, *major histocompatibility complex*, LTh2: linfocito T helper tipo 2, LB: linfocito B, LTc: linfocito T citotóxico, IL: interleuquina, Ig; immunoglobulina; TSLP: linfopoietina tímica del estroma, *thymical stromal lymphopoietin.* Figura propia.

## 2.7 FACTORES DE RIESGO

Aunque no existe una herencia genética mendeliana clásica asociada al desarrollo de la EEo, se ha evidenciado cierta asociación familiar.[97,121] Se ha observado un riesgo del 1,8 % a padecer EEo en caso de que algún familiar de primer grado la padezca y un mayor riesgo en caso de que el familiar afectado sea varón (padres y hermanos, 2,4 % y 3,5 %, respectivamente) que mujer (madre o hermana 0,6 %, 1,3 % respectivamente).[122]

La frecuencia de EEo entre gemelos homocigóticos es de un 41 % mientras que en gemelos dicigóticos es solo del 22 %, lo cual refuerza el componente genético en esta enfermedad. Aun así, la frecuencia entre hermanos es sólo de un 2,4 %, 10 veces menor si estos hermanos dejan de compartir el mismo ambiente.[122]

Existen varias hipótesis que sostienen la mayor afectación de esta enfermedad en el sexo masculino como por ejemplo la presencia de un SNP en el gen del receptor de la TSLP o la implicación del receptor de IL-13 en la respuesta inflamatoria, ambos codificados en los cromosomas sexuales.[123,124]

En el esófago de los pacientes con EEo se detecta una sobre-expresión muy acentuada de aproximadamente el 1 % del genoma comparado con individuos sanos o, incluso, con pacientes con esofagitis crónica. Este transcriptoma está altamente conservado a lo largo de los distintos fenotipos (edad, sexo, antecedentes alérgicos) siendo la eotaxina-3 el gen más sobre-expresado.

Mediante técnicas de genotipado se ha identificado un polimorfismo de base única (*single nucleotide polymorphism*, SNP) en el gen de la eotaxina-3 (+2496T>G, rs2302009) asociado a la EEo, aunque el alelo solo está presente en el 14% de los pacientes.[125] Más recientemente se ha encontrado otra asociación entre un SNP en el gen que codifica para la TSLP (rs3806932) y el riesgo de padecer EEo (OR = 0.54)[126] También se ha observado un aumento de la expresión en CAPN14[127], STAT 6 y SPINK5 y 7.[109]

El aumento de la incidencia y prevalencia de esta enfermedad en los últimos años sugiere que no sólo los factores genéticos están implicados; los factores ambientales también contribuyen en gran parte al desarrollo de la EEo, sobre todo aquellos a los que los pacientes están expuestos a edades tempranas como por ejemplo parto por cesárea, la ausencia de lactancia materna, estancia en la UCI, antibióticos, y también la ausencia de mascotas, el uso de IBPs…(**Figura 13**).

Se han observado asociaciones con las estaciones y el clima, observando brotes de EEo más agudos durante las épocas con más aeroalérgenos.[128,129] Tanto el consumo de tabaco y de AINEs como la infección por *Helicobacter pylori* parecen presentar un papel protector en pacientes con EEo[130–132] (**Figura 13**).

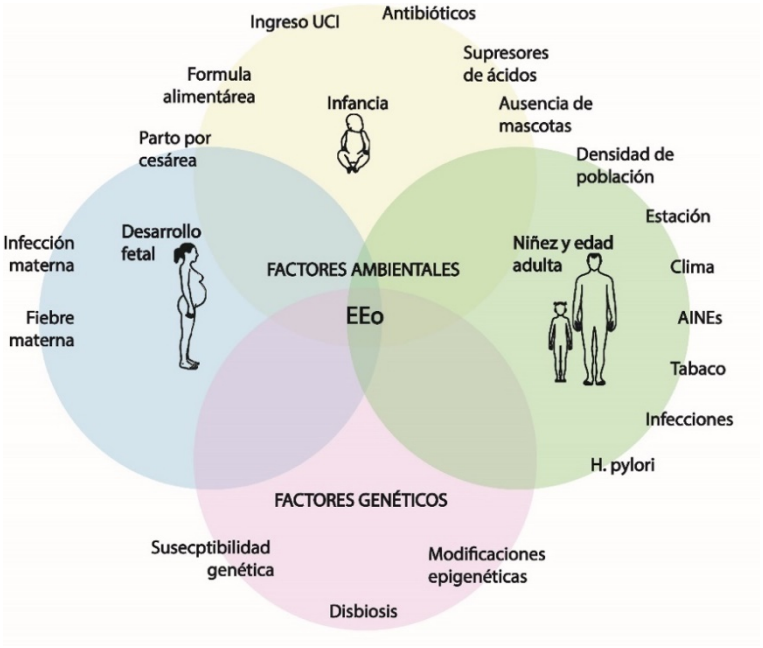


**Figura 13** | **Factores de riesgo en EEo.** Adaptado de Jensen and Dellon 2018 [133].

## 2.8 MODELOS EXPERIMENTALES

Para el estudio de los mecanismos etiopatogénicos de la EEo, se han desarrollado modelos murinos a partir de la exposición a alérgenos, como por ejemplo a *Aspergillus fumigatus,* ratones modificados genéticamente para la sobre-expresión de citoquinas producidas por linfocitos Th2, y la deficiencia de expresión de factores de transcripción o determinadas citoquinas.[134,135] Estos sistemas experimentales muestran una conexión entre el desarrollo de la inflamación eosinofílica en el tracto respiratorio y en el esófago no solo en respuesta a desencadenantes alérgicos externos sino también a factores intrínsecos como la expresión de citoquinas de tipo Th2. De hecho, la señalización por parte de linfocitos Th2 es necesaria para la inducción experimental de EEo dado que los ratones deficientes en IL-13 o con una depleción de STAT6 están parcialmente protegidos de la inducción de EEo.[136,137]

También se han puesto a puntos modelos *in vitro* en líneas celulares esofágicas, principalmente con la línea celular EPC2-hTERT. En esta línea se ha conseguido formar epitelios en estructura tridimensional mediante la técnica ALI e imitar la activación y el daño epitelial observado en EEo mediante el tratamiento con IL-13.[111,138]

# HIPÓTESIS

# HIPÓTESIS

"Las poblaciones inmunitarias citotóxicas $CD8^+$ así como la producción de inmunoglobulinas E y G participan activamente en la respuesta inflamatoria esofágica frente a alérgenos alimentarios en la esofagitis eosinofílica".

# OBJETIVOS

# OBJETIVOS

## OBJETIVO PRINCIPAL:

Identificar elementos celulares y humorales implicados en la respuesta alérgica presente en el epitelio esofágico de pacientes con esofagitis eosinofílica, y proponer biomarcadores que permitan identificar el mejor tratamiento para cada paciente.

## OBJETIVOS ESPECÍFICOS:

1. Ampliar el conocimiento sobre el potencial inmunológico del esófago humano mediante el fenotipado de las principales poblaciones de leucocitos en los distintos estratos a lo largo de este órgano.
2. Analizar la contribución de la población de linfocitos $CD8^+$ a la fisiopatología de la EoE mediante:
    a. Evaluación del número, activación y fenotipo de los linfocitos $CD8^+$ así como su modulación tras tratamiento.
    b. Análisis del perfil de expresión de genes relacionados con respuesta la citotóxica y su modulación tras el tratamiento.
    c. Estudio de respuestas epiteliales *in vitro* y *ex vivo*, a mediadores citotóxicos.
3. Identificar elementos de la inmunidad humoral y su contribución a la fisiopatología de la EoE mediante:
    a. Cuantificación de inmunoglobulinas en biopsias esofágicas
    b. Evaluación de la expresión de inmunoglobulinas y su modulación en la dieta de exclusión.
    c. Identificación del número y fenotipo de células $IgE^+$ o $IgG^+$ y su distribución en el esófago, así como su modulación mediante dieta de exclusión.

# CAPÍTULO 1

***"Characterization of immune cell populations in the human esophagus"***

# INTRODUCTION

Despite a seemingly simple appearance and function, the esophagus presents a complex neuromuscular structure and is an immunologically active organ. The defensive activity of the digestive system is divided into physical, chemical and immunological mechanisms. Within the esophagus, the epithelium establishes a tight physical barrier by a non-keratinized stratified squamous epithelial layer in which the basal stratum proliferates and moves upwards, constantly replacing the lining of the epithelium. Unlike other parts of the gastrointestinal tract, the esophageal epithelial layer lacks mucus-producing cells, secretory and absorptive functions, but it strongly contributes to the epithelial barrier function, restricting the entrance of antigens from the lumen to the organism by different protective mechanisms.[1]

In response to different physiological or pathological stimuli, the esophagus develops a quick and coordinated defensive response, mainly by recruiting immune cells. The immune system of the gastrointestinal tract detects constantly a wide variety of antigens, aeroallergens, and microorganisms, acquiring tolerance to them through innate and/or adaptive immune mechanisms. Immune activity also contributes to the maintenance of homeostasis by promoting epithelial barrier tightness and cell renovation[2], roles also expected to be developed in the esophageal epithelium.

In esophageal diseases of different etiology, a significant increase in the immune infiltrate is found in the epithelium that may affect epithelial integrity. The most common entities associated with esophageal immune activation are gastroesophageal reflux disease (GERD), eosinophilic esophagitis (EoE), lymphocytic esophagitis (LE), infectious esophagitis, motility disorders (such as achalasia), and congenital and tumoral diseases. Inflammatory esophageal disorders are characterized by specific and/or not specific histological hallmarks that may reflect different pathophysiological mechanisms, leading to esophageal dysfunction symptoms, associated with a significantly reduction in patient's quality of life.

The most frequent inflammatory condition of the esophagus is GERD with a prevalence of 5/1,000 inhabitants in developed countries[3], showing in some cases basal hyperplasia, papillary elongation and intraepithelial inflammation (granulocytes, eosinophils, and lymphocytes).[4,5] EoE, a-30-years-old esophageal inflammatory entity, has become the second more frequent esophageal disease, with an estimated prevalence in the USA and Europe of 29-41 cases per 100,000 inhabitants.[6] EoE is a chronic, local immune-mediated esophageal disease, characterized histologically by eosinophil-predominant inflammation,[7] mast cells and T lymphocytes.[8] Other chronic inflammatory diseases of the esophagus have recently been described such as LE, whose prevalence is still unclear and it is characterized by esophageal dysfunction and increased number of intraepithelial peripapillary lymphocytes without associated intraepithelial granulocytosis in the esophageal tissue.[9]

Despite the prevalence of esophageal disorders and the continuous discovery of new inflammatory entities of this organ, the specific role immune populations play in their pathophysiology is not fully described, as there is also limited information on cell counts and distribution of immunocytes in the esophagus in physiological conditions.[10,11] In fact, most studies are developed in a limited area of the tissue, as superficial biopsies (mainly containing epithelium and *lamina propria*) are obtained and no information is available on deeper layers, which may be critical to understand motility disorders as well. Moreover, reference tissues are frequently the adjacent areas of the esophagus of same patient in which no endoscopic alterations are identified. However, the lack of macroscopic findings does not assure histological normality, therefore, normal range values may not be properly obtained. In those studies, few immune cell populations, mainly eosinophils[12] and lymphocytes,[13] have been studied, but the presence of other resident cells such as mast cells and macrophages is poorly described.

To assure a proper diagnosis of esophageal immune-associated diseases, it is necessary to determine the normal range of the immune cell populations in the esophagus of healthy subjects. A more detailed description of the esophageal cellular

immune components may contribute to better discriminate between health and pathology, and to establish the histological definition of newly recognized entities. Considering the poor immunological characterization of the human esophagus in health, the specific aim of this study was to establish the content and distribution of the main immune cell populations throughout the different layers and length of this organ.

# EXPERIMENTAL DESIGN

The typification of immunological cells in the esophagus included the analysis of Th, Tc and B lymphocytes, mast cells, macrophages and eosinophils in the different esophageal layers: the epithelium (EP), the vascular papillae (VP), the *lamina propria* (LP), the *muscularis mucosae* (MM), and the muscularis externa (ME). The immune cell populations were analyzed in the proximal, middle and distal regions of the esophagus.

# METHODS

## 1 OBTENTION OF BIOLOGICAL SAMPLES

Esophageal samples were obtained from deceased patients from Hospital de la Santa Creu i de Sant Pau (Barcelona, Spain) and from Leuven University Hospital (Leuven, Belgium). Tissue sections were taken during the autopsy for histological examination, 6 hours after decease.

Inclusion criteria were the following: (1) absence of gastrointestinal pathologies (by clinical history), (2) absence of pathological findings during histological examination.

From each patient, transmural necropsies from proximal, medium and distal esophagus were taken and fixed with paraformaldehyde at 4 % during 24 h, dehydrated and embedded in paraffin following standard procedures.

## 2 ANALYTICAL PROCEDURES

### 2.1 STRUCTURAL ANALYSIS

Transmural tissue sections were cut at 5 µm, placed on microscope slides and stained with hematoxylin and eosin (H&E) following standard procedures. A histological examination was performed in all samples by an experienced investigator and

samples achieving optimal orientation (all esophageal layers visible) were selected for the analysis of immune cells.

## 2.2 PHENOTYPICAL ANALYSIS

Tissue sections were cut at 4 μm, deparaffined in xylene, gradually rehydrated by using decreasing concentrations of ethanol. Samples were processed for immunohistochemistry staining following standard procedures. Briefly, antigenic recovery was achieved by heat-induced epitope retrieval (HIER) in citrate buffer at pH6 or tris-EDTA at pH9 buffer (depending on the antibody) during 10 min at 120 ºC in an autoclave. Endogenous peroxidase activity was inactivated by adding Peroxidase-Blocking Solution (K4007/K4010, Agilent) and unspecific unions were blocked by adding Protein Block Serum-Free (X0909, DAKO). The type of the primary antibody used, the incubation conditions and the type of antigen retrieval method are indicated in **Table 1**. For the negative control, the primary antibody was omitted. Positive staining was revealed by the HRP-labeled polymer method (K4007/K4010, Agilent) followed by the staining with the peroxidase substrate 3,3'-diaminobenzidine tetrachloride (K4007/K4010, Agilent). Slides were counterstained with 50 % hematoxylin, dried and observed under an optical microscope.

**Table 1 | Primary antibodies used in the immunohistochemical study**

| Antibody | Target cell | Manufacturer | Dilution & incubation | Ag retrieval method |
|---|---|---|---|---|
| Monoclonal anti-CD3 (Clone PS1) | T lymphocytes | Leica (Novocastra) | 1/100 1 h RT | HIER EDTA |
| Monoclonal anti-CD4 (Clone 4B12) | Th lymphocytes | Master diagnostics | 1/50 ON 4 ºC | HIER EDTA |
| Monoclonal anti-CD8 [EP1150Y] | Tc lymphocytes | Abcam | 1/200 30′ RT | HIER Citrate |
| Monoclonal anti-CD20 (Clone L26) | B lymphocytes | DAKO | 1/500 30′ RT | HIER Citrate |
| Monoclonal anti-CD68 (Clone PG-M1) | Macrophages | DAKO | RTU 30′ RT | HIER Citrate |
| Monoclonal anti-MBP (Clone BMK-13) | Eosinophils | AbD Serotec | 1/50 2 h RT | PIER Proteinase K |
| Monoclonal anti-Tryptase (Clone AA1) | Mast Cell | DAKO | 1/200 30′ RT | HIER Citrate |

Th: T helper; Tc: T cytotoxic; RT: room temperature; RTU: ready to use; HIER: heat-induced epitope retrieval; PIER: proteolytic-induced epitope retrieval

### 2.3 QUANTIFICATION OF THE IMMUNE POPULATIONS

All analysis were carried out with a LEICA DMBL microscope, an OLYMPUS DP26 video camera and the computer program CellSens Standard 1.7. Immune cell types were counted in at least 10 fields of each layer and sample. No reliable counts could be performed of the submucosa layer due to its fragility.

The mean cellular density ($C_\rho$) was estimated as $C_\rho = \sum(Q/A)$, where Q is the total number of cells counted and A is the area determined for each picture. The results are expressed as number of cells per $mm^2$ of tissue.

## 3 STATISTICAL ANALYSIS

Data are expressed as median (range) or mean ± standard deviation. Normality of data distribution was tested by the D'Agostino and Pearson omnibus test. Outliers were identified by the Grubb's test. Normally distributed data were compared by One-Way Anova or Two-Way-Anova test, based on the number of independent

variables. Multiple comparisons were corrected using Tukey's multiple comparison test. Values of $p \leq 0.05$ were considered significant.

# RESULTS

## 1 STUDY POPULATION

A total of 9 individuals were included in the study. Mean age was 57 ± 11 years old and 33 % were female. Most common decease cause was cerebrovascular accident. Of each patient, transmural necropsies from the proximal, the middle and the distal esophagus were obtained.

## 2 STRUCTURAL ANALYSIS

The H&E staining showed a normal non-keratinized stratified squamous esophageal epithelium (EP) supported by connective tissue (*lamina propria*; LP) mainly constituted by collagenous and elastic fibers. In all esophageal portions, (proximal, middle and distal) lymphoid aggregates and scattered leukocytes were identified, especially in the epithelium and in the LP. *Muscularis mucosae* (MM) layer was identified discontinuous and in between the LP and the submucosal (SM) layer. Sero-mucosal glands were identified in the SM region and the *muscularis externa* (ME) was the thickest layer in the esophageal regions (proximal, middle and distal). Muscle types differences were observed when comparing the esophageal regions of the muscularis externa being entirely skeletal muscle in the proximal esophagus, mixed (smooth and skeletal muscle) in the middle and totally smooth in the lower portion (**Figure 1**).

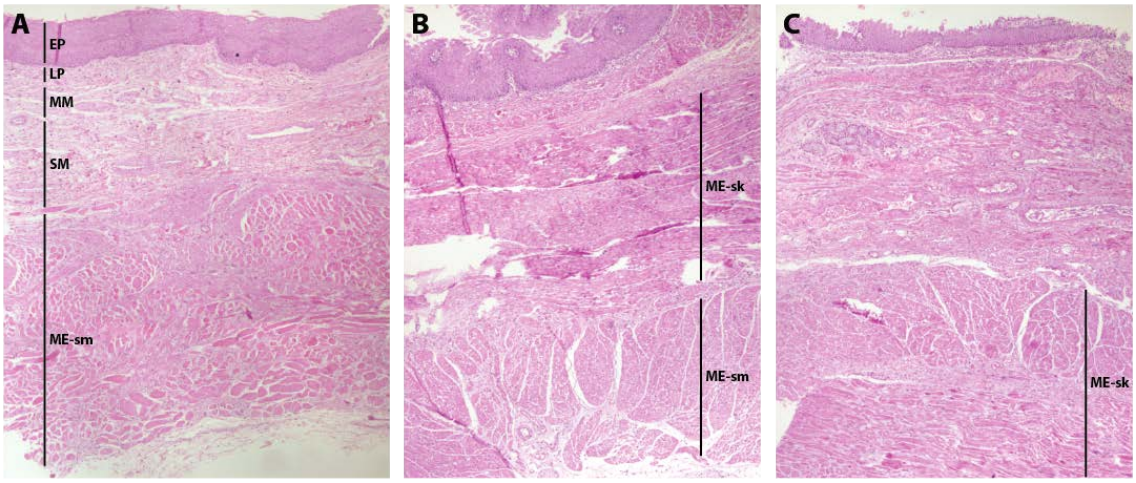


**Figure 1 | Transmural esophageal sections, stained with H&E. A**. Proximal B. Middle C. Distal. EP: epithelium, LP: *lamina propria*, MM: muscularis mucosae, SM: submucosa, ME: muscularis externa, sm: smooth and sk: skeletal

## 3 PHENOTYPICAL ANALYSIS

Significant differences were found in the amount of infiltrated cells in the esophageal layers ($p<0.0001$). As expected, due to its vascularization, the *vascular papilae* (VP) and the LP were the most infiltrated layers by all cell lineages analyzed (**Figure 2 A**). Lymphoid lineages stained revealed T lymphocytes ($CD3^+$), T helper lymphocytes ($CD4^+$), T cytotoxic lymphocytes ($CD8^+$) and B lymphocytes ($CD20^+$) as more abundant in the VP and LP followed by the EP, the MM and the ME (**Figure 2 B**). On the other hand, the myeloid lineages, macrophages ($CD68^+$) and mast cells ($tryptase^+$) were identified in all layers, predominantly in the VP and LP, followed by MM and ME, and the EP showed low infiltration of these cells (**Figure 2 C**).

For all cell types, the parapapillary and basal areas of the epithelium contained a greater percentage of the infiltrated cells than the rest of the EP. Cell counts are indicated for each esophageal portion and layer in **Table 2** and **Figure 5**. No differences were observed between sex or age ($p>0.05$).

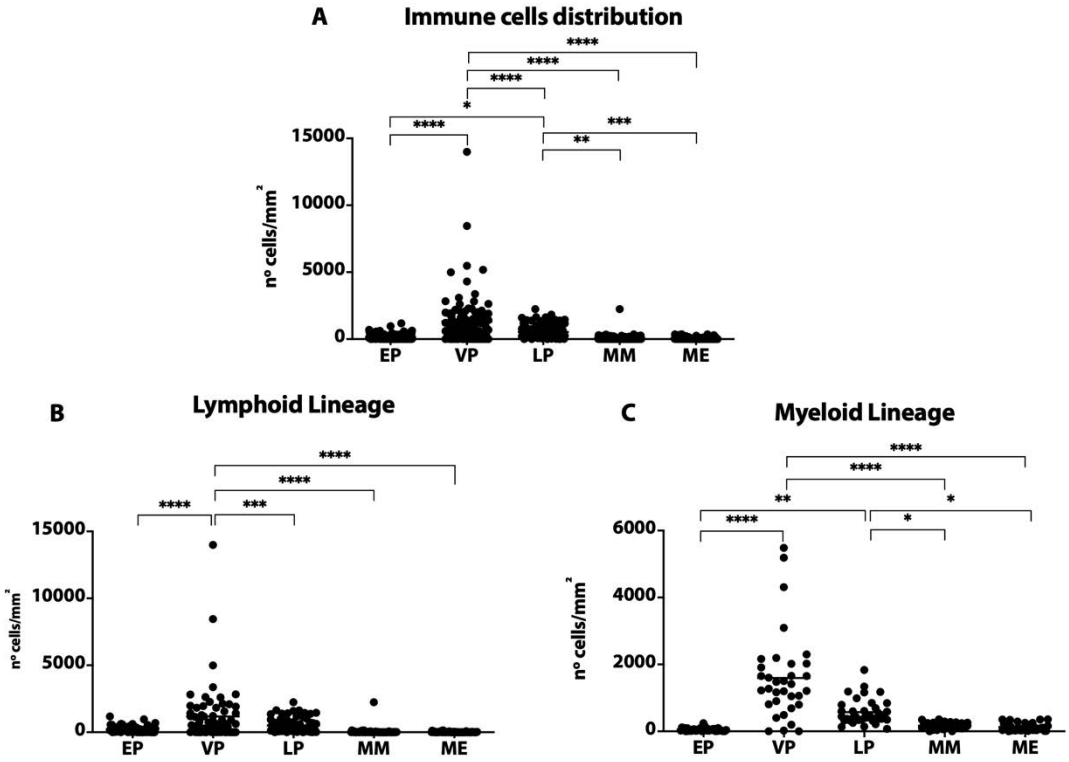


**Figure 2 | Resident immune cells distribution in the esophageal layers. Represented** results are the average of the 3 esophageal regions. A. Distribution of total immune cells B. Lymphoid lineage distribution C. Myeloid lineage distribution.

***Eosinophils:*** neither by MBP nor by H&E staining eosinophils were observed in any esophageal section or layer (**Table 2**).

***T Lymphocytes:*** T lymphocytes subtypes were found in the VP ($CD3^+$: 1,305 ± 999; $CD4^+$: 2,698 ± 3,457 and $CD8^+$: 401 ± 467 cells/$mm^2$), EP ($CD3^+$: 365 ± 231; $CD4^+$: 307 ± 274 and $CD8^+$: 248 ± 202 cells/$mm^2$) and LP ($CD3^+$: 700 ± 475; $CD4^+$: 877 ± 653 and $CD8^+$: 354 ± 332 cells/$mm^2$) of the esophagus. Significant differences were found between VP and EP and LP for $CD3^+$ and $CD4^+$ but not for $CD8^+$. For all cell subtypes, significant differences were found when comparing VP and LP layers with muscular layers (except LP for $CD4^+$ staining), MM ($CD3^+$: 26.2 ± 36.3; $CD4^+$: 48.6 ± 78.2 and $CD8^+$: 8.6 ± 11 cells/$mm^2$) and ME ($CD3^+$: 9.0 ± 12; $CD4^+$: 17.6 ± 28.7 and $CD8^+$: 36.8 ±3 9.6 cells/$mm^2$), where the infiltration was lower (**Figure 3 A, B and C**).

Proximal esophagus presenter a greater infiltration of $CD3^+$ cells when compared to distal esophagus (p=0.01). However when analyzing subpopulations no differences were observed between esophageal regions for $CD4^+$ and $CD8^+$.

***B Lymphocytes:*** the B lymphocyte was the less represented cell type in the esophagus (**Figure 3 D**). It was nearly absent in the EP (3.8 ± 6.8 cells/$mm^2$) and the ME (0.7 ± 2.1 cells/$mm^2$), and absent in the MM (0 cells/$mm^2$). However, a higher B cell number was found in the LP (92.7 ± 97.0 cells/$mm^2$) and VP (155.4 ± 258.2 cells/$mm^2$), as compared to EP, ME and MM. No differences were observed in B cell counts between esophageal regions.

***Macrophages:*** these large phagocytes were found widely infiltrated in the VP (1,406 ± 625.4 cells/$mm^2$), the LP (774 ± 454 cells/$mm^2$) and also in the deeper layers of the esophagus: the MM (154.3 ± 80.3 cells/$mm^2$) and the ME (139 ± 126 cells/$mm^2$) (**Figure 3 E**). In the EP (74.6 ± 59.9 cells/$mm^2$) lower cell counts were identified and no differences were observed between esophageal regions.

***Mast cells:*** tryptase positive cells followed the same pattern as macrophages (**Figure 3 F**). These cells displayed a significant greater infiltration in the VP layer, as compared with the other layers. Large cell counts were found in the VP (1,777 ± 1,730 cells/$mm^2$) followed by the LP (375 ± 113 cells/$mm^2$) and the muscular layers MM (176 ± 103 cells/$mm^2$) and ME (119 ± 94.3 cells/$mm^2$). In the EP (26.9 ± 21.0 cells/$mm^2$), mast cells were barely present. No differences were observed between esophageal regions.

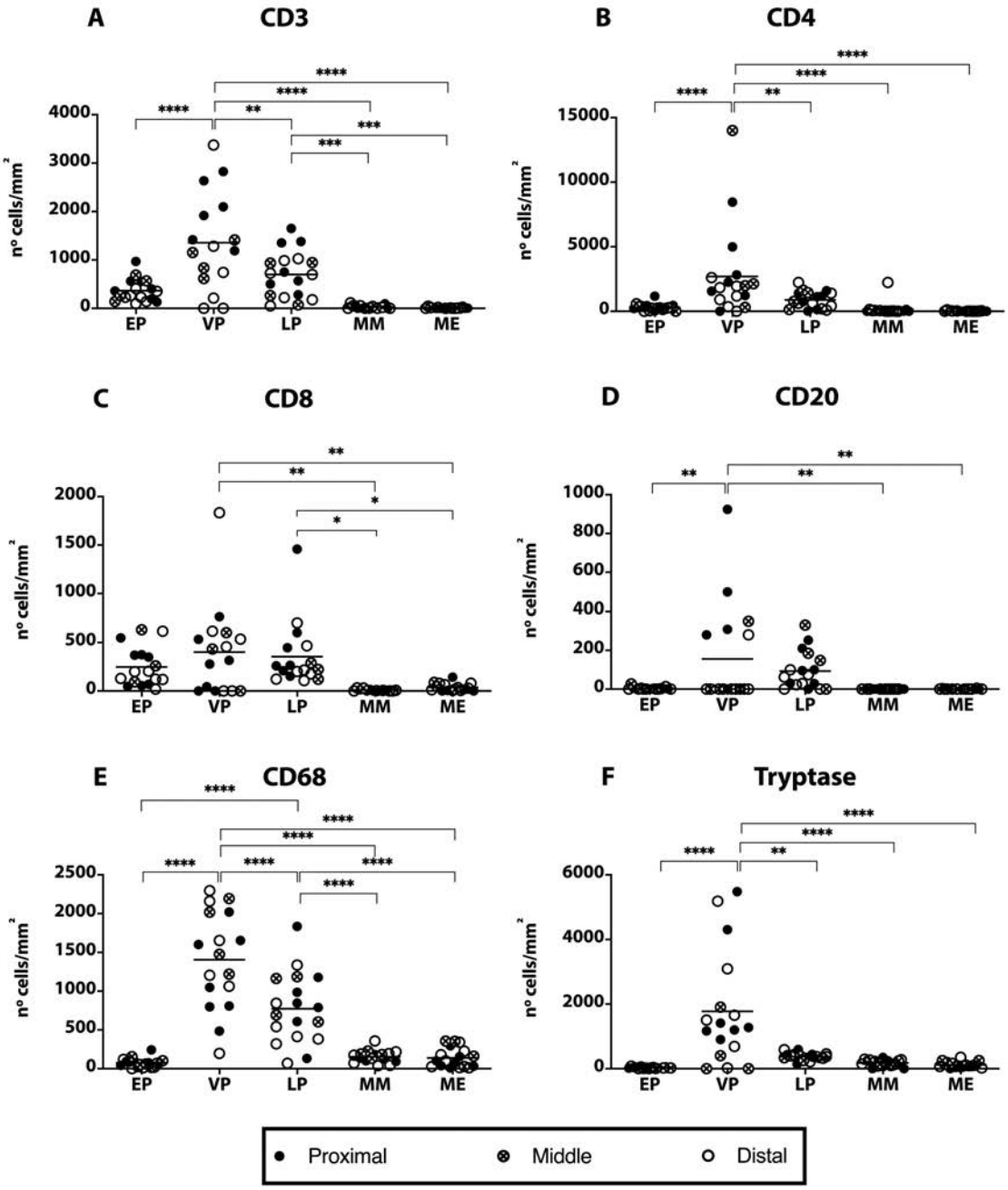


**Figure 3 | Immune cells distribution in esophageal layers in the three regions analyzed.** A. $CD3^+$ distribution B. $CD4^+$ distribution C. $CD8^+$ distribution D. $CD20^+$ distribution E. $CD68^+$ distribution D. $Tryptase^+$ distribution. Two-way ANOVA was performed for every cell type comparison. Tukey's multiple comparison test was used for adjusted p-value calculation (*p=0.05; **p=0.01;***p=0.001, ****p<0.0001).

For each layer of the esophagus (EP, VP, LP, MM and ME), significant differences were found in the number of each cell type and esophageal region (Figure 4).

In the EP (**Figure 4 A**), T lymphocytes ($CD3^+$, $CD4^+$ and $CD8^+$) were largely infiltrated, and significant differences were found when comparing T lymphocytes with other populations, such as B lymphocytes, macrophages and mast cells. A slightly greater infiltration was observed in the proximal region of the esophagus for B and T lymphocytes, as compared with middle and distal regions. However, only significant differences were detected for $CD3^+$ lymphocytes between proximal and distal regions. No variation was observed in the infiltration of T lymphocyte subtypes, macrophages or mast cells in any esophageal region.

The esophageal layer with the highest number of immunocytes was the VP (**Figure 4 B**), were $CD4^+$ was significantly the most abundant cell type.

Within the LP (**Figure 4 C**), T lymphocytes ($CD3^+$, $CD4^+$) and macrophages featured this tissue layer, where cytotoxic lymphocytes, B lymphocytes and mast cells were also present. A tendency of a greater infiltration in the proximal region was observed in T lymphocytes, both helper and cytotoxic populations. But, overall, no significant differences in the LP were found between proximal, middle and distal esophagus.

Muscular layers were characterized by myeloid lineage infiltration. While in MM (**Figure 4 D**) no significant differences in cellular infiltration were identifiable, in ME (**Figure 4 E**) significant differences between lymphoid lineage cells, macrophages and mast cells, were observed. Within the muscular layers, no differences were detected between esophageal regions.

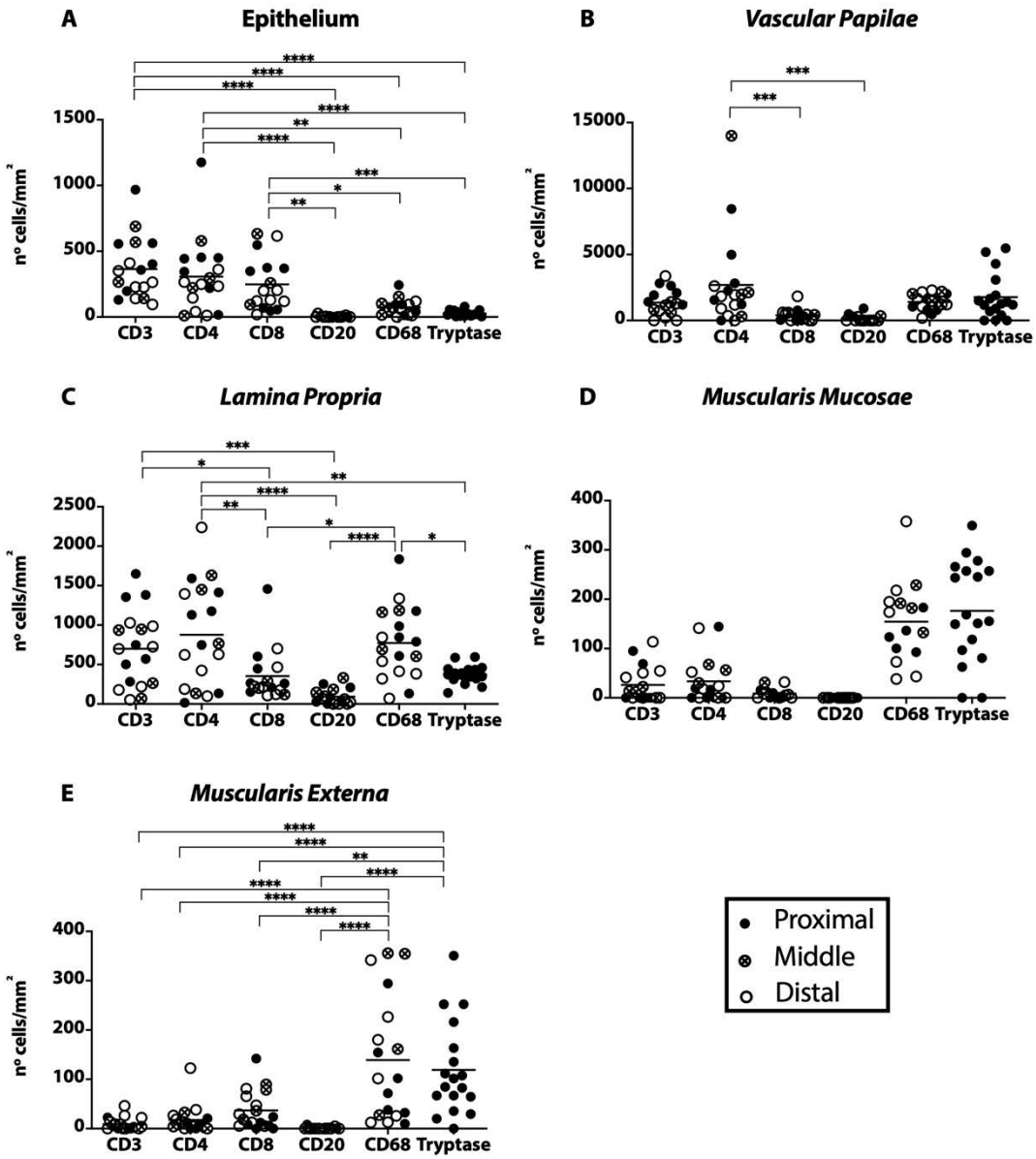


**Figure 4 | Immune cells distribution in esophageal layers in the three regions analyzed**. A. Epithelium B. *Vascular papilae* C. *Lamina propria* D. Muscularis mucosae E. Muscularis externa

**Table 2 | Immune cell counts in the different layers and regions of the esophagus.** Data are express as mean ± standard deviation.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Epithelium (cells/mm²)** | | | | | | | |
| | **CD3** | **CD4** | **CD8** | **CD20** | **CD68** | **Tryptase** | **MBP** |
| **Proximal** | 454 ± 280 | 440 ± 441 | 244 ± 214 | 3.1 ± 3.1 | 91 ± 73 | 34 ± 18 | 0 |
| **Middle** | 417 ± 255 | 278 ± 235 | 329 ± 276 | 7.8 ± 13 | 82 ± 61 | 15 ± 7.5 | 0 |
| **Distal** | 235 ± 106 | 194 ± 138 | 202 ± 211 | 2.2 ± 4.8 | 54 ± 45 | 31 ± 27 | 0 |
| ***Vascular papilae* (cells/mm²)** | | | | | | | |
| | **CD3** | **CD4** | **CD8** | **CD20** | **CD68** | **Tryptase** | **MBP** |
| **Proximal** | 2,014 ± 647.7 | 3,564 ± 3,286 | 323 ± 292 | 335 ± 347 | 1,203 ± 561.3 | 2,423 ± 1,956 | 0 |
| **Middle** | 1,004 ± 351.0 | 4,605 ± 6,317 | 345 ± 310 | 87.5 ± 175 | 1,727 ± 455.5 | 577.4 ± 906.2 | 0 |
| **Distal** | 896.6 ± 1,654 | 1,149 ± 956.3 | 574 ± 673 | 40.0 ± 105 | 1,430 ± 779.6 | 2,025 ± 1,861 | 0 |
| ***Lamina propria* (cells/mm²)** | | | | | | | |
| | **CD3** | **CD4** | **CD8** | **CD20** | **CD68** | **Tryptase** | **MBP** |
| **Proximal** | 927 ± 527 | 810 ± 695 | 522 ± 488 | 103 ± 96 | 911 ± 524 | 414 ± 103 | 0 |
| **Middle** | 555 ± 454 | 995 ± 684 | 211 ± 85.3 | 136 ± 136 | 912 ± 306 | 326 ± 57.3 | 0 |
| **Distal** | 490 ± 429 | 560 ± 463 | 215 ± 131 | 41 ± 39 | 559 ± 416 | 404 ± 113 | 0 |
| **Muscularis mucosae (cells/mm²)** | | | | | | | |
| | **CD3** | **CD4** | **CD8** | **CD20** | **CD68** | **Tryptase** | **MBP** |
| **Proximal** | 23 ± 41 | 7.2 ± 9.9 | 5.1 ± 7.3 | 0 | 127 ± 35.9 | 195 ± 134 | 0 |
| **Middle** | 12 ± 9.2 | 39 ± 30 | 19 ± 17 | 0 | 184 ± 39.6 | 207 ± 56.0 | 0 |
| **Distal** | 37 ± 25 | 39 ± 54 | 8.6 ± 13 | 0 | 157 ± 115 | 168 ± 85.4 | 0 |
| **Muscularis externa (cells/mm²)** | | | | | | | |
| | **CD3** | **CD4** | **CD8** | **CD20** | **CD68** | **Tryptase** | **MBP** |
| **Proximal** | 4.5 ± 8.2 | 3.0 ± 4.1 | 32 ± 55 | 1.2 ± 3.1 | 100 ± 98.4 | 86.8 ± 69.2 | 0 |
| **Middle** | 6.7 ± 6.0 | 12 ± 15 | 56 ± 34 | 0 | 224 ± 160 | 201 ± 60.6 | 0 |
| **Distal** | 24 ± 19 | 33 ± 46 | 40 ± 30 | 0.6 ± 1.7 | 129 ± 126 | 117 ± 107 | 0 |

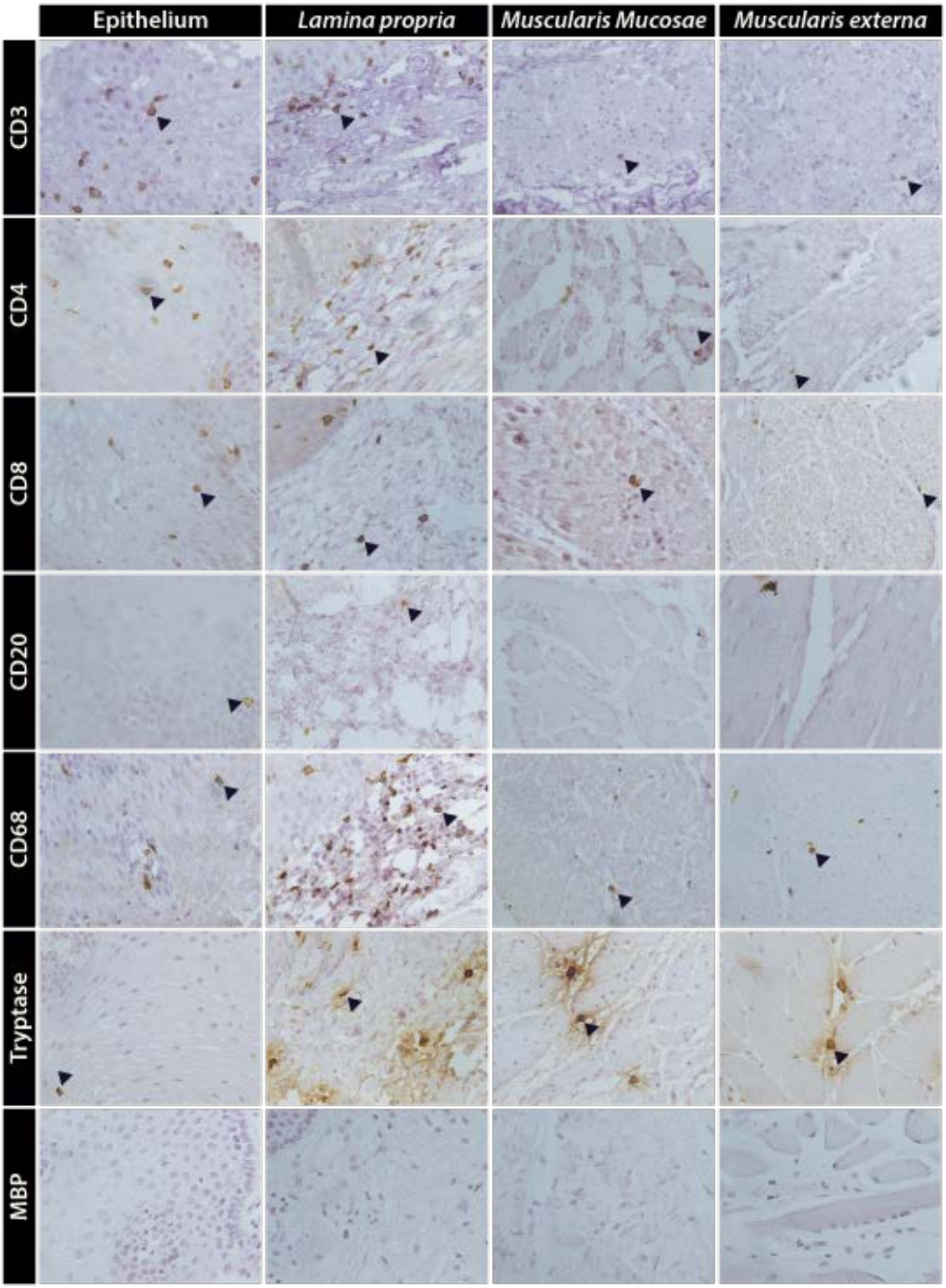


**Figure 5 | Representative images of the immune cells analyzed in different layers and regions of the esophagus.** Represented results are the average of the 3 esophageal regions. In each image, arrowhead points at positive staining.

