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NUEVAS DETERMINACIONES EN ESPUTO INDUCIDO: TIPOS DE EOSINÓFILOS E IgE LOCAL

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TESIS DOCTORAL

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TIPOS DE EOSINÓFILOS E IgE LOCAL

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A Sergio

The fact is that medicine must serve patients
and it is the responsibility of the profession to know
and understand what those needs are
and to insist that it is the standard to which we must aspire.
Dame Margaret Turner-Warwick

Lee y conducirás, no leas y serás conducido
Teresa de Jesús

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ABREVIATURAS

ACT	Test de control del asma
ROC	*Receiver Operator Characteristics*
ERS	*European Respiratory Society*
ECP	Proteína catiónica eosinofílica
EDN	Neurotoxina derivada del eosinófilo
EI	Esputo inducido
EPO	Peroxidasa eosinofílica
EPOC	Enfermedad pulmonar obstructiva crónica
Fe_{NO}	Fracción exhalada de óxido nítrico
FEV_1	Volumen espiratorio forzado en el primer segundo
GCI	Glucocorticoides inhalados
GEMA	Guía española del manejo del asma
GM-CSF	Factor estimulante de colonias de granulocitos y macrófagos
IL	Interleucinas
ILC2	Células linfoides innatas tipo 2
Ig	Inmunoglobulina
iEOS	Eosinófilos inflamatorios
IMC	Índice de masa corporal
iNOS	Sintasa de óxido nítrico inducible
LABA	Agonistas β adrenérgicos de acción rápida
MBP	Proteína básica mayor

MiniAQLQ	Versión reducida del cuestionario de calidad de vida de pacientes con asma
MPO	Mieloperoxidasa eosinofílica
PBS	Solución salina fosfatada
rEOS	Eosinófilos residentes
sIgE	Inmunoglobulina E específica
TSLP	Linfopoietina estromal tímica

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RESUMEN

El asma es una enfermedad crónica de las vías respiratorias que engloba varios fenotipos inflamatorios diferentes cuyas manifestaciones clínicas son similares. De ellos, el fenotipo T2 o eosinofílico supone aproximadamente la mitad de los casos y es el mejor conocido, pero todavía quedan muchos detalles por describir de su fisiopatología. La inflamación T2 que lo caracteriza está mediada por IL-5, IL-4 e IL-13 y la célula más representativa es el eosinófilo, ya que, a través de la liberación del contenido de sus gránulos, es el responsable de la sintomatología respiratoria. Sin embargo, teorías recientes defienden que los eosinófilos no son únicamente células citotóxicas, sino que también existen eosinófilos con funciones homeostáticas en diferentes órganos, como el intestino y el timo. En el caso del asma estudios preliminares han descrito la presencia de eosinófilos inflamatorios y homeostáticos en sangre de pacientes asmáticos y en pulmón de ratón.

Dentro de este fenotipo T2 se incluye el asma alérgica, en la que la esta inflamación se acompaña de la producción de IgE contra uno o varios alérgenos, que al unirse a su receptor de la superficie de los mastocitos provoca liberación de histamina y unos síntomas similares. Un concepto novedoso es el de alergia respiratoria local, una situación clínica en la que no es posible detectar biomarcadores de alergia a

nivel sistémico sino únicamente a nivel de los órganos y tejidos afectados, lo que ocasiona que algunos pacientes sean erróneamente catalogados como “no alérgicos”.

Diagnosticar el fenotipo T2 o eosinofílico es importante por sus implicaciones terapéuticas y pronósticas, siendo el método más preciso el recuento de eosinófilos en esputo inducido. Aunque es una técnica no disponible en todos los centros debido a que es laboriosa y requiere personal específicamente entrenado y dedicado, su importancia radica en que la correlación con otras muestras más accesibles (eosinófilos en sangre o FeNO) es débil y en que sobre ella se pueden determinar otros biomarcadores. Por todo ello, continuar investigando sobre sus diferentes utilidades no solo aporta información fenotípica si no también características específicas dentro de cada fenotipo y casi de cada paciente en particular. Esto permite alcanzar un grado de diagnóstico prácticamente personalizado de la inflamación bronquial, y por tanto guiar las decisiones terapéuticas con información fisiopatológica fundamentada.

El objetivo de esta tesis fue ampliar los conocimientos sobre la inflamación eosinofílica local en el asma mediante el uso de las muestras obtenidas tras la inducción de esputo para describir la identificación de tipos de eosinófilos en la vía aérea y la medición de IgE local. Para ello, se desarrollaron dos proyectos de carácter observacional prospectivo con recogida de datos sociodemográficos, antropométricos, clínicos, funcionales e inducción de esputo. Sobre la muestra obtenida se realizó determinación de los fenotipos de eosinófilos mediante citometría de flujo (estudio 1) y medición de IgE total y específica para Der p 1 (estudio 2).

Los resultados obtenidos fueron:

- Estudio 1: La citometría de flujo permitió definir dos poblaciones de eosinófilos llamadas E1 ($CD66b^{High}$, $CD15^{High}$) y E2 ($CD66b^{Low}$, $CD15^{Low}$). Los pacientes con eosinofilia mostraron un claro predominio de E1 sobre E2 en esputo inducido, pero en los neutrofílicos y paucigranulocíticos los porcentajes de E1 y E2 fueron similares. E1 se correlacionó con los niveles de IL-5 en el sobrenadante, la FeNO y los eosinófilos en sangre.

- Estudio 2: No se encontraron diferencias significativas entre pacientes con asma alérgica, no alérgica y controles sanos en los niveles de IgE total en esputo. Sin embargo, los niveles de IgE específica local fueron significativamente superiores en los asmáticos alérgicos (p=0,000), sin hallarse diferencias entre los controles y los asmáticos no alérgicos. Los niveles de IgE en suero y esputo, tanto total como específica, se correlacionaron de forma estadísticamente significativa.

Las conclusiones más relevantes de la presente Tesis Doctoral son que es posible tanto identificar tipos de eosinófilos como medir la IgE en el esputo inducido. Los eosinófilos inflamatorios y homeostáticos se pueden discriminar mediante citometría de flujo, siendo los primeros los más abundantes en pacientes con asma alérgica y eosinofilia bronquial. En esta muestra también es posible medir la IgE total y específica, siendo la segunda capaz de discernir a los pacientes con asma alérgica.

Palabras clave: *asma, alergia, eosinófilo, fenotipo, esputo inducido, citometría de flujo, inmunoglobulina E*

ABSTRACT

ABSTRACT

Asthma is a chronic airway disease that encompasses several different inflammatory phenotypes whose clinical manifestations are similar. T2-high or eosinophilic phenotype accounts for approximately half of the cases and is the best known, but many details of its pathophysiology remain to be described. The T2 inflammation is mediated by IL-5, IL-4 and IL-13 and the most representative cell is the eosinophil, since, through the release of the contents of its granules, it is responsible for the respiratory symptoms. However, recent theories argue that eosinophils are not only cytotoxic cells, but that there are also eosinophils with homeostatic functions in different organs, such as the intestine and thymus. In the case of asthma, preliminary studies have described the presence of inflammatory and homeostatic eosinophils in the blood of asthmatic patients and in mouse lung.

This T2 phenotype includes allergic asthma, in which this inflammation is accompanied by the production of IgE against one or more allergens, which upon binding to its receptor on the surface of mast cells causes histamine release and similar symptoms. A novel concept is that of local respiratory allergy, a clinical situation in which it is not possible to detect allergy biomarkers at the systemic level but only at the level of the affected organs and tissues, causing some patients to be erroneously labeled as "non-allergic".

Diagnosing the asthma phenotype is important because of its therapeutic and prognostic implications. The most accurate method is the induced sputum through the leucocyte count. Although this technique is not available in all centers because it is time-consuming and requires specifically trained and dedicated personnel, its importance lies in the fact that the correlation with other more accessible samples (eosinophils in blood or FeNO) is weak and that it allows the determination of other biomarkers. Therefore, further research on its different utilities not only provides phenotypic information but also specific characteristics within each phenotype and almost every patient in particular. This allows us to reach a degree of practically personalized diagnosis of bronchial inflammation, and therefore guide therapeutic decisions with informed pathophysiological information.