# DISCUSSION

The present study describes in detail the main immune phenotypes of the human esophagus in health. To our knowledge, this is the first study that quantifies these cell populations considering all tissue layers within the upper, middle and lower regions of this organ. This characterization may contribute to the establishment of reference values of esophageal immune cell populations from asymptomatic healthy subjects and may be useful in future research studies aimed at identifying the role of certain immune cell types in homeostasis and in defensive responses within the esophagus. Although a range of immune cells was identified in several esophageal layers, the VP and the LP were the most infiltrated stratums, which could be explained by its high degree of vascularization.

T and B lymphocytes are the most prevalent immune cells residing in the outer layers of the esophagus, contributing to maintain tissue homeostasis. T lymphocytes locate all along the esophagus, both helper and cytotoxic, especially as intraepithelial lymphocytes and has been described as altered in different esophageal diseases.[5] Besides, in some esophageal diseases such as EoE or GERD,[14,15] different mechanisms disrupt the epithelium, facilitating the entrance of antigens, which may trigger a local immune response. Intraepithelial lymphocytes, which work as a sentinel population, will act as an activator of innate or adaptive immune responses in case of infection or antigen penetration. In this study, we have identified that the T lymphocyte population is larger in the proximal than in the distal esophagus, which may be due to the antigen exposure, which occurs before in the proximal region of the esophagus.

B lymphocytes are barely infiltrated in the esophagus in heath. This population mainly resides in the ganglia, are physiologically present in the *lamina propia* and can be rapidly recruited in case of antigen penetration. Other cell populations are physiologically infiltrate in the healthy esophagus as mast cells that are commonly known for their role in allergy, yet they are involved in many other protective roles such as would healing, angiogenesis, defense against pathogens working as sentinel

cells. Other cells as macrophages, responsible for patrolling and eliminating potential pathogens, has been involved in other esophageal diseases[16,17] although its role is still understudied.

Different esophageal diseases are characterized by imbalance in these immune populations. In particular, in EoE the esophageal cell populations that densely infiltrate de *epithelium* and the *lamina propria* are eosinophils, followed by T lymphocytes, mast cells and B lymphocytes.[18]

Due to the difficulty in obtaining deeper layers of the esophagus, few studies analyze the submucosal and muscular layers. This may be of high interest for the study of motility disorders, as the high number of macrophages and mast cells within the muscular layers (observed in the present study) may contribute to physiological muscle activity and may also develop a role in disease. In fact, motor esophageal disorders also present immune cell imbalance. Particularly in achalasia, an increase in the CD3 lymphocyte population has been identified in the muscle layer, mainly CD8 that express the TIA-1 marker (a marker of degranulation). It also presents an increased infiltration of B lymphocytes[19] and mast cells, and this infiltration in the muscle has been associated with the loss of interstitial cells of Cajal and neuronal degeneration.[20]

The close interaction between epithelial and immune cells is key for barrier function maintenance. Epithelial damage represents an important trigger to develop and/or maintain an immune response in the esophagus. In fact, factors such as acid exposure stimulate the release of chemotactic factors from the epithelium to recruit immune cells and also disrupt intercellular junctions maintenance, leading to barrier dysfunction and facilitating antigen penetration. The impairment of the epithelial architecture, with dilated intercellular spaces within the squamous epithelium, in disorders such as non-erosive acid-damage and also in macroscopically normal EoE, also facilitates the diffusion of acid content and the access of luminal antigens into the epithelium, where antigens may trigger immune responses[21]. The role of the immune cells, as resident populations within the epithelium, during the first stages

of epithelial disruption has not been defined yet, and further studies are needed in order to identify whether immune activation enhances of dampens barrier alterations.

This study offers a basal characterization of esophageal immunocytes, placing altogether the main immune populations in the human esophagus, which has not been detailed before. These findings could be considered as reference values, which may also contribute to a better understanding of the esophageal immune system in health and disease.

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# CAPÍTULO 2

***"Contribution of CD8 lymphocytes to the pathophysiology of eosinophilic esophagitis and its modulation by dietary treatment"***

# INTRODUCTION

Eosinophilic esophagitis (EoE) is a chronic inflammatory disease of the esophagus, characterized by esophageal dysfunction and specific intraepithelial eosinophilic infiltration. EoE is considered to be an allergic disease and, similarly to other allergic conditions, is characterized by IL-5 and IL-13 production and considered to be modulated mainly by CD4 lymphocytes.[1] Notably, CD8, and not CD4 lymphocytes, are the predominant lymphocytic infiltrated population in the esophageal epithelium in EoE[2,3]. This cellular increase and the fact that no differences in the transcriptome are observed between allergic and non-allergic patients[4], suggests that CD8 lymphocytes are relevant contributors to the pathophysiology of EoE, despite its role is not well understood in this disease.

CD8 lymphocytes recognize cellular antigens from damaged epithelial cells[5], and are also able to recognize exogenous antigens from antigen presenting cells (APC), as they can also present exogenous antigens via MHC I through cross-presentation.[6–8] Classically, exogenous antigens are presented via MHC II to CD4 T lymphocytes, and intracellular antigens via MHC I to $CD8^+$ lymphoid cells (T cells, NK cells and NKT cells). Once CD8 lymphocytes recognize antigens, a rapid activation takes place for the destruction of infected cells through cytotoxic mechanisms.

Recent studies emphasize an additional role for CD8 lymphocytes in autoimmune and allergic disorders.[9–11] The activation of CD8 lymphocytes in allergen-driven responses starts after antigen recognition, leading to the generation of a diversity of CD8 subtypes (Tc1, Tc2, Tc9 and CD8 Treg), presenting different functions and effector molecules.[11] Tc1 cytotoxic T lymphocytes are the most widely characterized subpopulation, strongly induced by IL-2 and IL-12. They are responsible for intracellular pathogens clearance by the release of cytotoxic molecules, and the secretion of interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α). Some of these cells reside as memory T cells after activation.[12–14] Tc2 cytotoxic T lymphocytes are stimulated in the presence of IL-4[15] and are responsible for the exacerbation of Th2–mediated allergy mechanisms due to the production of IL-4, IL-

5 and IL-13.[16] This subtype, depending on milieu conditions, can acquire low or high cytotoxic profile.[13,17] Tc9 T lymphocytes are polarized by the presence of TGF-β and IL-4, and are responsible for the attenuation and suppression of Th2 responses and allergic diseases through the production of IL-9 and IL-10.[18,19] Finally, CD8 Treg lymphocytes, enhanced by TGF-β and IL-15, are responsible for the regulation of immune-regulatory responses through the production of TGF-β, IL-10, granzymes and perforines. They become functional in the late phase of the immune response after antigen re-encounter.[20–22]

In EoE, similarly to allergic airway inflammation, Th2 lymphocytes and IL-4, IL-5 and IL-13 production are considered pivotal. In recent studies, it has been described that CD8 cells are critical for a well-balanced response to inhaled allergen by enhancing IL-12 production and suppressing Th2-driven inflammation.[9] Moreover, not only in allergic airway inflammation, but also in atopic dermatitis CD8 lymphocytes are able to respond to antigens and also produce IL-13 and IL-5, contributing to eosinophilic recruitment and IgE production[16,23].

Therefore, different mechanisms can be developed by CD8 lymphocytes to contribute to esophageal inflammation and dysfunction in EoE. In this study, we hypothesized that CD8 lymphocytes contribute to EoE through cytotoxic mechanisms. We aimed to identify CD8 activity and the molecular elements contributing to epithelial damage and inflammation in EoE and its modulation by therapy.

# EXPERIMENTAL DESIGN

A prospective, multi-center observational study was designed in order to: (1) characterize the phenotype of the intraepithelial esophageal lymphocyte population in active EoE and in remission, and (2) identify the effect of cytotoxic mediators on the epithelium in both, *in vitro* in a human esophageal cell line and *ex-vivo* in human esophageal tissue (**Figure 1**).

**(1) CHARACTERIZATION OF ESOPHAGEAL CD8 LYMPOCYTES:**

Two experimental groups were studied: i) a case group of EoE patients with active disease and after treatment with either proton-pump inhibitors (PPIs) or a six-food elimination diet (SFED), and ii) a control group, including patients with esophageal dysfunction symptoms with or without gastro-esophageal reflux disease (GERD) and with histologically normal esophageal biopsies. Clinical assessment was reported and esophageal and duodenal biopsies were obtained in all participants. Samples for each experimental procedure were coded and analyzed blindly by one or two different investigators. The characterization of the lymphocytic population was performed as follows:

- Phenotypic profile of esophageal intraepithelial lymphocytes: staining for CD4 and CD8 lymphocyte markers as well as the CD2 activation marker were performed by immunohistochemistry or immunofluorescence.
- Gene expression profile: CD8-related genes of homing, cytotoxicity and inflammatory activity were analyzed by real-time quantitative PCR (qPCR).

The effect of therapy on lymphocyte infiltration and CD8-related gene expression was analyzed in all cases. Subgroups were divided based on the treatment administrated, either PPI or SFED.

**(2) CHARACTERIZATION OF THE EPITHELIAL RESPONSE TO CD8-ISOALTED MEDIATORS:**

The study was divided in two phases:

**PHASE 1**

A human esophageal cell line was phenotypically and functionally characterized at baseline and after stimulation as follows:

- Structural epithelial proteins and proinflammatory genes were analyzed by immunofluorescence and real-time qPCR to determine cell differentiation at baseline.
- The functionality of the esophageal barrier was evaluated by measuring the trans-epithelial electrical resistance (TEER), after the development of an air-liquid interface (ALI) cell culture model.
- Structural and pro-inflammatory gene expression quantification was assessed after stimulation with IL-13 to evaluate the suitability of the cell line as an *in-vitro* EoE model.
- Epithelial response to CD8-isolated mediators was measured by quantifying chemotactic, structural and pro-inflammatory genes by real-time qPCR.

**PHASE 2**

An *ex-vivo* study in human esophageal biopsies exposed to CD8-isolated mediators was performed as a more accurate approach to the esophageal epithelial activity in response to cytotoxic mediators. Biopsies obtained from EoE patients in remission were stimulated with IL-13 or CD8-isolated mediators and the expression of structural and pro-inflammatory genes was quantified by real-time qPCR.

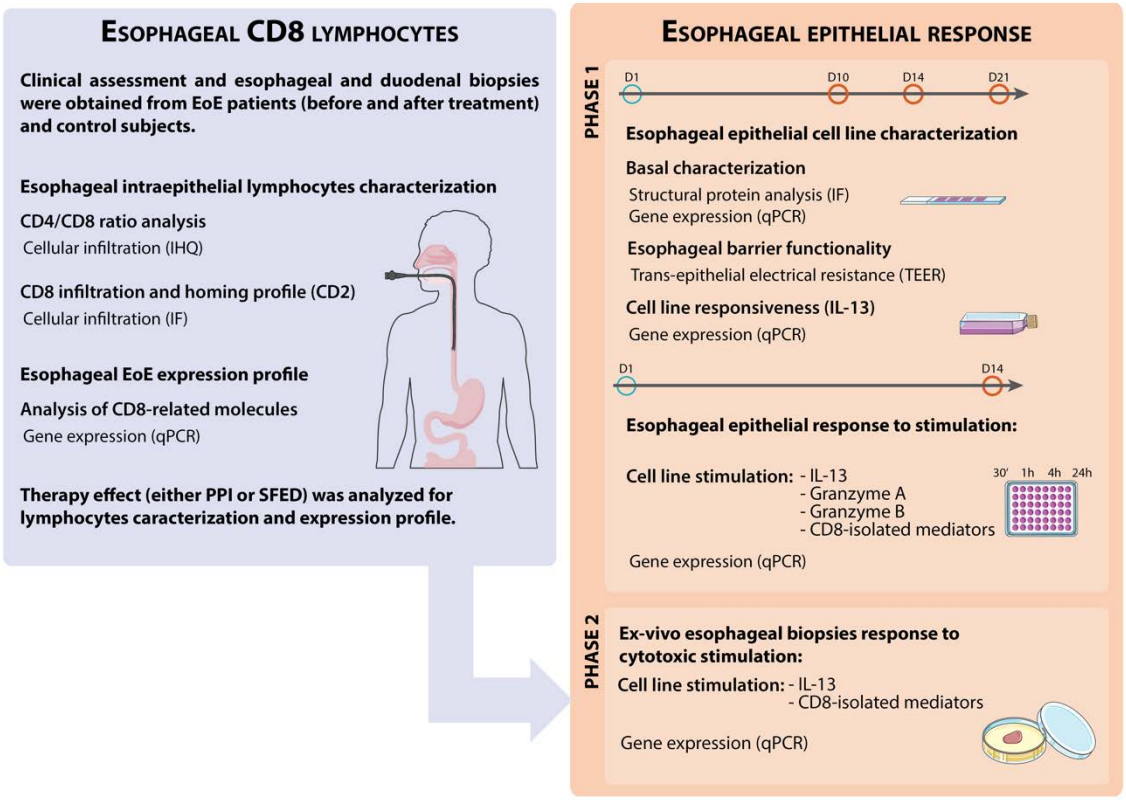


**Figure 1 | Chapter 2 experimental design and work-flow.** D: Day; IL-13: Interleukin 13; IHQ: Immunohistoquemistry; IF: Immunofluorescence; qPCR: quantitative PCR; FACS: Fluorescence-activated cell sorting; WB: Western Blot

# METHODS

## 1 SUBJECTS DESCRIPTION

### 1.1 STUDY POPULATION AND ETHICAL ASPECTS

Two groups of subjects (balanced by sex and age) were prospectively recruited from the Departments of Gastroenterology at Tomelloso General Hospital (Ciudad Real, Spain) and at Vall d'Hebron University Hospital (Barcelona, Spain):

a) Case cohort: consecutive adult EoE patients with a clinico-pathological diagnosis of active disease confirmed by: (1) presence of symptoms related to esophageal dysfunction and infiltration of 15 or more eosinophils per high-power field (hpf) in at least 1 of the esophageal epithelium biopsies; (2) no pathological eosinophilic infiltrate in gastric and duodenal biopsies; (3) other systemic and local causes of esophageal eosinophilia excluded[24]. No steroid treatment (neither oral, nasal airway nor swallowed) was allowed in the 8 weeks before the beginning of the study. Anti-H1 or inhaled β2-agonists and anticholinergic bronchodilator drugs were allowed in a stable dose during the study in cases of exacerbated rhinitis or asthma symptoms.

b) Control cohort: patients with symptoms of esophageal dysfunction with or without GERD and with normal endoscopy (except for not complicated endoscopic signs of erosive esophagitis related to GERD) and normal esophageal biopsies (no histological alterations), were included in the study. Subjects were not undergoing any treatment with PPI, corticosteroids or elimination diet.

The study was conducted in accordance with the principles of the Declaration of Helsinki and approved by the Institutional Review Board of La Mancha Centro General Hospital (acta nº 08/14) and the Ethics Committee at Vall d'Hebron University Hospital (PR(AG)94/2013). Informed consent was obtained from all participants.

## 1.2 CLINICAL EVALUATION AND TREATMENT PROTOCOL

### 1.2.1 SYMPTOMS EVALUATION

In all subjects, a general physical examination was performed. Symptoms of esophageal dysfunction were assessed before each endoscopy (both at basal level (case or controls) and after treatment) by a score validated for achalasia [25], as previously used in adult patients with EoE[26–28]. The score is based on the evaluation of the duration, intensity and frequency of dysphagia. The presence of other gastrointestinal symptoms was also collected for all study subjects.

### 1.2.2 ENDOSCOPY AND BIOPSY PROCEDURE

Upper endoscopy was performed at baseline for case and control groups and at the end of the 6-8 weeks treatment period (PPIs or elimination diet) for case group. All endoscopic procedures were performed under sedation with a flexible 9-mm-caliber Pentax EG-2770K gastroscope (Pentax of America, Montvale, NJ) with a 2.8-mm work channel. For the assessment of endoscopic features, white exudates, furrows, edema, fixed rings, crepe paper sign, short-segment stenosis, long-distance stenosis were recorded at each endoscopy following EREF validated questionnaire [29]. During endoscopy, 6 – 8 biopsies were obtained with a standard needle biopsy forceps (Endo Jaw FB-220U; Olympus Medical Systems, Tokyo, Japan) from upper and lower esophageal regions, preferably from visible lesions (if identified) for general histopathology evaluation to determine the response to treatment. No complications were observed in any patient during or after the endoscopic procedure.

### 1.2.3 ALLERGY TEST

All participants were examined in the allergy unit of the participant hospitals, where they underwent skin prick testing (SPTs) for a general panel of food and pneumoallergens. Particularly, SPTs against commercial food extracts (ALK-Abelló, Madrid, Spain) were performed on the forearm with disposable lancets (ALK-Abelló)

by pricking though a drop of the extract, which was then absorbed. Each drop was separated from the next by at least 2 cm. Reactions were recorded by measuring the largest diameter of the resulting wheal (in millimeters) at 15 minutes. Histamine (10 mg/mL) and saline solution were used as positive and negative controls, respectively. Results were considered positive if the wheal diameter was at least 3 mm.

### 1.2.4 RESPONSE DEFINITIONS

Clinical response and histological response were considered as defined previously in guidelines[24]: Clinical response to treatment defined according to the reduction of the dysphagia score >50 %; complete histological remission defined as a peak count of 0 to 5 eosinophils / hpf, in the highest infiltrated area and biopsy. Peak eosinophils count between 6 and 14 defined as a partial histological response in EoE patients and lack of response to treatment defined as peak counts of ≥15 eosinophils/hpf, at any area or esophageal region. Dysphagia score and EREF questionnaire were also obtained in all participants after the corresponding treatment period.

### 1.2.5 TREATMENT GROUPS

#### 1.2.5.1 PROTON PUMP INHIBITORS

EoE patients underwent 20 mg/12 h omeprazole treatment for a 6–8 weeks period. In cases of completed response, treatment was maintained, and endoscopic follow-up was performed every 6-12 months. Patients who did not respond to PPIs were withdrawn from the study and treated with oral corticosteroids or elimination diet.

#### 1.2.5.2 EMPIRICAL ELIMINATION DIET PROTOCOL

EoE patients underwent a six-food elimination diet (SFED) for a 6-8 weeks period, avoiding 6 potentially allergenic food groups: (1) cereals, including wheat, rice and corn, (2) milk and dairy products, (3) eggs, (4) fish and seafood, (5) legumes/soy and (6) nuts. Dietary restriction included processed foods containing ingredients obtained

from those foods. If coexisting GERD symptoms, PPIs were permitted in a stable dose during the empirical elimination diet evaluation. Supplements with amino acid-based formula adapted to oral consumption (Neocate Advance, 100g sachets, banana & vanilla favors; SHS International, Liverpool, UK) were administrated to complement the diet, if necessary. Information about permitted and not permitted food, instructions to read food labels carefully as well as direct contact with a reference doctor were provided to patients. A telephone number and an e-mail address were also provided in case of further doubts regarding the SFED. In cases of complete or partial histologic response, patients initiated sequential food re-challenge (**Figure 2**), where reintroduction of each food individually with subsequent endoscopic examinations and biopsies was performed. Patients were asked to consume each reintroduced food every day for a 6-8-week period. Due to its allergenic potential, wheat, milk and dairy products were, in ≥this order, the firsts foods reintroduced in all patients. The reintroduction of the remaining foods varied according to patient's preferences to normalize their diet as soon as possible. If patients responded positively (either complete or partially) after each single-food challenge, this food was considered to be well tolerated and maintained in the diet. On the contrary, if inflammation recurred (≥15 eosinophils/hpf), that food was removed permanently from the diet and considered an EoE trigger. Next food was immediately reintroduced with no washout period. When endoscopic findings were suspected of recurrence, the tested food was preventively removed until histological evaluation was performed. Patients who did not respond to SFED were withdrawn from the study and treated with oral corticosteroids or PPI.[30]

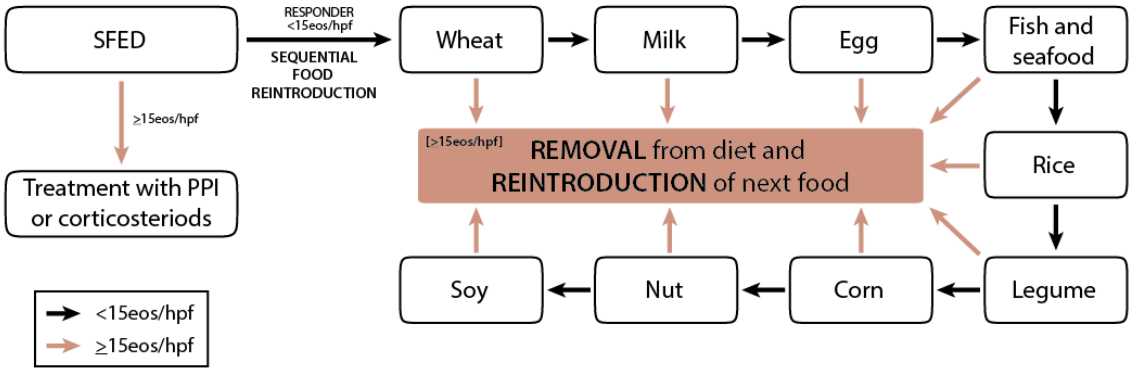


**Figure 2 | General scheme of the sequential food reintroduction protocol.** Wheat and milk were the first food to be reintroduced in all cases. The order of the remaining foods varied according to patient's preferences to normalize their diet as soon as possible. Black arrows indicate the following step when histological analysis showed <15 eosinophils / hpf. Red arrows indicate the following step when histological analysis showed ≥15 eosinophils / hpf. Eos: eosinophils

# 2 COLLECTION OF BIOLOGICAL SAMPLES

## 2.1 ESOPHAGEAL BIOPSIES

Four esophageal biopsies were fixed in 4 % paraformaldehyde (PFA), for further microscopic examination of tissue architecture and eosinophil infiltrate (by means of H&E staining) and additional immune cell characterization (by means of specific cell markers staining). Two biopsies were fixed in 500 µL of RNA Later (QIAGEN) and stored at -80 ºC until processed for RNA extraction. Two biopsies for the *ex-vivo* study were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) for a maximum of 10 minutes before exposure to mediators. Samples for each experimental procedure were coded and analyzed blindly by one or two different investigators.

## 2.2 BLOOD

Before the endoscopic procedure, 40 mL of blood samples were obtained from control subjects. Samples were maintained in ice for a maximum of 10 minutes before performing the isolation of the $CD8^+$ population.

# 3 HUMAN ESOPHAGEAL EPITHELIAL CELL LINE

This study was performed with the Het-1A cell line (ATCC CRL-2692), an immortalized primary esophageal epithelial cell line, originally obtained in 1986 from the esophageal epithelium of a 25 years-old black man. The Het-1A cells have adherent properties and are able to grow forming multi-layers [31].

Het-1A cells were maintained in DMEM supplemented with inactivated 10 % Fetal Bovine Serum (FBS), 1 % HEPES and 1 % Penicillin-Streptomycin (all reagents from Gibco). To promote cell adherence, growth surfaces were pre-coated with 0.01 mg/mL Fibronectin, 0.03 mg/ml Bovine Collagen type I, and 0.01 mg/mL BSA (all reagents from Sigma-Aldrich) dissolved in culture medium and incubated 24h at 37 ºC. Pre-coated surfaces can be stored at room temperature, light protected, for up to 1 month. Medium was renewed every 2 days. Cell dissociation, done weekly for maintenance or after stimulation, was performed with Trypsin-EDTA 0.05 % (Gibco) for 5 minutes at room temperature.

Cells were seeded at a density of 6.7 cells/mL and disrupted at 80-90 % of confluence. For basal characterization and stimulation analysis, 0.9 µM calcium was added to the maintenance medium. After 1-week, $Ca^{2+}$ supplement was increased up to 1.8 µM in order to favor cell differentiation.

# 4 ANALYTICAL PROCEDURES IN TISSUE SPECIMENS

## 4.1 EOSINOPHIL COUNTS

PFA-fixed biopsies were embedded paraffin following standard procedures and routinely processed as follows: tissues were sectioned (5 µm thick), deparaffined in xylene, gradually rehydrated by using a battery of decreasing ethanol concentrations. H&E staining was performed and eosinophils were counted in the most densely inflamed areas in three non-overlapping hpf at 400X magnification (total area measured of 0.238 $mm^2$) under an optical microcope. Peak eosinophil counts per hpf was calculated from each sample by an experienced pathologist.

## 4.2 IMMUNE CELL PHENOTYPE

### 4.2.1 IMMUNOFLUORESCENCE TISSUE STAINING

Tissues were sectioned (5 µm thick), deparaffined in xylene, and gradually rehydrated by using a battery of decreasing ethanol concentrations. Antigenic recovery was achieved by heat-induced antigen retrieval using an autoclave in citrate buffer at 120 ºC pH 6 for 10 minutes. To avoid unspecific signal in the tissue the protein block serum-free reagent (DAKO) was added. Incubation took place using specific primary antibodies against human antigens (**Table 1**). As negative control, the primary antibody was omitted. Slides were washed with Phosphate Buffer Saline (PBS) and incubated with secondary antibody diluted in protein blocking solution for 30 minutes, protected from light at room temperature (RT). After incubation, cells were washed and counterstained with 4'6-diamidino-2-phenylindole (DAPI) for 10 minutes protected from light at RT. Finally, tissue sections were covered with mounting media (Life technologies) and a coverglass. Positive stained cells were counted at hpf choosing randomly 10 fields (180.7 x 110.1 µm) of each sample and individual. The mean cellular density was expressed as number of cells per hpf. All analysis were carried out with an Olympus BX61 microscope, an OLYMPUS DP26 video camera and the computer program CellSens Standard 1.7.

### 4.2.2 IMMUNOHISTOCHEMISTRY TISSUE STAINING

Tissues were sectioned (5 µm thick), deparaffined in xylene, and gradually rehydrated by using a battery of decreasing ethanol concentrations. CD4 and CD8 stainings were processed by validated immunohistochemistry procedures at the pathology department of HUVH (**Table 1**) and analyzed by two experienced investigators in the Translational Mucosal Immunology laboratory of VHIR. Positive stained cells were counted at hpf choosing randomly 10 fields (180.7 x 110.1 µm) of each sample and subject. The mean cellular density was expressed as number of cells per hpf. All analysis were carried out with an optical Olympus BX61 microscope, an OLYMPUS DP26 video camera and the computer program CellSens Standard 1.7.

**Table 1 | Antibodies and experimental conditions used for immunofluorescence and immunohistochemistry procedures.**

| Antibody | Host and target | Manufacturer and reference | Conditions |
|---|---|---|---|
| Primary antibodies | | | |
| Anti-CD2 | Mouse anti-human | Novus Biologicals NBP1-47690 | 1:100 ON 4 ºC |
| Anti- CD4 | Rabbit anti-human | Roche 790-4423 | PD department routine |
| Anti-CD8α | Rabbit anti-human | Roche 790-4460 | PD department routine |
| Anti-CD8α | Rabbit anti-human | Abcam ab93278 | 1:100 60 min RT |
| Secondary antibodies | | | |
| Alexa Fluor 488 | Goat anti-rabbit | Invitrogen A11070 | 1:500 30 min RT |
| Alexa Fluor 594 | Goat anti-mouse | Invitrogen A11020 | 1:500 30 min RT |
| Labelled polymer HRP | Goat anti-rabbit | Roche 760-500 | PD department routine |

RT: room temperature, ON: over-night, PD: pathology department

## 4.3 GENE EXPRESSION ANALYSIS

### 4.3.1 RNA ISOLATION

RNA was isolated at Parque Científico de Madrid. MirVana™ mRNA Isolation Kit (Ambion) was used following manufacturer's intructions. Samples integrity and concentrations was determined using Bioanalyzer.

### 4.3.2 cDNA SYNTHESIS AND QUANTITATIVE REAL TIME PCR

cDNA synthesis was performed using 1 µg of total RNA using the High Capacity Reverse Transcription Reagents Kit (Thermo Fisher Scientific), following manufacturer's instructions. RNase-free $H_2O$ was used as negative control. Each assay number is available at Applied Biosystems™ (**Table S 1**).

Quantitative real-time PCR (qPCR) was performed with TaqMan Low-Density Arrays (Applied Biosystems) in a 384-well format and spotted on a microfluidic card. For this, each reverse primer was used at a final concentration of 900nM, and a Taq-Man™ MGB probe was used (6-FAM dye-labeled; Applied Biosystems) with a final concentration of 250 nM. Each assay is gene-specific and was designed to span exon-

exon junctions. Thermal cycling conditions were 2 min at 50 ºC, 10 min at 95 ºC, followed by 40 cycles of denaturation at 95 ºC for 15 s, and annealing and extension at 60 ºC for 1 min in an ABI PRISM® 7900 HT Sequence Detection System (Applied Biosystems). This procedure was replicated twice for each gene and each sample. Water was used as negative control. Results were validated in another cohort by qPCR with TaqMan™ Fast Universal PCR Master Mix (2X) (Applied Biosystems) and analyzed on an ABI PRISM® 7500 FAST Sequence Detection System (Applied Biosystems).

Data were analyzed by the $2^{\Delta\Delta Ct}$ method for the treatment effect comparison, using basal condition as reference sample [32]. Relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method; were ΔCt=Ct target gene – Ct endogenous gene and ΔΔCt = ΔCt patient – ΔCt control expressed as fold change. Expression levels of target genes were normalized to the following endogenous genes: 18S, GAPDH, PGK1, GUSB, and PPIA expression.

## 4.4 STATISTICAL ANALYSIS

Data are expressed as median (range) or mean ± standard deviation. Data distribution was analyzed by means of the D'Agostino and Pearson omnibus normality test. GraphPad Prism 8.0 software was used for all statistical analysis. Normally distributed parametric data was compared by the paired or unpaired Student's *t* test (two-tailed). Otherwise, the Mann-Whitney *U* test (for unpaired) or the Wilcoxon *t* test (for paired) was used. Relationship between biological and clinical variables was assessed by Pearson correlation if data followed a normal distribution or by Spearman's rho correlation, if data was semi-quantitative or did not follow normality. Values of $p \leq 0.05$ were considered significant and were adjusted for multiple comparisons using the Benjamini and Hochberg method and the application of correction is indicated in each table and figure legends.

# 5 ANALYTICAL PROCEDURES IN EPITHELIAL CELLS

## 5.1 CHARACTERIZATION OF THE EPITHELIAL CELL LINE AT BASELINE CONDITIONS

Expansion and maintenance of the Het-1A cells was performed and experiments carried out in passages 8-14. Cell viability was calculated by counting cells with Trypan Blue dye in a Neubauer chamber with the following formula: Viability (%) = nº alive cells / (nº alive cells + nº death cells) x 100. For the phenotypic and functional characterization, immunofluorescence staining procedures in well chambers, measurement of TEER in transwells and quantification of gene expression by qPCR was performed as follows:

- Well chamber slides were seeded at a density of 200,000 cells/$cm^2$, with supplemented DMEM medium. Structural and cell junction proteins were characterized after the culture reaches 100 % of confluence, at 10, 14 or 21 days after seeding. Then, the cells were fixed in 4 % PFA and stained.
- Transwells were seeded at a density of 100,000, 200,000 400,000 and 600,000 cells/$cm^2$, with supplemented DMEM medium. The ALI model was performed and apical medium was extracted at day 7 after seeding in order to expose cells to the air [33]. To qualitative evaluate the cell growth, cells were fixed in 4 % PFA at 14 or 21 days after seeding and stained with H&E.
- Het-1A cells were seeded at a density of 10,000 cells/$cm^2$ in 48-well plates, with supplemented DMEM medium. Gene expression analysis was performed after the culture reached 100 % confluence at day 10, and also at day 14 and 21 after seeding. Then, cells were harvested, RNA was preserved in RLT Buffer (QIAGEN) - β-mercaptoethanol (100:1) and stored at -80 ºC.

## 5.2 CHARACTERIZATION OF THE EPITHELIAL RESPONSE TO CYTOTOXIC MEDIATORS

Experimental conditions are summarized in **Table 2** and **Figure 3**.

### 5.2.1 EoE IN-VITRO MODEL IN CELL LINE.

Forty-eight-wells plates were seeded at a density of 10,000 cells/cm$^2$, with supplemented DMEM medium. Experiments were performed after the culture reached the adequate confluence at 10, 14 or 21 days after seeding. Then, to partially reproduce the effect inflammatory mediators induce in the esophageal epithelium, and as a positive control of epithelial responsiveness[34], HET-1A cells were exposed to IL-13 (200-13, Peprotech) at 100 ng/mL and harvested after 24 hours. RNA of cell pellets were stabilized in RLT lysis buffer (RNAeasy Mini Kit, Qiagen) with β-mercaptoethanol (100:1) and stored at -80 ºC until further extraction and analysis.

### 5.2.2 CELL LINE EXPOSURE TO CD8-ISOLATED MEDIATORS

Forty-eight-wells plates were seeded at a density of 10,000 cells/cm$^2$, with supplemented DMEM medium. Experiments were performed after the culture reached the adequate confluence at day 14 after seeding. To identify the effect of CD8-isolated mediators on esophageal epithelium, cells were exposed to cytotoxic mediators (**Table 2**) and harvested at different time points: 30 minutes, 1 hour, 4 hours or 24 hours. RNA of cell pellets were stabilized in RLT lysis buffer with β-mercaptoethanol (100:1) and stored at -80 ºC until further extraction and analysis.

### 5.2.3 BIOPSY CULTURE

Esophageal biopsies were placed 100 uL of supplemented DMEM and exposed to mediators and collected at 4 hours. Biopsies were preserved in RNAlater buffer (Qiagen) and stored at - 80 ºC until further RNA extraction and analysis.

**Table 2 | Mediators used for Het 1-A cell line exposure**

| Stimuli | Trading and reference | Vehicle | Concentration |
|---|---|---|---|
| **IL-13** | Preprotech 200-13 | BSA 0.1 % + $H_2O$ | 100 ng/mL |
| **Granzyme A** | Enzo ALX-200-605 | 0.9 % NaCl | 192 nM |
| **Granzyme B** | Enzo ALX-200-602 | 0.9 % NaCl | 30 nM |
| **CD8-isolated mediators** | | DMEM | 1:2 |

IL: Interleukin; BSA: Bovine Serum Albumin; NaCl: Sodium chloride. CD8-isolated mediators were obtained from lysed CD8 cells, isolated from blood.

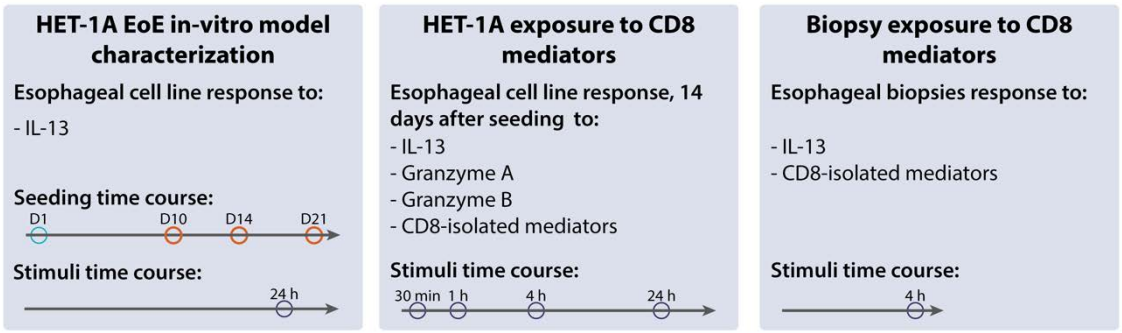


**Figure 3 | Experimental design of the conditions used to identify the effect of CD8-isolated mediators on esophageal epithelial cells.** CD8-isolated mediators were obtained from lysed CD8 cells, isolated from blood.

## 5.3 ANALYSIS OF CELL STRUCTURE AND PHENOTYPE

### 5.3.1 HEMATOXILIN & EOSIN STAINING

Cells grown in transwell chambers (Costar) were washed in 37 ºC PBS (Gibco), fixed with 4 % PFA for 10 minutes and washed with PBS. The transwell membrane was cutted and embedded in paraffin. Samples were cut at 4 µm and stained with hematoxylin & eosin and further observed in an optical Olympus BX61 microscope, an OLYMPUS DP26 video camera and the computer program CellSens Standard 1.7.

### 5.3.2 IMMUNOFLUORESCENCE STAINING OF CULTURED CELLS

Cells grown in well-chamber slides (Sarstedt) were washed in 37 ºC PBS (Gibco), fixed with methanol-acetone 1:1 for 10 minutes and washed with cold PBS. To avoid unspecific signal, samples were incubated for 2 hours with protein blocking solution (DAKO). Next, cells were incubated with the primary antibody (**Table 3**) diluted in

protein blocking solution, ON in a wet chamber at 4 ºC. The following day, cells were washed with cold PBS and incubated with the secondary antibody, diluted in protein blocking solution for 30 minutes in the dark at RT. After incubation, cells were washed with PBS and incubated during 10 minutes at RT with DAPI to counterstain nuclei. Finally, samples were washed with PBS and covered with a glass and mounting media (Life technologies).

**Table 3 | Antibodies and experimental conditions used for immunofluorescence.**

| Reactives | Host and target | Manufacturer and reference | Conditions |
|---|---|---|---|
| **Microfilament staining** | | | |
| **Phalloidin** | - | Sigma-Aldrich P1951 | 1:500 30min RT |
| **Primary antibodies** | | | |
| **Anti-Desmoglein 1** | Mouse anti-human | Merk Millipore MABT118 | 1:25 ON 4 ºC |
| **Anti-Zonula 1** | Mouse anti-human | Invitrogen 33-9100 | 1:100 ON 4 ºC |
| **Anti-Occludin** | Rabbit anti-human | Invitrogen 71-1500 | 1:100 ON 4 ºC |
| **Anti-Cytokeratin** | Mouse anti-human | Santa Cruz sc-51582 | 1:200 ON 4 ºC |
| **Anti-Vimentin** | Goat anti-human | Santa Cruz sc-7557 | 1:200 ON 4 ºC |
| **Secondary antibodies** | | | |
| **Alexa Fluor 488** | Goat anti- mouse | Invitrogen A11017 | 1:500 30 min RT |
| **Alexa Fluor 488** | Chicken anti-goat | Invitrogen A21467 | 1:500 30 min RT |
| **Alexa Fluor 594** | Goat anti-rabbit | Invitrogen A11072 | 1:500 30 min RT |
| **Alexa Fluor 647** | Chicken anti-mouse | Invitrogen A21463 | 1:500 30 min RT |

Ab: Antibody; RT: Room Temperature; ON: Over-night

## 5.4 BARRIER FUNCTION ASSESSMENT

To avoid the influence of temperature variability in TEER measurements, cells growing in transwells plates were equilibrated from 37 ºC to room temperature for at least 15 minutes before starting TEER monitoring. In order to clean and optimize the electrodes before TEER measurement, electrodes of the epithelial voltohmmeter (EVOM) were immersed into ethanol 70 % for 5 minutes, PBS for 5 minutes and cell culture medium for 10 minutes. For TEER measurement, one electrode was placed in the upper and the other in the lower compartment of the transwells. The measurement procedure consisted in measuring the resistance across the layer at least 3 times. As base blank, the resistance of the semipermeable membrane was measured in a pre-coated transwell containing culture media without cells. The

resistance is inversely proportional to the effective area of the semipermeable membrane. The final TEER values are reported in units of Ω·cm$^2$ and calculated as: $TEER_{reported} = (R_{sample} - R_{blank}) \times MA$, being R the resistance in ohms and MA the membrane area in cm$^2$.

## 5.5 CD8$^+$ LYMPHOCYTES ISOLATION

### 5.5.1 PERIPHERAL BLOOD MONONUCLEAR CELLS ISOLATION

Isolation of peripheral blood mononuclear cells (PBMC) was performed from 8 mL of freshly collected heparinized blood diluted in 27 mL of PBS-EDTA 2 mM. Diluted samples were placed carefully over 15 mL of Ficoll-Histopaque and a density gradient centrifugation was performed, for 30 minutes, 24 ºC at 1,000 g. Blood components get distributed along the tube according to their gradient of density, as illustrated in **Figure 4**. PBMCs layer was aspirated and transferred into a new tube containing 40 mL of PBS-EDTA 2 mM + 2 % FBS. Cell suspension was centrifuged at 120 g for 10 minutes at 24 ºC resuspended in 2 mL of PBF-EDTA-FBS and the number of cells were counted for each isolation.

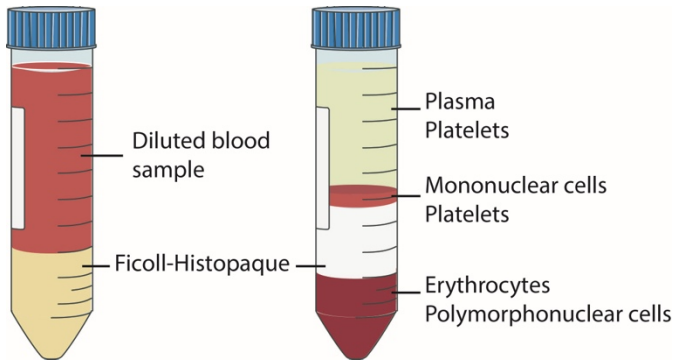


**Figure 4 | Illustration of Ficoll - Histopaque gradient, used for cell separation.**

### 5.5.2 CD8$^+$ CELLS PURIFICATION

The CD8$^+$ population was separated from total PBMCs using a positive isolation kit (Milteny Biotech), following manufacturer's instructions. Briefly, both cells and reagents were kept cold during all purification steps to prevent capping the

antibodies on cell surface and non-specific cell labeling. The PBMCs cell suspension was centrifuged at 300 g for 10 minutes at 4 ºC and resuspended in 80 µL of MACs isolation buffer per up to $10^7$ cells. Then, 20 µL of CD8 microbeads were added per up to $10^7$ cells and incubated for 15 minutes (2-8 ºC). Cells were washed and diluted in 500 µL of MACs isolation buffer.

Following manufacturer instructions, according to number of total cells, a LS column was placed in the magnetic MACs separator and prepared by rising 3 mL of MACs isolation buffer. Sample suspension was placed onto the column, unlabeled cells passed through and were discarded. Then the column was washed 3 times with 3 mL of MACs isolation buffer. After, the column was removed from the magnetic field, 5mL of buffer were added and immediately flushed out to recover the $CD8^+$ cell population. Finally, isolated cells were counted and purity was analyzed by FACS and immunofluorescence.

### 5.5.3 OBTENTION OF $CD8^+$ CELLS LYSATE

The cytoplasmatic content from $CD8^+$ cells was extracted by mechanical lysis. Cells were centrifuged and resuspended in 1 mL of DMEM. The suspension was homogenized through a 27G needle for 20 times and sonicated using an ultrasonic ice-bath for 30 minutes. Lysates were frozen at -80 ºC, defrosted and centrifuged at 1200 g for 10 minutes at 4 ºC. Supernatant was aliquoted and stored at -80 ºC until used for stimulation experiments. Total protein isolated was quantified using Bradford protein assay (BioRad) and cytotoxic proteins from the extract were identified through Western Blot.