The aim of this thesis was to extend the knowledge of local eosinophilic inflammation in asthma by using samples obtained after sputum induction to describe the identification of eosinophil subsets in the airway and the measurement of local IgE. For this purpose, two prospective observational projects were developed with the collection of sociodemographic, anthropometric, clinical, functional and sputum induction data. On the sample obtained, eosinophil phenotypes were determined by flow cytometry (study 1) and measurement of total and specific IgE for Der p 1 (study 2).

The results obtained were:

- Study 1: Flow cytometry allowed the definition of two eosinophil populations called E1 (CD66bHigh, CD15High) and E2 (CD66bLow, CD15Low). Patients with eosinophilia showed a clear predominance of

E1 over E2 in induced sputum, but in neutrophilic and paucigranulocytic patients the percentages of E1 and E2 were similar. E1 correlated with IL-5 levels in the supernatant, FeNO and blood eosinophils.

- Study 2: No significant differences were found between patients with allergic asthma, non-allergic asthma and healthy controls in sputum total IgE levels. However, local specific IgE levels were significantly higher in allergic asthmatics (p=0.000), with no differences found between controls and non-allergic asthmatics. Serum and sputum IgE levels, both total and specific, were statistically significantly correlated.

The most relevant conclusions of this Doctoral Thesis are that it is possible to identify eosinophil subsets and to measure total and specific IgE in induced sputum. Flow cytometry allows discriminating inflammatory and homeostatic eosinophils in this biological sample, the former being the most abundant in patients with allergic asthma and bronchial eosinophilia. In this sample it is also possible to measure total and specific IgE, the latter being able to discern patients with allergic asthma.

Keywords: *asthma, allergy, eosinophil, phenotype, induced sputum, flow cytometry, inmunoglobulin E*

1. Introducción

1.1. EL ASMA BRONQUIAL

1.1.1. DEFINICIÓN Y EPIDEMIOLOGÍA

El asma se caracteriza por una inflamación bronquial crónica que provoca la aparición de los síntomas característicos: disnea, tos y opresión torácica (1). Las causas de esta inflamación son variadas, pero en general se considera que es una combinación de factores genéticos y exposicionales (2) y debido a ello, es posible encontrar diferentes patrones inflamatorios entre pacientes, en lo que hoy se conoce como fenotipos(3). La presencia de inflamación crónica en las vías aéreas provoca hiperrespuesta bronquial (4),que es una reactividad exagerada a diferentes sustancias (como alérgenos o irritantes), y obstrucción al flujo aéreo, que en el asma suele ser de tipo reversible, ya sea espontáneamente o tras la administración del tratamiento (5).

El asma bronquial es la enfermedad obstructiva bronquial más prevalente y se calcula que en la actualidad afecta a más de 350 millones de individuos, variando entre países (6) debido a la heterogeneidad entre métodos y criterios diagnósticos. Los costes socioeconómicos del asma son muy importantes, ya que puede afectar a individuos de todas las edades y no es infrecuente en niños y en adultos en edad laboral, por lo que supone también una importante carga de forma indirecta.

1.1.2. FISIOPATOLOGÍA

La inflamación propia del asma bronquial (figura 1) puede ser idiopática o desencadenada por diferentes exposiciones, como a los virus, los alérgenos, o la contaminación. Las primeras células implicadas en la activación de la cascada inflamatoria son las células del epitelio bronquial, que liberan alarminas (linfopoietina estromal tímica o TSLP, IL-33 o IL-25) a partir de las cuales se activan las células dendríticas que actúan como presentadoras de antígenos para linfocitos T y células linfoides innatas. Estas células, a través de citocinas e interleucinas, activan el reclutamiento y la activación de leucocitos (especialmente eosinófilos, pero también neutrófilos, basófilos y mastocitos) que perpetúan la inflamación y que pueden llegar a generar remodelado de las vías respiratorias. Este remodelado supone una modificación permanente de las diferentes estructuras de la pared bronquial, como engrosamiento de la membrana basal, fibrosis subepitelial, hipertrofia e hiperplasia del músculo liso e hiperplasia de las glándulas mucosas (7). Estos cambios son los responsables de los

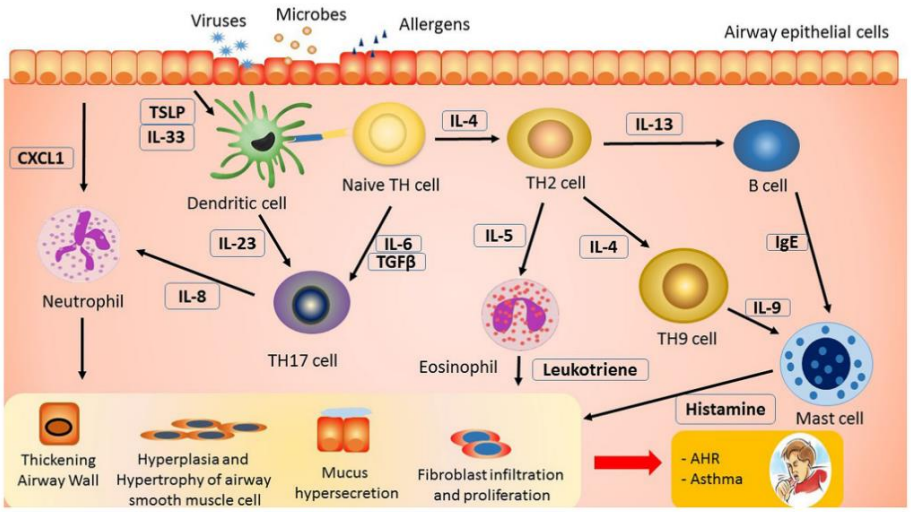

Figura 1. Cascada inflamatoria en el asma(106)

síntomas del asma de larga evolución, como son la obstrucción irreversible al flujo aéreo o la hipersecreción mucosa.

1.1.3. DIAGNÓSTICO

El diagnóstico de asma se basa en la demostración objetiva de alguna de las principales características del asma –la inflamación, la hiperrespuesta bronquial o la reversibilidad de la obstrucción al flujo aéreo– en una persona con síntomas sugestivos. En el algoritmo de la actual Guía Española del Manejo del Asma (GEMA 5.2) (2)(figura 2) se puede observar cómo la espirometría suele ser la técnica inicial de elección, ya que permite demostrar la obstrucción al flujo aéreo, la reversibilidad de la misma mediante la prueba broncodilatadora o el

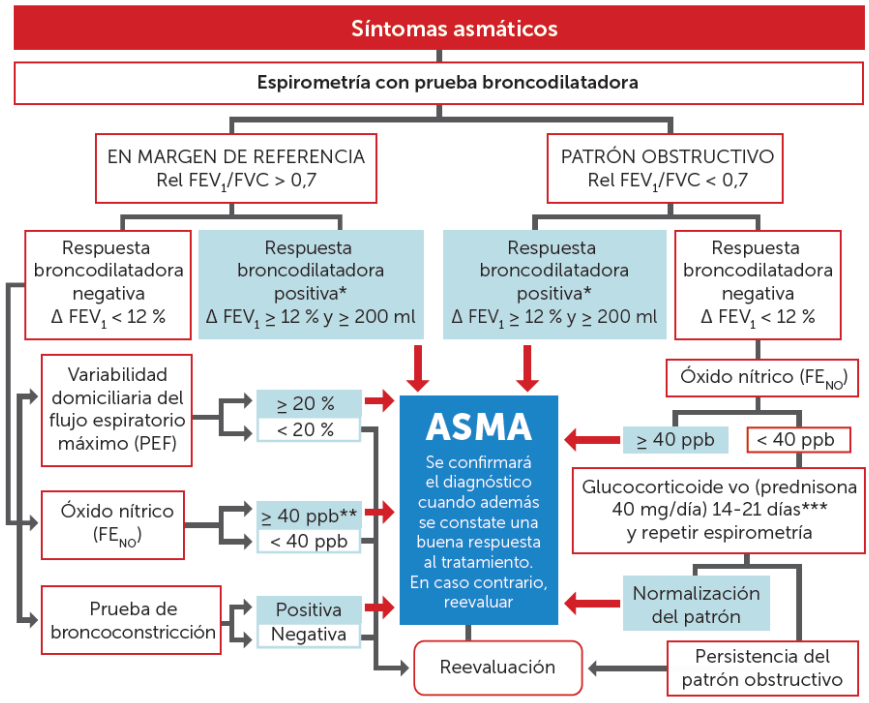

Figura 2. Algoritmo diagnóstico de asma según la GEMA 5.2 (2)