### 5.5.4 ANALYSIS OF $CD8^+$ PHENOTYPE

Cell phenotype and the presence of cytotoxic mediators from isolated cells was confirmed by different technical procedures.

#### 5.5.4.1 IMMUNOFLUORESCENCE STAINING OF CELLS IN SUSPENSION

Cell pellets ($1.5 \cdot 10^5$ cells) were washed in PBS supplemented with 2 % FBS and centrifuged at 300 g for 7 min at 4 ºC in round bottom polystyrene tubes. When permeabilization was required, in the case of cytoplasmic proteins (**Table 4**), cell pellets were pre-treated with BD Cytofix/Cytoperm reagent (BD Biosciences) for 20 minutes at 4 ºC and washed twice with Perm/Wash buffer (BD wash, BD Biosciences). Cell pellets were incubated with primary antibody for 1 hour on ice (**Table 4**). Primary antibodies were previously diluted in PBS + 2 % FBS buffer for non-permeabilized samples, and with BD wash buffer for permeabilized samples. After incubation, cells were washed with PBS twice and incubated for 30 minutes at 4 ºC light protected with the appropriate secondary antibody (**Table 4**). Cells were washed twice, either with PBS + 2 % FBS or with BD wash buffer. Finally, cells were resuspended in 100 µL of PBS and added to a cytofunnel apparatus attached to a glass slide and centrifuged in a cytocentrifuge at 200 g for 6 minutes. For cytospin preparations, stained cells in suspension were fixed with 4 % paraformaldehyde for 5 minutes and incubated for 10 minutes with DAPI, washed twice with PBS and mounted with Prolong gold antifade mounting media (Invitrogen). Samples were kept at 4 ºC light protected until analysis.

#### 5.5.4.2 FLOW CYTOMETRY

Immunofluorescence staining of cells in suspension was performed as described above in section 5.5.4.1. Stained cells were collected and analyzed on a FacsCalibur Flow Cytometer (BD biosciences). Analysis was performed quickly after the staining. Additionally, to assess cell viability, 10 µL of propidium-iodure were added to a viability control tube (without primary and secondary antibody) in a 200 µL of final volume.

**Table 4 | Antibodies and experimental conditions used for immunofluorescence staining of cells in suspension.**

| Reactives | Host and target | Manufacturer and reference | Conditions |
|---|---|---|---|
| **Nuclei staining** | | | |
| **Propidium-iodure** | - | Sigma-Aldrich P4864 | 50 µg/mL |
| **DAPI** | - | Life technologies D1306 | 10 ng/mL |
| **Primary antibodies** | | | |
| **Anti-granzyme A** | Mouse anti-human | AbD Serotec MCA 2117 | 1:25 BD Cf/Cp |
| **Anti-granzyme B** | Mouse anti-human | Novus Biologicals NBP1-97525 | 1:25 BD Cf/Cp |
| **Anti-CD8α** | Rabbit anti-human | Abcam ab 93278 | 1:500 PBS+FBS |
| **Secondary antibodies** | | | |
| **Alexa Fluor 488** | Goat anti-rabbit | Invitrogen A11070 | 1:500 PBS+FBS |
| **Alexa Fluor 594** | Goat anti-mouse | Invitrogen A11010 | 1:500 BD Cf/Cp |
| **Alexa Fluor 647** | Chicken anti-mouse | Invitrogen A21463 | 1:500 BD Cf/Cp |

BD Cf/Cp: BD Cytofix/Cytoperm; PBS: Phosphate Buffer Saline; FBS: Fetal Bovine Serum

#### 5.5.4.3 WESTERN BLOT

The cell lysate from the $CD8^+$ isolated cells (10 mg of protein) was separated by electrophoresis and blotted to a polyvinylidene fluoride (PVDF) membrane (ThermoFisher Scientific). After blocking with 5 % solution of non-fat milk (BioRad), membranes were incubated with primary antibody (**Table 5**). After washing with TBS Tween, membranes were incubated with specific secondary antibodies for 1 hour at RT and, finally, with SuperSignal TM West Femto Maximum Sensitivity Substrate reagent (Thermo scientific). Images were digitized with Odyssey Fc 2800. LI-COR.

**Table 5 | Antibodies and experimental conditions used for Western blot**

| Reactives | Host and target | Manufacturer and reference | Conditions |
|---|---|---|---|
| **Primary antibodies** | | | |
| **Anti-granzyme A** | Mouse anti-human | AbD Serotec MCA 2117 | 1:250 O/N 4 ºC |
| **Anti-granzyme B** | Mouse anti-human | Novus Biologicals NBP1-97525 | 1:250 O/N 4 ºC |
| **Anti-cyclophilin 1** | Rabbit anti-human | Proteintech 10720-1-AP | 1:1250 2h RT |
| **Secondary antibodies** | | | |
| **Secondary Antibody, HRP** | Goat anti-rabbit | ThermoFiser Scientific, 32460 | 1:2500 30 min RT |
| **Secondary Antibody, HRP** | Goat anti-mouse | ThermoFiser Scientific, 32430 | 1:2500 30 min RT |

AB: Antibody; HRP: Horsearadish peroxidase; O/N: Over-night; RT: Room Temperature

## 5.6 GENE EXPRESSION

### 5.6.1 RNA ISOLATION

RNA Isolation protocol was performed with different kits depending on the needs of each experiment, as detailed:

#### 5.6.1.1 CELL LINE

Cell pellets were homogenized through a 20G (0.9 mm) needle 10 times. After disruption, sample was eluted in RNeasy mini columns (QIAGEN) according to manufacturer instructions. Finally, RNA was eluted in 20 µL of RNase-free water and quantified using Nanodrop™. Samples were stored at -80 ºC until processed for analysis.

#### 5.6.1.2 CULTURED ESOPHAGEAL BIOPSIES

One single biopsy from each subject was homogenized through 3 cycles of 5-second-of FastPrep™ in a lysing matrix D tube (MP Biomedicals). After disruption it was eluted in RNaquous micro kit (Life technologies) according to manufacturer instructions. Finally, RNA was eluted in 20 µL of RNase-free water and quantified using Nanodrop™. Samples were stored at -80 ºC until processed for analysis.

### 5.6.2 cDNA SYNTHESIS AND QUANTITATIVE REAL TIME PCR

cDNA synthesis was performed using 0.5 µg of total RNA using the High Capacity Reverse Transcription Reagents kit (Thermo Fisher Scientific), following manufacturer's instructions. RNase-free H2O was used as negative control.

Transcriptional analysis was assessed by qPCR using validated TaqMan Gene Expression Assays (Thermo Fisher Scientific) on an ABI PRISM® 7500 FAST Sequence Detection System (Applied Biosystems). Due to the low abundance of some specific genes analyzed in esophageal samples, pre-amplification of cDNA was performed before qPCR when required (**Table S 1**) with the PreAmp Master Mix following manufacturer instructions (Applied Biosystems). Analyzed genes are shown in

supplementary **Table S 1**. After analyzing their stability, GAPDH, PPIA and 18S genes were used as endogenous control for data normalization. Quantification of gene expression was performed in a minimum of three different passages (Biological replicates) and each sample was run in triplicate (technical replicates), including negative controls for the reverse transcription the qPCR.

Data were analyzed by the $2^{\Delta\Delta Ct}$ method (using basal condition as reference sample) for the treatment effect comparison [32]. Relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method; were ΔCt=Ct target gene – Ct endogenous gene and ΔΔCt = ΔCt stimulated cells – ΔCt vehicle expressed as fold change.

## 5.7 STATISTICAL ANALYSIS

Data are expressed as median (range) or mean ± standard deviation. Data distribution was analyzed by means of the D'Agostino and Pearson omnibus normality test. GraphPad Prism 8.0 software was used for all statistical analysis. When sample followed normality, one-way ANOVA was performed when comparing between groups, time points and treatments. When comparing each sample with its vehicle one sample Student's *t* test and Wilcoxon test were used for statistical analysis. Values of $p \leq 0.05$ were considered significant and were adjusted for multiple comparisons using the Benjamini and Hochberg method and the application of correction is indicated in each table and figure legends.

# RESULTS

## 1 HUMAN SAMPLES

### 1.1 STUDY POPULATION

Twenty-one control subjects and 21 EoE patients were included in the study. Clinical characteristics of the experimental subjects are given in **Table 6**. No differences were observed in age or sex between the study groups ($p>0.05$) and the mean duration of symptoms for this group exceeded 5 years. No eosinophil was found in the esophageal epithelium of subjects from the control group, while a significant infiltration was observed in esophageal biopsies from the EoE patients group. Within the gastric and duodenal samples, all experimental groups showed similar eosinophil counts, as part of the leukocyte population in these anatomical regions. The predominant symptoms in EoE patients were dysphagia and food impaction. On the other hand, the control group presented a wider range of symptoms as reported in **Table 6**.

During endoscopy, a greater percentage of subjects in the control group presented a normal mucosal appearance when compared to EoE patients ($p=0.0036$). In the control group, erosive esophagitis was the most common finding, while in the EoE group longitudinal furrows and white plaques were the most frequent findings. Esophageal rings were a common finding in both groups.

Personal background of atopy was more common in the EoE group ($p>0.05$), especially allergic rhinitis ($p=0.06$), food sensitivity ($p=0.0014$) and bronchial asthma ($p=0.0034$). Family background of atopy was similar in both cohorts.

**Table 6 | Clinical and demographic characteristics of participants.** Values represent the mean ± SD, median (range) or nº individuals (percentage). Some patients did not report information on atopy, indicated as data not available.

| | | Control (n=21) | EoE (n=21) | p value |
|---|---|---|---|---|
| | **Age (years)** | 41.4 ± 3 | 38.4 ± 3 | 0.50 |
| | **Sex** | Male: 15 (71 %) | Male: 14 (67 %) | 0.99 |
| | **Time of evolution (months)** | - | 61 ± 44 | - |
| | **Eosinophil infiltration** | 0 | 51 (15 - 100) | - |
| **Symptoms at first endoscopy** | **Abdominal pain** | 3 (14 %) | 0 | - |
| | **Heartburn** | 7 (33 %) | 2 (9.5 %) | 0.28 |
| | **Dysphagia** | 13 (62 %) | 17 (81 %) | 0.31 |
| | **Food impaction** | **5 (24 %)** | **18 (86 %)** | **0.0001** |
| | **Vomiting** | 0 | 2 (9.5 %) | - |
| | **Regurgitation** | **7 (33 %)** | **1 (5 %)** | **0.0045** |
| | **Functional dyspepsia** | 3 (14 %) | 0 | - |
| | **Weight loss** | 0 | 1 (5 %) | - |
| **Endoscopy: mucosal appearance** | **Normal** | **13 (62 %)** | **3 (14 %)** | **0.0036** |
| | **White plaques** | 0 | 11 (52 %) | - |
| | **Rings** | **3 (14 %)** | **14 (67 %)** | **0.0013** |
| | **Edema** | 0 | 4 (19 %) | - |
| | **Longitudinal furrows** | 0 | 15 (71 %) | - |
| | **Stenosis** | 0 | 3 (14 %) | - |
| | **EE g. A** | 3 (14 %) | 0 | - |
| | **EE g. B** | 2 (9.5 %) | 0 | - |
| **Personal background of atopy** | **No atopy** | 9 (43 %) | 4 (19 %) | 0.18 |
| | **Allergic rhinitis** | 7 (33 %) | 14 (67 %) | 0.06 |
| | **Drug sensitivity** | 1 (5 %) | 0 | - |
| | **Atopic dermatitis** | 1 (5 %) | 1 (5 %) | 0.99 |
| | **Food sensitivity** | **1 (5 %)** | **11 (52 %)** | **0.0014** |
| | **Bronchial asthma** | 0 | 8 (38 %) | - |
| | **Data not available** | 4 (19 %) | 0 | - |
| **Family background of atopy** | **No atopy** | 15 (71 %) | 16 (76 %) | 0.99 |
| | **Allergic rhinitis** | 0 | 2 (9.5 %) | - |
| | **Drug sensitivity** | 0 | 1 (5 %) | - |
| | **Food sensitivity** | 0 | 1 (5 %) | - |
| | **Bronchial asthma** | 0 | 1 (5 %) | - |
| | **Data not available** | 6 (29 %) | 3 (14 %) | 0.45 |

EE g A/B: Erosive Eophagitis grade A/B.

The twenty-one active EoE patients who underwent treatment (SFED or PPI) reduced significantly the peak of eosinophils infiltrated in the mucosa from 51 (15-100) to 0 (0-10) (**Figure 5**).

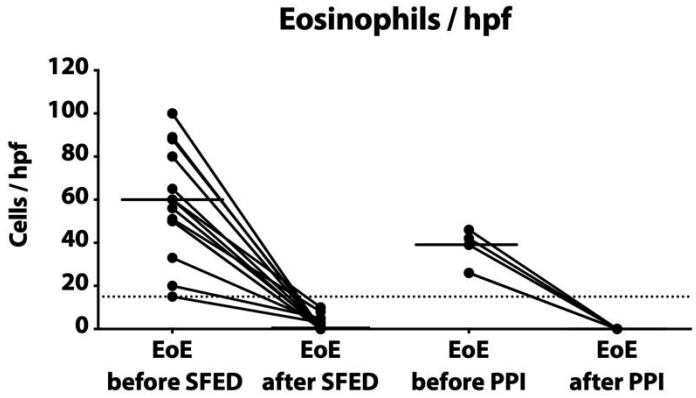


**Figure 5 | Eosinophil intraepithelial infiltration in EoE patients before and after treatment.** Dotted line represents the diagnosis cut-off value of 15 eosinophils/hpf. Horizontal bars represent median value. tx: treatment.

In **Table 7,** clinical and histological characteristics of EoE patients before and after treatment are summarized. Dysphagia score was significantly reduced ($p<0.0001$) in EoE patients after treatment. Also, a higher percentage of active dysphagia (>5) was observed in EoE patients before treatment ($p=0.0013$).Additionally, treatment did also reduce the EREF score although not reaching statistical significance ($p=0.0625$). Further, a non-significant trend was observed in achieving normal mucosal appearance after SFED. Endoscopic features such as white plaques, oedema and longitudinal furrowings were significantly reduced ($p<0.05$)

**Table 7 | Clinical characteristics of participants before and after SFED**. Values represent the mean ± SD, median (range) or nº individuals (percentage).

| | | EoE before tx (n=21) | EoE after tx (n=21) | p- value |
|---|---|---|---|---|
| **Dysphagia score** | **Mean** | **8.1 ± 0.9** | **4.8 ± 0.4** | **<0.0001** |
| | **<5** | **6 (30 %)** | **17 (85 %)** | **0.002** |
| | **≥5** | **14 (70 %)** | **3 (15 %)** | **0.0013** |
| **Endoscopy mucosal appearance** | **EREF score** | 3.5 (1-5) | 1 (0-5) | 0.0625 |
| | **Normal** | 3 (14 %) | 8 (38 %) | 0.16 |
| | **White plaques** | 11 (52 %) | 0 | - |
| | **Rings** | 14 (67 %) | 11 (52 %) | 0.53 |
| | **Edema** | 4 (19 %) | 2 (9.5 %) | 0.66 |
| | **Longitudinal furrows** | **15 (71 %)** | **3 (14 %)** | **0.0004** |
| | **Estenosis** | 1 (5 %) | 1 (5 %) | >0.99 |

tx: Treatment

From the 21 EoE patients of the study, 5 responded to PPI and 16 to SFED. In the SFED-responders subgroup, the most common triggering foods were milk and cereals (**Table 8**). The majority of patients had only 1 or 2 triggering food out of the SFED.

**Table 8 | Triggering foods in EoE participants who underwent SFED treatment.** Values represent nº individuals (percentage).

| Triggering foods | | Nº Triggering foods / patient | |
|---|---|---|---|
| **Cereals** | 9 (56 %) | **1** | 5 (31 %) |
| **Milk** | 10 (62.5 %) | **2** | 2 (12.5 %) |
| **Legumes/soy** | 6 (37.5 %) | **3** | 5 (31 %) |
| **Egg** | 5 (31 %) | **>3** | 4 (25 %) |
| **Fish & seafood** | 5 (31 %) | | |
| **Nuts** | 5 (31 %) | | |

## 1.2 INTRAEPITHELIAL LYMPHOCYTES

Given the role allergy plays in EoE, intraepithelial lymphocytes were assessed as $CD4^+$ and $CD8^+$ cell counts and CD4/CD8 ratio in active disease compared to controls (**Figure 6** and **Figure 7**).

No differences in $CD4^+$ lymphocytes or in the CD4/CD8 ratio were found comparing control and active EoE patients. However, a significant increase was found for $CD8^+$ lymphocytes (**Figure 6**).

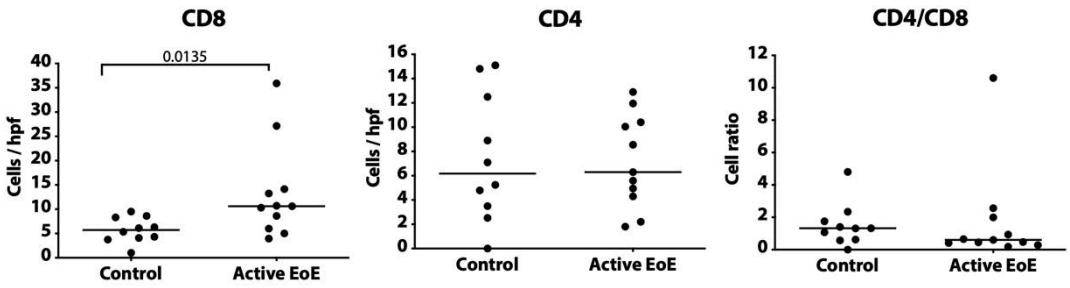


**Figure 6 | $CD8^+$ cells, $CD4^+$ and double positive cell counts in esophageal samples in patients with active EoE and in control subjects.** Data are expressed as mean ± SD. Graphs represent data of 8–10 individuals. Mann-Whitney *U* test was used to compare groups. Horizontal bars indicate median values.

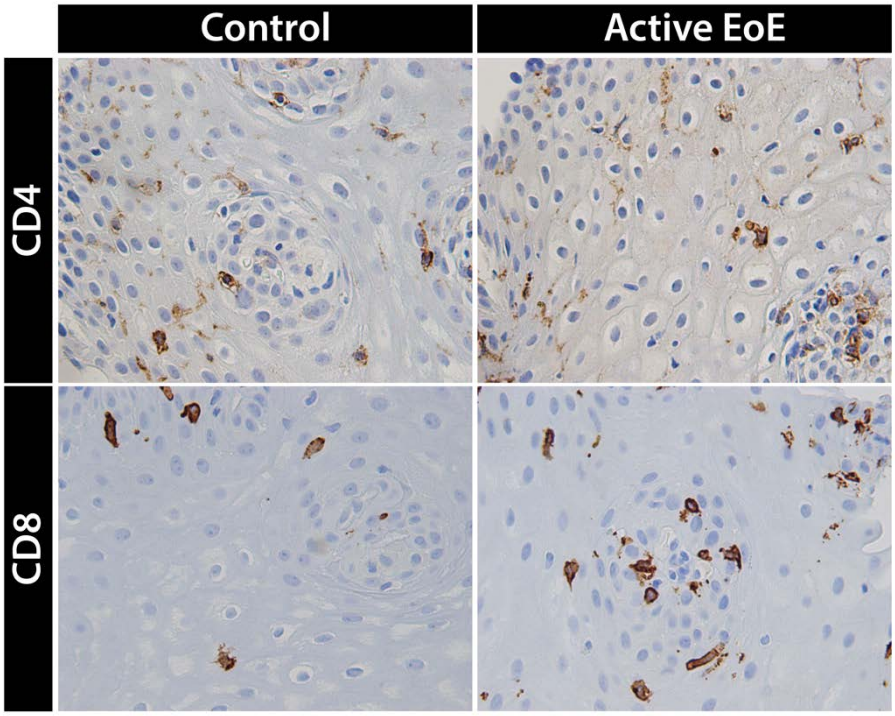


**Figure 7 | Representative images of $CD8^+$ and $CD4^+$ cells in esophageal samples in active EoE and control groups.** Hematoxylin counterstaining in blue, CD8 and CD4 cells in brown. Magnification 400X.

The effect of two different treatments on lymphocytes counts was analyzed in the two subgroups receiving PPI or SFED (**Figure 8**). PPI-treated patients showed no significant differences in the cellular infiltration after therapy. SFED treatment significantly decreased the number of infiltrated $CD4^+$ lymphocytes ($p=0.04$) and slightly decreased the $CD8^+$ lymphocytes counts ($p>0.05$). No biological differences were found in the CD4/CD8 ratio.

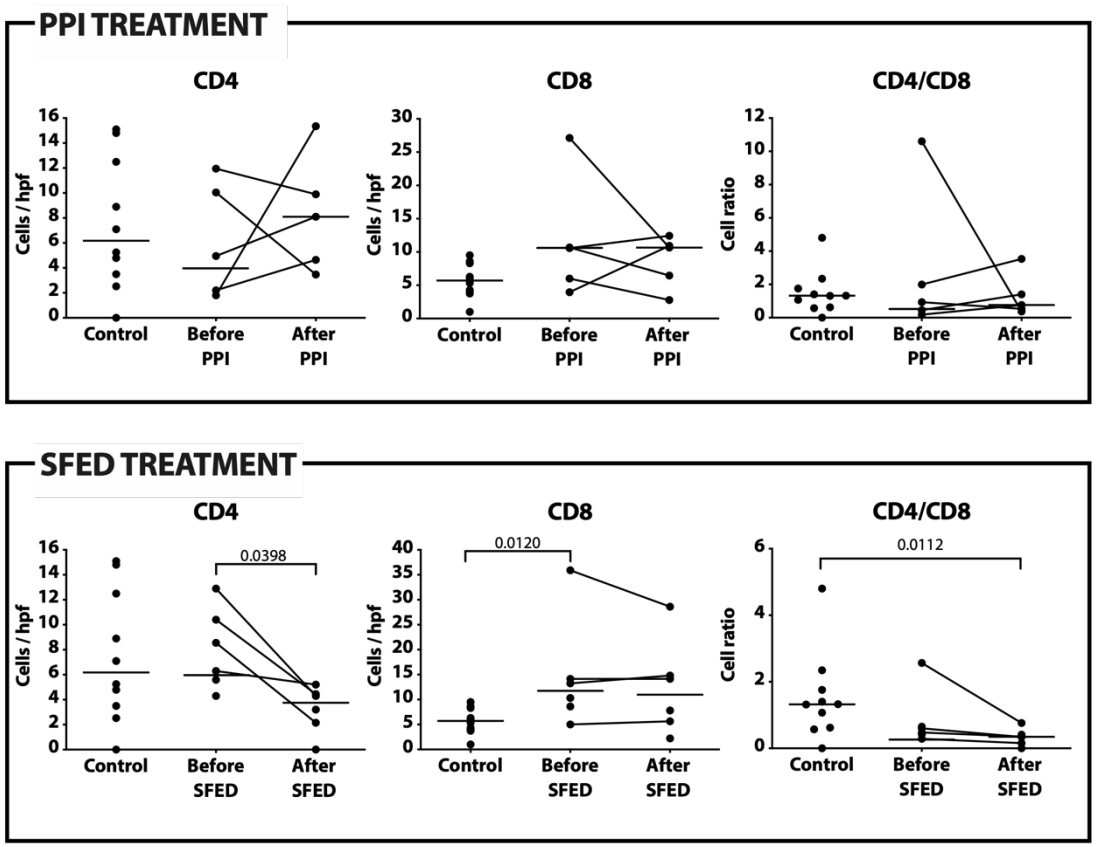


**Figure 8 | $CD8^+$, $CD4^+$ and CD4/CD8 ratio in esophageal samples from EoE patients before and after PPI treatment or six-food elimination diet (SFED) and in control subjects.** Data are expressed as mean ± SD. Graphs represent data of 5–10 individuals. Comparison between EoE patients before and after treatment was performed using the paired Wilcoxon t test. Comparison between EoE patients (before or after) with control subjects was performed using the Mann-Whitney *U* test. Horizontal bars indicate median values.

In the subgroup of EoE patients responders to SFED, the number of cells expressing the CD8α chain and the CD2 marker of active-homing lymphocyte were analyzed (**Figure 9** and **Figure 10**). All, CD8, CD2 and double positive cells were significantly over-infiltrated in active EoE patients (before SFED) when compared to the control group. This increase was normalized after therapy to control values ($p<0.005$) (**Figure 9**).

In control subjects, 76 % of the recruited cells ($CD2^+$) cells were $CD8^+$, being the remaining 24 % of other subsets of active lymphocytes. In EoE patients this percentage increased up to 84 %. Although the number of $CD2^+$ cells decreased after treatment, the proportion of $CD8^+$ from the total recruited cells remained at 84 % suggesting the maintenance of CD8 active phenotpye despite remision. However, no statistical differences are observed.

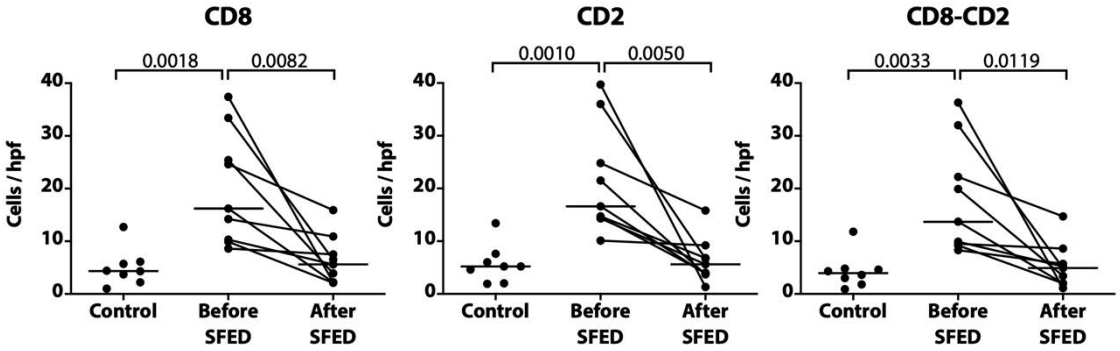


**Figure 9 | CD8α⁺ cells, CD2⁺ and double positive cell counts in esophageal samples in patients with EoE before and after six-food elimination diet (SFED) and in control subjects.** Data are expressed as mean ± SD. Graphs represent data from 8–10 individuals. To compare EoE patients before and after treatment, the paired Wilcoxon t test was used. The Mann-Whitney *U* test was used to compare EoE patients (before or after) with control subjects. Horizontal bars indicate median values.

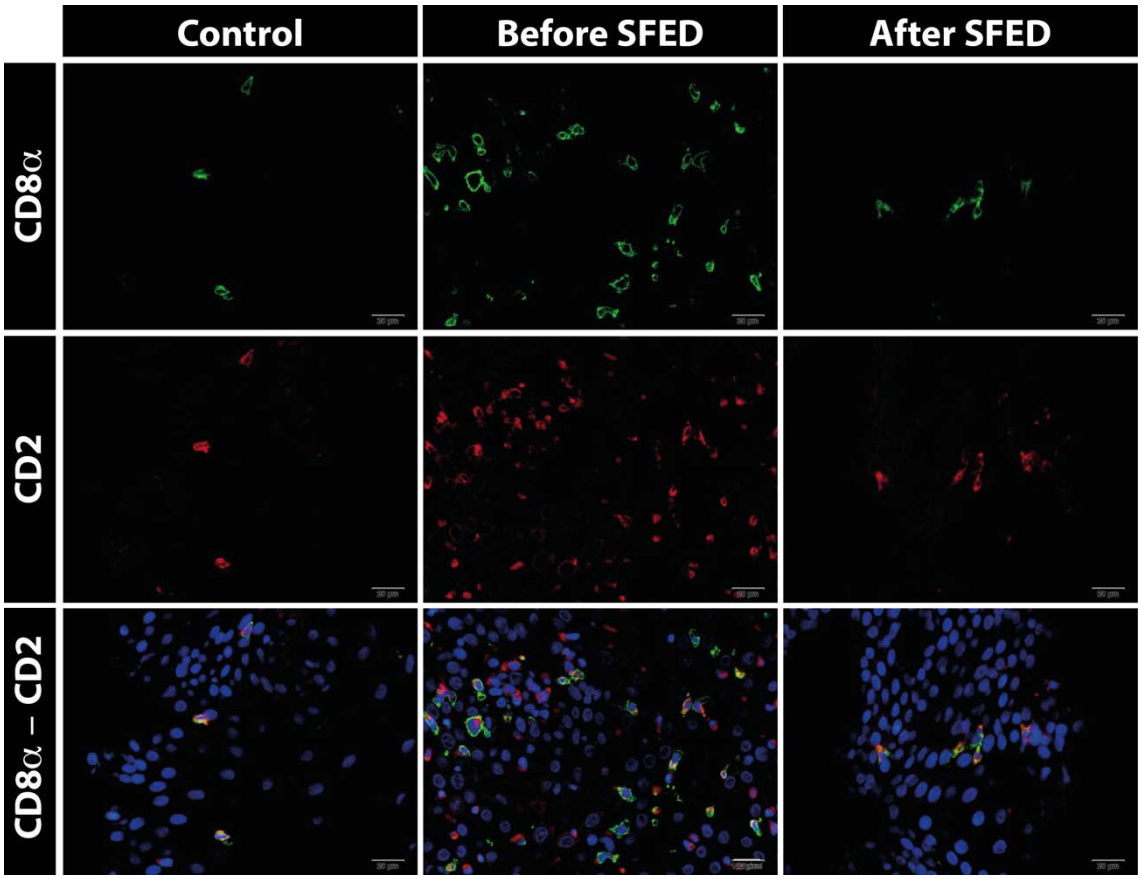


**Figure 10 | Representative images for CD8α, CD2 and double positive cells in esophageal samples in EoE patients before and after SFED and in the control group.** DAPI counterstaining identified in blue, CD8α cells in green and CD2 cells in red. Magnification 400X.

The number of $CD8^+$ lymphocytes positively correlated with the number of $CD2^+$ ($r^2$=0.68; FDR=1.02E-13) and $CD4^+$ ($r^2$=0.49; FDR=0.05) lymphocytes. When analyzing biological and clinical associations a positive correlation between the number of eosinophils with $CD2^+$ cells ($r^2$=0.83, FDR=3.58E-06) and with $CD8^+$ cells ($r^2$= 0.58; FDR=2.36E-05) was observed but no correlation was observed with $CD4^+$ cells. Additionally, the number of $CD2^+$ cells positively correlated with dysphagia score ($r^2$=0.68; FDR=0.02)

### 1.3 EXPRESSION OF LYMPHOCYTE-ASSOCIATED MOLECULES IN THE ESOPHAGEAL TISSUE

The expression of CD8-related molecules was analyzed comparing active EoE and a control cohort as well as the effect of SFED therapy on those molecules in esophageal (**Table S 2**) and duodenal (**Table S 3**) biopsies.

The expression of **lymphocyte surface markers**, *CD4* and *CD8* genes were overexpressed in active EoE compared with controls, not decreasing significantly after SFED treatment (**Figure 11**). In duodenal samples, no differences were observed in those genes (**Table S 3**).

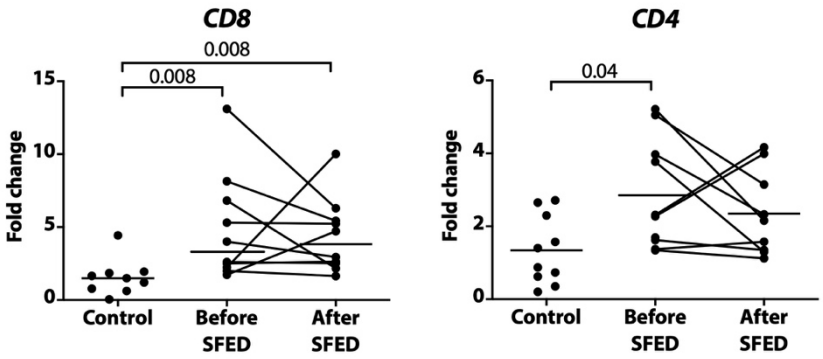


**Figure 11 | Gene expression of lymphocyte surface markers in esophageal biopsies from EoE patients before and after six food elimination diet (SFED) and control subjects.** Relative expression was analyzed by qPCR. To obtain the fold-change value for each sample the ratio between target gene and the average of reference genes (18S, PPIA; PGK1, GAPDH, GUSB) was calculated for each sample and then normalized to the average of the control group. Horizontal bars indicate mean or median values and significant p values are indicated in the graphs.

The expression of **genes involved in lymphocyte homing (Figure 12),** *ITGA4* (Integrin $\alpha$4)*, MADCAM1* (Mucosal Vascular Addressin Cell Adhesion Molecule 1), *ICAM1* (Intercellular Adhesion Molecule 1) and *ICAM2* (Intercellular Adhesion Molecule 2) were increased in the esophagus of active EoE patients compared with controls. SFED treatment reduced the expression of *MADCAM1* compared to active EoE, however not reaching statistical significance (p=0.08), and it did not reach values of the control group. In addition, SFED reduced *ICAM1* expression to control values and *ICAM2* expression close to control values, while no differences in *ITGA4* were found. Additionally, gene expression was similar in all groups in the duodenal mucosa (**Table S 3**).

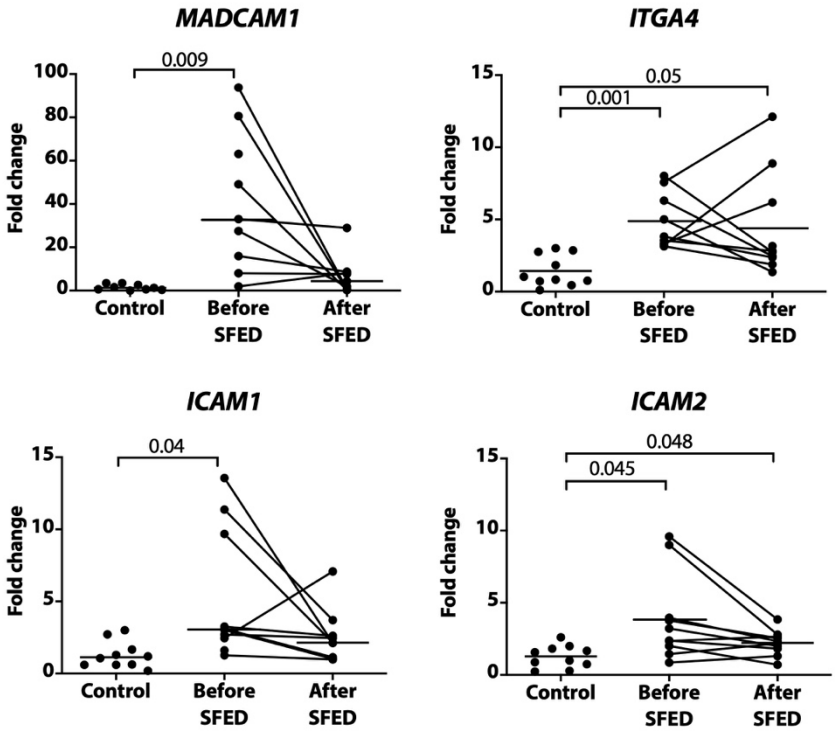


**Figure 12 | Gene expression of lymphocyte homing molecules in esophageal biopsies from EoE patients before and after six food elimination diet (SFED) and control subjects.** Relative expression was analyzed by qPCR. To obtain the fold-change value for each sample the ratio between target gene and the average of reference genes (18S, PPIA; PGK1, GAPDH, GUSB) was calculated for each sample and then normalized to the average of the control group. Horizontal bars indicate mean or median values and significant p values are indicated in the graphs.

**The expression of the MHCI-related genes** *MICA* (MHC Class-I Polypeptide-Related Sequence A) and *MICB* (MHC Class-I Polypeptide-Related Sequence B) was analyzed. *MICA* expression was heterogeneous in active EoE and SFED modulated it to a more homogeneous gene expression profile, without decreasing to control values (p=0.02). *MICB* was overexpressed in active EoE and SFED treatment did not modify this profile (**Figure 13**). For both genes no differences were observed between groups in the duodenum (**Table S 3**).

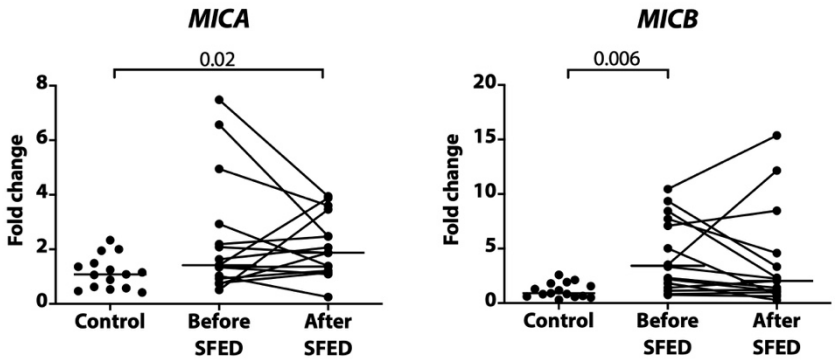


**Figure 13 | Gene expression of MHCI antigen presenting molecules in esophageal biopsies from EoE patients before and after six food elimination diet (SFED) and control subjects.** Relative expression was analyzed by qPCR. To obtain the fold-change value for each sample the ratio between target gene and the average of reference genes (18S, PPIA; PGK1, GAPDH, GUSB) was calculated for each sample and then normalized to the average of the control group. Horizontal bars indicate mean or median values and significant p values are indicated in the graphs.

### Gene expression of the CD8-related molecules associated with cytotoxic activity

*GZMA* (Granzyme A), *GZMB* (Granzyme B), *PRF1* (Perforin 1), *GNLY* (Granulysin) and *GRN* (Granulin) was increased in the esophagus of EoE patients when compared to controls, although only *GZMA* and *GNLY* presented statistical significance (**Figure 14**). SFED treatment did not modify gene expression except for the increase in *GRN* in EoE respect to controls. No differences were found in duodenal samples between the experimental groups (**Table S 3**).

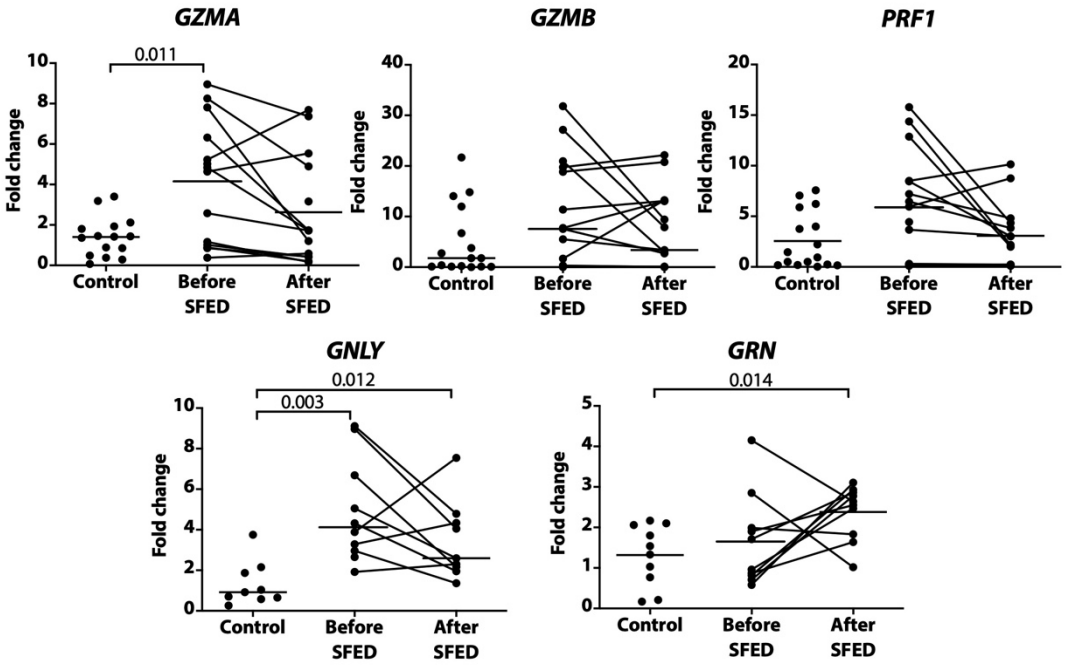


**Figure 14 | Gene expression of molecules with cytotoxic activity in esophageal biopsies from EoE patients before and after six food elimination diet (SFED) and control subjects.** Relative expression was analyzed by qPCR. To obtain the fold-change value for each sample the ratio between target gene and the average of reference genes (18S, PPIA; PGK1, GAPDH, GUSB) was calculated for each sample and then normalized to the average of the control group. Horizontal bars indicate mean or median values and significant p values are indicated in the graphs.

## 1.4 EXPRESSION OF PROINFLAMATORY MOLECULES IN THE ESOPHAGEAL TISSUE

As previously described in the literature, the expression of the eosinophil chemotactic chemokines**,** *CCL11* (C-C Motif Chemokine Ligand 11, Eotaxin-1), *CCL24* (C-C Motif Chemokine Ligand 24, Eotaxin-2) and *CCL26* (C-C Motif Chemokine Ligand 26, Eotaxin-3) was increased in the esophagus in active EoE compared to controls (**Figure 15**). SFED reduced chemokines gene expression although CCL24 did not reach statistical significance. However, despite gene expression modulation, all molecules remained elevated after SFED therapy. No differences in duodenal samples were observed between the experimental groups (**Table S 3**).

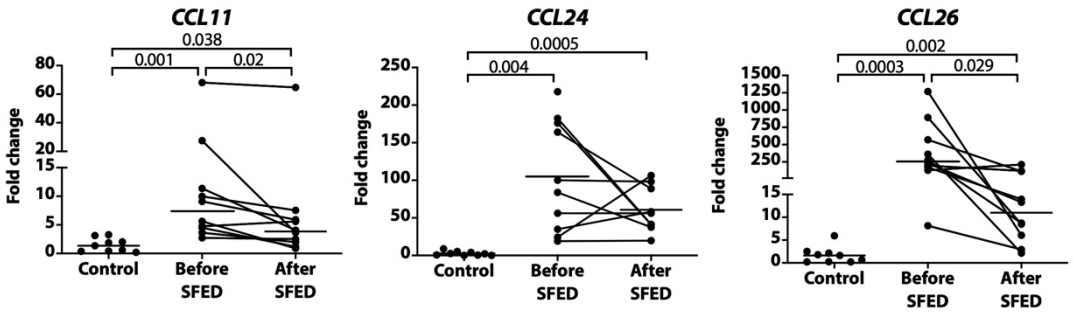


**Figure 15 | Gene expression of eosinophil chemotactic molecules in esophageal biopsies from EoE patients before and after six food elimination diet (SFED) and control subjects.** Relative expression was analyzed by qPCR. To obtain the fold-change value for each sample the ratio between target gene and the average of reference genes (18S, PPIA; PGK1, GAPDH, GUSB) was calculated for each sample and then normalized to the average of the control group. Horizontal bars indicate mean or median values and significant p values are indicated in the graphs.

Gene expression of the interleukins *IL-10* (Interleukin 10), *IL-12B* (Interleukin 12B), *IL-13* (Interleukin 13) and *IL-15* (Interleukin 15) was significantly higher in active EoE when compared to controls. *IL-13* presented a significant reduction after SFED, and *IL-15*, *IL-10* and *IL-12B* were reduced although not reaching statistical significance (**Figure 16**). No differences were observed in the duodenum between the experimental groups (**Table S 3**).