tratamiento con glucocorticoides, o la hiperrespuesta bronquial en las pruebas de broncoprovocación.

La medición de la fracción exhalada de óxido nítrico (FeNO) permite evidenciar la inflamación local, y las medidas seriadas del flujo espiratorio máximo determinar la variabilidad de la función pulmonar. Todas estas exploraciones pueden tener falsos positivos y falsos negativos, lo que dificulta confirmar o descartar el diagnóstico de asma en algunos pacientes.

1.1.4. TRATAMIENTO Y CONTROL

El tratamiento del asma es principalmente inhalado y hoy contamos con tres familias de fármacos inhalados disponibles: los glucocorticoides, los agonistas β adrenérgicos y los antimuscarínicos (2). Estos dos últimos pueden presentarse como moléculas de corta duración (como el salbutamol o la terbutalina, y en el segundo caso el bromuro de ipratropio) que se utilizan como rescate, y de larga duración, que se utilizan asociadas a los glucocorticoides inhalados como tratamiento de mantenimiento (en el caso de los anticolinérgicos, solo están aprobados en el tratamiento del asma el tiotropio y el glicopirronio). Habitualmente el tratamiento del asma se administra escalonadamente y se ajusta a las necesidades de cada paciente (2). Los casos leves pueden utilizar únicamente tratamientos de rescate, ya sea mediante broncodilatadores de corta duración, o combinaciones de glucocorticoides inhalados con agonistas β adrenérgicos de larga duración (LABA o *long acting beta adrenergics*) de acción rápida que aportan no solo alivio sintomático si no también efecto antiinflamatorio

(8). Los siguientes escalones terapéuticos consisten en el uso de glucocorticoides inhalados de mantenimiento, con la adición sucesiva de LABA y/o LAMA (*long acting antimuscarinics*). Cuando se considera necesario, por vía oral se puede añadir montelukast (9), un antileucotrieno, o azitromicina (10), un antibiótico de tipo macrólido con efecto inmunodulador. En los pacientes más graves es necesario añadir anticuerpos monoclonales (11), a día de hoy disponibles anti inmunoglobulina E (omalizumab), anti IL-5 (mepolizumab y reslizumab) y su receptor (benralizumab), anti IL-4 (dupilumab) y anti TSLP (tezepelumab). En la actualidad la terapia de mantenimiento con glucocorticoides sistémicos se reserva como última opción terapéutica debido a sus efectos adversos a largo plazo.

El concepto de control del asma es una de las claves para el adecuado manejo y tratamiento de estos pacientes. El control adecuado del asma está determinado por la ausencia de síntomas y de exacerbaciones, pero también por una reducción tanto del riesgo futuro de pérdida de función pulmonar progresiva como de los efectos adversos del tratamiento. El control también permite definir el grado de gravedad de cada paciente ya que esta depende de la cantidad de tratamiento necesario para alcanzarlo y exige una revaloración periódica de la adecuación del mismo a la situación del asma, que característicamente es muy variable (12). Una gran mayoría de los pacientes asmáticos pueden alcanzar un control correcto con GCI solos o en combinación, pero alrededor de un 10% tienen lo que se denomina asma grave, de los cuales el 50% presentan mal control (13). El asma grave requiere medidas adicionales, empezando por la determinación del fenotipo inflamatorio al que pertenece el paciente de cara a valorar si podría beneficiarse de un tratamiento específico e incluso seleccionar el de mayor posibilidad de éxito terapéutico (14).