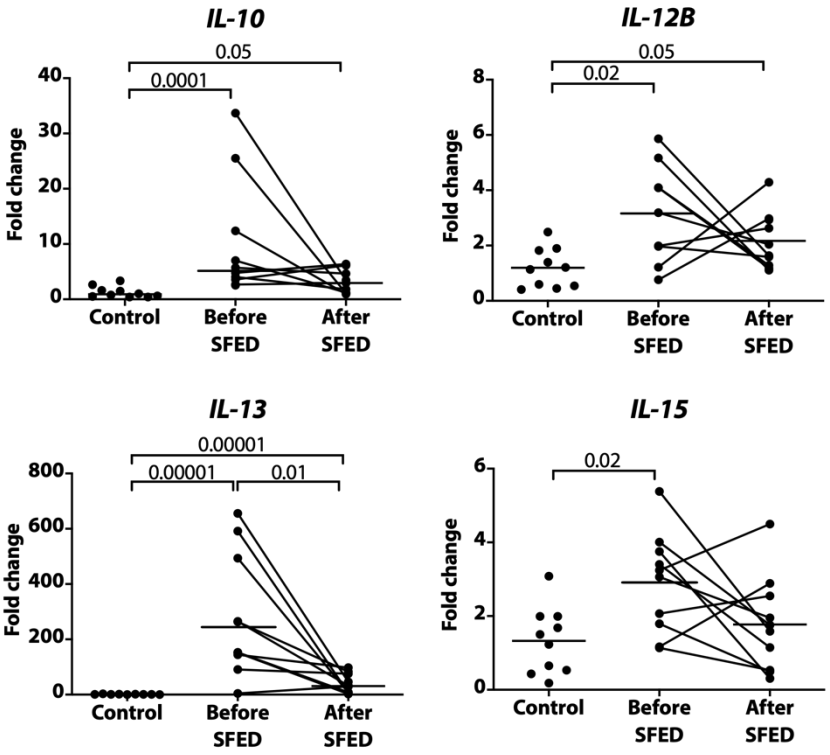


**Figure 16 | Expression of interleukines in esophageal biopsies from EoE patients before and after six food elimination diet (SFED) and control subjects.** Relative expression was analyzed by qPCR. To obtain the fold-change value for each sample the ratio between target gene and the average of reference genes (18S, PPIA; PGK1, GAPDH, GUSB) was calculated for each sample and then normalized to the average of the control group. Horizontal bars indicate mean or median values and significant p values are indicated in the graphs.

A panel of proinflammatory ligands and receptors for T cell positioning and attraction was analyzed, including *CCR8* (C-C Motif Chemokine Receptor 8), *CCL18* (C-C Motif Chemokine Ligand 18), *TGF-β, IFN-γ* and *TNF-α*. *CCR8, CCL18* and *TGF-β* were overexpressed in active EoE patients respect to controls. All genes were reduced after SFED, yet only *CCL18* expression reached statistical significance (**Figure 17**). No differences in *INF-γ* and *TNF-α* were found in the esophagus and no differences in duodenal biopsies were observed in any of these genes between the experimental groups (**Table S 3**).

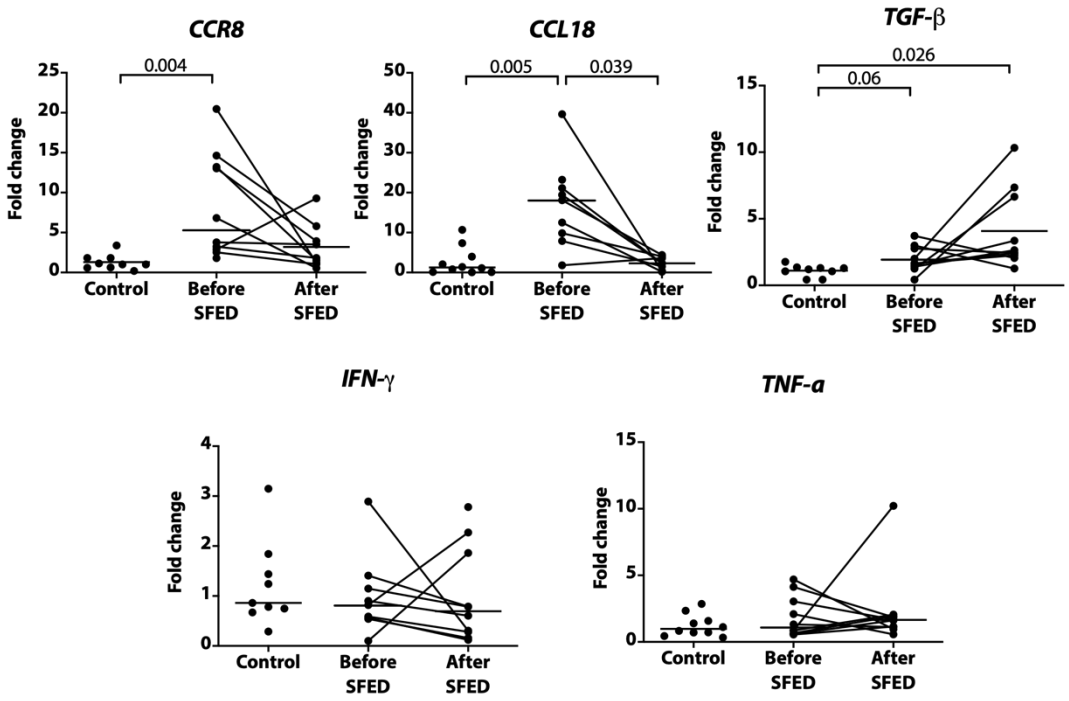


**Figure 17 | Expression of pro-inflammatory genes in esophageal biopsies from EoE patients before and after six food elimination diet (SFED) and control subjects.** Relative expression was analyzed by qPCR. To obtain the fold-change value for each sample the ratio between target gene and the average of reference genes (18S, PPIA; PGK1, GAPDH, GUSB) was calculated for each sample and then normalized to the average of the control group. Horizontal bars indicate mean or median values and significant p values are indicated in the graphs.

## 1.5 EXPRESSION OF CATHEPSIN MOLECULES IN THE ESOPHAGEAL TISSUE

The cathepsins associated with protease activity *CTSC* (Cathepsin C), central coordinator for activation of many serine proteases in immune cells, *CTSB* (Cathepsin B), enhancer of proteases involved in autophagy and catabolism, and *CTSS* (Cathepsin S) responsible for degradation antigenic proteins to peptides for MHCII presentation, were analyzed. All three genes were significantly overexpressed in active EoE when compared to controls. SFED induced a reduction in the expression of *CTSC* and *CTSS,* while an increase was observed for *CTSB* (**Figure 18**). No differences in duodenal samples were identified between the experimental groups (**Table S 3**).

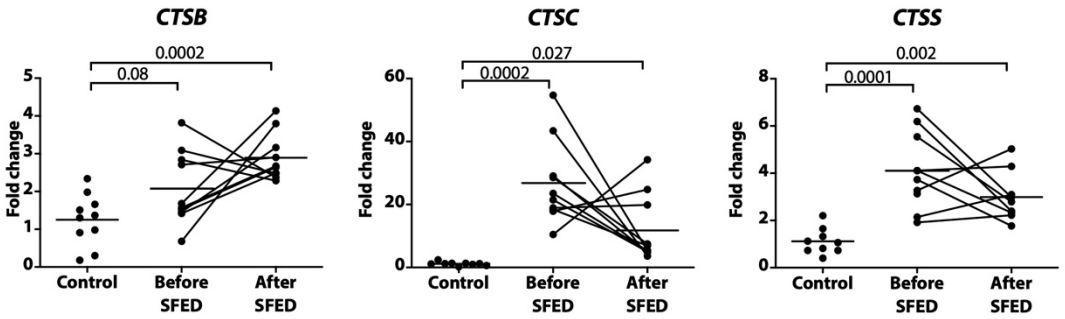


**Figure 18 | Gene expression of cathepsins in esophageal biopsies from EoE patients before and after six food elimination diet (SFED) and control subjects.** Relative expression was analyzed by qPCR. To obtain the fold-change value for each sample the ratio between target gene and the average of reference genes (18S, PPIA; PGK1, GAPDH, GUSB) was calculated for each sample and then normalized to the average of the control group. Horizontal bars indicate mean or median values and significant p values are indicated in the graphs.

## 1.6 BIOLOGICAL AND CLINICAL CORRELATIONS

To gain deeper insight into lymphocytes' role in EoE pathophysiology, the association analysis between gene expression of lymphocyte-related genes and major clinical manifestations and histological findings was performed (**Table 9**).

Eosinophils/hpf and CCL26 (Eotaxin-3) showed a significant positive correlation coefficient with all eosinophil chemotactic proteins, lymphocytes surface molecules expression, $CD8^+$ cell counts, homing molecules, cytotoxic granules expression, hypersensitivity-related pro-inflammatory mediators, antigen presenting molecules and cathepsines with protease activity.

$CD8^+$ cells/hpf positively correlated with Eotaxin 3, homing molecules and cytotoxic granules expression. It also correlated with hypersensitivity-related pro-inflammatory mediators as well as antigen presenting molecules and cathepsins with protease activity.

*CD8* and *GZMA* gene expression positively correlated with all eosinophil chemotactic proteins. It also correlated with *CD4* expression but not CD4 infiltrate, with homing molecules, cytotoxic granules, hypersensitivity-related pro-inflammatory mediators. Both, *CD8* and *GZMA* also correlated with antigen presenting molecules and cathepsins with protease activity.

**Table 9 | Biological and clinical correlations**.

c: cells; hpf: high power field; ToE: Time of Evolution (months), DSS: Dysphagia Score

| | | **Eosinophils/hpf** | | ***CCL26*** | | **CD8⁺ c/hpf** | | ***CD8*** | | ***GZMA*** | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **r** | **FDR** | **r** | **FDR** | **r** | **FDR** | **r** | **FDR** | **r** | **FDR** |
| Eosinophil chemotactic proteins | CCL11 | **0.61** | **4.8E-04** | **0.47** | **0.017** | 0.48 | 0.08 | **0.55** | **0.005** | **0.56** | **0.007** |
| | CCL24 | **0.52** | **0.004** | **0.68** | **3.2E-04** | 0.50 | 0.07 | **0.58** | **0.003** | **0.63** | **0.002** |
| | CCL26 | **0.80** | **1.8E-07** | - | - | **0.72** | **0.01** | **0.56** | **0.004** | **0.52** | **0.012** |
| Lymphocyte subtype | CD8 | **0.44** | **0.02** | **0.56** | **0.003** | 0.43 | 0.13 | - | - | **0.51** | **0.012** |
| | CD4 | **0.43** | **0.022** | **0.48** | **0.015** | 0.37 | 0.19 | **0.61** | **0.003** | **0.63** | **0.002** |
| Homing molecules | MADCAM | **0.75** | **6.3E-06** | **0.65** | **6.9E-04** | **0.75** | **0.04** | **0.51** | **0.012** | **0.62** | **0.002** |
| | ITGA4 | **0.63** | **2.2E-04** | **0.64** | **6.4E-04** | **0.58** | **0.01** | **0.70** | **4.2E-04** | **0.83** | **9.9E-07** |
| | ICAM 1 | **0.53** | **0.003** | **0.60** | **1.4E-03** | 0.48 | 0.07 | **0.69** | **5.2E-04** | **0.59** | **0.005** |
| | ICAM 2 | **0.48** | **0.007** | **0.41** | **0.036** | 0.31 | 0.24 | **0.53** | **0.007** | **0.62** | **0.002** |
| Cytotoxic granules | GZMA | 0.19 | 0.22 | **0.52** | **0.008** | **0.53** | **0.03** | **0.51** | **0.011** | - | - |
| | GZM B | 0.14 | 0.34 | **0.50** | **0.010** | 0.28 | 0.22 | **0.60** | **0.002** | **0.81** | **7.6E-10** |
| | PRF1 | 0.16 | 0.29 | **0.60** | **1.4E-03** | **0.42** | **0.08** | **0.39** | **0.054** | **0.88** | **1.8E-13** |
| | GNLY | **0.54** | **0.003** | **0.67** | **4.2E-04** | 0.29 | 0.29 | **0.66** | **1.2E-03** | **0.42** | **0.05** |
| | GRN | 0.02 | 0.91 | 0.00 | 0.982 | -0.01 | 0.98 | **0.43** | **0.03** | **0.07** | **0.75** |
| Pro-inflammatory mediators | IL-10 | **0.70** | **2.1E-05** | **0.70** | **2.1E-04** | **0.64** | **0.02** | **0.56** | **0.005** | **0.55** | **0.008** |
| | IL-12B | **0.45** | **0.014** | **0.63** | **9.2E-04** | **0.56** | **0.04** | **0.55** | **0.005** | **0.41** | **0.055** |
| | IL-13 | **0.81** | **0.0001** | **0.92** | **5.3E-11** | **0.64** | **0.02** | **0.63** | **0.002** | **0.56** | **0.006** |
| | IL-15 | **0.55** | **0.002** | **0.61** | **1.1E-03** | 0.39 | **0.16** | **0.47** | **0.016** | **0.47** | **0.026** |
| | CCR8 | **0.68** | **6.5E-05** | **0.67** | **4.0E-04** | **0.60** | **0.03** | **0.52** | **0.010** | **0.45** | **0.04** |
| | CCL18 | **0.76** | **3.0E-06** | **0.63** | **1.1E-03** | 0.37 | 0.18 | **0.40** | **0.053** | **0.43** | **0.04** |
| | IFN-γ | 0.20 | 0.30 | 0.79 | 0.8 | 0.03 | 0.93 | 0.23 | 0.28 | -0.13 | 0.6 |
| | TGF-β | 0.15 | 0.45 | **0.39** | **0.042** | 0.22 | 0.44 | **0.41** | **0.037** | 0.14 | 0.57 |
| | TNF-a | 0.21 | 0.30 | 0.24 | 0.4 | 0.14 | 0.59 | 0.04 | 0.59 | 0.17 | 0.51 |
| Antigen presenting molecules | MICA | 0.15 | 0.33 | 0.28 | 0.17 | -0.18 | 0.43 | 0.15 | 0.47 | 0.08 | 0.64 |
| | MICB | **0.40** | **0.006** | **0.66** | **4.6E-04** | **0.61** | **0.01** | **0.35** | **0.075** | **0.52** | **0.002** |
| Cathepsins with protease activity | CTSB | 0.22 | 0.233 | **0.42** | **0.031** | 0.16 | 0.57 | **0.59** | **0.003** | 0.30 | 0.17 |
| | CTSC | **0.75** | **3.4E-06** | **0.81** | **1.7E-06** | **0.60** | **0.02** | **0.70** | **4.4E-04** | **0.67** | **8.7E-04** |
| | CTSS | **0.65** | **1.8E-04** | **0.78** | **8.5E-06** | **0.50** | **0.08** | **0.50** | **0.077** | **0.69** | **8.4E-04** |
| Histology | Eos/hpf | - | - | **0.80** | **1.7E-06** | - | - | **0.44** | **0.027** | 0.19 | 0.3 |
| | CD8⁺ c/hpf | - | - | **0.72** | **1.2E-03** | - | - | 0.43 | 0.08 | **0.53** | **0.012** |
| | CD2⁺ c/hpf | - | - | **0.73** | **9.9E-04** | - | - | 0.39 | 0.12 | **0.71** | **0.005** |
| | CD4⁺ c/hpf | - | - | 0.80 | 0.36 | - | - | 0.00 | 1 | -0.30 | 0.530 |
| Clinical features | DSS | - | - | **0.76** | **3.9E-04** | - | - | 0.15 | 0.56 | -0.12 | 0.6 |
| | ToE | - | - | -0.28 | 0.907 | - | - | -0.09 | 0.81 | -0.06 | 0.84 |

c: cells; hpf: high power field; ToE: Time of Evolution (months), DSS: Dysphagia Score

# 2 IN VITRO EOE MODEL

## 2.1 CELL LINE DIFFERENTIATION CHARACTERISTICS

To perform stimulation analysis, the differentiation status of the Het-1A cell line was analyzed at days 10, 14 and 21 after seeding. Although no statistical differences were observed, cell viability (96.3 - 97.3 %) was slightly higher at confluence (10 days post seeding) and at day 14 when compared with day 21 (**Figure 19**).

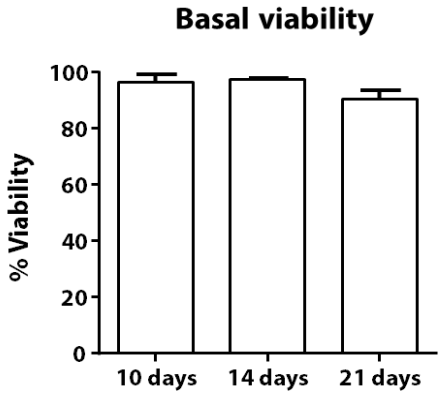


**Figure 19 | Cell viability of Het-1A cell line at days 10, 14 and 21 after seeding.** Graphs represent results from 2 independent experiments. Data are expressed as median ± standard error.

To assess the formation of a functional epithelial barrier upon air exposure (ALI experimental protocol), TEER was monitored during a 3-week period. Although cell resistance slightly increased in the 10 first days, a significant reduction was observed after day 14 (**Figure 20 A**).

To visualize the structure of the cell layer, transwells were cut and stained with hematoxylin and eosin (H&E) after 14 (data not shown) and 21 days of culture (**Figure 20 B**). After air exposure, Het-1A cells developed a multilayer stratified epithelium over the transwell membranes. However, tight contact was not functionally established between the cells in the different layers, as indicated by the TEER functional values.

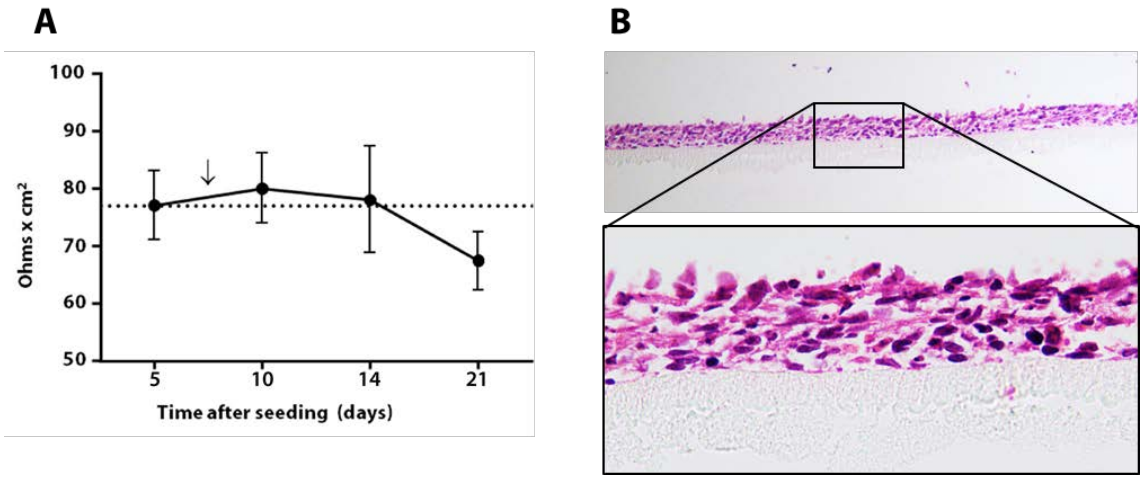


**Figure 20 | A. Representation of TEER measurements from day 5 to day 21 after seeding.** Arrow indicates the point for air exposure (day 7). Dotted line represents blank resistance (0.4 pore semipermeable membrane). **B. Representative H&E stainings of ALI cultures at day 21 after seeding.** Images magnification: top 100X, bottom 400X.

Gene expression analysis was performed to analyze the transcriptional profile during differentiation of molecules associated with apoptosis (*CASP8*, *FAS* and *BCL2*), eosinophil chemotactic proteins (*CCL11*, *CCL24* and *CCL26*), tight and adherent junctions (*CDH26, OCLN CLDN1, ZO-1* and *ZO-3*), structural genes and proteases (*SYNPO* and *CAPN14*) inflammatory cytokines and chemokines (*POSTN, TSLP* and *CXCL8*) and antigen presenting molecules (*MICB).* All genes were expressed at any time point and no differences were identified between days (**Figure 21**).

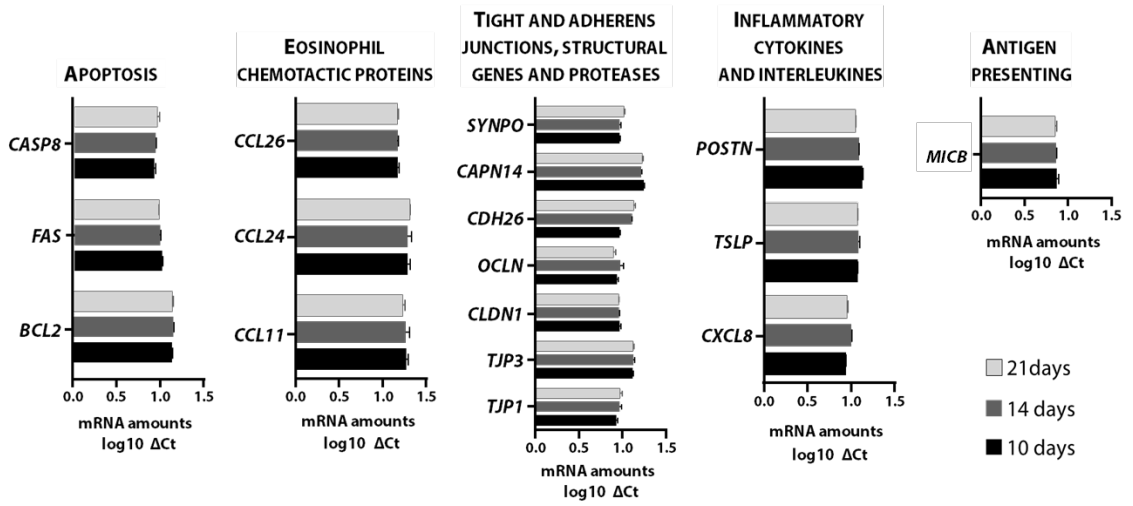


**Figure 21 | Gene expression of structural pro-inflammatory and apoptosis related molecules in the Het-1A cell line.** Relative mRNA amount expression by qPCR represented as log10ΔCt values normalized to endogenous genes (*PPIA*. *GAPDH* and *18S*). Graphs represent data from 1 experiment (run in triplicate for each gene and condition). *CASP8*: Caspase 8. *FAS*: Fas cell surface death receptor. *BCL2*: B-cell lymphoma 2. *MICB*: Major Histocompatibility Complex I Sequence B. *POSTN*: Periostin *TSLP*: Thymic Stromal Lymphopoietin. *CXCL8*: Chemokine ligand 8. *SYNPO*: Synaptopodin. *CAPN14*: Calpain 14. *CDH26*: Cadherin 26. *OCLN*: Occludin. *CLDN1*: Claudin 1. *ZO-3*: Zonula-Occludens 3. *ZO-1*: Zonula-Occludens 1. *CCL26*: Chemokine ligand 26. *CCL24*: Chemokine ligand 24. *CCL11*: Chemokine ligand 11.

In order to analyze cell differentiation, immunofluorescence staining of structural proteins was performed at days 14 and 21 after seeding. At day 10 no staining could be performed since the cell line did not achieve an optimal attachment to the growth surface.

Cells morphology, based on structural proteins such as tight-junction and desmosome proteins, appeared to be not fully differentiated. Furthermore, phalloidin staining was not homogeneous all over the cell monolayer. No differences were observed between 14 and 21 days for any of the proteins stained (**Figure 22**).

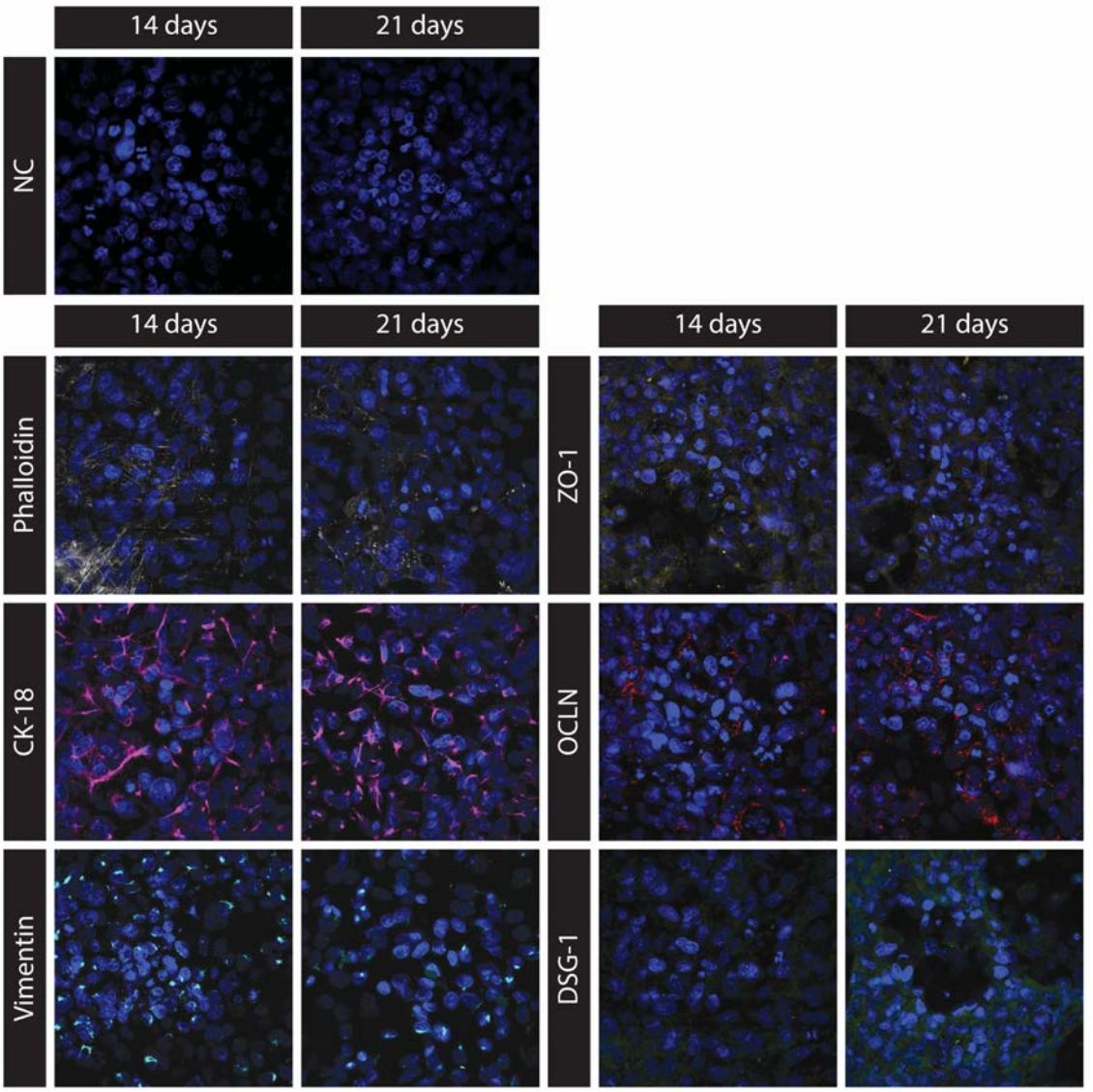


**Figure 22 | Representative immunofluorescence images of structural proteins in Het-1A cells.** Tight-junction proteins and desmosome proteins stainings of Het-1A cell line at 14 and 21 after seeding. NC: Negative Control. Phalloidin (white). CK-18: Citokeratin 18 (magenta). Vimentin (cyan). ZO-1: Zonula-occludens 1 (yellow). OCLN: Occludin (red). DSG-1: Desmoglein 1 (green). Images are representative of 3 independent experiments. Magnification 600X.

In order to validate the *in vitro* EoE model in the HET-1A cell line and the ability of these cells to respond to IL-13 (due to its role in the pathophysiology of EoE), cells were exposed to this cytokine and its responsiveness was analyzed.

For apoptosis-related genes we observed an overexpression at 14 days of differentiation. As previously described in different cell lines[34], IL-13 exposure increased the eosinophil chemotactic proteins *CCL11* (Eotaxin-1) and *CCL26* (Eotaxin-3) gene expression in all time points tested. However, *CCL24* (Eotaxin-2) slightly

increases respect to basal expression. Inflammatory cytokines and interleukin genes were overexpressed, mainly at day 14, although *POSTN* showed a time-dependent decreased response to IL-13 exposure. Tight and adherens junctions were homogenously expressed, and only TJP3 was highly overexpressed at day 10, and *TJP1 OCLN* and *CLDN1* showed an increased response to IL-13 at day 14. The structural gene *SYNPO* showed overexpression at days 10 and 14, while down-regulation of *CAPN14* was observed in all conditions. The expression of the antigen presenting molecules *MICB* was not modified, however decreased values were detected at day 21 (**Figure 23**).

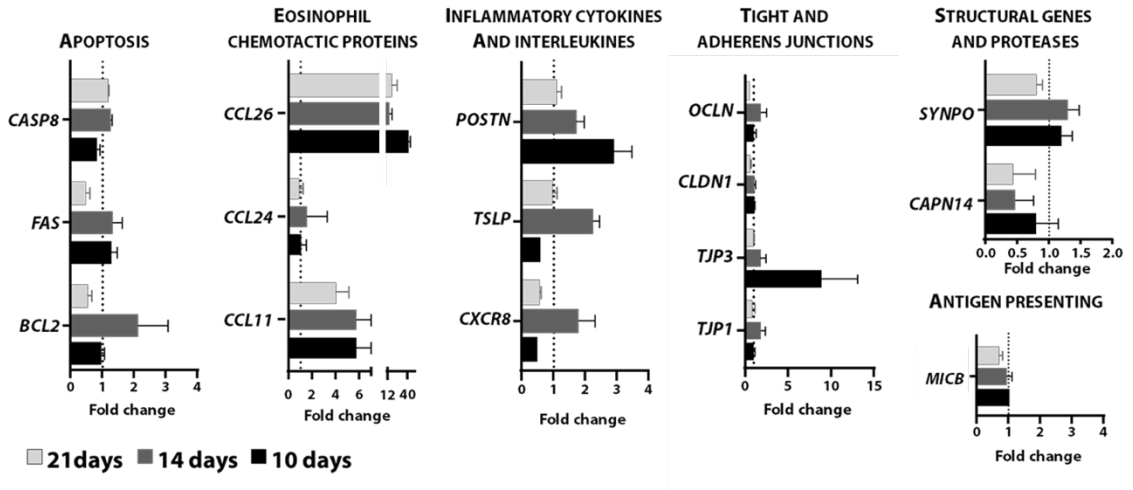


**Figure 23 | Expression of apoptosis related genes, eosinophil chemotactic proteins, inflammatory cytokines and interleukines, tight and adherens junctions, structural genes and proteases and antigen presenting molecules after stimulation of the Het-1A cell line with IL-13.** Apoptosis related genes (*CASP8. FAS* and *BCL2*). Eosinophil chemotactic proteins (*CCL11. CCL24* and *CCL26),* inflammatory cytokines and interleukins (*CXCR8. TSLP* and *POSTN*). Tight and adherens junctions (*TJP1. TJP3. CLDN1. OCLN* and *CLDN1*), structural genes and proteases (*CAPN14* and *SYNPO*) and antigen presenting molecules (*MHCI.*) were analyzed. Relative expression was analyzed by qPCR. To obtain the fold-change value for each sample the ratio between target gene and the average of reference genes (18S, PPIA and GAPDH) was calculated for each sample and then normalized to the average of the control group. Data are expressed as median ± SD. Graphs represent data from 2 independent experiments. Dotted line indicates vehicle-treated cell expression.

## 2.2 CELL LINE RESPONSE TO GRANZYMES EXPOSURE

The HET-1a cell line was then exposed to CD8-isolated mediators at 14 days after confluence. Cellular viability was not affected by granzyme exposure, only a slight reduction was observed at 24 hours of stimulation (**Figure 24**).

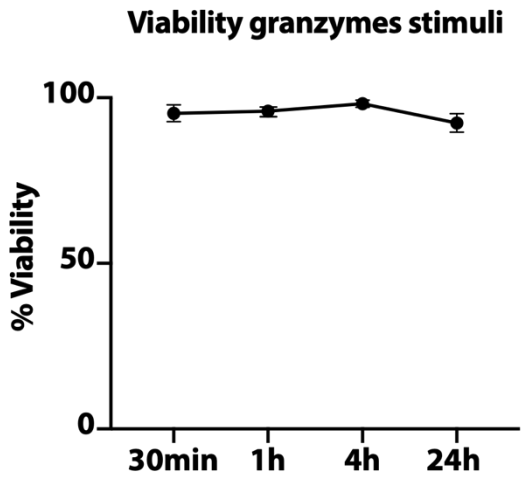


**Figure 24 | Cellular viability after stimulation with granzymes A and B**. Data are expressed as median ± SED. Graphs represent data from 5 independent experiments.

Individual exposure to granzyme A increased the expression of *CCL24* and *CCL26* at 30 min and of *CCL11*, *CCL26* and *OCLN* at 4 hours after stimulation, however, these results were obtained in only 1 single experiment. On the other hand, individual exposure to granzyme B induced an increase only in *CCL24* at 30 min. None of these differences reached statistical significance (p>0.05; **Figure 25**)

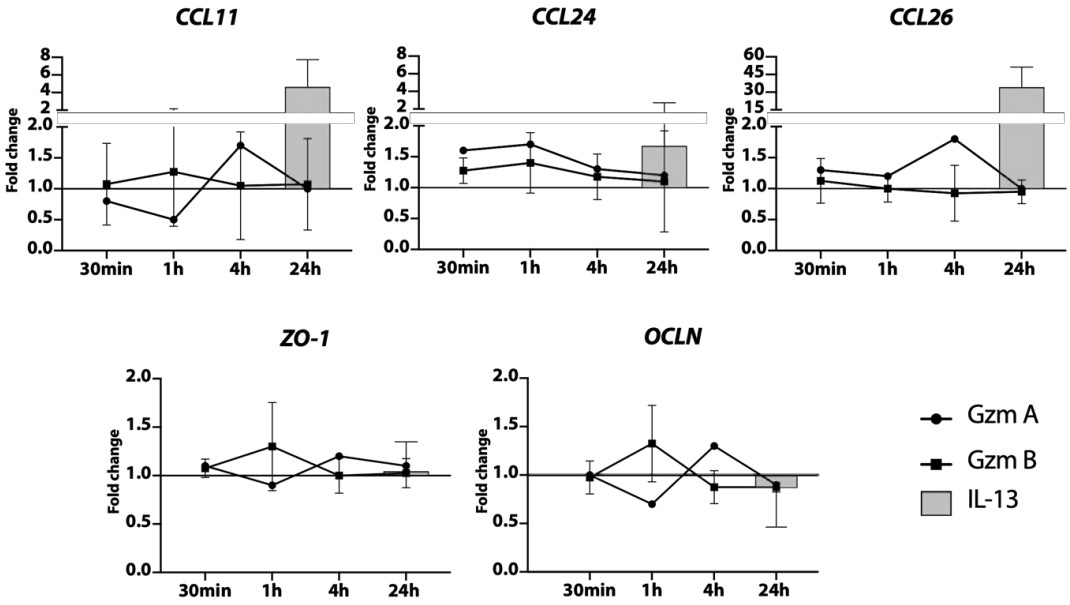


**Figure 25 |** Gene **expression of eosinophil chemotactic proteins** (*CCL11*, *CCL24* and *CCL26*) and cellular junction proteins (*ZO-1* and *OCLN*) after exposure of the Het-1A cell line to granzyme A, granzyme B or IL-13 (as positive control of the response). Results are expressed in fold-change value respect to vehicle (dotted line at y=1), as mean ± SD. Graphs representing granzyme A belong to 1 experiment and graphs for granzyme B belong to 4 independent experiments. All p values were not significant (p>0.05).

Due to the limited access to commercial granzymes and the slight response identified in the cell line, a new source of CD8 mediators was used. For that, a protocol was developed for isolating CD8-isolated mediators from $CD8^+$ cells obtained from blood, to further reproduce the cytotoxic milieu observed in the esophageal epithelium during active EoE.

## 2.3 PHENOTYPE OF ISOLATED $CD8^+$ LYMPHOCYTES

The phenotype of isolated $CD8^+$ cells from blood was evaluated by flow cytometry and immunofluorescence. Purity of cell suspension was always around 95 %. Additionally, 44 ± 2 % of the cells were positive for granzyme A and 96 ± 4 % of cells were positive for granzyme B (**Figure 26**). Notably, two distinct profiles of granzyme A fluorescence intensity (high and low) were observed in $CD8^+$ cells by immunofluorescence (**Figure 27**), which could explain the difficulty in separating the 2 populations by flow cytometry. In concordance with the flow cytometry data, all isolated cells were positive for granzyme B staining (**Figure 26** and **Figure 27**).

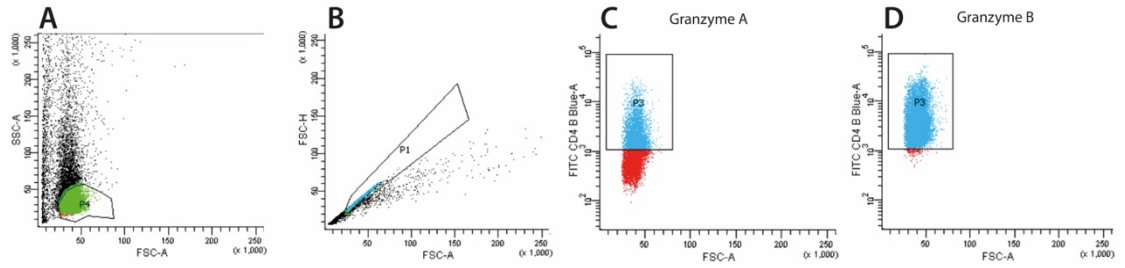


**Figure 26 | Representative images of dot plots from flow cytometry analysis of isolated $CD8^+$ lymphocytes.** A. Selection of lymphocytes by size and granularity. B: Selection of lymphocyte single cells. C. Granzyme A positive cells. D. Granzyme B positive cells.

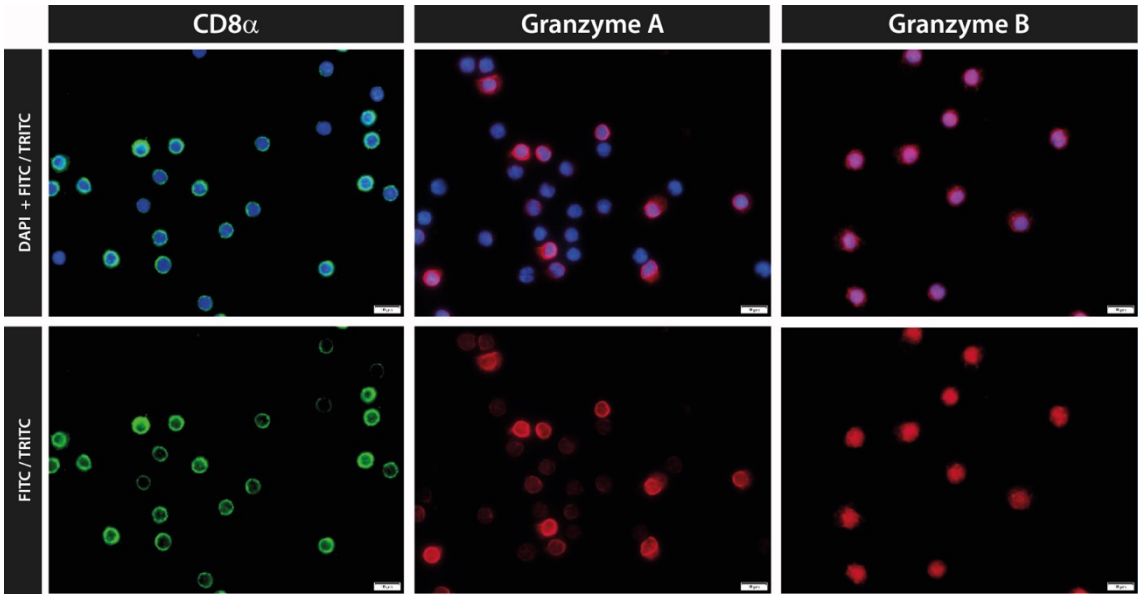


**Figure 27 | Immunofluorescence of isolated CD8⁺ lymphocytes.** $CD8^+$ positive cells are visualized in green, granzymes in red and DAPI for nuclei counterstaining in blue. Magnification 400X.

Both, granzyme A and granzyme B were identified in the CD8-isolated mediators lysate by WB (**Figure 28**), ensuring their presence in the medium of these cytotxic mediators for further incubation of epithelial cells.

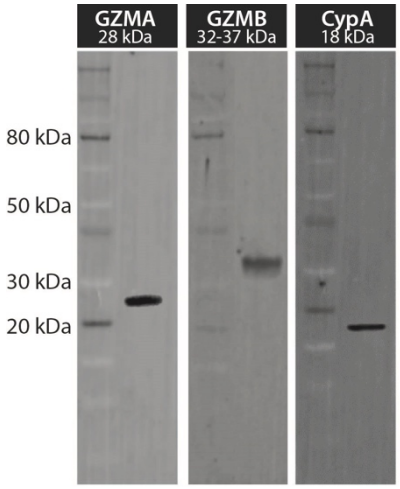


**Figure 28 | Western Blot of CD8-isolated mediators**. Detection of granzyme A at 28kDa, granzyme B at 32-37kDa and CypA as a control gene at 18kDa.

## 2.4 CELL LINE RESPONSE TO CD8-ISOLATED MEDIATORS

The response of Het-1A cells to CD8-isolated mediators lysate was analyzed after incubation for 24h. IL-13 was used as a positive control of epithelial stimulation. Cell exposure to IL-13 or CD8 lysate did not modify cell viability (**Figure 29**).

Gene expression of eosinophil chemotactic proteins (*CCL11*, *CCL24* and *CCL26*), apoptosis (*BCL2*, *FAS* and *CASP8*), tight and adherent junctions (*CDH26*, *ZO-1*), structural genes (*SYNPO*, *FLG*), extracellular matrix proteases and its inhibitors (*CAPN14, SPINK5, SPINK7*), pro-inflammatory cytokines and interleukins (*CXCL8, TSLP, IL-33*), cell adhesion (*POSTN*) and antigen presentation (*MHC* I), were evaluated.

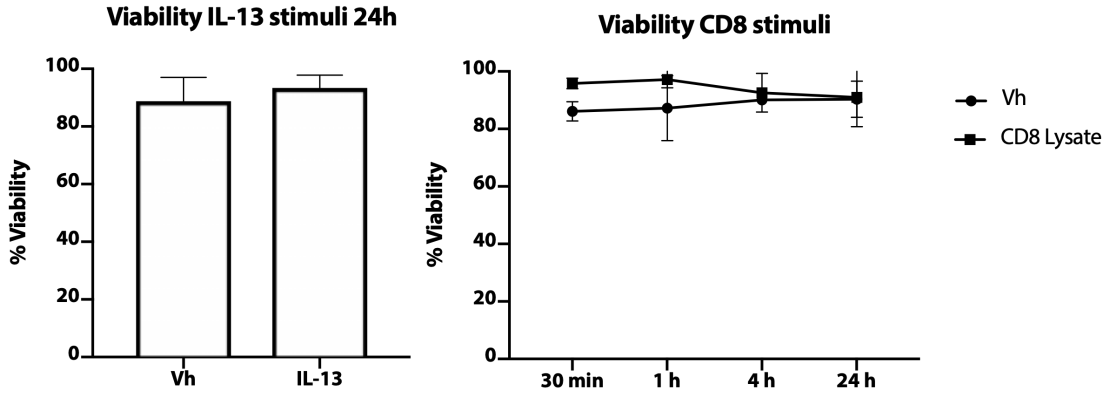


**Figure 29 | Cell line viability.** Left, viability after 24h of exposure to IL-13 and vehicle. Right, viability of Het-1A during the time-course exposure to CD8-isoalted mediators. Graphs represent results from 3-12 independent experiments. Data are expressed as mean ± SEM.

Exposure of epithelial cells to IL-13 reproduced the increase in *CCL11*, *CCL24* and *CCL26* ($p<0.05$) when compared to vehicle, as well as induced a reduction in *FLG* at 24 h ($p>0.05$). For the other genes no differences were observed after IL-13 stimulation.

Exposure of epithelial cells to CD8-isoalted mediators during 1h induced a significant increase in *CCL11* gene expression, as compared with vehicle ($p<0.01$). Additionally, the lysate reduced the expression of *BCL2, CAPN4* and *FLG* genes ($p>0.05$). (**Figure 30**).

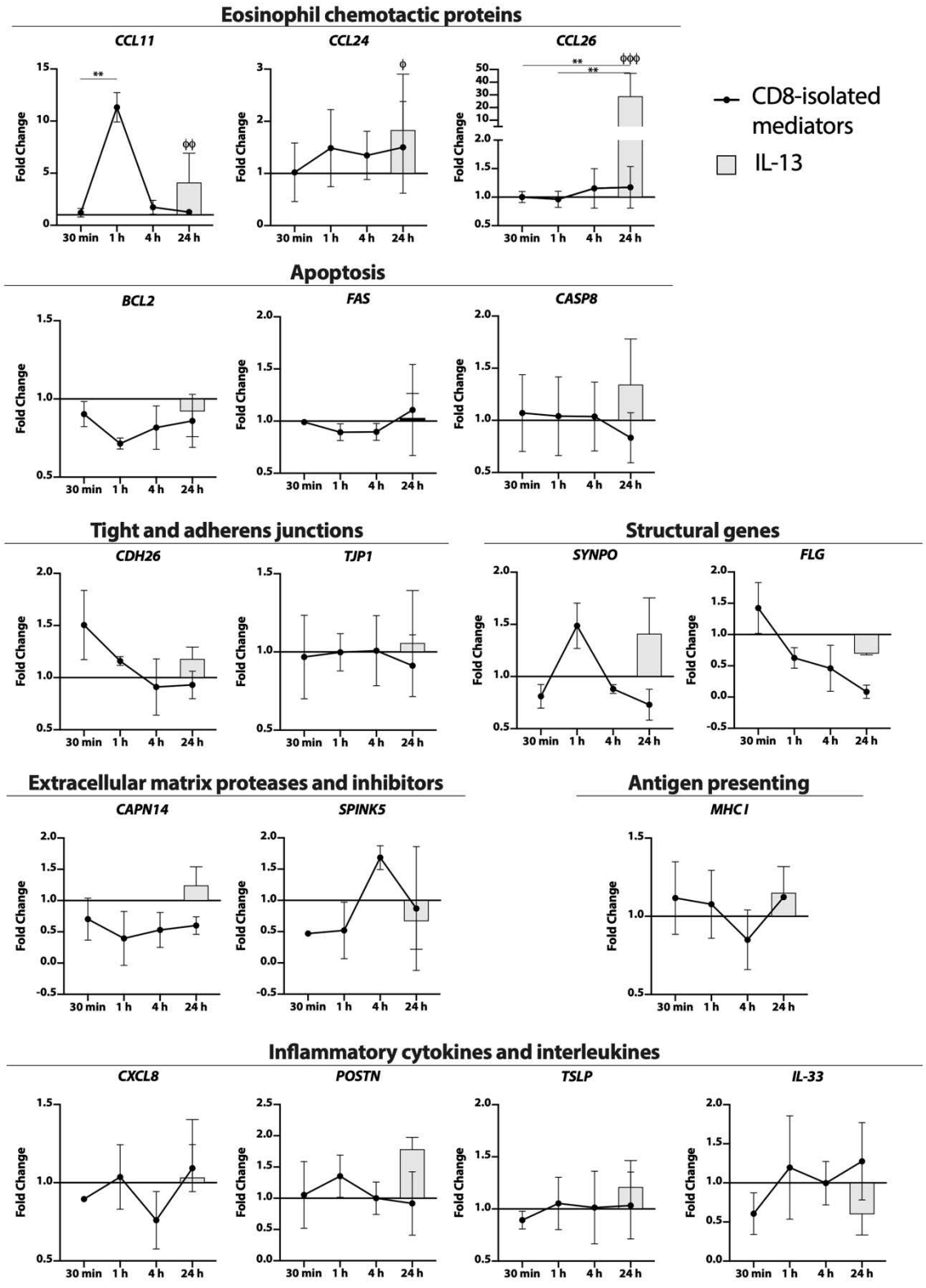


**Figure 30 | Time-course analysis of gene expression of after stimulation with IL-13 and CD8-isolated mediators.** Cells were treated with IL-13 during 24h or with CD8-isolated mediators for the indicated time points. Gene expression was analyzed by qPCR. Fold-change was calculated by normalizing the data with the endogenous control genes (*GAPDH* and *PPIA*) and comparing each time point to cells at the same time point treated with vehicle. Graphs represent data from 3-12 independent experiments for *CCL11*, *CCL24*, *CCL26* and *TJP1* and from 3 different independent experiments for the other genes. Data are expressed as mean ± standard error. Statistical analysis was performed by one-way ANOVA when comparing between time points

* p<0.05, ** p<0.01. One sample t and Wilcoxon test were used for statistical analysis when comparing each sample with its vehicle. Φ p<0.01, ΦΦ p<0.001 ΦΦΦ p<0.0001. *CCL11*: Chemokine ligand 11, *CCL24*: Chemokine ligand 24, *CCL26*: Chemokine ligand 26, *BCL2:* B-cell lymphoma 2, *FAS:* Fas cell surface death receptor, *CASP8*: Caspase 8, *CDH26*: Cadherin 26, *TJP1: Tight-Junction Protein* 1, *SYNPO*: Synaptopodin, *FLG*: Filaggrin, *CAPN14*: Calpain 14, *SPINK5*: serine peptidase inhibitor, Kazal type 5, SPINK7: serine peptidase inhibitor, Kazal type 7, *MHC I*: Major Histocompatibility Complex I, *CXCL8:* Chemokine ligand 8, *POSTN*: Periostin, *TSLP*: Thymic Stromal Lymphopoietin, *IL-33*: Interleukin 33.

# 3 CULTURED BIOPSIES

## 3.1 TISSUE RESPONSE TO CD8-ISOLATED MEDIATORS

To characterize the response of esophageal tissue to IL-13 or CD8-isolated mediators exposure, esophageal biopsies were cultured for 4 hours. Gene expression of the same panel previously analyzed was quantified, including eosinophil chemotactic protein *(CCL11, CCL24 and CCL26)*, apoptosis *(BCL2, FAS and CASP8)*, tight and adherent junctions *(DSG-1, ZO-1, ZO-3, OCLN, CLDN1 and CDH26)*, structural genes *(SYNPO and FLG)*, extracellular matrix proteases and its inhibitors *(CAPN14, SPINK5 and SPINK7)*, pro-inflammatory cytokines and interleukins *(CXCL8, TSLP and IL-33),* cell adhesion *(POSTN)* and antigen presentation *(MHC I)*.

Exposure of biopsies to IL-13 induced the expected gene expression changes (colored in grey behind the graphs of **Figure 31**) for eosinophil chemotactic proteins, apoptosis, extracellular matrix proteases and its inhibitors, *TSLP*, adhesion molecules, antigen presenting molecules, structural genes and tight and adherens junctions.

Exposure of biopsies to the CD8-isolated mediatores induced changes in the expression of apoptosis genes, extracellular matrix proteases and inhibitors, antigen presenting molecules, structural genes and tight and adherens junctions.

These results are only observational, as due to the small sample size no statistical analysis could be performed.

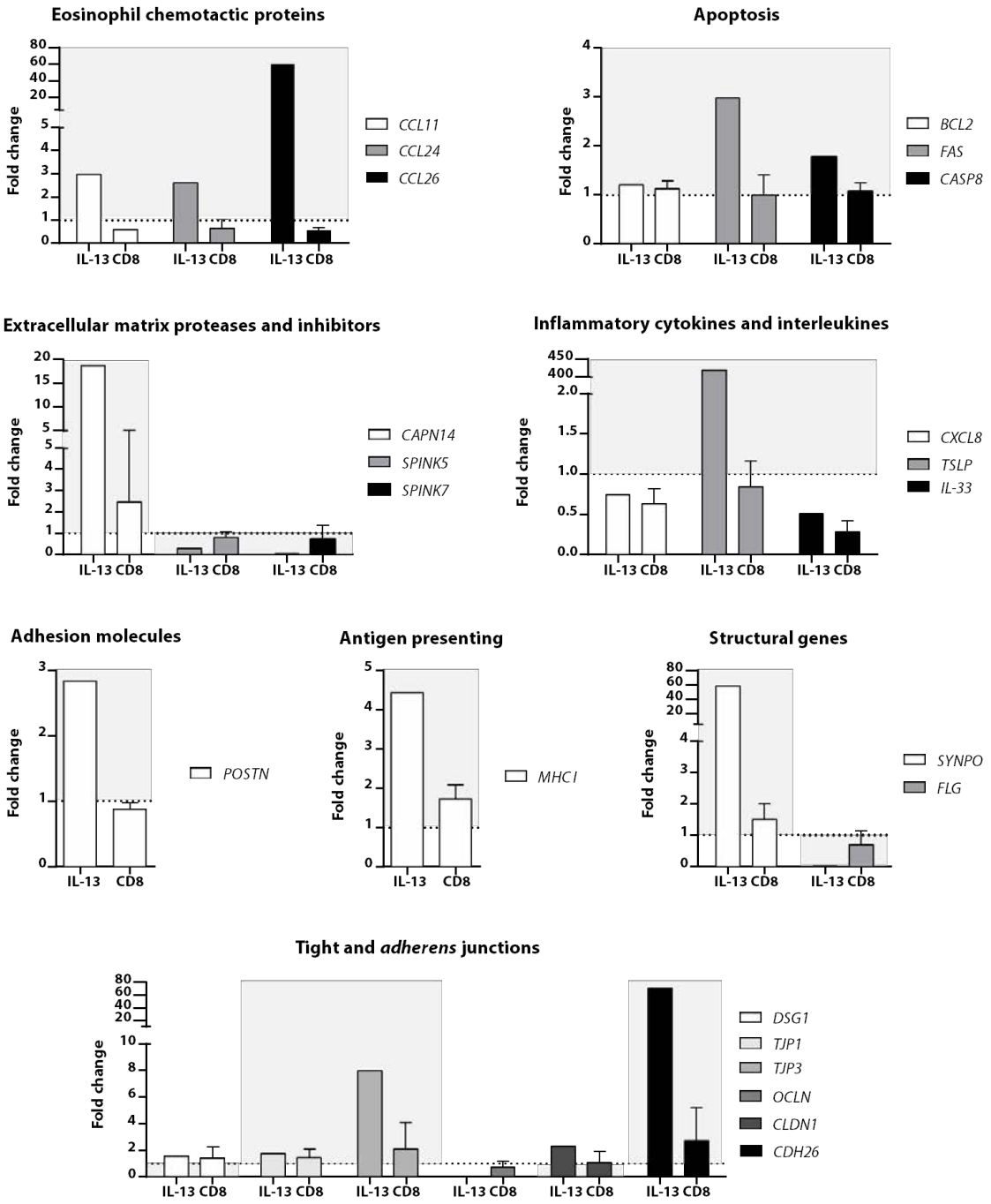


**Figure 31 | Gene expression in esophageal tissue after stimulation with IL-13 or CD8-isolated mediators.** Biopsies were treated with IL-13 or CD8-isolated mediators for 4 hours. Gene expression was analyzed by qPCR. Fold-change was calculated by normalizing the data with the endogenous control genes (*GAPDH, 18S* and *PPIA*) and comparing each time point to biopsies at the same time point treated with vehicle. Graphs represent data from 1-2 independent experiments. Data are expressed as mean ± standard error. *CCL11*: Chemokine ligand 11, *CCL24*: Chemokine ligand 24, *CCL26*: Chemokine ligand 26, *BCL2:* B-cell lymphoma 2, *FAS:* Fas cell surface death receptor, *CASP8*: Caspase 8, *CAPN14*: Calpain 14, *SPINK5*: serine peptidase inhibitor, Kazal type 5, SPINK7: serine peptidase inhibitor, Kazal type 7, *CXCL8:* Chemokine ligand 8, *TSLP*: Thymic Stromal Lymphopoietin, *IL-33*: Interleukin 33, *POSTN*: Periostin, *MHC I*: Major Histocompatibility Complex I, *SYNPO*: Synaptopodin, *FLG*: Filaggrin, *DSG-1:* Desmoglein 1, *TJP1: Tight-Junction Protein* 1, *TJP3: Tight-Junction Protein* 3, *OCLN*: Occludin, *CLDN1*: Claudin 1, *CDH26*: Cadherin 26.

# DISCUSSION

This is the first study that specifically aims at identifying the role of $CD8^+$ cells in the pathophysiology of EoE. The results not only confirms active homing of $CD8^+$ cells but also the overexpression of its related mediators in EoE and its modulation after elimination diet therapy. Additionally, the expression of these CD8-related mediators correlates with disease markers such as intraepithelial eosinophil numbers and the main eosinophil chemotactic protein in EoE, Eotaxin 3. These findings clearly point at the CD8 population as developing a role in the pathophysiology of EoE.

Although previously published data[2,3,35] reported an increase in CD4 lymphocytes in EoE when compared with controls, the present study did not observe such increment. The CD4/CD8 ratio did not reveal significant differences in active EoE patients as observed in other studies[3], as well as no differences were identified after treatment, with neither PPI nor SFED, suggesting that the CD4/CD8 ratio is not an optimal biological marker of lymphocyte activity in the esophageal tissue in EoE. On the contrary, the present study observed a greater CD8 infiltration in EoE patients as published previously which also correlated with eosinophilic infiltration and *CCL26* expression (60 % and 70 % respectively). Taking into account that in murine allergic asthma models has been observed that CD8 enhances eosinophil tissue infiltration and their ability to regulate eotaxin production[36], CD8 may be implicated in the initiation of the allergic response and may also contribute to eosinophil recruitment.

The infiltration of CD8 lymphocytes was also modulated by SFED treatment and not by PPI treated patients. Yet, Mousavinab et al.,[37] only observed a reduction after SFED in CD8 but not in CD4. In addition to the infiltrated modulation, only CD8 positively correlated with eosinophilic infiltration. In parallel with our study, Aguilar-Pimentel et al.,[38] in an asthma animal model, observed a high number of $CD8^+$ cells with high cytotoxic activity. This seems to compensate for a lower number of activated CD4, which is apparently linked to the exposition to low doses of allergens. Based on our findings and observations from other studies, we hypothesize that the continued exposure to triggering allergens in PPI-treated patients, although being in

histological remission, is responsible for the differential infiltration behaviour when comparing PPI to SFED subgroups. Still, a larger sample size is needed to confirm this differential behaviour.

*CD8* expression did not follow the same pattern as that of cell infiltration, as it was significantly overexpressed in active EoE before SFED and, unlike $CD8^+$ cells, did not decrease after treatment. One plausible explanation could be the phenotype of activation that remains in the tissue after SFED, together with $CD2^+$ expression, as double positive cells could be expressing at a higher rate the CD8 surface marker than that expressed in controls.

Regarding the expression study, all homing molecules were overexpressed in active EoE when compared to control. Also, their reduced expression after treatment suggests an active recruitment of immune cells from bloodstream. Although CD4 cells counts did not correlate with *CD4* gene expression, *CD4* expression is positively associated with *ICAM1* and *ITGA4* (data not shown). This may indicate that CD4 T cells are being recruited but maybe not as significantly as other lymphocytes subtypes. The *CCL18* cytokine, which displays chemotactic activity for naïve T cells (*CD4* and *CD8*), is also significantly overexpressed in active EoE and is reduced after SFED.

Rubio et al.,[39] analyzed CD8 activation in a cohort of patients with compound lymphocytic esophagitis–eosinophilic esophagitis through TIA1 and granzyme B staining, demonstrating the activation of this cell subtype. In the present study, we used CD2 as a marker of homing of active lymphocytes and we observed an increase in both, CD2 and double positive CD2-CD8 in active EoE and its reduction after SFED. These results, together with the positive correlation analysis between CD2 and CD8, emphasize the fact that main infiltrated lymphocytes are $CD8^+$. Still, a double staining for CD4-CD2 should be performed in order to confirm this observation.

In order to better understand the CD8 contribution to EoE, the present study analyzed a detailed panel of cytotoxic-related molecules and its modulation by SEFD. It is

important to highlight that significant differences were exclusively found in esophageal and not in duodenal biopsies. Hereby, this further supports that CD8-associated mechanisms in EoE are also disease-specific and restricted to the esophagus. Yet, performing a CD4-CD8 immunohistochemical phenotypic analysis in duodenal samples would be necessary to further confirm our findings.

CD8 antigen presenting molecules *(MICA and MICB)* were overexpressed in active EoE, highlighting the active recruitment of these cells. Nonetheless, after treatment both molecules presented different profile, which may be due to its differential role in the activation of T lymphocytes, NK and NKT, which has not been elucidated yet. Although only granzyme A *(GZMA)* and granulysin *(GNLY)* presented significant differences, all cytotoxic granules are overexpressed in active EoE. Except for granulin, all cytotoxic proteins contained in CD8 cytoplasmic granules were reduced after SFED. This, together with its correlation with EoE tissular biomarkers such as *CCL11*, *CCL24* and *CCL26* and pro-inflammatory mediators, suggest they have an active role in the inflammatory response that is taking place in the esophageal epithelium in active EoE.

Several pro-inflammatory interleukines related to cytotoxic lymphocyte maturation (IL-12B), regulation of cytotoxic T cells and iNKT growth and survival (IL-15) and suppression of Th1 cytokines and MHC II (IL-10) are overexpressed in EoE patients and modulated after SFED, which suggest an active role of T cytotoxic lymphocytes in EoE pathophysiology. The *CCR8* gene, related with the proper positioning of activated T cells in antigenic challenge sites, is also overexpressed in EoE patients and reduced after SFED reinforcing CD8 lymphocytes role. Additionally, *IL-10* is positively associated with *MICB* (data not shown), which may indicate that antigen presentation is in mostly carried out by MHCI and not MHCII. On the other side, no differences were observed for *TNF-α* and *IFN-γ*, related to viral and microbial infections and Tc1 activity, enhancing the hypothesis of EoE being an allergic disease, implicating Tc2, Tc9 or $CD8^+$ reg. In fact, all three subpopulations are enhanced by IL-4, IL-4 and TGFβ and TGFβ and IL-15 respectively, and all were found to be overexpressed in EoE

samples. Moreover, their effector molecules were also overexpressed in EoE patients such as IL-5, IL-13, IL-4, TGFβ and IL-10. Also, is worth noting that IL-10, IL-15 correlate with granzyme A and CD8 but not IFN-γ. Therefore, given the pro-inflammatory cytokines and the interleukines profiles determined here, EoE CD8 subpopulations are more likely to be either Tc2, Tc9 or CD8+ reg, a hypothesis that has not been yet tested.

The EoE in-vitro model could not be successfully developed, as the Het-1A cell line did not seem to be enough differentiated to create a stratified functional epithelial layer. Response to IL-13 was successful, however at lower rates than described in the literature, although in different cell lines.[34] Even structural staining such as phalloidin was not homogeneous all over the cell monolayer, which may occur due to the difficulties of this primary immortalized cell line to attach to the growth surface. Despite the lack of strong evidence for differentiation, at 14 days post-seeding expression of tight junction proteins was slightly increased, viability was higher, and cells seemed to be better attached to the growth surface displaying an elongated morphology. Conversely, at 21days post-seeding more gaps were observed between cells. Therefore, 14 days post-seeding was selected as the most appropriate time point for stimulation. Despite manufacturer's instructions[31] recommend not to use FBS complemented medium due to growth factors' inhibition, we observed a better cell growth and differentiation when compared to cells grown in Bronchial epithelial growth medium (BEGM) with supplements and growth factors and DMEM without FBS (data now shown). Yet, fibroblast co-culture or different medium supplementation could be also of interest in order to have a better set-up of the Het-1A culture. In order to conclude on the usefulness of this experimental model, cell differentiation should be better achieved or other esophageal epithelial cell line tested.

Individual exposure to commercial granzymes concentrations was chosen based on literature[40–42], yet no significant modulations were observed. GzmA was used in a high concentration, still GzmB concentration could have been increased. However,

the vast majority of studies used 30nM, while 75nM[43] lead to a 50-60 % of cell death, which would not allow to set up the experimental conditions to develop the *in vitro* assessment. While cytotoxic granule extraction was confirmed after FACS and WB analysis, lysates exposure seemed not to strongly affect gene expression, therefore an enzyme-activity assay should be performed in order to assure the correct activity of the cytototic extract. An important limitation of this approach we should consider that could justify the low response is that granule mediators from systemic $CD8^+$ cells may not mimic the cytotoxic profile of intraepithelial CD8 in EoE. Hence, we acknowledge that a more accurate approach and additional tests are needed before concluding on the effect of cytotoxic mediators on epithelial activity. Nonetheless, *CCL24* shows a similar profile when comparing IL-13 stimulation and cytotoxic stimulation at any time points. In *CCL26* expression, we observed an increase although not comparable with that achieved with IL-13 exposure. Further, *CCL11* expression significantly increased after 1 hour of exposure, which may suggest an effect of activated CD8 lymphocytes in recruiting eosinophils at early stages of EoE. In cultured biopsies, stimulation with IL-13 reproduced EoE pattern in all genes except *CXCL8* and *IL-33*. Although with CD8 lysate stimulation the behavior of some genes was similar, no significant overexpression of *CCL11*, *CCL24* and *CCL26* was observed. Again, before concluding, samples size should be increased and, importantly, enzymatic activity studies should be performed to confirm that the granzymes (and other CD8-derived molecules) obtained in the extract were indeed active.

In conclusion, this study demonstrates the implication of the CD8 population to the esophageal inflammatory response in EoE and highlights the need for developing additional studies to better describe its implication in EoE pathophysiology. A deeper analysis of this cell type and its specific function with the epithelial environment will contribute to better define its role in esophageal dysfunction and also its activity in allergic diseases.

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# CAPÍTULO 3

***"Elevated esophageal IgE and IgG4 against food allergens in eosinophilic esophagitis"***

# INTRODUCTION

Eosinophilic esophagitis (EoE) is a chronic inflammatory disease of the esophagus and constitutes the most prevalent cause of chronic esophagitis after gastroesophageal reflux disease (GERD). Over a decade ago, several studies defined EoE as an allergic disorder mainly driven by Th2 cells.[1–3] The contribution of allergy to EoE etiopathogenesis is further supported by the higher prevalence of concomitant atopic disorders and allergic comorbidities[4,5] and the efficacy of dietary therapy with either amino acids-based elemental diet[6], which achieves complete remission in >90 % of patients, and empirical elimination diets, which has been shown to be effective in 50 – 70 % of the patients.[7–9]

Similarly to allergic diseases, the cellular infiltrate in the esophageal epithelium is characterized by eosinophils and mast cells (MC), and the over-expression of certain cytokines such as IL-5 and IL-13,[10,11] and MC-related proteases, all demonstrated to reverse after dietary treatment.[12] Further, B cells are in higher counts in both, the epithelium and the *lamina propria* in pediatric patients along with an increase in IgE mRNA production.[13] Therefore, humoral activity seems to be critical in EoE pathophysiology.

Despite EoE association with allergy, no efficacy has been observed neither when using skin-prick guided diets in adults, only showing around a 30 % of response,[14] nor when patients were treated with the mast cell stabilizer cromolyn sodium, which does not offer therapeutic efficacy.[15] Lack of clinical improvement has also been observed with omalizumab, a humanized anti-IgE treatment. The low number of studies performed with omalizumab showed a regression of symptoms and significant but partial reduction of tissue eosinophils, mast cells and tissue IgE. Still, no histological remission was ever achieved in contrast with other commonly used drugs (corticosteroids, diet and IBPs).[16–18]

After omalizumab failure, the allergic response in EoE was suggested to be mainly driven by IgG4 and not by IgE, as tissular and seric IgG4 and specific IgG4 against food in serum were identified[17]. Similarly, elevated IgG4 levels in esophageal samples

of peadiatric EoE were identified, which correlated with eosinophil counts and transcriptomic EoE features.[19] Also, few studies tried to use IgG4 detection as a predictor for guided elimination diets but no conclusive results have been obtained.[20].

Even though current studies are pointing out the importance of humoral response in EoE, no conclusive data has been reported yet. Prospective and interventional studies are mandatory in order to further clarify IgG4 relevance. Additionally, despite no efficacy has been observed with omalizumab, IgE may still have an important role in EoE as intraepithelial mast cells bearing IgE are present in EoE[13,21]

Therefore, the aim of the present study was to further evaluate esophageal humoral activity and its contribution to the pathophysiology of EoE in adults. This research was designed to assess the production and distribution of IgE and IgG4 against food allergens and its modulation by dietary restriction. The application of this approach may contribute to the development personalized dietary interventions based on the production of specific immunoglobulins in the esophagus.

# EXPERIMENTAL DESIGN

A prospective multi-center observational longitudinal case-control study was designed in order to: (1) set up an immunoglobulin extraction and quantification protocol, and (2) quantify immunoglobulins in esophageal biopsies and blood samples in active EoE and in remission. Two cohorts were recruited: i) case cohort of active EoE patients before and after empirical elimination diet treatment; ii) control cohort of patients with symptoms of esophageal dysfunction with or without gastro-esophageal reflux disease (GERD), and histologically normal esophageal biopsies.

Clinical assessment and biological samples (esophageal biopsies and blood) were obtained in all participants. The total IgG and total and specific IgE and IgG4 were analyzed in tissue and blood samples to evaluate both local and systemic humoral activity as follows (**Figure 1**):

#### (1) SET UP OF IMMUNOGLOBULIN EXTRACTION

The quantification of immunoglobulins in esophageal biopsies and blood samples was evaluated in patients with active EoE and controls. A protocol for immunoglobulin extraction and quantification from biopsies was developed and immunoglobulins measured by ImmunoCAP taking into account dilution buffer, optimal concentration and protein recovery.

#### (2) IMMUNOGLOBULIN QUANTIFICATION

The quantification of immunoglobulins in esophageal biopsies and blood samples was performed after 6-8 week empirical elimination diet treatment in EoE patients to evaluate both local and systemic humoral activity and its predictive value to identify a causative food triggers of the disease. The study focused on a cohort of patients who responded at least to milk and/or gluten exclusion diet.

Presence of Ig was confirmed to further characterize immunoglobulin-bearing cells.

- Immunolgobulin quantification in esophageal biopsies and blood was performed by ImmunoCAP

- Identification of immunoglobulin-bearing cells in esophageal biopsies was performed by immunofluorescence
- Ultrastructural analysis of the epithelium and the main immune infiltrate by transmission electron microscopy

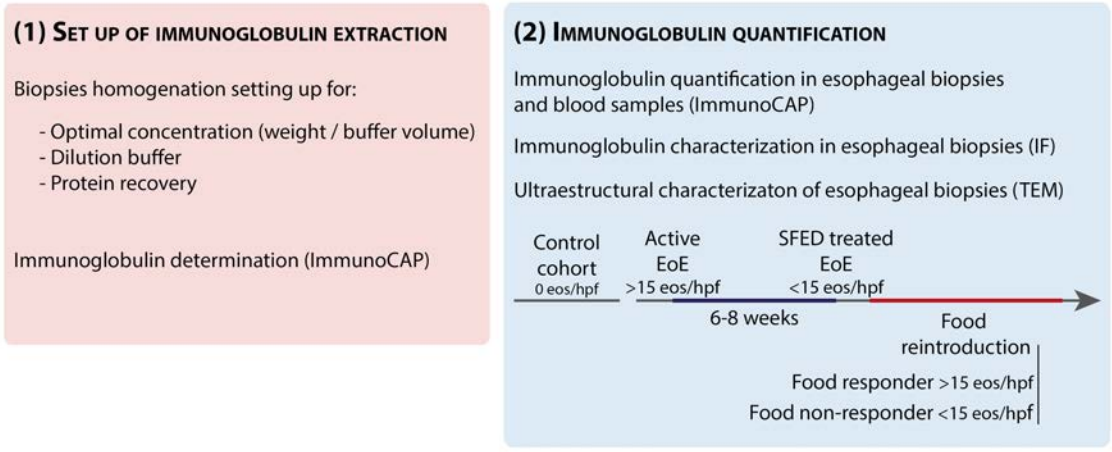


**Figure 1 | Experimental design chapter 3.** IF: immunofluorescence; TEM: transmission electron microscopy; hpf: high power field; SFED: six food elimination diet, Eos: eosinophils

# METHODS

## 1 SUBJECTS DESCRIPTION AND ETHICAL ASPECTS

### 1.1 STUDY POPULATION AND ETHICAL ASPECTS

Two groups of subjects (balanced by sex and age) were prospectively recruited from the Department of Gastroenterology at Tomelloso General Hospital (Ciudad Real, Spain) and at Vall d'Hebron University Hospital (HUVH, Barcelona, Spain):

a) Case cohort: consecutive adult EoE patients with a clinico-pathological diagnosis of active disease confirmed by: (1) presence of symptoms related to esophageal dysfunction and infiltration of 15 or more eosinophils per high-power field (hpf) in at least 1 of the esophageal epithelium biopsies; (2) no pathological eosinophilic infiltrate in gastric and duodenal biopsies; (3) other systemic and local causes of esophageal eosinophilia excluded [21]. Only patients who responded to a SFED were included in the study.

No steroids treatment (either oral, nasal, airway or swallowed) was allowed in the 8 weeks before the begging of the study. If existed GERD concomitance symptoms, proton-pump inhibitors (PPI) were permitted in a stable dose without change before and after empirical elimination diet evaluation. Anti-H1 or inhaled β2-agonists and anticholinergic bronchodilator drugs were allowed in a stable dose during the study in cases of exacerbated rhinitis or asthma symptoms.

b) Control cohort: patients with esophageal dysfunction symptoms with or without GERD and with normal endoscopy (except for non-complicated endoscopic signs of erosive esophagitis related to GERD) and normal esophageal histological analysis of biopsies. All subjects were not undergoing any treatment with PPI, corticosteroids or elimination diet.

The study was conducted in accordance with the principles of the Declaration of Helsinki and approved by the Institutional Review Board of La Mancha Centro General

Hospital (acta nº 08/14) and Ethics Committee at Vall d'Hebron University Hospital (PR(AG)94/2013). Informed consent was obtained from all participants.

## 1.2 CLINICAL EVALUATION AND TREATMENT PROTOCOL

### 1.2.1 SYMPTOM EVALUATION

Symptom evaluation was performed as described in section 1.2.1. of chapter 2. Briefly, symptoms of esophageal dysfuncion were assessed before each endoscopy by a validated score.[23] The score is based on the duration, intensity and frequency of dysphagia.

### 1.2.2 ENDOSCOPY AND BIOPSY PROCEDURE

Endoscopy and biopsy procedure was performed as described in section 1.2.2. of chapter 2. Briefly, upper endoscopy was performed at baseline for case and control groups and at the end of the 6-8 elimination diet. Endoscopy features were recorded following EREF validated questionnaire.[24] Biopsies were obtained from upper and lower esophageal regions, preferably from visible lesions.

### 1.2.3 ALLERGY TEST

Atopy background was recorded and allergy tests were performed as described in section 1.2.3. of chapter 2. Briefly, all participants were examined in the allergy unit of the participant hospitals, where they underwent skin prick testing (SPTs) for a general panel of food and pneumoallergens.

### 1.2.4 RESPONSE DEFINITIONS

Response definitions were performed as described in section 1.2.4. of chapter 2. Clinical and histological response were considered as defined in guidelines.[21]

### 1.2.5 EMPIRICAL ELIMINATION DIET PROTOCOL

Empirical elimination diet protocol was performed as defined in section 1.2.5. of chapter 2. Briefly, EoE patients underwent a six-food elimination diet (SFED) for a 6-8 weeks period, avoiding 6 potentially allergenic food groups: (1) gluten, (2) milk and dairy products, (3) eggs, (4) fish and seafood, (5) legumes/soy and (6) nuts, as well as processed foods containing them. Due to its allergenic potential, gluten and milk and dairy products were, in this order, the firsts foods reintroduced in all patients. The remaining foods reintroduction varied according to previous results and the patient's preferences to normalize their diet as soon as possible. If patients responded positively (either complete or partial) after each single-food challenge, this food was considered to be well tolerated and maintained in the diet. On the other hand, if inflammation recurred (≥15 eosinophils/hpf), the challenged food was removed permanently from the diet and considered an EoE trigger. Next food was immediately reintroduced with no washout period. Patients who did not respond to SFED were withdrawn from the study and treated with oral corticosteroids or PPI[8] (**Figure 2**).

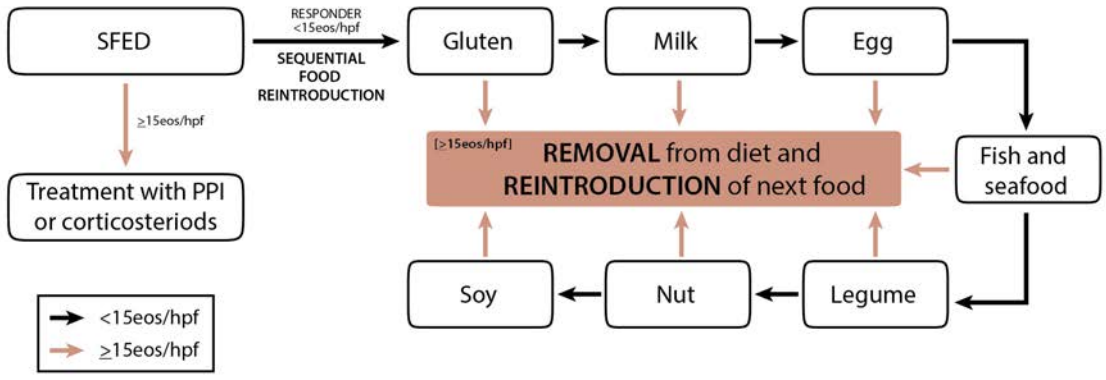


**Figure 2 | General scheme of the sequential food reintroduction protocol.** Wheat and milk were the first food to be reintroduced in all cases. The order of the remaining foods varied according to patient's preferences to normalize their diet as soon as possible. Black arrows indicate the following step when histological analysis showed <15 eosinophils / hpf. Red arrows indicate the following step when histological analysis showed ≥15 eosinophils / hpf. Eos: eosinophils

### 1.3 COLLECTION OF BIOLOGICAL SAMPLES

#### 1.3.1 BIOPSIES

Four biopsies were fixed in 4% PFA, for further microscopic examination of tissue architecture, eosinophil infiltrate (by means of H&E staining) and additional immune cell characterization (by means of specific cell marker staining). Between 4-10 biopsies were obtained for immunoglobulin extraction and fixed in 500 µL of AllProtect (QIAGEN) and stored at -80ºC until processed for immunoglobulin isolation and protein quantification. Two additional biopsies were collected for ultrastructure analysis by transmission electron microscopy (TEM) and were immediately fixed in 2.5% glutaraldehyde (EM grade, Merck), 2% paraformaldehyde (PFA) in 0.1M PBS (Sigma Aldrich) (pH 7.4) and stored at 4ºC until processed. Samples for each experimental procedure were coded and analyzed blindly by one or two different investigators.

#### 1.3.2 BLOOD

Ten mL of blood were obtained before every endoscopic procedure. Samples were centrifuged at 1,200 g for 10 minutes, and plasma (supernatant) was collected and stored at -40 ºC.

## 2 ANALYTICAL PROCEDURES

### 2.1 IMMUNOGLOBULIN EXTRACTION PROTOCOL

For the extraction of the protein extract from esophageal biopsies, a protocol was developed as follows: samples fixed in AllProtect Tissue Reagent were processed for immunoglobulin extraction (**Figure 3**). First, biopsies were defrosted on ice and washed three times with PBS. The excess of PBS was eliminated with filter paper and samples were weighted. Biopsies were placed in a lysing matrix D tube (MP Biomedicals) with 400 µL of PBS (pH 7.4) and 2 µL of protease inhibitor cocktail (PI) (ThermoScientific) for every 10 mg of tissue.

Next, 5 tissue homogenization cycles in a lysis matrix D tube (MPBio) (speed 6.5 for 5 seconds at Fastprep-24 5G) were performed. In all cases samples were kept in ice and a spin was performed between cycles. The supernatant from the lysis procedure was then pipetted to a new tube (A).

Then, 300 µL of PBS-PI was added to the lysis tube and a new homogenization cycle was performed. Supernatant was transferred again to tube A. This step was repeated 2 times, but each time the supernatant was transferred to a new tube: tube B and C respectively. Finally, biopsy homogenates were centrifuged at 2,000 g for 10 minutes at 4ºC and supernatant was stored at -80ºC until further analysis of Ig and total protein content.

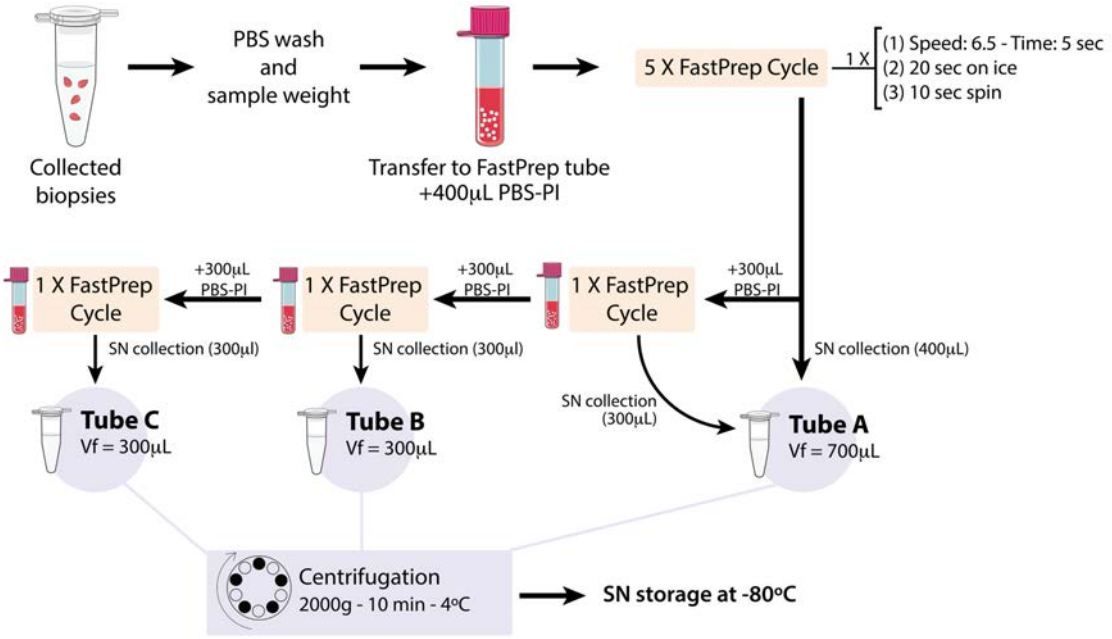


**Figure 3 | Scheme of immunoglobulin extraction protocol.** PBS-PI: phosphate-buffered saline with protease inhibitors; SN: supernatant; Vf: final volume

## 2.2 PROTEIN DETERMINATION

Protein quantification was performed with the Quick Stard Bradford Protein Assay (BioRad) following manufacturer instructions. Briefly, 5 µL of standard value and each sample were added in duplicate to a microplate. Then, 250 µL of tempered 1X dye reagent was added to each well. After 5 minutes of incubation, absorbance was

measured at 595 nm in an ELx800 spectrophotometer microplate reader (BIO-TEK Instruments, INC).

## 2.3 IMMUNOGLOBULIN QUANTIFICATION

### 2.3.1 IMMUNOGLOBULINS AGAINST FOOD ALLERGENS

Upon finalization of the SFED, patients were divided in 2 groups based on their response: i) **food responders,** EoE patients in which the reintroduced food has been identified as trigger of eosinophilic infiltration by SFED; and ii) **food-non-responders**, EoE patients in which the reintroduced food did no trigger eosinophilic infiltration. Gluten and milk were identified as triggers in more than 50% of EoE patients.

As the humoral activity to gluten and milk was analyzed in detail, a panel of allergens were selected to quantify specific IgE and IgG4 in the esophageal tissue and in blood against gluten (f79), casein (f78) and milk (f2), and as negative control chicken (f83) and bee (i1) were tested by ImmunoCAP (all reagents and instrument fromThermoFisher). Quantification of immunoglobulins (specific IgE and specific IgG4; total IgE, IgG4 and IgG) in biopsies and blood was performed by means of different methodological procedures, as follows.

### 2.3.2 SPECIFIC IgE IN TISSUE AND BLOOD

Specific IgE from esophageal tissue and blood was determined by the enzyme-linked immunofluorescent assay ImmunoCAP 1000/250 (IM-CAP-01/02) at HUVH clinical laboratories, according to the validated procedure. Specific IgE E against gluten (f79), casein (f78), milk (f2), and chicken (f83) were tested. Biopsies homogenates were not diluted, while blood samples were diluted if necessary when were outside the measuring range of 0.35-100 kU/L. Results are expressed as kUA/L.

Specific IgE ImmunoCAP detection range is found between 0.1 and 100, therefore all values under 0.1 were not considered in the analysis. Those individuals under 0.1

were excluded from the statistical analysis. Since IgE levels from almost all control individuals were under 0.1, to evaluate statistical differences with the control group, a One sample t test to analyze differences of each group with detection cut-off (0.1).

### 2.3.3 SPECIFIC IgG4 IN TISSUE AND BLOOD

Specific IgG4 from esophageal tissue and blood were determined by enzyme-linked immunofluorescent assay ImmunoCAP 1000/250 (IM-CAP-01/02) at HUVH clinical laboratories, according to the validated procedure. Specific IgE against gluten (f79), casein (f78) and chicken (f83) were tested. Biopsies homogenates were not diluted, while blood samples were diluted if necessary when were outside the measuring range of 0-300 µgA/L. Results are expressed as µgA/mL.

### 2.3.4 TOTAL IgE IN TISSUE AND BLOOD

Total IgE from esophageal tissue and blood were determined by enzyme-linked immunofluorescent assay ImmunoCAP 1000/250 (IM-CAP-01/02) at HUVH clinical laboratories, according to the validated procedure. Biopsies homogenates and blood samples were diluted if necessary when were outside measuring range of 2-5000 kU/L. Results are expressed as kUA/L.

### 2.3.5 TOTAL IgG4 IN TISSUE AND BLOOD

**Tissue**: Total IgG4 from tissue was quantified by ELISA (SEA234Hu, Cloud-Clone Corp) according to manufacturer's protocol. Biopsies homogenates were not diluted. Results are expressed as ng/mL.

**Blood:** Total IgG4 from plasma was measured by turbidimetry at HUVH clinical laboratories according to the validated procedure. Results are expressed as mg/dL.

### 2.3.6 TOTAL IgG IN TISSUE AND BLOOD

**Tissue**: Total IgG from tissue was quantified by enzyme-linked immunosorbent assay (ELISA) (E80-104, Bethyl) according to manufacturer's protocol. Biopsies homogenates were not diluted. Results are expressed as ng/mL.

**Blood:** Total IgG from plasma was measured by turbidimetry at HUVH clinical laboratories according to the validated procedure. Results are expressed ad mg/dL.

## 2.4 EOSINOPHIL COUNTS

Samples fixed in formalin were routinely processed for paraffin inclusion and sectioning as described in section 4.1. of Chapter 2. Peak eosinophil counts per hpf was calculated from each sample by an experienced pathologist.

## 2.5 IMMUNOFLUORESCENCE STAINING

Tissue sections were processed as described in section 4.2.1. of Chapter 2. Incubation took place using specific primary antibodies against human antigens. **Table 1** illustrates the primary antibody used in the study, the dilutions in which where used and the incubation period.

**Table 1 | Antibodies and experimental conditions used for immunofluorescence procedures.**

| Antibody | Host and target | Manufacturer and reference | Conditions |
|---|---|---|---|
| **Primary antibodies** | | | |
| Anti- IgE | Rabbit anti -human | Dako A 0094 | 1:100 ON 4 ºC |
| Anti-IgG | Rabbit anti -human | Abcam ab181236 | 1:100 ON 4 ºC |
| Anti-Tryptase | Mouse anti -human | Dako M 7052 | 1:100 30 min RT |
| Anti-CD38 | Mouse anti -human | Dianova BSH-7347-100 | 1:100 ON 4 ºC |
| **Secondary antibodies** | | | |
| Alexa Fluor 488 | Goat anti -rabbit | Invitrogen A11070 | 1:500 30min RT |
| Alexa Fluor 594 | Goat anti -mouse | Invitrogen A11020 | 1:500 30min RT |

ON: over-night, RT: room temperature

### 2.6 QUANTITATIVE ANALYSIS OF THE CELLULAR INFILTRATION

Positive stained cells were counted in 10 fields randomly chosen for each sample. Cell infiltration was expressed as the number of positive cells per hpf. All analysis were carried out with an Olympus BX61 microscope, an OLYMPUS DP26 video camera and the computer program CellSens Standard 1.7.

### 2.7 ULTRASTRUCTURAL ANALYSIS

Fixed samples were dehydrated and embedded in Epon's resin following standard procedures. Thin sections were cut at 5 µm, stained with toluidine blue and observed under a light microscope to assure optimal orientation of the biopsy and to select the area for subsequent TEM analysis. Once selected, ultrathin (70 – 90 nm) esophageal sections were performed with a Leica Ultracut UCT microtome (Leica Micro-systems GmbH), placed on grids (100mesh), contrasted and finally examined using a TEM JEM-1400 (Jeol Ltd., Tokyo, Japan) equipped with a CCD Gatan ES1000 W Erlangshen camera.

An observational study was carried out in all experimental groups to describe the structure of the esophageal epithelium and to identify the location and degranulation profile of eosinophils, mast cells and plasma cells, identified based on their specific morphology.

## 3 STATISTICAL ANALYSIS

Data are expressed as median (range) or mean ± standard deviation. Data distribution was analyzed by means of the D'Agostino and Pearson omnibus normality test. Normally distributed parametric data were compared by the paired or unpaired Student's *t* test (two-tailed). Otherwise, the Mann-Whitney *U* test (for unpaired) or the Wilcoxon signed-rank test (for paired) was used. One sample t test was used for statistical analysis when comparing each sample with a specific value, using GraphPad Prism 8.0 software. For defining a cutoff in Ig quantification, ROC

curve analysis was performed using all analyzed population and sensibility and specificity was calculated for the case cohort. Relationship between biological and clinical variables was analyzed by Spearman's rho correlation. Values of $p \leq 0.05$ were considered significant and were adjusted for multiple comparisons using the Benjamini and Hochberg method and the application of correction is indicated in the corresponding table and figure legends.

# RESULTS

## 1 STUDY POPULATION

A total of 21 control subjects and 27 EoE patients were included in the study. Clinical and demographic characteristics of the experimental groups are summarized in **Table 2**.

No differences were observed in age or sex between the study groups ($p>0.05$) and the mean duration of symptoms of EoE patients group exceeded 5 years. No esophageal eosinophilic infiltration was found in control subjects group, while a great infiltration was observed in esophageal biopsies from EoE patients. Within the gastric and duodenal samples, all experimental groups showed similar eosinophil counts, as part of the leukocyte population in these anatomical regions.

Predominant symptoms in EoE patients were dysphagia and food impaction. On the other hand, subjects from the control group presented a wider range of symptoms including dysphagia and food impaction, but also heartburn, regurgitation, abdominal pain and diarrhea.

During endoscopy examination, a greater percentage of subjects in the control group presented a normal mucosal appearance compared to EoE patients ($p=0.001$). While in control group erosive esophagitis are the most common findings, in EoE group prevailed white plaques and longitudinal furrows. Esophageal rings were observed in both groups, with higher prevalence in the EoE group.

Regarding atopy background, both, personal and family background of atopy was more common in the EoE group ($p<0.05$).

**Table 2 | Clinical and demographic characteristics of participants.** Values represent the mean ± SD, median (range) or nº individuals (percentage).

| | | Control subjects (n=21) | EoE patients (n=27) | p-value |
|---|---|---|---|---|
| | **Age (years)** | 42.9 ± 15 | 38.2 ± 14 | 0.28 |
| | **Sex** | Male: 12 (57 %) | Male: 18 (67 %) | 0.56 |
| | **Time of evolution (months)** | - | 65 (7 - 235) | - |
| | **Eosinophil infiltration** | 0 ± 0 | 70 ± 42 | - |
| | **Disphagia score** | - | 7.8 ± 0.8 | - |
| **Symptoms at first endoscopy** | **Abdominal pain** | 3 (14 %) | 0 | - |
| | **Heatburn** | 8 (38 %) | 4 (15%) | 0.1 |
| | **Halitosis** | 1 (5 %) | 0 | - |
| | **Dysphagia** | 13 (62 %) | 18 (67 %) | 0.77 |
| | **Food impaction** | 6 (29 %) | 27 (100 %) | **<0.0001** |
| | **Diarrhea** | 3 (14 %) | 0 | - |
| | **Iron deficiency** | 1 (5 %) | 0 | - |
| | **Vomiting** | 1 (5 %) | 3 (11 %) | 0.62 |
| | **Chest pain** | 1 (5 %) | 1 (4 %) | >0.99 |
| | **Epigastric pain** | 1 (5 %) | 0 | - |
| | **Functional dyspeptia** | 1 (5 %) | 0 | - |
| | **Regurgitation** | 6 (29 %) | 1 (4 %) | **0.03** |
| | **Weight loss** | 0 | 1 (4 %) | - |
| **Endoscopy Mucosal Appearance** | **Normal** | 13 (62 %) | 4 (145 %) | **0.001** |
| | **White plaques** | 0 | 16 (59 %) | **<0.0001** |
| | **Rings** | 3 (14 %) | 10 (37 %) | 0.11 |
| | **Edema** | 0 | 12 (44.5 %) | - |
| | **Longitudinal furrows** | 0 | 20 (74 %) | - |
| | **Estenosis** | 0 | 2 (7.5 %) | - |
| | **EE g. A** | 3 (14 %) | 0 | - |
| | **EE g. B** | 2 (9.5 %) | 0 | - |
| **Personal background of atopy** | **No atopy** | 13 (62 %) | 4 (15 %) | **0.001** |
| | **Allergic rhinitis** | 7 (33 %) | 18 (67 %) | **0.04** |
| | **Drug sensitivity** | 1 (5 %) | 1 (4 %) | >0.99 |
| | **Atopic dermatitis** | 1 (5 %) | 3 (11 %) | 0.62 |
| | **Food sensitivity** | 1 (5 %) | 13 (48 %) | **0.001** |
| | **Bronchial asthma** | 0 | 3 (11 %) | - |
| **Family background of atopy** | **No atopy** | 21 (100 %) | 18 (67 %) | **0.003** |
| | **Allergic rhinitis** | 0 | 4 (15 %) | - |
| | **Drug sensitivity** | 0 | 1 (4 %) | - |
| | **Food sensitivity** | 0 | 4 (15 %) | - |
| | **Bronchial asthma** | 0 | 3 (11 %) | - |

EE g A/B: Erosive Eophagitis grade A/B.

In **Table 3**, clinical and histological characteristics of EoE patients before and after SFED are summarized. SFED significantly reduced the peak of intraepithelial eosinophils ($p<0.0001$). Regarding clinical improvement, SFED significantly reduced the disphagia score ($p=0.0053$) and also the percentage of active dysphagia (>5) in the EoE group ($p=0.04$).

Additionally, SFED did also reduce the EREF score in active EoE, however not reaching statistical significance. Normal mucosal appearance was achieved after SFED ($p=0.0001$) and endoscopic features such as white plaques, edema and longitudinal furrows were also significantly reduced ($p<0.05$).

**Table 3 | Clinical characteristics of participants before and after SFED.** Values represent the mean ± SD, median (range) or the nº of individuals (percentage).

| | | EoE before SFED | EoE after SFED | p- value |
|---|---|---|---|---|
| **Eosinophil infiltration (n=27)** | | 65 (9 - 160) | 3 (0 - 15) | **<0.0001** |
| **Dysphagia score (n=22)** | **Mean** | 7.8 ± 0.8 | 4.8 ± 0.7 | **0.0053** |
| | **<5** | 4 (18 %) | 10 (48 %) | 0.1 |
| | **≥5** | 18 (82 %) | 11 (52 %) | **0.04** |
| **Endoscopy mucosal appearance (n=27)** | **EREF Score** | 3.5 (0 - 5) | 1 (0 - 5) | 0.0625 |
| | **Normal** | 4 (15 %) | 18 (67 %) | **0.0001** |
| | **White Plaques** | 16 (59 %) | 0 | - |
| | **Rings** | 10 (37 %) | 9 (33 %) | >0.99 |
| | **Edema** | 12 (44 %) | 1 (4 %) | **0.007** |
| | **Longitudinal furrows** | 20 (74 %) | 1 (4 %) | **<0.0001** |
| | **Estenosis** | 2 (7.5 %) | 1 (4 %) | >0.99 |

The number and type of food groups included in the SFED that were identified to induce clinical symptoms and histological eosinophil infiltration in EoE patients are represented in **Table 4**. The most common triggering foods were milk and gluten. The majority of patients had only 1 or 2 triggering food, out of the 6 groups of food tested.

**Table 4 | Triggering foods in the EoE cohort.** Values represent the nº of individuals (percentage).

| Triggering foods | | Nº triggering foods / patient | |
|---|---|---|---|
| Gluten | 7 (32%) | 1 | 14 (52 %) |
| Milk | 18 (82 %) | 2 | 6 (22 %) |
| Legumes/soy | 3 (14 %) | 3 | 1 (4 %) |
| Egg | 3 (14 %) | >3 | 1 (4 %) |
| Fish & seafood | 1 (5 %) | | |
| Nuts | 0 | | |

## 2 IMMUNOGLOBULIN EXTRACTION PROTOCOL

As no previous protocol has been validated for specific immunoglobulin isolation and quantification from esophageal biopsies and no reference values are available, only samples from active EoE that were known to respond to dietary exclusion were used in the protocol set up. The protocol was developed and specific IgE and IgG4 measured, as both immunoglobulins have been identified as increased in esophageal samples from EoE patients by mRNA gene expression[13] and by protein quantification through Luminex 100 system and ELISA.[19]

The tested conditions for the extraction protocol are described below.

### 2.1 DETECTABLE IMMUNOGLOBULIN CONCENTRATION

To determine the minimal tissue weight needed for homogenization to further detect specific immunoglobulins, several biopsies were used (increasing amounts of biopsy per volume unit of buffer were tested) and homogenates assayed for specific IgG4 anti-casein determination. Samples from patients with active EoE both, responders and non-responders to milk dietary restriction, were selected for this set up.

As indicated in **Table 5**, all samples (controls and EoE patients responders and non responders to milk exclusion) showed detectable concentration of IgG4 anti-casein, above the values detected in the vehicle (PBS+PI). Responders showed values over 10 times higher than the control group. No association between biopsy weight and IgG4 concentration was identified ($p>0.05$) (**Figure 4**). Although no variable

contributed to set the experimental conditions, a minimum of 4 biopsies were selected for isolating immunoglobulins from esophageal samples.

**Table 5 | Detection of IgG4 anti-casein in esophageal biopsies at different tissue weight (biopsy weight/mL buffer)**. Vh values indicate the mean of 3 independent analysis.

| Sample | Group | Nº of biopsies | Wet weight (mg) | Concentration (mg biopsy / mL) | Protein concentration (mg/mL) | IgG4 anti-casein (ngA / mL) |
|---|---|---|---|---|---|---|
| 1 | CTL | 2 | 3.7 | 4.1 | 0.098 | 54.4 |
| 2 | CTL | 2 | 4.4 | 4.8 | 0.028 | 5.96 |
| 3 | CTL | 3 | 3.1 | 3.4 | 0.052 | 21.8 |
| 4 | CTL | 3 | 4.6 | 4.8 | 0.093 | 13.1 |
| 5 | CTL | 3 | 5.3 | 5.8 | 0.132 | 21.1 |
| 6 | CTL | 4 | 6 | 7.1 | 0.174 | 29.7 |
| 7 | CTL | 4 | 6.1 | 6.6 | 0.164 | 38.0 |
| 8 | CTL | 5 | 8.1 | 8.5 | 0.059 | 12.9 |
| 9 | CTL | 6 | 7 | 7.8 | 0.318 | 15.7 |
| 10 | EoE milk + | 3 | 10 | 16.7 | - | 449 |
| 11 | EoE milk + | 4 | 4 | 44.4 | 0.114 | 233 |
| 12 | EoE milk + | 4 | 4.4 | 4.6 | 0.082 | 170 |
| 13 | EoE milk + | 4 | 11.3 | 11.9 | 0.101 | 62.2 |
| 14 | EoE milk + | 4 | 11.3 | 14.1 | - | 290 |
| 15 | EoE milk + | 5 | 16.2 | 17.1 | 0.255 | 146 |
| 16 | EoE milk + | 6 | 8.9 | 9.7 | 0.126 | 329 |
| 17 | EoE milk + | 6 | 11.5 | 12.5 | 0.287 | 1,637 |
| 18 | EoE milk + | 7 | 6.9 | 7.7 | 0.089 | 453 |
| 19 | EoE milk + | 8 | 7 | 9.3 | 0.176 | 15.8 |
| 20 | EoE milk + | 8 | 10.8 | 12.7 | 0.185 | 132.7 |
| 21 | EoE milk + | 8 | 15.3 | 17.0 | 0.253 | 217 |
| 22 | EoE milk + | 9 | 20 | 50.0 | - | 248 |
| 23 | EoE milk - | 2 | 4 | 4.0 | - | 20.3 |
| 24 | EoE milk - | 2 | 4.5 | 9.0 | - | 10.7 |
| 25 | EoE milk - | 4 | 11.3 | 14.1 | 0.363 | 499 |
| 26 | EoE milk - | 4 | 14.5 | 15.3 | 0.189 | 312 |
| 27 | EoE milk - | 5 | 10 | 10.0 | - | 16.8 |
| 28 | EoE milk - | 7 | 13.2 | 14.3 | 0.366 | 7.66 |
| 29 | Vh- | - | - | - | 0 | 6 |

CTL: Control group, EoE milk +: EoE patients responder to milk, EoE milk -: EoE patient non responder to milk,Vh. Vehicle

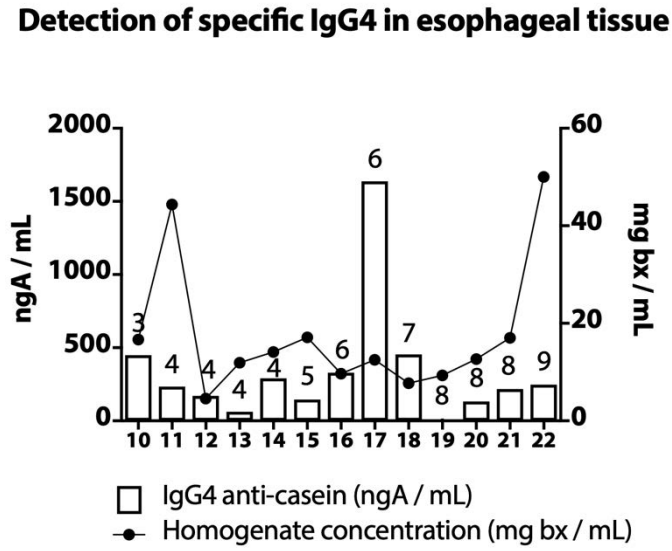


**Figure 4| Concentration of IgG4 anti-casein and tissue homogenates from active EoE patients (10-22).** Graph represents specific IgG4 concentration in bars (left axis) and concentration biopsy homogenation in black dots (right axis) according to different number of biopsies indicated above the bar.

## 2.2 OPTIMAL PROTEIN EXTRACTION

In order to fully recover the protein fraction from the esophageal samples, three consecutive extractions were performed and labeled as A, B and C (as detailed in methods section 3.1.). As expected, the highest protein concentration was obtained in the first extraction, while the second and third extractions gave 12 and 48 times less concentration, respectively, than the first one (**Table 6**).

**Table 6 | Protein determination in consecutive extraction samples.**

| | A | B | C |
|---|---|---|---|
| **Protein concentration (µg/mL)** | 384± 33.0 | 33.0± 33.1 | 8.05 ± 16.1 |

Values are expressed as Mean ± SD

## 2.3 SPECIFIC IG DETERMINATION IN CONSECUTIVE EXTRACTIONS

To further support the inclusion of the second consecutive extraction, specific IgG4 was measured in biopsy homogenates from patients that responded to milk and/or gluten exclusion. Specific IgG4 to casein and gluten and a negative control (chicken) were assayed in extractions A and B (**Table 7**).

Specific IgG4 was identified in both fractions from EoE patients responders to milk and gluten, being the concentration in the second one between 4 - 18 times lower than in the first extraction. Non-responder patients did not show differences between consecutive extractions.

**Table 7 | Specific IgG4 concentration in consecutive extractions.**

| Sample | Consecutive extraction | Response to milk exclusion | IgG4 anti-casein (ngA / mL) | Response to gluten exclusion | IgG4 anti-gluten (ngA / mL) |
|---|---|---|---|---|---|
| **1** | **A** | Yes | 648 | Yes | 281.8 |
| | **B** | | 50.6 | | 5.4 |
| **2** | **A** | Yes | 248 | No | - |
| | **B** | | 59.4 | | - |
| **3** | **A** | No | 10.7 | No | - |
| | **B** | | 7.1 | | - |
| **Vh** | | | 6 | | 1 |

Vh: Vehicle; -: Not tested

# 3 IMMUNOGLOBULIN QUANTIFICATION IN ESOPHAGEAL TISSUE

## 3.1 IMMUNOGLOBULIN E

Total IgE was significantly higher in active EoE when compared with the control group. SFED significantly reduced total esophageal IgE, however not reaching the values detected in the control group (**Figure 5**).

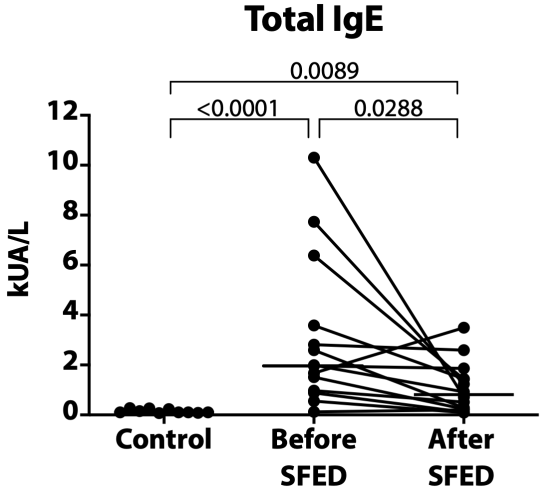


**Figure 5 | Total IgE in EoE before and after SFED and controls in esophageal samples.** A total of 10 individuals were included in the control group and 14 in the EoE group. For EoE patients before and after SFED, paired Student's *t* test was used to analyze IgE concentration following normal distribution and Wilcoxon test for those who did not follow. The groups EoE (before or after) were compared with control subjects and analyzed using unpaired Student *t* test if values followed a normal distribution or Mann-Whitney test if values did not. Horizontal bars indicate the mean or median values.

## 3.2 SPECIFIC IMMUNOGLOBULIN E

In active EoE, patients that responded to gluten exclusion showed higher concentration of IgE against gluten in the esophageal tissue than the controls and the non-responders (**Figure 6**). Diet exclusion reduced local IgE, however not reaching statistical significance.

The negative control (IgE anti-chicken) was not detected in any of the experimental groups.

Similarly to the IgE profile detected against gluten, IgE against milk was significantly higher in active EoE that responded to dietary exclusion as compared with the control

group. After SFED, EoE patients significantly reduced the esophageal IgE concentration, although not all reaching the control values. When analyzing the presence of IgE against casein, similar results were found, however this time SFED significantly reduced the concentration of specific IgE in the esophagus. Notably, in non-responders to milk, IgE anti-casein was comparable to the control group.

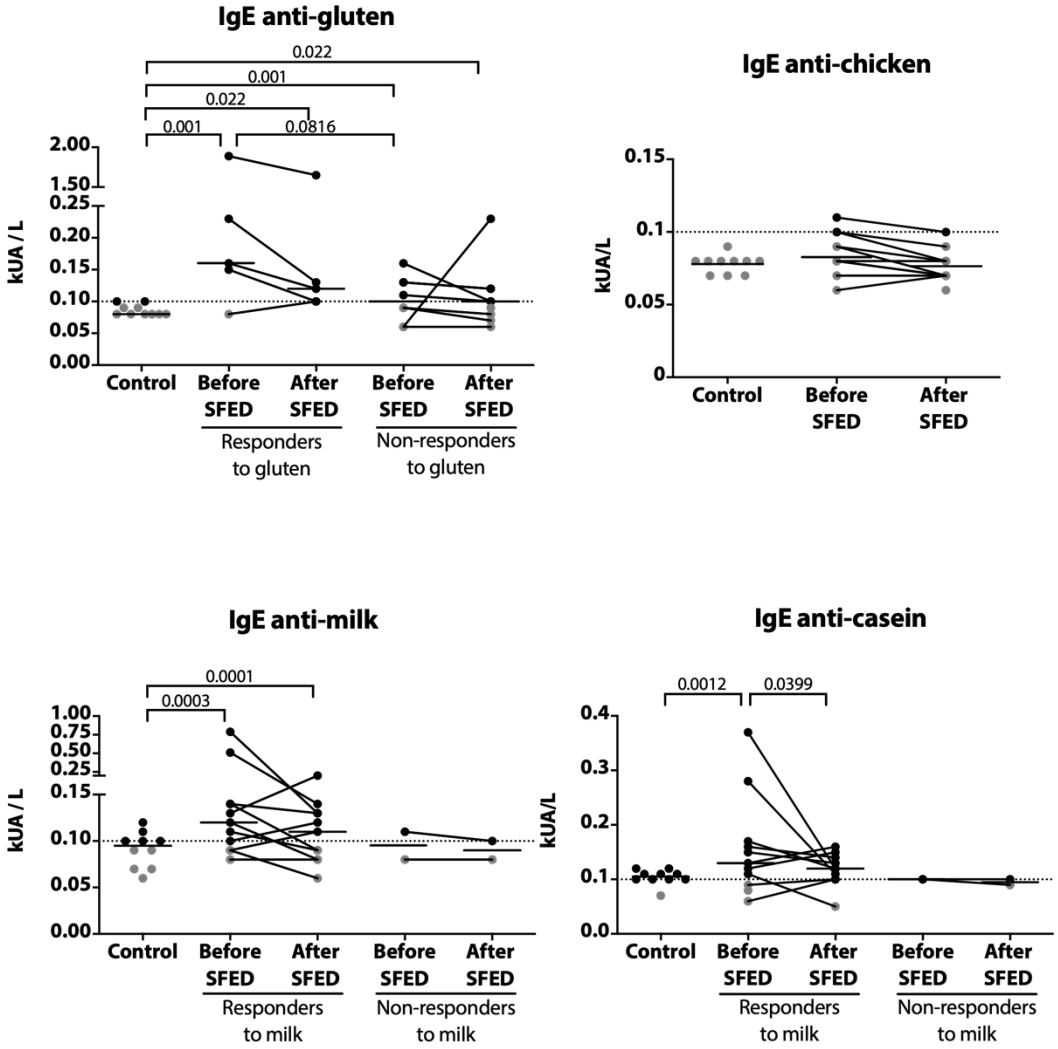


**Figure 6 | Concentration of specific IgE in EoE patients before and after SFED and in control subjects.** A total of 10 individuals were included in the control group and 14 in the EoE group before and after SFED. Chicken is tested as a negative control. Those individuals under 0.1 are represented in the graph in grey and were excluded from the statistical analysis. For EoE patients before and after SFED Paired t test was used IgE concentration following normal distribution and Wilcoxon test for those who did not follow. EoE patients (before or after, responders or non-responders) compared with control subjects were analyzed with unpaired Student's *t* test if values followed a normal distribution or the Mann-Whitney *U* test if values did not. Horizontal bars indicate mean or median values.

## 3.3 IMMUNOGLOBULIN G

Total esophageal IgG showed a significant increase in active EoE when compared to the control group, which decreased after SFED although not reaching statistical significance. IgG4 was also higher in active EoE than in controls, however differences were not significant, and SFED decreased this concentration to similar values to the control group. (**Figure 7**).

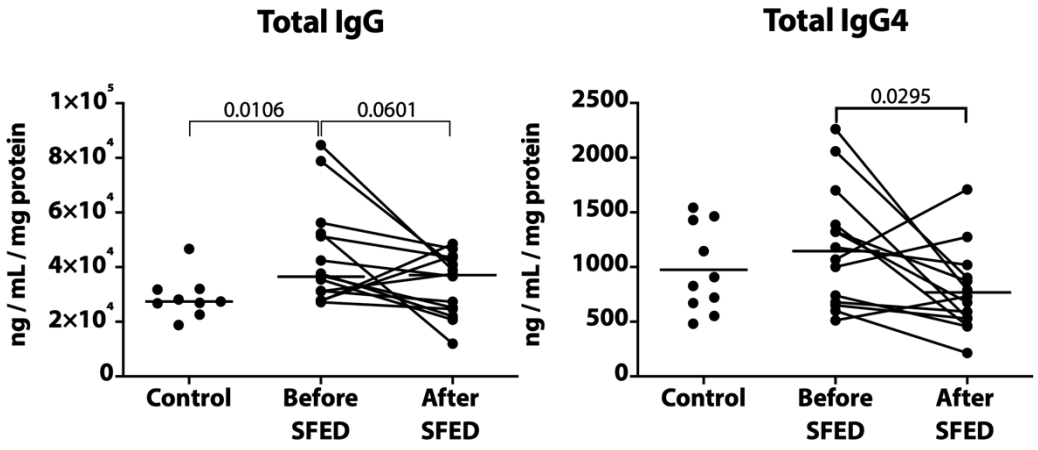


**Figure 7 | Concentration of total IgG and IgG4 in EoE patients before and after SFED and in control subjects.** A total of 10 individuals were included in the control group and 14 in the EoE group. For EoE patients before and after SFED, paired Student's *t* test was used to analyze IgG and IgG4 concentration following normal distribution and Wilcoxon test for those who did not follow. The groups EoE (before or after) were compared with control subjects and analyzed using unpaired Student *t* test if values followed a normal distribution or Mann-Whitney test if values did not. Horizontal bars indicate the mean or median values.

## 3.4 SPECIFIC IMMUNOGLOBULIN G4

IgG4 anti-gluten in the esophageal tissue was higher in active EoE (gluten-responders) than in controls and was reduced after the SFED intervention (**Figure 8**). Significantly higher tissular IgG4 anti-gluten was also found in gluten-non-responders before SFED as compared with the control group, values not modified by the dietary exclusion.

IgG4 anti-casein in the esophageal tissue was also higher in active EoE (milk-responders) and the SFED treatment lead to a two different profile of outcome: 58 % of the patients reduced IgG4 concentration to control values and 42 % did not

change IgG4 production in the tissue. Few non-responders were assayed, and no analysis could be performed with those results.

The negative control test proved no identification of IgG4 against chicken in any of the samples tested.

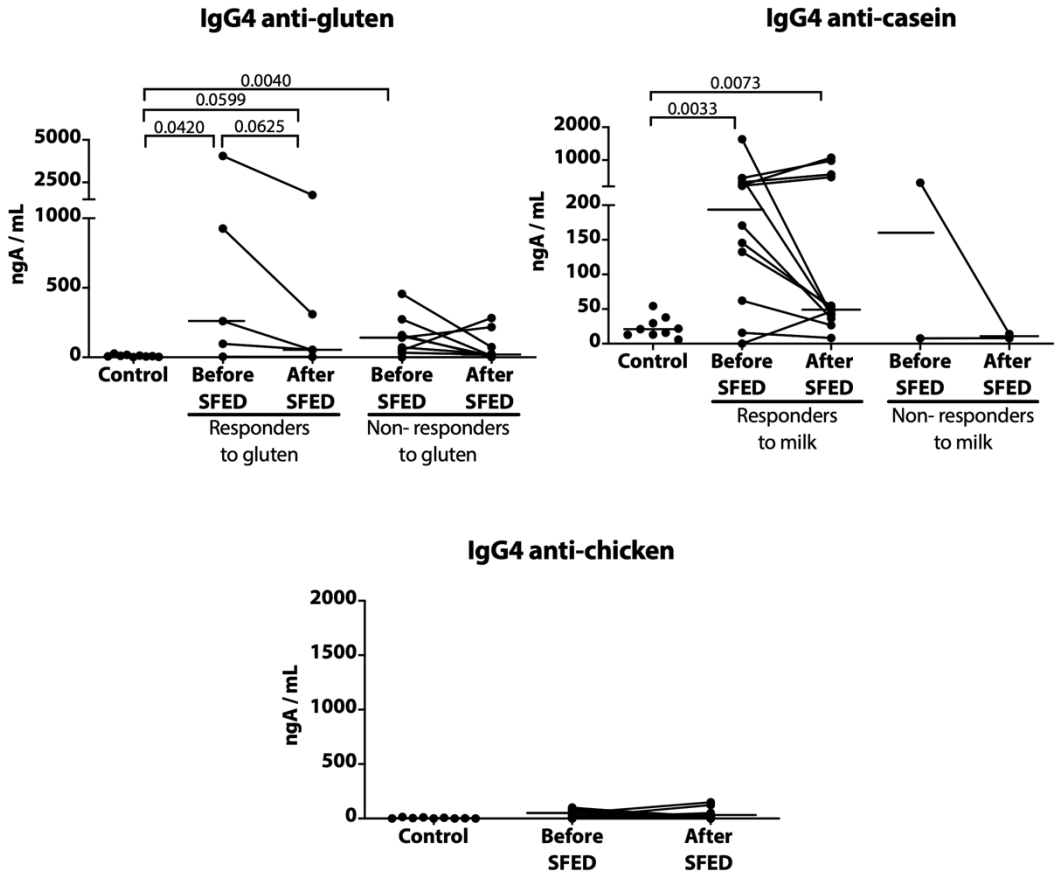


**Figure 8 | Concentration of specific IgG4 in EoE patients before and after SFED and in control subjects.** A total of 10 individuals were included in the control group and 14 in the EoE group before and after SFED. Chicken is tested as a negative control. Those individuals under 0.1 are represented in the graph in grey and were excluded from the statistical analysis. For EoE patients before and after SFED Paired t test was used IgE concentration following normal distribution and Wilcoxon test for those who did not follow. EoE patients (before or after, responders or non-responders) compared with control subjects were analyzed with unpaired Student's *t* test if values followed a normal distribution or the Mann-Whitney *U* test if values did not. Horizontal bars indicate mean or median values.

# 4 SENSIBILITY AND SPECIFICITY

Considering the usefulness of these data to further establish novel biomarkers to identify triggering food in EoE patients, a ROC analysis was performed to assess the appropriate cutoff of the technique considering both, control group and active EoE group. Then, sensibility and specificity only considering the active EoE group was calculated by identifying the true positive, true negative, false positive and false negative.

## 4.1 IMMUNOGLOBULIN E

Gluten IgE AUC was 0.84 and the cut-off was established at 0.14 kUA/L. Milk IgE AUC was 0.78, and casein IgE AUC 0.71; for both immunoglobulins the cut-off was established at 0.115 kUA/L (**Figure 9**). The positive likelihood ratio (LR), an index for diagnostic efficiency, was considered for gluten LR strong, for milk LR moderated and for casein LR weak.

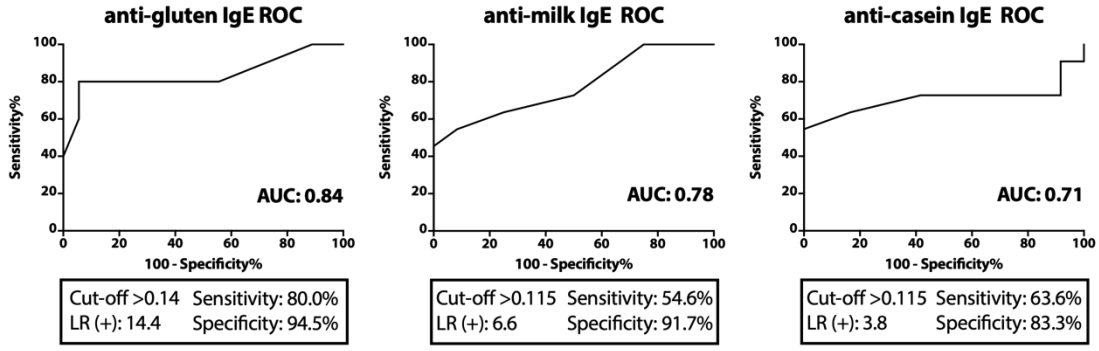


**Figure 9 | ROC curves for specific IgEs, gluten, milk and casein.** AUC, Cut-off value, positive likelihood ratio, sensitivity and specificity are given.

With the determined cut-off identified in ROC analysis, sensibility and specificity were estimated in basal active EoE patients, results are shown in **Table 8**. Total milk IgE was also analyzed by considering a true positive in at least one of the two milk antigens analyzed.

**Table 8 | Estimated sensibility and specificity of IgE determination in basal active EoE patients**. Cutoff values established by ROC analysis.

| | anti-gluten IgE | anti-milk IgE | anti-casein IgE | total milk IgE |
|---|---|---|---|---|
| **Sensitivity** | 0.67 | 0.54 | 0.64 | 0.82 |
| **Specificity** | 0.88 | 1 | 1 | 1 |

## 4.2 IMMUNOGLOBULIN G

Gluten IgG4 AUC was 0.75 and the cutoff was established at 210 ngA/mL. Casein IgG4 AUC was 0.80 and the better cutoff was 58.33 ngA/mL (**Figure 10**). In this case, both likelihood ratios (LR) were considered moderated.

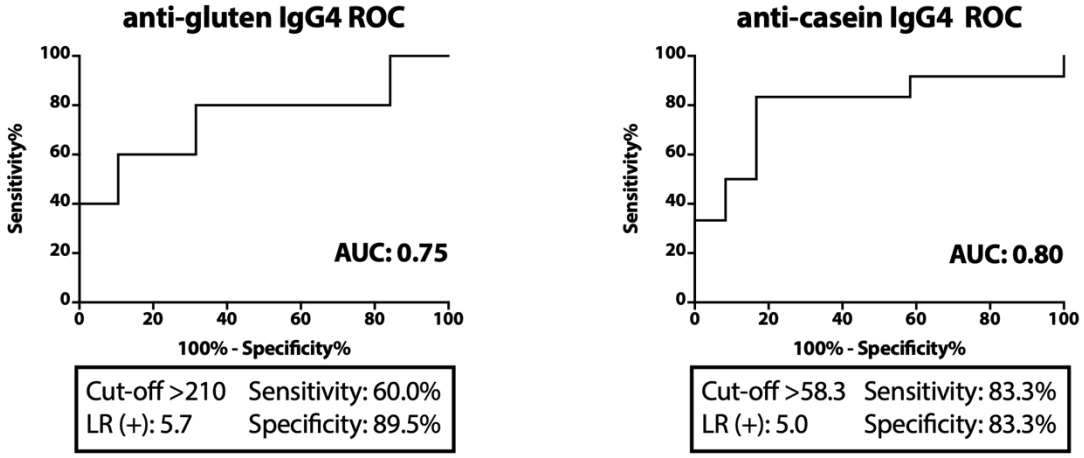


**Figure 10 | ROC analysis for specific IgG4 gluten and milk.** AUC, Cut-off value, positive likelihood ratio, sensitivity and specificity are given.

With the determined cutoff identified in the ROC analysis, sensibility and specificity were estimated in active EoE patients (before SEFD), results are shown in **Table 9**.

**Table 9 | Estimated sensibility and specificity of IgG4 determination in active EoE patients**. Cutoff values established by ROC analysis.

| | anti-gluten IgG4 | anti-casein IgG4 |
|---|---|---|
| **Sensitivity** | 0.8 | 0.83 |
| **Specificity** | 0.78 | 0.5 |

In order to achieve the higher reliability for tissular immunoglobulin detection the sensitivity and specificity of the combination of immunoglobulins in active EoE patients were analyzed. The combined sensibility and specificity was estimated, considering a true positive in at least one of the two immunoglobulins analyzed. Even so, no improvement in sensibility and specificity respect to individual analysis was achieved (**Table 10**)

**Table 10 | Estimated sensibility and specificiy for the combination of IgE and IgG4 in basal active EoE patients.** Cutoff values were established by ROC analysis.

| | anti-gluten | anti-casein |
|---|---|---|
| **Sensitivity** | 0.8 | 1 |
| **Specificity** | 0.78 | 0.5 |

# 5 IMMUNOGLOBULIN DETERMINATION IN BLOOD

To further assess that the observed changes in immunoglobulin concentrations in biopsies was specific of the esophageal tissue, and not a systemic response, total IgE, IgG and IgG4 were analyzed in control subjects and EoE patients before and after treatment (**Figure 11**).

## 5.1 IMMUNOGLOBULIN E

Significant differences were observed in total IgE in blood between control subjects and EoE patients (either before and after SFED), similar to what was found in esophageal tissue (**Figure 11**). However, this total IgE increase in patients was found to be influenced by allergic comorbidities and not by tissue eosinophil infiltration. The correlation analysis showed that 72% of the IgE increase were associated with allergic comorbidities (**Table 11**).

## 5.2 IMMUNOGLOBULIN G

No differences were observed between groups in total IgG or in total IgG4 in blood (**Figure 11**). For both of them, no relationship was found with eosinophilic infiltrate or allergic comorbidities (**Table 11**).

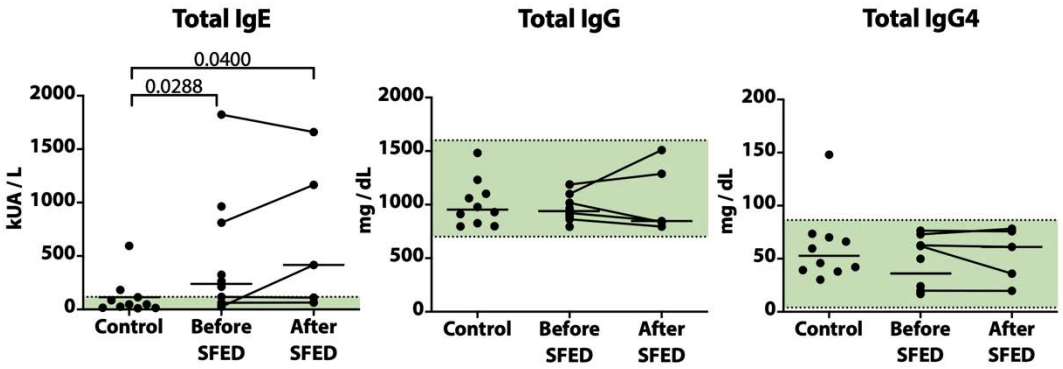


**Figure 11** | **Concentration of blood total IgE, total IgG and IgG4 in EoE patients before and after SFED and in control subjects.** A total of 10 individuals were included in the control group, 10 in the EoE group before and 5 in the EoE after SFED. Green background limits reference values for each immunoglobulin. Paired t test was used IgE concentration following normal distribution and Wilcoxon test for those who did not follow. EoE patients (before or after, responders or non-responders) compared with control subjects were analyzed with unpaired Student's *t* test if values followed a normal distribution or the Mann-Whitney *U* test if values did not. Horizontal bars indicate mean or median values.

**Table 11 | Biological and clinical correlations between blood immunoglobulins, intraepithelial eosinophil counts and allergic comorbidities.** Statistical analysis were performed by the Spearman correlation test and p-values are corrected by the Benjamini-Hochberg test.

| | | **Eosinophils** | **Allergy** | | | | |
|---|---|---|---|---|---|---|---|
| **IgE** | R | 0.33 | **0.724** | | | | |
| | p | 0.11 | **3.62E-05** | | | | |
| | | Eosinophils | Allergy | | | Eosinophils | Allergy |
| **IgG** | R | -0.10 | -0.37 | **IgG4** | R | -0.37 | -0.12 |
| | p | 0.59 | 0.12 | | p | 0.082 | 0.61 |

# 6 BIOPSIES – BLOOD IMMUNOGLOBULIN CORRELATION

Although no increase in IgG was observed and the increase in total IgE was associated to allergic comorbidities, specific immunoglobulins in paired biopsies – blood samples of active EoE patients were analyzed.

## 6.1 IMMUNOGLOBULIN E

For specific immunoglobulins, IgE against gluten presented higher values in biopsies than in blood, for those individuals who responded to gluten elimination in SFED (**Figure 12**). For those who did not, only 2 patients presented tissular specific IgE values over the threshold limit. It is worth mentioning that none of the blood values was above the positivity threshold (0.35 kUA/L).

For IgE anti-milk, all samples analyzed belonged to the responders group. Patients 1 and 5 were above detection limit for both, blood and biopsies. Patient 4 presented higher blood IgE concentration but was not positive. Patients 2 and 3 showed detectable blood IgE anti-milk, however in biopsies patient 2 present really low levels and patient 3 presents levels even higher than blood.

For IgE anti-casein, all samples analyzed belonged to the responders group. Concentration of specific IgE was higher in biopsies than in blood for all individuals except for one. Subjects 2 and 3 showed similar profile of IgE anti milk and casein (biopsy vs blood).

Finally, no correlation was observed between blood – IgE and tissue – IgE (data not shown).

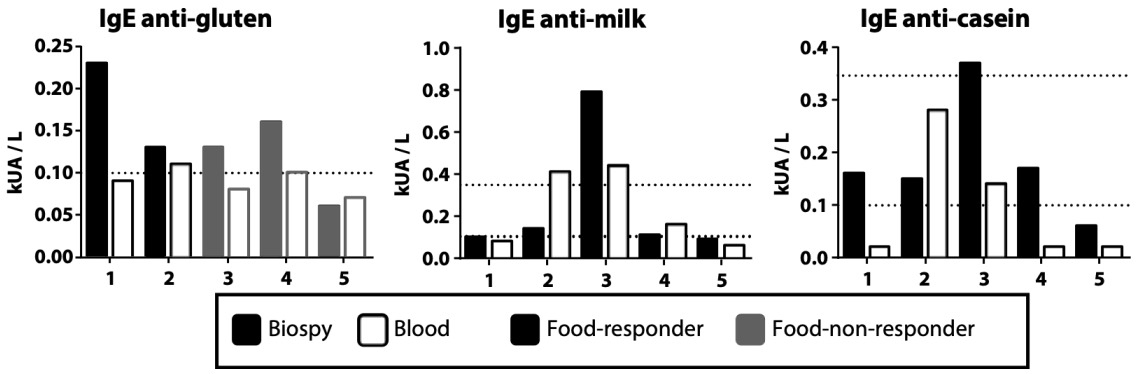


**Figure 12 | Biopsy (tissue homogenates) and blood specific IgE quantification in active EoE patients.** Full bars represent tissue homogenates and empty bars represent blood. Black color is for foods detected in SFED and grey for non-responder. Five patients were included in this analysis, all participants represented individually. Biopsy is represented with colored bars and blood with empty bars. Black color is for individuals who responded in the SFED to the specified food and grey for non-responder individuals

## 6.2 IMMUNOGLOBULIN G4

IgG4 anti-gluten presented the same profile for both, responders and non-responders (**Figure 13**). In IgG4 anti -casein was detected in biopsies and not in blood, except for individual 3, who presented higher levels of blood IgE against casein. No correlation was identified between IgG4 in biopsies and blood for the specific antigens tested (data not shown).

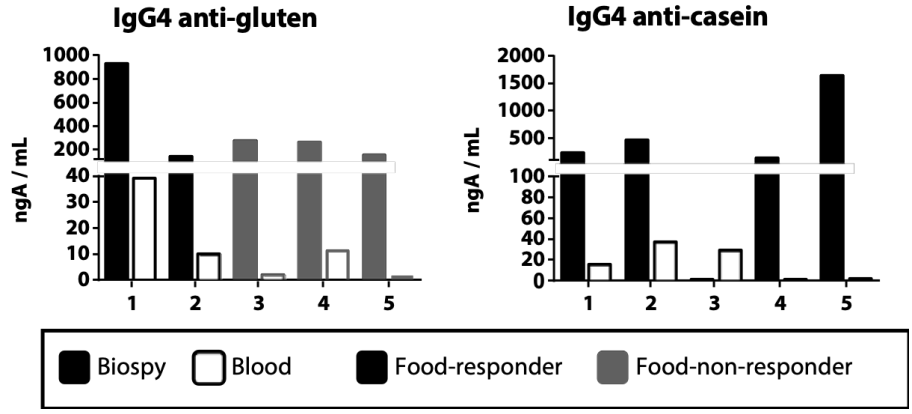


**Figure 13 | Biopsy (tissue homogenates) and blood specific IgG4 quantification in active EoE patients**. Full bars represent tissue homogenate and empty bars represent blood. Black color is for foods detected in SFED and grey for non-responders. Five patients were included in this analysis, all participants represented individually. Biopsy is represented with colored bars and blood with empty bars. Black color is for individuals who responded in the SFED to the specified food and grey is for nor non-responder individuals.

# 7 IMMUNOGLOBULIN CHARACTERIZATION

Aiming to analyze the immunoglobulin distribution in the tissue and for phenotyping the cells responsible for the increased immunoglobulin in the tissue extracts, we performed immunofluorescence analysis for immunoglobulin subtype (IgE – IgG), mast cell s and plasma cells.

## 7.1 IMMUNOGLOBULIN E

IgE was vastly infiltrated in active EoE patients respect to control group, present in both plasma cells and mast cells. Plasma cells were only present in *vascular papilae and lamina propia* while mast cells were present in all stratums (**Figure 14**). When the cell count was performed, a significant increase in $IgE^+$cells, mast cells or $IgE^+$mast cells were observed in EoE patients before SFED when compared to the control group (**Figure 15**). This infiltration was reduced after SFED treatment, although it did not reach the values in the control group. Notably, $IgE^+$ mast cells were almost absent in the epithelium of control subjects, while it was present not only in active illness but also, and reduced, in patients after SFED.

$IgE^+$ plasma cells were infiltrated exclusively in the *vascular papillae* and *lamina propria*. No cell counts could be performed for IgE and plasma cells because not all samples were deep enough to have representative *lamina propria*.

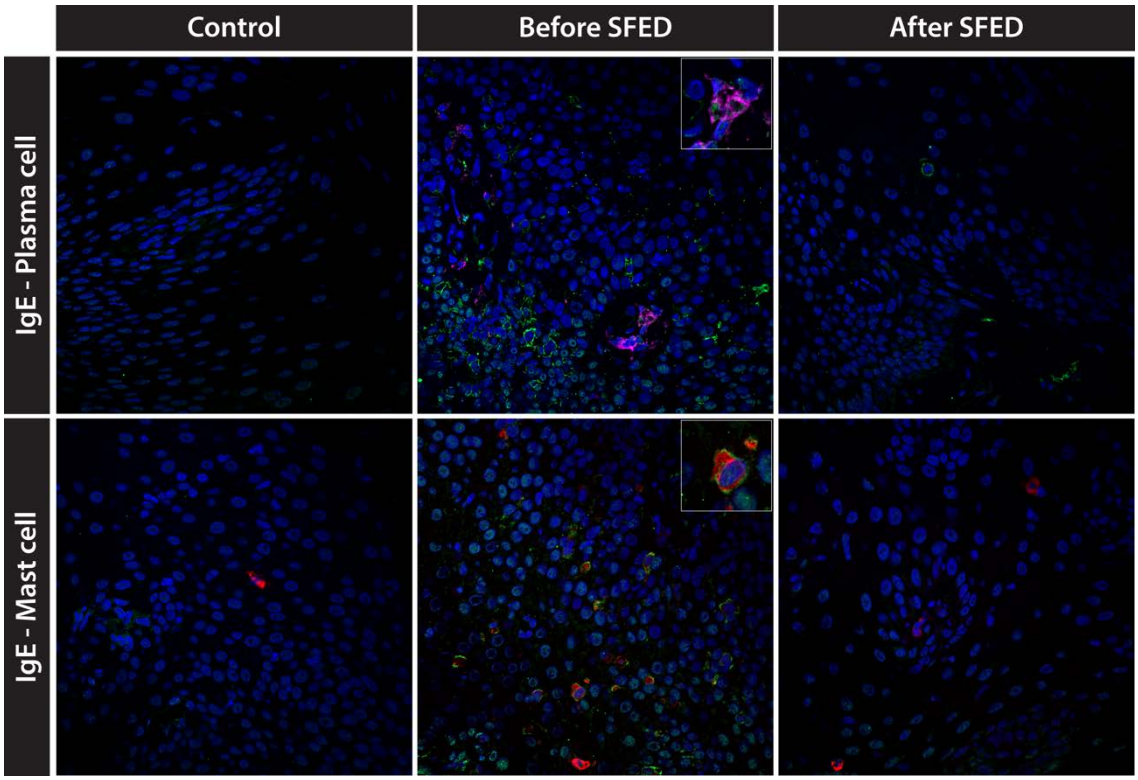


**Figure 14 | Representative images of IgE$^+$ cells, plasma cells and mast cells in esophageal samples from EoE patients** before and after SFED and from the control group. DAPI counterstaining is dyed in blue, IgE$^+$ cells in green, plasma cells in violet and mast cells in red. Magnification 400X.

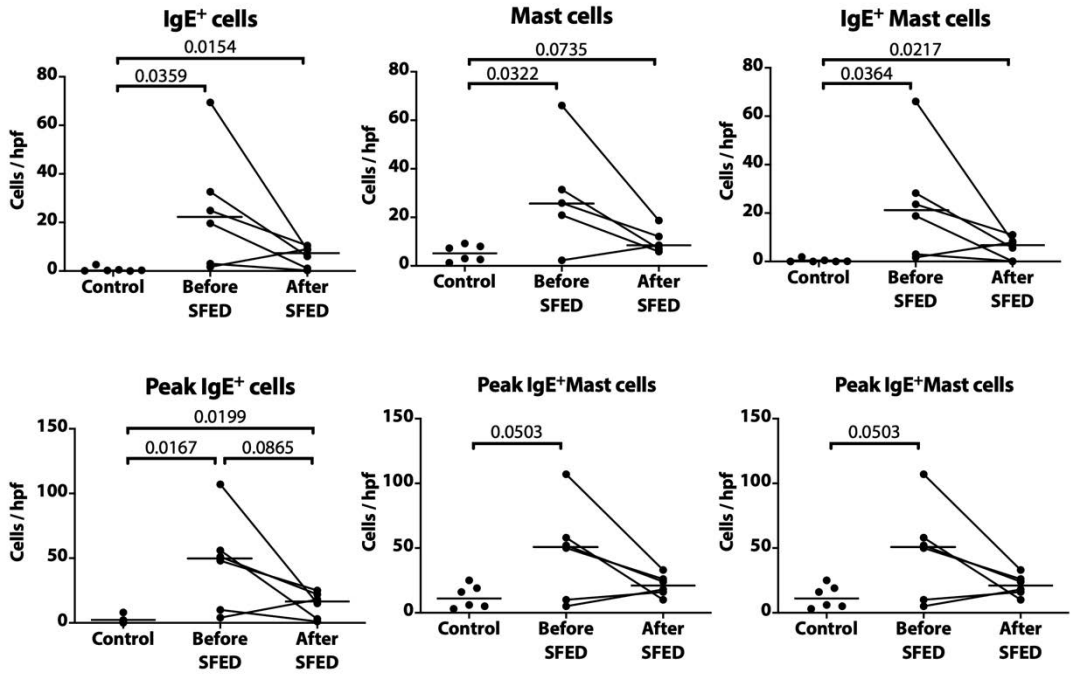


**Figure 15| IgE$^+$ cells, mast cells and double positive cell counts in esophageal samples in patients with EoE before and after six-food elimination diet (SFED) and in control subjects.** Data are expressed as median ± range. Graphs represent data of 6 – 7 individuals. For comparison EoE patients before and after SFED Wilcoxon t test was used. Mann-Whitney t test was used to compare EoE patients (before or after) with control subjects. Horizontal bars indicate median values.

## 7.2 IMMUNOGLOBULIN G

In active EoE patients, IgG staining showed large amount of IgG diffused throughout the epithelium, not allowing cell counting (**Figure 16**). This diffused immunoglobulin was not observed in either the control group or patients after SFED. In EoE patients, mast cells bearing IgG were not observed either in the epithelium or lamina propria. $IgG^+$ cells were predominantly plasma cells, located in the lamina propria. However, IgG positive cells were also observed infiltrated in the epithelium of EoE patients, and those cells were not identified as mast cells or plasma cells.

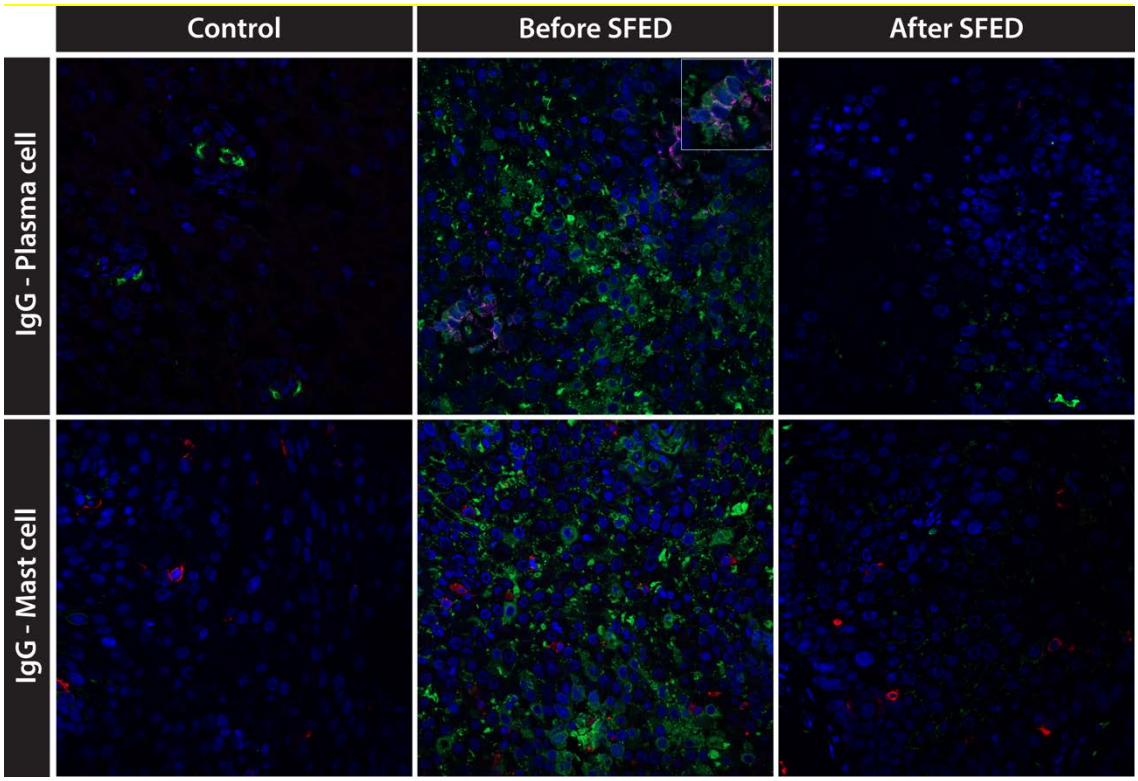


**Figure 16 | Representative images of $IgG^+$ cells, plasma cells and mast cells in esophageal samples from EoE patients before and after SFED and from the control group.** DAPI counterstaining is dyed in blue, $IgG^+$ cells in green, plasma cells in violet and mast cells in red. Magnification 400X.

## 8 CLINICO-BIOLOGICAL ASSOCIATIONS

To get a closer insight into humoral mechanisms and to better understand the esophageal antibody activity in EoE, we analyzed the association between immunoglobulin concentration and major clinical manifestations and histological.findings. No significant correlations were observed after FDR correction between any of the analyzed parameters. Only a trend is observed between intraepithelial eosinophil counts and allergic comorbidities, tissular total IgE and $IgE^+$ cells/hpf as well as $IgE^+$ mast cells/hpf and allergic comorbidities. Also a tendency is observed between tissular total IgG and IgG4 (**Table 12)**.

**Table 12 | Clinical and biological correlations**

| | Eosinophi | | | | | | Tissue total IgE (kUA / L) | | | | | | Tissue total IgG (ngA / mL / mg prot) | | | | | | Tissue total IgG4 (ngA / mL / mg prot) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $r^2$ | p | FDR | C | Bf | Af | $r^2$ | p | FDR | C | Bf | Af | $r^2$ | p | FDR | C | Bf | Af | $r^2$ | p | FDR | C | Bf | Af |
| **Eosinophils / hpf** | - | - | | - | - | - | 0.3 | 0.08 | 0.11 | 10 | 10 | 10 | 0.0 | 0.97 | 0.97 | 10 | 10 | 10 | 0.2 | 0.35 | 0.45 | 10 | 10 | 10 |
| **Time of evolution (months)** | 0.2 | 0.39 | 0.70 | - | 27 | - | -0.3 | 0.28 | 0.36 | - | 10 | - | -0.3 | 0.24 | 1.09 | - | 10 | - | -0.1 | 0.71 | 0.71 | - | 10 | - |
| **Dysphagia Score** | 0.2 | 0.20 | 0.60 | - | 24 | 23 | 0.4 | 0.06 | 0.10 | - | 10 | - | 0.1 | 0.55 | 1.65 | - | 10 | - | 0.5 | 0.05 | 0.23 | - | 10 | - |
| **Tissue total IgE (kUA / L)** | 0.3 | 0.08 | 0.34 | 10 | 10 | 10 | - | - | | - | - | - | 0.0 | 0.91 | 1.02 | 10 | 10 | 10 | 0.1 | 0.71 | 0.80 | 10 | 10 | 10 |
| **Tissue total IgG (ngA / mL / mg prot)** | 0.0 | 0.97 | 1.10 | 10 | 10 | 10 | 0.0 | 0.91 | 0.91 | 10 | 10 | 10 | - | - | | - | - | - | 0.4 | **0.02** | 0.14 | 10 | 10 | 10 |
| **Tissue total IgG4 (ngA / mL / mg prot)** | 0.2 | 0.35 | 0.79 | 10 | 10 | 10 | 0.1 | 0.71 | 0.80 | 10 | 10 | 10 | 0.4 | **0.02** | 0.14 | 10 | 10 | 10 | - | - | | - | - | - |
| **$IgE^+$ cells / hpf** | 0.1 | 0.85 | 1.10 | 6 | 5 | 5 | 0.5 | **0.03** | 0.15 | 6 | 5 | 5 | 0.0 | 0.85 | 1.10 | 6 | 5 | 5 | 0.4 | 0.10 | 0.19 | 6 | 5 | 5 |
| **Mast cells / hpf** | 0.0 | 0.98 | 0.98 | 6 | 5 | 5 | 0.5 | 0.05 | 0.12 | 6 | 5 | 5 | 0.1 | 0.67 | 1.50 | 6 | 5 | 5 | 0.4 | 0.08 | 0.23 | 6 | 5 | 5 |
| **$IgE^+$ mast cells / hpf** | 0.1 | 0.84 | 1.25 | 6 | 5 | 5 | 0.5 | **0.04** | 0.13 | 6 | 5 | 5 | 0.1 | 0.84 | 1.26 | 6 | 5 | 5 | 0.4 | 0.09 | 0.20 | 6 | 5 | 5 |
| **Allergic Comorbidities** | 0.3 | **0.048** | 0.43 | 21 | 27 | 0 | 0.5 | **0.01** | 0.06 | 10 | 10 | 0 | -0.1 | 0.77 | 1.38 | 10 | 10 | 0 | 0.2 | 0.32 | 0.48 | 10 | 10 | 0 |

Hpf:; high power field; prot: protein; p: p-value; C: Control; Bf: EoE patients before SFED; Af: EoE patients after SFED

# 9 ULTRASTRUCTURAL ANALYSIS

Aiming at characterizing the cells associated with humoral activity, biopsies were observed to define morphological changes and possible modulation by SFED.

## 9.1 EPITHELIUM

Greater intracellular spaces were observed in active EoE patients when compared to the control group (**Figure 17**). SFED treatment was able to reduce these alterations in those responder patients, as observed when comparing SFED responders and non-responders (**Figure 17**).

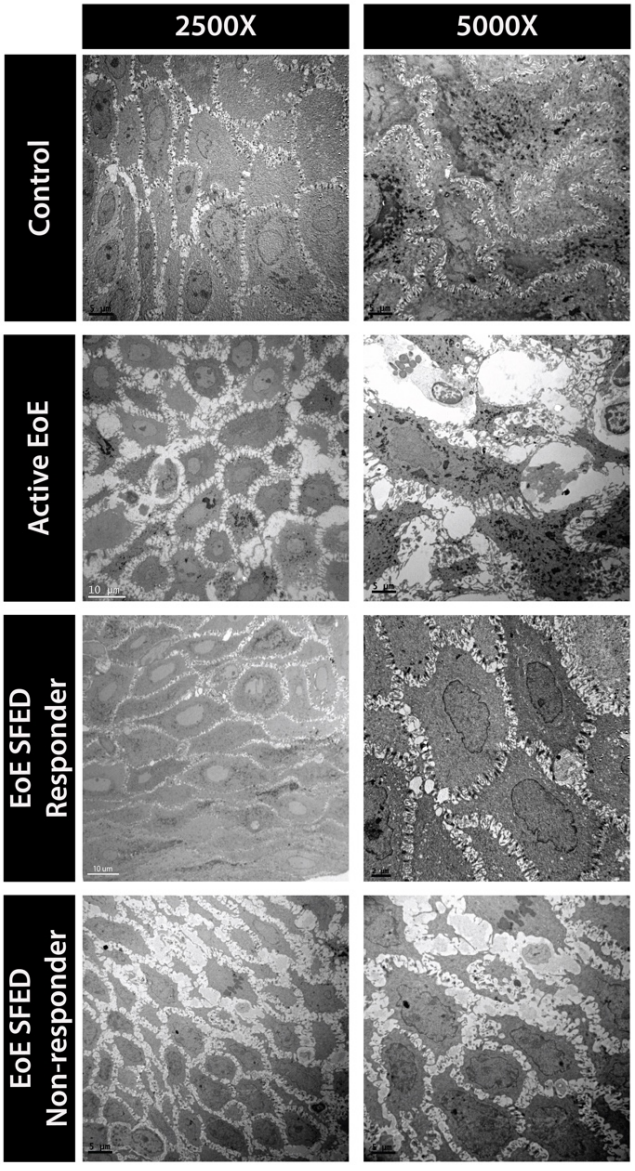


**Figure 17 | Epithelial esophageal morphology in the experimental groups. Magnification 2,500 - 5,000x**

## 9.2 EOSINOPHILS

Eosinophils could only be identified in EoE patients and not in control group (**Figure 18**). A large infiltration of eosinophils with clear signs of degranulation of cytoplasmic granules was observed in active EoE patients. Few eosinophils were found in EoE responders which acquired an apoptotic profile as long as no cellular membrane was observed (**Figure 18**).

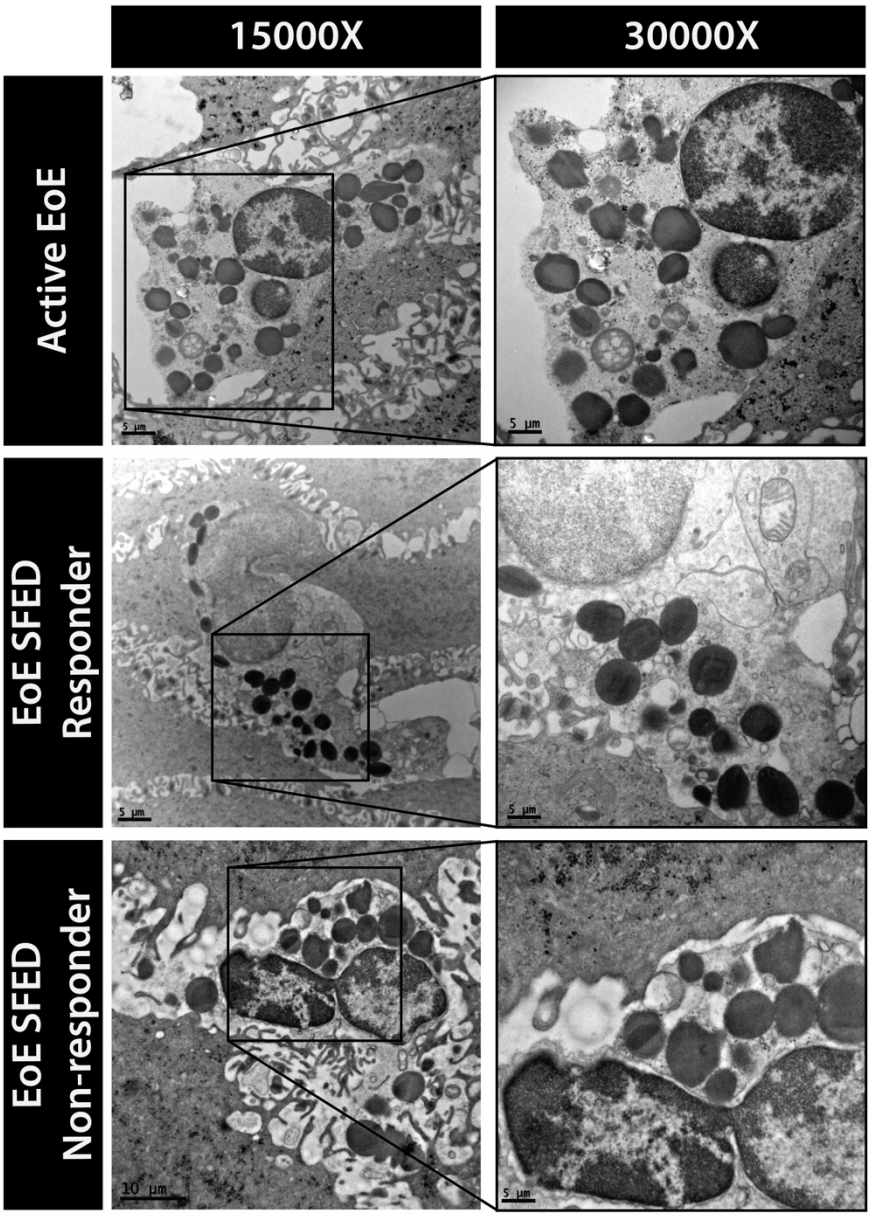


**Figure 18 | Profile of activation of eosinophils in EoE patients before and after SFED. Magnification 15,000-30,000x**

## 9.3 MAST CELL

Although mast cells were identified in all analyzed groups by IF, it identification by TEM was possible only in control and active EoE groups. Mast cells in active EoE presented a more active and degranulated profile than in the control group (**Figure 19**).

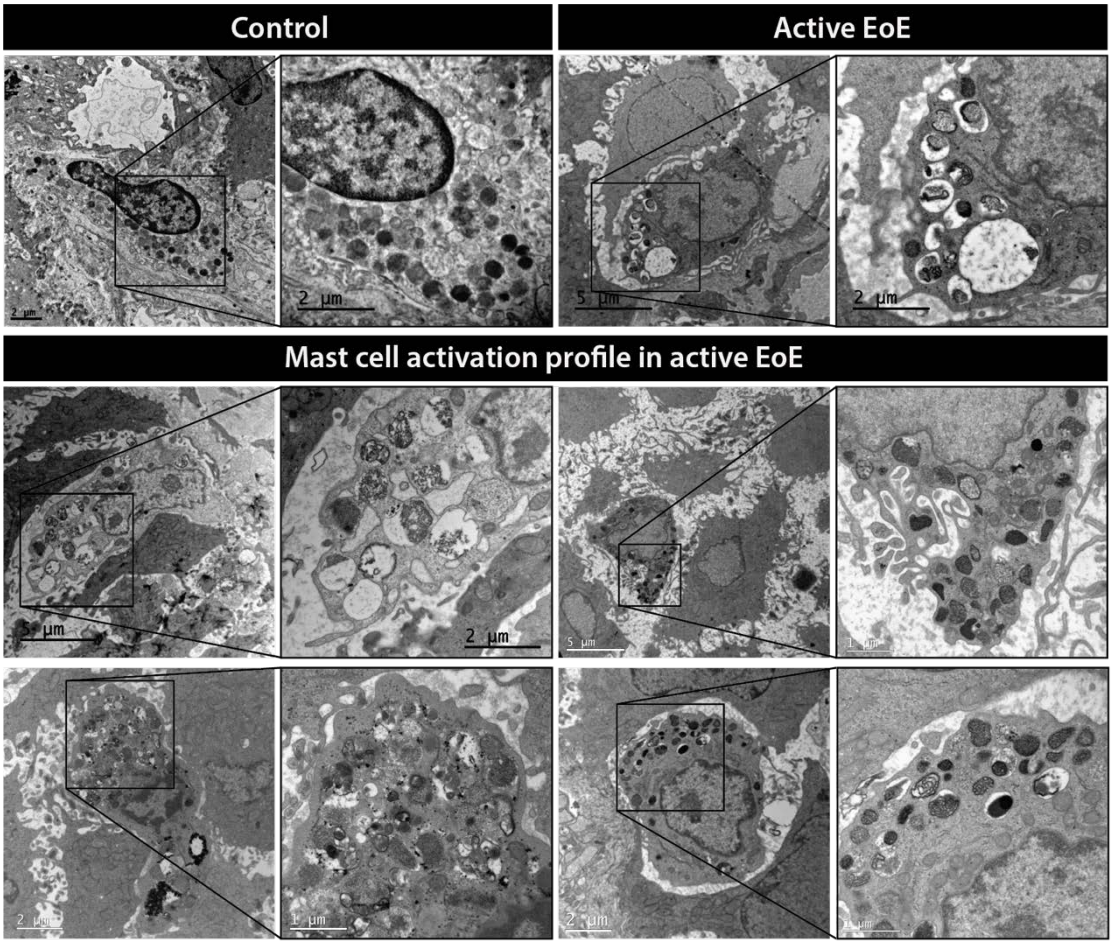


**Figure 19 | Mast cell ultraestructure in control group and active EoE patients. Magnification 10,000-25,000x**

## 9.4 PLASMA CELL

Plasma cells were only observed in active EoE patients, in parallel with IF results. Moreover in in active EoE, proximity between plasma and mast cells was identified (**Figure 20**.)

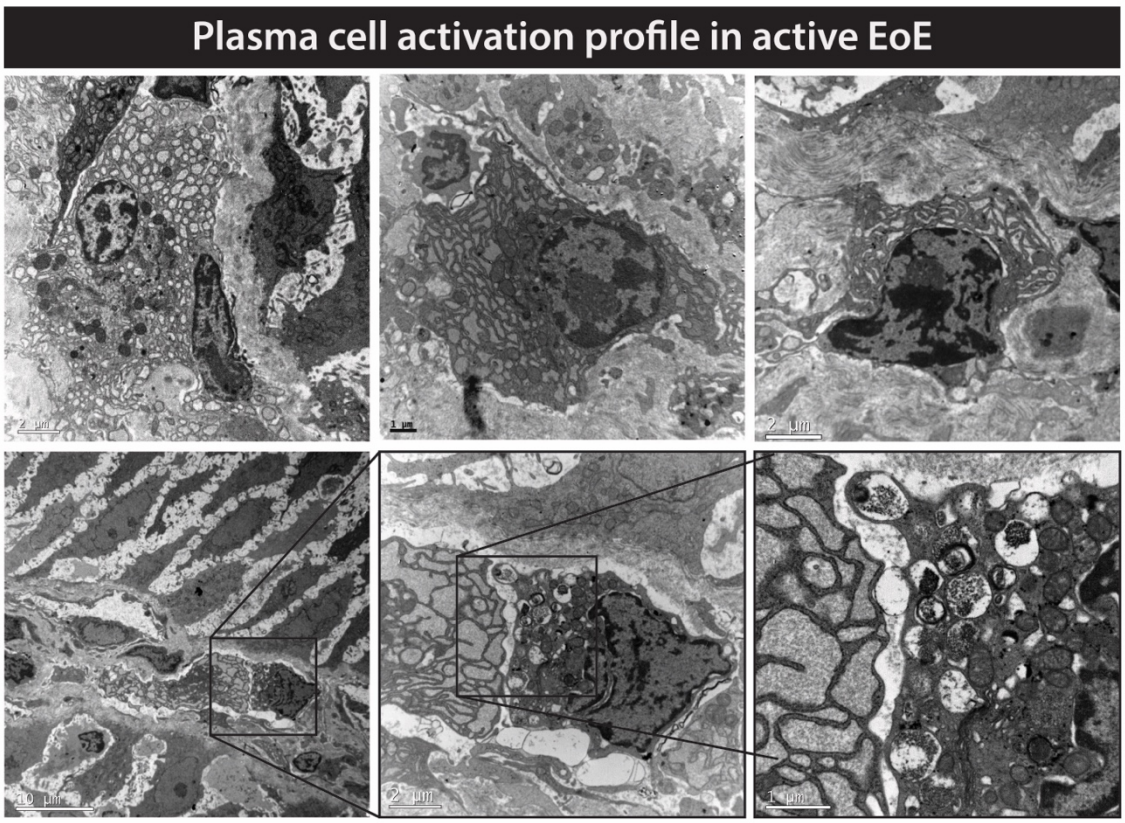


**Figure 20 | Plasma cell activation profile in active EoE patients (top images). Plasma cell and mast cell proximity (bottom images). Magnification 2,500-30,000x**

# DISCUSSION

This study provides clear evidences of the esophageal production of IgE and IgG against food components and its modulation by dietary exclusion, in parallel with histological and clinical improvement. The phenotype of IgE and IgG positive cells revealed that mast cells are the predominant IgE positive cells, which are absent in control subjects. Moreover, the identification of plasma cells positive for IgG or IgE, demonstrates the presence of active antibody-producing cells in the esophageal *lamina propria*, a population that decreases after dietary exclusion. All these findings demonstrate that humoral activity is an active pathophysiological mechanism in EoE.

The methodological limitations of the protocol assayed in this study avoided us from fully validating the experimental conditions. The difficulty in obtaining multiple biopsies from the same patient was the first limitation for setting up the method. Not only had the number of biopsies influenced the outcome, but the size of each biopsy was also key. As plasma cells locate in the *lamina propria*, superficial biopsies may not yield enough amount of immunoglobulin to be detected. This may explain the lack of linearity between the number of biopsies or biopsy concentration and protein concentration and the amount of specific immunoglobulin detected, as shown in the experimental protocol set up section. Furthermore, as EoE displays patchy inflammatory infiltrate in the tissue, a small number of biopsies may not be sufficient to collect samples that fully represents the immune activity, as happens for eosinophil counts needed for diagnosis.[25] A more detailed study to finally validate the proposed protocol is needed to be able to develop new diagnosis techniques useful for designing dietary therapies.

Anti-milk and gluten specific immunoglobulins were selected in this study because those are the most potentially allergenic foods triggering EoE in our country, as was represented in our cohort. An increased concentration of specific IgE and IgG4 has been observed in esophageal samples of active EoE patients as compared with the control group. Despite control group was quite homogeneous, specific immunoglobulin values from some EoE patients overlapped. Further, EoE patients

who tolerated the analyzed food still had elevated values of the specific immunoglobulins when compared to controls for both immunoglobulins but for IgE anti-milk who present control value.

Similar studies analyzed immunoglobulin concentration in esophageal biopsies [17,24], but only observed IgG4 and not IgE differences between EoE and controls. Aiming to understand the differences with our results we hypothesize that (1) EoE patients may not be in the same evolution point, especially if we are considering the existence of an IgE to IgG4 transition but also (2) extraction methodology and set up is decisive. Deep and patchy biopsies as well as rigorous and conscientiously set up of immunoglobulin extraction may have been pivotal, especially for detecting differences in IgE. Also, ImmunoCAP equipment, specialized in immunoglobulin detection, was crucial since allowed us to modify the instrument settings for different dilutions. Nonetheless, the establishment of a cut-off value for immunoglobulin positivity with this technique as well as the better way to express the obtained results was challenging. Considering that the Phadia standard curve is not lineal and the values obtained belong to the lowest part of the curve and protein determination is 3 logarithms higher than immunoglobulin we decided to express results as raw data. Besides, of importance to support our decision is that EoE is a patchy illness.

Both, specific IgE and IgG are potentially reliable biomarkers for food identification when analyzing sensitivity and specificity. Specific gluten IgE showed a good ROC (over 0.8), good specificity and sensibility and an excellent likelihood ratio (14). On the other hand, anti-milk/casein-IgE did not present a ROC curve as good as gluten. Both antigens presented low sensibility and low LR, but high specificity. On the contrary, IgG4 presented a better ROC curve for casein – IgG4, also with good specificity and sensibility (>0.80). Yet, anti-gluten IgG4 presented a ROC under 0.8 and a poorer sensitivity.

Besides, analyzing both immunoglobulins all together did not increase technique sensibility and specificity. Specifically, in gluten a sensitivity of 0.8 and specificity of 0.78 was achieved, and a sensitivity of 1 for milk. However, it loses specificity when

comparing with anti-milk IgE alone. Consider other cut-off values may be of interest in other to achieve a better specificity and avoid eliminating from diet not-allergenic food.

We acknowledge that the small size of participants (n=22), especially when analyzing subgroups (EoE milk-non-responder group n=2) is a weakness for this study. Still, results are promising, so it is the AUC considering the small sample size. Further studies are needed in order to better define this novel diagnostic tool.

Albeit an increase in intraepithelial $IgE^+$ mast cells in pediatric and adult EoE patients was already described [20], we provided additional data regarding both, IgE and IgG immunoglobulins also expressed in plasma cells. In concordance with published data we observed an increase in $IgE^+$ mast cells in EoE patients, and their decrease after SFED. However, is important to emphasize that while the density of mast cells was similar in control group and EoE after SFED, very few $IgE^+$ mast cell were observed in controls while almost all mast cells in EoE patients after SFED were $IgE^+$. This could be a sign of active mast cells in surveillance, or it could be that only 6-8 weeks may not be sufficient to eliminate all inflammatory activity markers. An additional immunoglobulin quantification after one year of diet maintenance would be needed to better analyze the behavior of the immunoglobulin production after SFED.

It is important to highlight that differences in specific IgE and IgG4 were only observed in tissue samples and not in blood further supporting the need of developing methodological approaches to identify local immune activation, not detected in systemic circulation.

Although no clinical – biological correlation was observed between immunoglobulin detection and histopathological variables in this study, we still contemplate an indirect association. In fact, no $IgG^+$ mast cells were observed in EoE patients which suggest a chronicity or different roles for immunoglobulin subtypes, where IgG4 would be expressed as a protection from immediate hypersensitivity reaction[26]. We suggest humoral response is triggering the inflammation process from the very beginning, acting against food allergens which are able to cross a damaged

esophageal epithelium[27,28]. The lack of correlation may be explained considering immunoglobulins would not be responsible for the eosinophilic infiltration or the tissue damage. In future studies, clinical – biological correlations could be performed considering atopy or seasonal variations, which was not taken into account in the present study.

Few studies have addressed, however IgE, the main mediator of hypersensitivity type-1 allergic responses. It is true that SPT have failed in identifying antigens for elimination diets but is important to highlight that only happened in adults.[14,29]. In pediatric population, the antigen identification has been quite successful, probably because the analysis is performed in earlier stages of the disease[30]. Moreover, our data suggest that a local response rather than systemic response is associated with esophageal inflammation in EoE, and none of the specific tested immunoglobulins (either IgE or IgG) has been identified in blood. SPT technique is a test driven for systemic responses which will not be able to detect a local allergic response. Aditionally, clinical trials with omalizumab have not been effective in inducing a complete histological remission. Nevertheless, they have shown significant reduction of tissue eosinophils, mast cells and tissue IgE, supporting the hypothesis that IgE has a role in the development although it might not be the main mediator. Another important factor is oral immunotherapy, which has been reported to increase the risk of developing EoE in a 2.7% of the patients[31].

The implication of IgG4 in explaining EoE pathophysiology has gained more attention as its identification in the esophageal mucosa and its increase compared to control subjects was recently reported[17,19]. Although it is not yet clear if physiologic IgG4 is linked to the development of tolerance to allergens or whether it might be linked to other epiphenomenons, several roles may explain IgG as IgE antagonizer[32]: (1) IgG4 is able to recombine and create bispecific antibodies which lack de ability to cross-link allergens and do not form immune complexes (2) IgG can also downregulate the activation of mast cells and basophils by cross-linking FcεRI and FCγR3 with allergen – complexed specific IgE and IgG. (3) IgG4 is also able to disrupt C1q binding site, impeding complement activation and (4) it is able to hinder mast cell and basophil

degranulation in competition with specific IgE for allergen binding as "blocking antibody". Nonetheless, if tolerance to allergens is taking place in EoE patients, not only tissue IgG should be detected and observed but also mast cells should be linked to IgG. Conversely, in this study we only observed $IgG^+$ plasma cells. On the other hand, IgG4 has high affinity for the activating receptor FcγRI, and it is expressed in monocytes, macrophages, dendritic cells and granulocytes. So, both immunoglobulins should be farther studied future research in order to determine their contribution to EoE physiology.

In conclusion, our study confirms that EoE is a local and not a systemic disease, emphasizing the need of developing new diagnostic tools as well as more efficacious therapeutic interventions. EoE pathophysiology is not fully elucidated and different stages may exist as well as the implication of different cell types. Within immune activation, humoral mechanisms seem to significantly contribute to EoE development, however further studies are needed to determine whether local Ig production is a primary mechanism or if humoral activity is the consequence of esophageal barrier dysfunction. Finally, this study suggests that specific IgE and IgG4 determination are potential novel biomarkers for predicting effective clinical and histological response to food elimination in EoE patients.

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# DISCUSIÓN GENERAL

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La esofagitis eosinofílica (EEo) es una enfermedad inflamatoria crónica que provoca un deterioro considerable en la calidad de vida de los pacientes, tanto niños como adultos, y cuya prevalencia sigue en aumento desde que se definió como entidad independiente. Ello es debido al desconocimiento sobre su fisiopatología, la dificultad en su diagnóstico y las diferentes terapias a las que se someten a los pacientes puesto que no existen biomarcadores predictivos de respuesta. Actualmente, y tras varios consensos entre expertos, su diagnóstico se basa en la presencia de síntomas de disfunción esofágica y en el recuento de 15 o más eosinófilos intraepiteliales por campo de gran aumento, así como la exclusión de otras enfermedades que cursan con infiltrado eosinofílico.

Aunque la EEo se identifica como una enfermedad alérgica, los mecanismos que desencadenan respuestas de hipersensibilidad no están establecidos. La respuesta inflamatoria existente en el tejido esofágico generada por eosinófilos, mastocitos y linfocitos Th2, se caracteriza por la producción de mediadores capaces de alterar el epitelio esofágico y promover el reclutamiento de células defensivas. En estos pacientes, se han descrito alteraciones en las uniones de las células epiteliales esofágicas y una menor expresión del gen de la Desmogleina 1[138], mermando así la principal barrera defensiva de este órgano, lo que facilitaría la entrada de agentes externos al epitelio esofágico. Sin embargo, se desconoce si existe una alteración epitelial previa que facilite el desarrollo de respuestas inflamatorias, dada la capacidad del epitelio esofágico en reclutar leucocitos al tejido.

La presente tesis se desarrolla con el objetivo de determinar la implicación de los componentes inmunitarios citotóxicos en la fisiopatología de la EEo y su interacción con el epitelio esofágico, así como la evaluación de la producción de inmunoglobulinas frente a alérgenos en el esófago. Ambos tipos de mecanismos inmunitarios coexisten en esta enfermedad, aunque se desconoce si una prevalece sobre otra y cómo el tratamiento con inhibidor de la bomba de protones o con dieta de exclusión regula ambas respuestas.

Para ampliar el conocimiento sobre el potencial inmunitario del esófago, puesto que no existen valores de referencia completos en humanos, se ha desarrollado un primer estudio para cuantificar las principales poblaciones inmunitarias considerando todas las capas de tejido dentro de las regiones superior, media e inferior de este órgano. Se ha identificado un mayor número de linfocitos T en la región esofágica superior y se han definido valores que pueden puede ser útiles para futuros estudios destinados a identificar la función de células inmunitarias en homeostasis y en las respuestas defensivas en el esófago.

Las enfermedades virales, bacterianas o producidas por patógenos se caracterizan por una respuesta citotóxica mediada por linfocitos T CD8[28], mientras que las respuestas de hipersensibilidad como el asma o la dermatitis atópica se caracterizan por un aumento de IgE específica contra el alérgeno causante y un aumento y activación de linfocitos CD4[139]. Aunque ambas respuestas son generadas por estirpes celulares diferentes, varios estudios han demostrado un papel crucial de las células CD8 en la inflamación alérgica, así como en la regulación de la IgE en las vías respiratorias de modelos murinos [140,141]. Estas células CD8 se han asociado con una evolución clínica más favorable en pacientes con dermatitis atópica[142] así como en modelos experimentales de inflamación alérgica en las vías respiratorias[141]. Aunque se desconoce con exactitud su función, e incluso podría llegar a ser dual[140,143,144], se ha demostrado que las respuestas mediadas por IgE, con un perfil de citoquinas tipo Th2, pueden ser moduladas, según el estado de tolerancia al alérgeno, por varios tipos celulares[145]. La hipótesis más aceptada para la implicación directa de los linfocitos CD8 en la modulación de la respuesta alérgica es la presentación cruzada de antígenos, donde antígenos externos quedarían expuestos a las células $CD8^+$ mediante MHC I. Estos antecedentes, junto al aumento de linfocitos CD8 intraepiteliales en la EEo[103,146], respaldan la hipótesis de su implicación en la fisiopatología de esta enfermedad alérgica. Los resultados observados en esta tesis indican una contribución significativa en la enfermedad activa, que remite tras la dieta de exclusión. Esta función se relaciona con la capacidad citotóxica de la población, en base al aumento en la expresión de ciertos mediadores de la población

CD8. Sin embargo, podría ejercer un papel regulador, que es necesario estudiar en futuros estudios.

De hecho, Allakhverdi *et al.,*[143] observaron en modelos murinos la depleción de CD8 producía a una respuesta más exacerbada en respuestas alérgicas tardías así como un mayor infiltrado de eosinófilos en el tejido. Además, observaron que los CD8 tienen la capacidad de regular la producción de eotaxina en un modelo de asma alérgico. En esta tesis, se ha identificado una correlación positiva entre la expresión de eotaxinas o el número de eosinófilos (herramienta diagnóstica en la EEo) y el número de linfocitos CD8 infiltrados en el tejido. Además, la falta de modulación de linfocitos tras tratamiento con IBP en este estudio sugiere que la exposición continua al antígeno (dado que no excluyen alimentos, a priori) sería la causante de mantener el infiltrado de linfocitos a pesar de la remisión de eosinófilos, lo que apuntaría a que contribuyen de manera activa al reclutamiento inicial de eosinófilos. Esto sugiere que, en esta enfermedad, también de origen alérgico, las células CD8 podrían estar llevando a cabo el mismo papel regulatorio que en respuestas alérgicas tardías, asma o inflamación de vías respiratorias.

Considerando el perfil de expresión observado en este y otros estudios, la presencia de IL-5, IL-13, IL-10 e IL-15 en la EEo predominaría la regulación de la respuesta humoral frente a la respuesta citotóxica, caracterizada por IFN-γ y TNF-α, dos citoquinas de baja relevancia para esta enfermedad. Sin embargo, una vez la respuesta alérgica se ha establecido, las células CD8 pueden mantener o desarrollar un papel pro-inflamatorio dado su potencial en producir citoquinas con perfil Th2. El mecanismo que definirá el tipo de respuesta de estas células CD8 todavía no ha sido dilucidado. Además, en dichas respuestas tardías, se ha observado un elevado infiltrado de las células CD8 memoria específicas de alérgeno, inducidas por fenómenos de presentación cruzada que se dan en la respuesta alérgica[141], mecanismos que no han sido estudiados aún en la EEo.

El papel en etapas tardías de las células CD8 ha sido descrito principalmente en modelos murinos, donde bien pueden tener una función protectora, incrementando

la producción de IL-12 que regularía las células dendríticas[147] o bien incrementarían la severidad de la respuesta alérgica mediada por IgE mediante la producción de IL-13[148,149]. En esta tesis, ambas interleuquinas se han visto aumentadas, por lo que todavía queda por esclarecer si son las células CD4 las productoras de IL-13 y las células CD8 desarrollan una función anti-inflamatoria mediante la producción de IL-12, o bien si ambos tipos de linfocitos están exacerbando la respuesta alérgica mediante la producción de IL-13.

En respuestas alérgicas, el papel protector de la IgG4, disminuyendo la IgE y reduciendo el exceso de respuesta pro-inflamatoria, ha sido ampliamente descrito. La IgG4 también tiene la habilidad de bloquear lugares de unión de IgE en células cebadas como mastocitos, así como de unirse a receptores FCγ inhibitorios. Así pues, la IgG4 es la immunoglobulina responsable de adquirir tolerancia alérgica. Rosenberg et al., observaron la inexistencia de correlación entre IgG4 y marcadores mastocitarios como carboxipeptidasa A3 (CPA3) sugiriendo que los mastocitos no están llevando a cabo de manera primaria la respuesta pro-inflamatoria[150]. Tras los resultados de este estudio, confirmando mediante inmunofluorescencia que los mastocitos no están unidos a IgG pero sí a IgE sería necesario reconsiderar la hipótesis actual puesto que la IgG no parece estar bloqueando el reconocimiento de IgE en células cebadas y ambos anticuerpos se encuentran elevados al analizar inmunoglobulinas específicas contra antígenos alimentarios. Por lo tanto, la producción local de IgE se daría en etapas tempranas, aunque, a pesar de evolucionar hacia una respuesta de tipo IgG4, no cesaría la producción de ésta dado que se identifica en pacientes adultos con elevado tiempo de evolución.

A pesar de que se ha visto un ligero aumento de IgE específica en suero, ello no es suficiente como herramienta predictiva para definir la pauta dietética de la misma manera que el tratamiento con anti-IgE no es efectivo en pacientes con EEo[105,115]. De forma similar, la identificación de IgG4 específica en sangre tampoco se ha consolidado como una herramienta predictiva para la exclusión dietética[151]. Asimismo, la falta de eficacia de las dietas de exclusión basada en *pricks* cutáneos de

alergia no excluye la participación de la IgE en la EEo, sino que reforzaría el hecho de que la respuesta alérgica se produce únicamente en el esófago.

Estudios previos han observado un aumento de IgG4 específica, a pesar de no determinar los alimentos a los que los pacientes responden, mientras otros, a pesar de determinar los alimentos, no consiguieron identificar claramente IgG4 específica[105,152]. Es importante destacar que, hasta el momento, no se han realizado estudios de IgE específica en biopsias esofágicas. En este estudio, sin embargo, centrado en los alérgenos más frecuentes (leche y gluten), se ha identificado la producción de IgE específica frente a los alimentos en aquellos pacientes que respondieron clinica e histológicamente al tratamiento con dieta de eliminación. Además, se ha observado dicho aumento tanto en IgG4 como IgE[153], sugiriendo la implicación de ambas en esta respuesta alérgica local y no sistémica.

Los resultados de esta tesis sugieren que la EEo no es una enfermedad Th2 clásica. El reclutamiento y la activación de linfocitos CD8 son característicos en la EEo y la presencia de IgE e IgG4 especifica en dichos pacientes confirma la actividad humoral local. La presencia de estas immunoglubulinas específicas podría validarse como biomarcador definitivo para diseñar terapias personalizadas en función del tipo de immunoglobulina presente en el esófago. Asimismo, destaca la necesidad de desarrollar técnicas diagnósticas dirigidas a identificar biomarcadores locales, en el tejido esofágico, que indiquen el estado inflamatorio en el esófago y contribuyan a un rápido diagnóstico. El desarrollo exitoso de esa metodología contribuirá significativamente a reducir el gasto sanitario y a mejorar la calidad de vida de los pacientes.

# CONCLUSIONES

# CONCLUSIONES

Los resultados obtenidos en esta tesis doctoral han dado lugar a las siguientes conclusiones:

1. El esófago humano contiene poblaciones inmunitarias de linfocitos T colaboradores, linfocitos T citotóxicos, linfocitos B, macrófagos y mastocitos dispersos a lo largo de todo el esófago y en todas sus capas anatómicas.

2. En homeostasis, el epitelio en el tercio superior esofágico contiene mayor número de linfocitos T que el tercio inferior.

3. En la EEo activa, el aumento en el número de linfocitos T intraepiteliales citotóxicos disminuye tras el tratamiento con dieta de exclusión y no con inhibidor de la bomba de protones.

4. En la EEo activa, la expresión génica de moléculas asociadas a la actividad de linfocitos T citotóxicos, NK y NKT correlaciona positivamente con el número de eosinófilos intraepiteliales y con la expresión de eotaxina 3.

5. La estimulación ex vivo del epitelio esofágico con IL-13 reproduce la activación observada en la EEo, mientras que la línea epitelial esofágica Het-1A requiere de mayor diferenciación para la generación de un modelo in-vitro de EEo reproducible.

6. El tejido esofágico en pacientes con EEo produce de forma significativa IgE e IgG4 específicas frente a alérgenos alimentarios, las cuales se reducen en los pacientes que responden a la dieta de exclusión. Estos hallazgos respaldan el potencial de estas Ig específicas como biomarcadores para el diseño de dietas de eliminación dirigidas en estos pacientes.

7. La detección de IgE e IgG4 específias en el esófago no en sangre destaca la necesidad de desarrollar técnicas diagnósticas focalizadas en el tejido y no sistémicas.

8. El análisis de la ultraestructura esofágica en la EEo revela un aumento del espacio paracelular epitelial, eosinófilos y mastocitos con un perfil de degranulación significativo en el epitelio y células productoras de anticuerpos en la lamina propria. Estas alteraciones revierten parcialmente en pacientes que responden al tratamiento con dieta de eliminación.

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# ANEXOS

# ANEXO 1

## MATERIAL SUPLEMENTARIO CAPÍTULO 2

**Table S 1 | Gene expression assay probes used for qPCR analysis**. PreA. indicates pre-amplification of cDNA. A. *In-vivo* study. B. *In-vitro* model. C. *Ex-vivo* model.

| Gene assay | Gene name | TaqMan assay | PreA. | A | B | C |
|---|---|---|---|---|---|---|
| 18S | Eukaryotic 18S rRNA | Hs99999901_s1 | No | x | x | x |
| ACTB | Actin Beta | Hs99999903_m1 | No | x | | |
| BCL2 | BCL2 Apoptosis Regulator | Hs00608023_m1 | No | | x | x |
| CAPN14 | Calpain 14 | Hs00871882_m1 | Yes | | x | x |
| CASP8 | Caspase 8 | Hs01018151_m1 | No | | x | x |
| CCL11 | C-C Motif Chemokine Ligand 11 | Hs00237013_m1 | Yes | x | x | x |
| CCL18 | C-C Motif Chemokine Ligand 18 | Hs00268113_m1 | No | x | | |
| CCL24 | C-C Motif Chemokine Ligand 24 | Hs00171082_m1 | Yes | x | x | x |
| CCL26 | C-C Motif Chemokine Ligand 26 | Hs00171146_m1 | No | x | x | x |
| CCR8 | C-C Motif Chemokine Receptor 8 | Hs00174764_m1 | No | x | | |
| CD4 | CD4 Molecule | Hs01058407_m1 | No | x | | |
| CD8 | CD8a Molecule | Hs00233520_m1 | No | x | | |
| CDH26 | Cadherin 26 | Hs00902321_m1 | No | | x | x |
| CLDN1 | Claudin 1 | Hs00221623_m1 | No | | | x |
| CTSB | Cathepsin B | Hs00947433_m1 | No | x | | |
| CTSC | Cathepsin C | Hs00175188_m1 | No | x | | |
| CTSG | Cathepsin G | Hs00175195_m1 | No | x | | |
| CTSK | Cathepsin K | Hs00166156_m1 | No | x | | |
| CTSL1 | Cathepsin L 1 | Hs00377632_m1 | No | x | | |
| CTSL2 | Cathepsin L 2 | Hs00952036_m1 | No | x | | |
| CTSS | Cathepsin S | Hs00175407_m1 | No | x | | |
| CTSW | Cathepsin W | Hs00175160_m1 | No | x | | |
| CXCL8 | C-X-C Motif Chemokine Ligand 8 | Hs00174103_m1 | No | | x | x |
| DSG-1 | Desmoglein 1 | Hs00355084_m1 | Yes | | | x |
| FAS | Fas Cell Surface Death Receptor | Hs00236330_m1 | No | | x | x |
| FLG | Filaggrin | Hs00856927_g1 | Yes | | x | x |
| GAPDH | Glyceraldehyde-3-Phosphate Dehydrogenase | Hs02758991_g1 | No | x | | |
| | | Hs99999905_m1 | No | | x | x |
| GNLY | Granulysin | Hs00246266_m1 | No | x | | |
| GRN | Granulin Precursor | Hs00963707_g1 | No | x | | |
| GUSB | Glucuronidase Beta | Hs99999908_m1 | No | x | | |
| GZM B | Granzyme B | Hs01554355_m1 | No | x | | |
| GZMA | Granzyme A | Hs00989184_m1 | No | x | | |
| ICAM 1 | Intercellular Adhesion Molecule 1 | Hs00164932_m1 | No | x | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| ICAM 2 | Intercellular Adhesion Molecule 2 | Hs00609563_m1 | No | x | | |
| IFNG | Interferon Gamma | Hs99999041_m1 | No | x | | |
| IL-10 | Interleukin 10 | Hs99999035_m1 | No | x | | |
| IL-12B | Interleukin 12B | Hs99999037_m1 | No | x | | |
| IL-15 | Interleukin 15 | Hs99999039_m1 | No | x | | |
| IL-33 | Interleukin 33 | Hs04931857_m1 | Yes | | x | x |
| ITGA4 | Integrin Subunit Alpha 4 | Hs00168433_m1 | No | x | | |
| MADCAM | Mucosal Vascular Addressin Cell Adhesion Molecule 1 | Hs00369968_m1 | No | x | | |
| MICA | MHC Class I Polypeptide-Related Sequence A | Hs00792195_m1 | No | x | | |
| MICB | MHC Class I Polypeptide-Related Sequence B | Hs00792952_m1 | No | x | x | x |
| OCLN | Occludin | Hs01049883_m1 | No | | | x |
| PGK1 | Phosphoglycerate Kinase 1 | Hs99999906_m1 | No | x | | |
| POSTN | Periostin | Hs01566750_m1 | No | | x | x |
| PPIA | Cyclophilin A | Hs99999904_m1 | No | | | x |
| PRF1 | Perforin 1 | Hs00169473_m1 | No | x | | |
| SPINK5 | Serine Peptidase Inhibitor. Kazal Type 5 | Hs00928570_m1 | Yes | | x | x |
| SPINK7 | Serine Peptidase Inhibitor. Kazal Type 7 | Hs00261445_m1 | Yes | | x | x |
| SYNPO | Synaptopodin | Hs00200768_m1 | No | | x | x |
| TGFB1 | Transforming Growth Factor Beta 1 | Hs99999918_m1 | No | x | | |
| TJP1 | Tight Junction Protein 1 | Hs01551861_m1 | No | | x | x |
| TJP3 | Tight Junction Protein 3 | Hs00274276_m1 | No | | | x |
| TSLP | Thymic Stromal Lymphopoietin | Hs00263639_m1 | No | | x | x |

**Table S 2 | Esophageal gene expression.** Control and Before SFED: ϕ between Control and After SFED: ¥. between Before and After SFED: #. 1 symbol p<0.05; 2 symbols p<0.01; 3 symbols p<0.001; 4 symbols p<0.0001. Data are expressed as mean + SD or median (range)

| | Control | Before SFED | After SFED | p-value |
|---|---|---|---|---|
| CD8 | 1.49 (0.04 – 4.43) | 3.31 (1.73 - 13.1) | 3.84 (1.65 – 10.0) | ϕϕ; ¥¥ |
| CD4 | 1.34 ± 0.94 | 2.86 ± 1.51 | 2.35 ± 1.17 | ϕ |
| MADCAM1 | 1.30 (0.08 – 3.47) | 33.0 (1.95 – 93.8) | 4.36 (0.26 – 29.0) | ϕϕ |
| ITGA4 | 1.43 ± 1.09 | 4.89 ± 1.94 | 4.40 ± 3.55 | ϕϕ; ¥ |
| ICAM1 | 1.14 (0.19 - 3.00) | 3.07 (1.27 - 13.6) | 2.15 (0.98 - 7.08) | ϕ |
| ICAM2 | 1.28 ± 0.78 | 3.86 ± 3.03 | 2.22 ± 0.86 | ϕ; ¥ |
| MICA | 1.08 (0.42 - 2.34) | 1.42 (0.51 – 7.48) | 1.88 (0.25 - 3.94) | ¥ |
| MICB | 0.90 (0.31 - 2.59) | 3.34 (0.76 - 10.5) | 2.05 (0.28 – 15.4) | ϕϕ |
| GZMA | 1.41 ± 0.99 | 4.14 ± 3.01 | 2.63 ± 2.67 | ϕ |
| GZMB | 1.78 (0.01 - 21.7) | 7.54 (0.03 - 31.8) | 3.38 (0.03 - 22.2) | - |
| PRF1 | 1.42 (0.09 – 3.94) | 0.97 (0.11 – 2.40) | 0.89 (0.27 – 6.84) | - |
| GNLY | 0.92 (0.26 – 3.75) | 4.11 (1.92 – 9.11) | 2.60 (1.36 – 7.55) | ϕϕ; ¥ |
| GRN | 1.32 ± 0.75 | 1.65 ± 1.14 | 2.38 ± 0.67 | ¥ |
| IFN-γ | 0.86 (0.29 – 3.15) | 0.82 (0.10 – 2.89) | 0.70 (0.12 – 2.78) | - |
| TGF-β | 1.10 ± 0.43 | 1.94 ± 0.96 | 4.08 ± 2.98 | ϕ; ¥¥ |
| IL-10 | 0.90 (0.42 - 3.34) | 5.25 (0.25 - 33.7) | 3.0 (0.94 - 6.38) | ϕϕϕ; ¥ |
| IL-12β | 1.20 ± 0.71 | 3.15 ± 1.78 | 2.17 ± 1.02 | ϕ |
| IL-15 | 1.33 ± 0.90 | 2.90 ± 1.35 | 1.77 ± 1.29 | ϕ |
| CCR8 | 1.01 (0.20 – 3.39) | 5.31 (1.8 – 20.5) | 1.82 (0.56 – 9.28) | ϕϕ |
| CCL18 | 1.25 (0.08 - 10.7) | 18.10 (1.82 - 39.6) | 2.32 (0.20 - 4.40) | ϕϕ; # |
| CTSB | 1.25 ± 0.68 | 2.08 ± 0.97 | 2.89 ± 0.63 | ¥¥¥ |
| CTSC | 1.16 ± 0.60 | 26.6 ± 13.3 | 11.8 ± 10.7 | ϕϕϕ; ¥ |
| CTSG | 2.89 ± 3.14 | 93.5 ± 69.6 | 42.0 ± 53.6 | ϕϕ |
| CTSK | 0.82 (0.23 – 7.68) | 4.54 (1.58 – 13.6) | 5.30 (2.4 – 14.3) | ϕ; ¥¥ |
| CTSL1 | 1.56 (0.02 - 3.21) | 7.41 (2.89 - 14.2) | 3.14 (1.28 - 13.5) | ϕϕϕ; ¥ |
| CTSL2 | 1.3 (0.14 – 3.61) | 2.43 (0.51 – 8.15) | 3.87 (0.28 – 5.14) | ¥ |
| CTSS | 1.11 ± 0.55 | 4.09 ± 1.62 | 2.99 ± 1.05 | ϕϕϕ; ¥¥ |
| CTSW | 1.09 (0.14 – 3.21) | 2.43 (0.13 – 7.11) | 2.60 (0.21 – 6.38) | - |
| CCL11 | 1.36 (0.20 – 3.27) | 7.36 (2.71 – 68.2) | 3.85 (0.90 – 64.7) | ϕϕϕ; #; ¥ |
| CCL24 | 2.71 ± .2.94 | 105.8 ± 73.7 | 60.83 ± 30.2 | ϕϕ; ¥¥¥ |
| CCL26 | 1.63 (0.23 – 5.96) | 254.0 (8.14 – 1269) | 11.02 (2.16 – 213.2) | ϕϕϕ; #; ¥¥ |

**Table S 3 | Duodenal gene expression.** No statistical differences were identified between study groups.

| | Control | Before SFED | After SFED | p-value |
|---|---|---|---|---|
| CD8 | 1.05 ± 0.32 | 0.75 ± 0.23 | 1.04 ± 0.66 | p > 0.05 |
| CD4 | 1.11 ± 0.50 | 0.86 ± 0.37 | 0.86 ± 0.39 | p > 0.05 |
| MADCAM1 | 1.12 ± 0.52 | 1.93 ± 1.55 | 1.72 ± 1.45 | p > 0.05 |
| ITGA4 | 1.09 ± 0.42 | 1.20 ± 0.30 | 1.01 ± 0.22 | p > 0.05 |
| ICAM1 | 1.01 (0.31 - 2.79) | 0.85 (0.34 - 1.48) | 0.91 (0.08 - 3.60 | p > 0.05 |
| ICAM2 | 1.07 ± 0.44 | 0.80 ± 0.23 | 0.73 ± 0.14 | p > 0.05 |
| MICA | 1.06 ± 0.37 | 1.08 ± 0.74 | 1.03 ± 0.43 | p > 0.05 |
| MICB | 1.15 ± 0.54 | 1.35 ± 0.73 | 1.59 ± 0.45 | p > 0.05 |
| GZMA | 1.12 ± 0.62 | 0.86 ± 0.23 | 0.72 ± 0.36 | p > 0.05 |
| GZMB | 1.23 ± 0.66 | 0.82 ± 0.37 | 0.71 ± 0.29 | p > 0.05 |
| PRF1 | 1.01 (0.64 - 1.74) | 1.00 (0.20 - 4.21) | 1.5 (0.83 - 2.12) | p > 0.05 |
| GNLY | 0.98 (0.55 - 2.4) | 1.59 (0.37 - 1.96) | 1.27 (0.40 - 2.09) | p > 0.05 |
| GRN | 1.11 ± 0.47 | 0.96 ± 0.37 | 0.97 ± 0.33 | p > 0.05 |
| IFN-γ | 1.2 ± 0.76 | 0.81 ± 0.43 | 0.69 ± 0.51 | p > 0.05 |
| TGF-β | 0.87 (0.72 - 1.93) | 1.21 (0.38 - 1.85) | 1.21 (0.89 - 1.59) | p > 0.05 |
| IL-10 | 0.69 ± 0.51 | 0.81 ± 0.43 | 0.69 ± 0.51 | p > 0.05 |
| IL-12β | 0.83 (0.67 - 2.53) | 1.3 (0.64 - 3.16) | 1.47 (0.64 - 2.68) | p > 0.05 |
| IL-15 | 1.07 ± 0.40 | 0.77 ± 0.17 | 0.52 ± 0.30 | p > 0.05 |
| CCR8 | 1.34 ± 0.84 | 2.37 ± 2.05 | 2.62 ± 1.80 | p > 0.05 |
| CCL18 | 1.09 ± 0.48 | 1.58 ± 0.80 | 1.33 ± 0.50 | p > 0.05 |
| CTSB | 1.04 ± 0.30 | 0.91 ± 0.29 | 1.18 ± 0.10 | p > 0.05 |
| CTSC | 1.03 ± 0.26 | 1.03 ± 0.33 | 1.14 ± 0.14 | p > 0.05 |
| CTSG | 1.22 ± 0.82 | 2.61 ± 1.51 | 1.78 ± 0.72 | p > 0.05 |
| CTSK | 1.00 (0.54 - 1.87) | 0.80 (0.43 - 1.34) | 0.81 (0.01 - 1.19) | p > 0.05 |
| CTSL1 | 1.04 ± 0.33 | 1.12 ± 0.34 | 1.18 ± 0.52 | p > 0.05 |
| CTSL2 | 1.21 ± 0.82 | 1.39 ± 0.84 | 1.49 ± 0.33 | p > 0.05 |
| CTSS | 1.00 (0.58 - 1.47) | 0.91 (0.83 - 1.31) | 1.19 (0.17 - 1.41) | p > 0.05 |
| CTSW | 1.42 ± 01.03 | 1.43 ± 1.05 | 1.07 ± 0.61 | p > 0.05 |
| CCL11 | 1.31 ± 1.04 | 1.43 ± 0.84 | 0.83 ± 0.32 | p > 0.05 |
| CCL24 | 1.09 ± 0.45 | 0.99 ± 0.26 | 0.78 ± 0.38 | p > 0.05 |
| CCL26 | 1.17 (0.35 - 2.15) | 1.81 (0.35 - 4.89) | 1.35 (0.77 - 3.12) | p > 0.05 |

# ANEXO 2

# PUBLICACIONES COMPLEMENTARIAS

## ARTÍCULOS ORIGINALES, REVIEWS Y PRESENTACIONES A CONGRESOS

### 1. ARTÍCULOS ORIGINALES

**Toll-like receptors-mediated pathways activate inflammatory responses in the esophageal mucosa of adult eosinophilic esophagitis**

Arias Á, Vicario M, Bernardo D, Olalla JM, **Fortea M**, Montalban-Arques A, Martínez-Fernández P, González-Castro AM, Mota-Huertas T, Arias-González L, Lucendo AJ.

Clin Transl Gastroenterol. 2018 Apr 25;9(4):147. doi: 10.1038/s41424-018-0017-4.

**Decreased TESK1-mediated cofilin 1 phosphorylation in the jejunum of IBS-D patients may explain increased female predisposition to epithelial dysfunction**

Rodiño-Janeiro BK, Martínez C, **Fortea M**, Lobo B, Pigrau M, Nieto A, González-Castro AM, Salvo-Romero E, Guagnozzi D, Pardo-Camacho C, Iribarren C, Azpiroz F, Alonso-Cotoner C, Santos J, Vicario M.

Sci Rep. 2018 Feb 2;8(1):2255. doi: 10.1038/s41598-018-20540-9.

**miR-16 and miR-103 impact 5-HT(4) receptor signalling and correlate with symptom profile in irritable bowel syndrome**

Wohlfarth C, Schmitteckert S, Härtle JD, Houghton LA, Dweep H, **Fortea M**, Assadi G, Braun A, Mederer T, Pöhner S, Becker PP, Fischer C, Granzow M, Mönnikes H, Mayer EA, Sayuk G, Boeckxstaens G, Wouters MM, Simrén M, Lindberg G, Ohlsson B, Schmidt PT, Dlugosz A, Agreus L, Andreasson A, D'Amato M, Burwinkel B, Bermejo JL, Röth R, Lasitschka F, Vicario M, Metzger M, Santos J, Rappold GA, Martinez C, Niesler B

Sci Rep. 2017 Oct 31;7(1):14680. doi: 10.1038/s41598-017-13982-0.

**Downregulation of mucosal mast cell activation and immune response in diarrhoea-irritable bowel syndrome by oral disodium cromoglycate: A pilot study.**

Lobo B, Ramos L, Martínez C, Guilarte M, González-Castro AM, Alonso-Cotoner C, Pigrau M, de Torres I, Rodiño-Janeiro BK, Salvo-Romero E, **Fortea M**, Pardo-Camacho C, Guagnozzi D, Azpiroz F, Santos J, Vicario M.

United European Gastroenterol J. 2017 Oct;5(6):887-897. doi:0.1177/2050640617691690

**Dietary treatment modulates mast cell phenotype, density, and activity in adult eosinophilic oesophagitis.**

Arias Á, Lucendo AJ, Martínez-Fernández P, González-Castro AM, **Fortea M**, González-Cervera J, Yagüe-Compadre JL, Mota-Huertas T, Vicario M

Clin Exp Allergy. 2016 Jan;46(1):78-91. doi: 10.1111/cea.12504.

**Increased humoral immunity in the jejunum of diarrhoea-predominant irritable bowel syndrome associated with clinical manifestations.**

Vicario M, González-Castro AM, Martínez C, Lobo B, Pigrau M, Guilarte M, de Torres I, Mosquera JL, **Fortea M**, Sevillano-Aguilera C, Salvo-Romero E, Alonso C, Rodiño-Janeiro BK, Söderholm JD, Azpiroz F, Santos J.

Gut. 2015 Sep;64(9):1379-88. doi:10.1136/gutjnl-2013-306236. Epub 2014 Sep 10.

## 2. REVIEWS

**Mucosal pathobiology and molecular signature of epithelial barrier dysfunction in the small intestine in irritable bowel syndrome.**

González-Castro AM, Martínez C, Salvo-Romero E, **Fortea M**, Pardo-Camacho C, Pérez-Berezo T, Alonso-Cotoner C, Santos J, Vicario M J

Gastroenterol Hepatol. 2017 Jan;32(1):53-63. doi:10.1111/jgh.13417. Review.

## 3. PRESENTACIONES A CONGRESOS

**Effect of a mucoprotectant (xyloglucan + pea protein and tannins + xylo-oligosaccharide) on intestinal permeability and mucosal microinflammation in the jejunum of irritable bowel syndrome with diarrhea.**

**M Fortea**; C Alonso-Cotoner; A Nieto; C Galán; B Lobo; E Expósito; M Pigrau; R Farré; D Guagnozzi; AM González-Castro; M Vicario; J Santos.

United European Gastroenterology Week 2019 (UEGWeek), October 2019, Barcelona, Spain. House of European Gastroenterology. Poster presentation

**Acute stress triggers IBS-like miRNA-mediated regulation of barrier function in the jejunum of healthy volunteers**

Rodiño-Janeiro BK; Pigrau M; Nieto A; Salvo- Romero E; Lobo B; González-Castro AM; **Fortea M**; Pardo-Camacho C; de Torres I; Martinez C; Guagnozzi D; Niesler B; Azpiroz F; Vicario M; Santos J; Alonso-Cotoner C.

Digestive Disease Week 2018 (DDW). June 2018, Washington DC, Estados Unidos de América. American Gastroenterological Association.

**Desmosome associated genes improve predictability of IBS compared to clinical variables.**

Martinez C; Mosquera JL; Rodiño-Janeiro BK; **Fortea M**; Lobo B; Pigrau M; González-Castro AM; Salvo-Romero E; Pardo-Camacho C; Guagnozzi D; Niesler B; Azpiroz F; Alonso-C C; Vicario M; Santos J.

Digestive Disease Week 2018 (DDW). June 2018, Washington DC, Estados Unidos de América. American Gastroenterological Association.

**Acute stress impacts clock genes and barrier integrity in the intestinal mucosa in health.**

Pigrau M; Rodiño-Janeiro BK; Salvo-Romero E; Nieto A; Hernández- Palet L; Pribic T; Gallart M; Lobo B; González-Castro AM; **Fortea. M;** Pardo-Camacho C; Guagnozzi D; Martínez C; Pérez-Berezo T; Iribarren C; de Torrés I; Azpiroz F; Vicario M; Alonso-Cotoner C; Santos J.

United European Gastroenterology Week 2017 (UEGWeek), October 2017, Barcelona, Spain. House of European Gastroenterology. Oral presentation

**Stress regulates specific sex-related molecular alterations in epithelial barrier regulatory genes in the jejunal mucosa of healthy volunteers.**

Rodiño-Janeiro BK; Pigrau M; Nieto A; Pribic T; Hernández- Palet L; Salvo-Romero E; Lobo B; González-Castro AM; **Fortea M**; Gallart M; Pardo-Camacho C; de Torrés I; Martínez C; Guagnozzi D; Pérez-Berezo T; Azpiroz F; Vicario M; Santos J; Alonso-Cotoner C.

United European Gastroenterology Week 2017 (UEGWeek), October 2017, Barcelona, Spain. House of European Gastroenterology. Oral presentation

**Modulation of CD8+ cells infiltration and activity in eosinophilic esophagitis by six-food elimination diet**

Marina Fortea, Alfredo J. Lucendo, Ángel Arias, Ana M. González-Castro, Danila Guagnozzi, Beatriz Lobo, Fernando Azpiroz, Carmen Alonso, Javier Santos, María Vicario

Falk Simposium October 2017. Belín, Germany. FALK, 3rd award. Oral Presentation

**Increased Functional Toll-like Receptors in the Esophageal Mucosa of Adult Patients with Eosinophilic Esophagitis.**

Arias A; Vicario M; Bernardo D; Olalla JM; **Fortea M**; Montalbán-Arqués A; Martínez-Fernández P; González-Castro AM; Mota-Huertas T; Arias-González L; Lucendo AJ

Falk Simposium October 2017. Belín, Germany. FALK, Poster Presentation

**Modulation of CD8+ cells infiltration and activity in eosinophilic esophagitis by six-food elimination diet.**

**Fortea M**; Lucendo AJ; Arias A; González-Castro AM; Guagnozzi D; Lobo B; Azpiroz F; Alonso-Cotoner C; Santos J; Vicario M.

4th CURED Research Conference 2017. October 2017. Cincinnati, Estados Unidos de América. CURED. Poster presentation.

**Acute stress impacts clock genes and barrier integrity in the intestinal mucosa in health.**

Pigrau M; Rodiño-Janeiro BK; Salvo-Romero E; Nieto A; Hernández- Palet L; Pribic T; Gallart M; Lobo B; González-Castro AM; **Fortea. M;** Pardo-Camacho C; Guagnozzi D; Martínez C; Pérez-Berezo T; Iribarren C; de Torrés I; Azpiroz F; Vicario M; Alonso-Cotoner C; Santos J.

Digestive Disease Week 2017 (DDW). May 2017, Chicago, Estados Unidos de América. American Gastroenterological Association. Poster presentation

**Integrated multi-omic analysis reveals female predominance of deregulated mucosal actin depolymerization by decreased TESK1-mediated CFL1-phosphorylation in IBS-D.**

Rodiño-Janeiro BK; Martínez C; Fortea-Guillamón M; Lobo B; Pigrau M; González-Castro AM; Salvo-Romero E; Pardo-Camacho C; Iribarren C; Guagnozzi D; Azpiroz F; Alonso-Cotoner C; Santos J; Vicario M.

**Stress regulates specific sex-related molecular alterations in epithelial barrier regulatory genes in the jejunal mucosa of healthy volunteers.**

Rodiño-Janeiro BK; Pigrau M; Nieto A; Pribic T; Hernández- Palet L; Salvo-Romero E; Lobo B; González-Castro AM; **Fortea M**; Gallart M; Pardo-Camacho C; de Torrés I; Martínez C; Guagnozzi D; Pérez-Berezo T; Azpiroz F; Vicario M; Santos J; Alonso-Cotoner C.

Digestive Disease Week 2017 (DDW). May 2017, Chicago, Estados Unidos de América. American Gastroenterological Association. Poster presentation

**Gender-related differential methylation patterns of the corticotropin releasing factor gene in the intestinal mucosa may relate to female predominance in diarrhea-prone bowel syndrome**

BK Rodiño-Janeiro; I Palma; **M Fortea**; E Salvo-Romero; B Lobo; M Pigrau; AM González-Castro; C Martínez; F Azpiroz; M Vicario; J Santos; C Alonso-Cotoner.

COST Action BM 1106 GENIEUR Final Conference, March 2016, Heidelberg, Alemania

COST Action. Poster presentation

**Decreased proinflamatory profile and increased corticotropin releasing factor in mucosal eosinophils in association with clinical manifestations diarrhea-prone irritable bowel sydrome**

E Salvo-Romero; C Martínez; B Lobo; M Pigrau; M Casado-Bedmar; A Sánchez-Chardi; AM González-Castro; BK Rodiño-Janeiro; **M Fortea**; C Pardo-Camacho; F Azpiroz; C Alonso-Cotoner; J Santos; M Vicario.

COST Action BM 1106 GENIEUR Final Conference, March 2016, Heidelberg, Alemania

COST Action. Poster presentation

**Increased intestinal mucosal production of immunoglobulin G in diarrhea-predominant irritable bowel syndrome**

C Pardo-Camacho; AM González-Castro; B Lobo; C Alonso-Cotoner; **M Fortea**; E Salvo-Romero; M Casado-Bedmar; BK Rodiño-Janeiro; F Azpiroz; J Santos; M Vicario.

COST Action BM 1106 GENIEUR Final Conference, March 2016, Heidelberg, Alemania

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**Decreased proinflamatory profile and increased corticotropin releasing factor in mucosal eosinophils in association with clinical manifestations diarrhea-prone irritable bowel sydrome**

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United European Gastroenterology Week 2015 (UEGWeek), October 2015, Barcelona, Spain. House of European Gastroenterology. Poster presentation

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**Modulation of CD8+ cells infiltration and activity in eosinophilic oesophagitis by six-food elimination diet**

**M Fortea**; A Lucendo; A Arias; AM González-Castro; D Guagnozzi; F Azpiroz; C Alonso-Cotoner; J Santos; M Vicario.

United European Gastroenterology Week 2015 (UEGWeek), October 2015, Barcelona, Spain. House of European Gastroenterology. Poster presentatio

**Paired transcriptomic and proteomic profiling analysis of the intestinal mucosa identifies similar biological pathways in diarrhoea-prone irritable bowel syndrome**

BK Rodiño-Janeiro; C Martínez; B Lobo; M Pigrau; AM González-Castro; **M Fortea**; M Casado-Bedmar; C Pardo-Camacho; F Azpiroz; C Alonso-Cotoner; M Vicario; J Santos.

United European Gastroenterology Week 2015 (UEGWeek), October 2015, Barcelona, Spain. House of European Gastroenterology. Oral presentation

**Increased antibody response in the intestinal mucosa of diarrhoea-prone irritable bowel syndrome in association with psychological stress and abdominal pain**

AM González-Castro; C Pardo-Camacho; B Lobo; C Alonso-Cotoner; **M Fortea**; E Salvo-Romero; M Casado-Bedmar; BK Rodiño-Janeiro; F Azpiroz; J Santos; M Vicario.

International Society of Psychoneuroendocrinology Congress, September2015, Edimburgo Reino Unido. International Society of Psychoneuroendocrinology. Poster presentation

**Activación de la población CD8$^+$ intraepitelial en la esofagitis eosinofílica y su modulación mediante dieta de exclusión de seis alimentos**

**M Fortea**; A Lucendo; A Arias; AM González-Castro; B Lobo; D Guagnozzi; F Azpiroz; C Alonso-Cotoner; J Santos; M Vicario.

Congreso de la Sociedad Española de Patología Digestiva 2015 (SEPD 2015). Junio 2015, Sevilla, Spain. Sociedad Española de Patología Digestiva. Oral presentation

**Actividad secretora del eosinófilo en la mucosa yeyunal en el síndrome del intestino irritable: valoración mediante un modelo in vitro**

E Salvo-Romero; M Casado-Bedmar; B Lobo; BK Rodiño-Janeiro; AM González-Castro; **M Fortea**; F Azpiroz; C Alonso-Cotoner; J Santos; M Vicario.

Congreso de la Sociedad Española de Patología Digestiva 2015 (SEPD 2015). Junio 2015, Sevilla, Spain. Sociedad Española de Patología Digestiva. Oral presentation

**Caracterización del infiltrado celular, análisis de su ultraestructura y de mecanismos de actividad humoral en la colitis ulcerosa en remisión**

AM González-Castro; B Lobo; A Keita; M Pigrau; **M Fortea**; C Pardo; J Santos; J Söderholm; M Vicario.

Congreso de la Sociedad Española de Patología Digestiva 2015 (SEPD 2015). Junio 2015, Sevilla, Spain. Sociedad Española de Patología Digestiva. Poster presentation

**El género femenino determina un patrón diferencial en la metilación del factor liberador de corticotropina a nivel yeyunal: implicaciones en el síndrome del intestino irritable**

BK Rodiño-Janeiro; I Palma; **M Fortea**; E Salvo-Romero; B Lobo; M Pigrau; AM González-Castro; C Martínez; F Azpiroz; M Vicario; J Santos; C Alonso-Cotoner.

Congreso de la Sociedad Española de Patología Digestiva 2015 (SEPD 2015). Junio 2015, Sevilla, Spain. Sociedad Española de Patología Digestiva. Poster presentation

**Clinical benefit and intestinal mucosal transcriptome modulation after long-term mast cell stabilization with oral disodium cromoglycate in diarrhea-predominant irritable bowel síndrome (IBS-D) patients**

B Lobo; M Pigrau; C Martínez; AM González-Castro; M Guilarte; I de Torres; E Salvo-Romero; BK Rodiño-Janeiro; **M Fortea**; C Alonso-Cotoner; F Azpiroz; M Vicario; J Santos.

NeuroGASTRO 2015, Junio 2015, Estambul, Turquía. European Society of Neurogastroenterology & Motility. Oral presentation

**Gender-related differential methylation patterns of the corticotropin releasing factor gene in the intestinal mucosa may relate to female predominance in diarrhea-prone irritable bowel syndrome**

BK Rodiño-Janeiro; I Palma; **M Fortea**; E Salvo-Romero; B Lobo; M Pigrau; AM González-Castro; C Martínez; F Azpiroz; M Vicario; J Santos; C Alonso-Cotoner.

NeuroGASTRO 2015, Junio 2015, Estambul, Turquía. European Society of Neurogastroenterology & Motility. Oral presentation

**Down-regulation of intestinal inflammatory transcriptome after long-term treatment with oral mast cell stabilizer disodium cromoglycate, in diarrea-predominant irritable bowl síndrome (IBS-D) patients is associated with clinical improvement**

B Lobo; M Pigrau; C Martínez; AM González-Castro; M Guilarte; I de Torres; E Salvo-Romero; BK Rodiño-Janeiro; **M Fortea**; C Alonso-Cotoner; M Vicario; J Santos.

Digestive Disease Week 2015, May 2015, Washington, Estados Unidos de América. American Gastroenterological Association. Poster presentation

**Jejunal mucosal eosinophils show higher corticotropin-releasing hormone content in association with clinical manifestations in diarrhea-prone irritable bowel syndrome**

E Salvo-Romero; C Martínez; B Lobo; M Pigrau; A Sánchez-Chardi; AM González-Castro; BK Rodiño-Janeiro; **M Fortea**; F Azpiroz; C Alonso-Cotoner; J Santos; M Vicario.

Digestive Disease Week 2015, May 2015, Washington, Estados Unidos de América. American Gastroenterological Association. Oral presentation

**Effects of dietary treatment over the mast cell populations and gene expression in esophageal mucosa of adults with eosinophilic esophagitis**

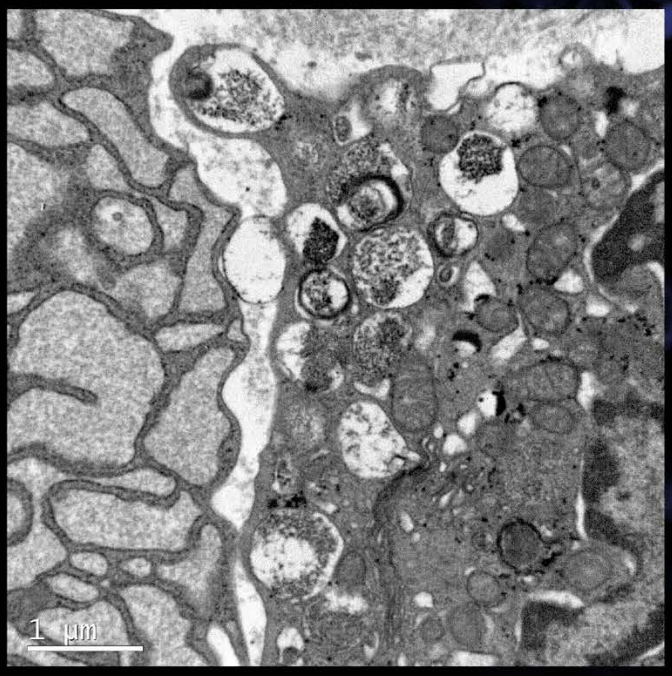
A. Arias; A.J. Lucendo; P. Martínez-Fernández; A.M. González-Castro; **M. Fortea**; J. González-Cervera; J.L. Yagüe-Compadre; T. Mota-Huertas; M. Vicario

United European Gastroenterology Week (UEG Week), October 2014, Vienna, Austria. House of European Gastroenterology. Poster presentation

**Molecular, structural and ultrastructural analyzes of eosinophils in the jejunal mucosa of irritable bowel syndrome patients**

E Salvo-Romero; **M Fortea**; A Sánchez-Chardi; B Lobo; AM González-Castro; A Cardoso; F Azpiroz; J Santos; M Vicario.

18th International Microscopy Congress, July 2014, Prague, República Checa.



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