1.1.5. FENOTIPOS INFLAMATORIOS

Durante las últimas décadas la clasificación del asma ha ido variando en fenotipos clínico-inflamatorios (15), fenotipos únicamente inflamatorios o incluso endotipos (16). Sin embargo, la disponibilidad de tratamientos biológicos específicos ha ido derivando la tendencia hacia la definición de dos fenotipos inflamatorios, que se diferencian por la presencia o no de inflamación de tipo T2: asma T2 (o T2 elevado) y asma no T2 (o T2 bajo).

La inflamación T2 se caracteriza por la activación de las células innatas linfoides de tipo 2 (ILC2) tras diferentes estímulos sobre el epitelio bronquial. Como respuesta, estas células producen citocinas (principalmente IL-5) que estimulan el reclutamiento y la activación de eosinófilos y por tanto el término T2 a veces se utiliza como sinónimo de fenotipo eosinofílico. Los eosinófilos, mediante la liberación del contenido de sus gránulos citoplasmáticos, producen inflamación local y los síntomas propios del asma (17). El asma alérgica suele considerarse T2 debido a que habitualmente comparte parte de esta cascada inflamatoria, si bien en el caso de la alergia la exposición a un alérgeno provoca que los linfocitos Th2 estimulen a los linfocitos B a través de IL-4 y la IL-13 a producir inmunoglobulina E (IgE) específica. Esta inmunoglobulina se une a su receptor en la superficie de los mastocitos, y provoca su degranulación en exposiciones posteriores al alérgeno.

El fenotipo no T2 (18) es peor conocido y engloba pacientes pauciinflamatorios, o con inflamación de tipo Th1 y Th17. Por el momento solo se dispone de tratamiento biológico para los casos de inflamación T2 y por ello la tendencia en la práctica clínica diaria es

priorizar la identificación de estos pacientes para una posible terapia dirigida posterior.

1.1.6. MEDICIÓN DE LA INFLAMACIÓN: EL ESPUTO INDUCIDO

Determinar la presencia o no de inflamación eosinofílica y su alcance no solo sirve para establecer el fenotipo, si no que tiene valor para establecer el pronóstico, el control y predecir la respuesta terapéutica, especialmente a los glucocorticoides y los anticuerpos monoclonales.

Existen diferentes métodos para identificar la inflamación T2 en un paciente asmático, siendo las más habituales el nivel de eosinófilos en sangre y la FeNO. Sin embargo, estos métodos son predictores poco precisos (19,20) de la inflamación eosinofílica en el esputo inducido (SI), que es la técnica mejor validada para definir los fenotipos inflamatorios bronquiales.

La inducción de esputo consiste en la nebulización de suero hipertónico a concentraciones crecientes para fluidificar las secreciones del paciente, que las expulsa mediante ejercicios de tos vigorosa (21). Antes de iniciar el procedimiento se administra un broncodilatador y se espera diez minutos a que actúe su efecto. Mediante un nebulizador ultrasónico se administran suero hipertónico a concentraciones de 3, 4 y 5%. Tras cada nebulización se realiza higiene buconasal para prevenir la contaminación de la muestra por células orofaríngeas y se estimula al paciente para que tosa vigorosamente y deposite el material expectorado en un bote estéril. Antes de administrar la siguiente concentración se comprueba mediante espirometría ausencia de deterioro de la función pulmonar, que obligaría a detener el procedimiento. La inducción finaliza en el momento en el que se

obtiene una muestra visualmente adecuada o una vez realizadas las tres nebulizaciones correspondientes.

En las primeras 2h posteriores a su obtención, el esputo se procesa seleccionando los tapones de moco y tratándolos con ditiotreitol y solución salina fosfatada (PBS). La suspensión celular se filtra y se homogeniza con métodos físicos y/o químicos. Mediante un hemocitómetro y la tinción de azul de tripano se calculan el número total de células por gramo de esputo, el porcentaje de células vivas -que definen la viabilidad de la muestra- y el de células escamosas orofaríngeas, que informan de la contaminación por secreciones de vía aérea superior. Con estos datos se calcula la calidad de la muestra. Tras centrifugar el preparado celular, se obtiene el sedimento celular y el sobrenadante.

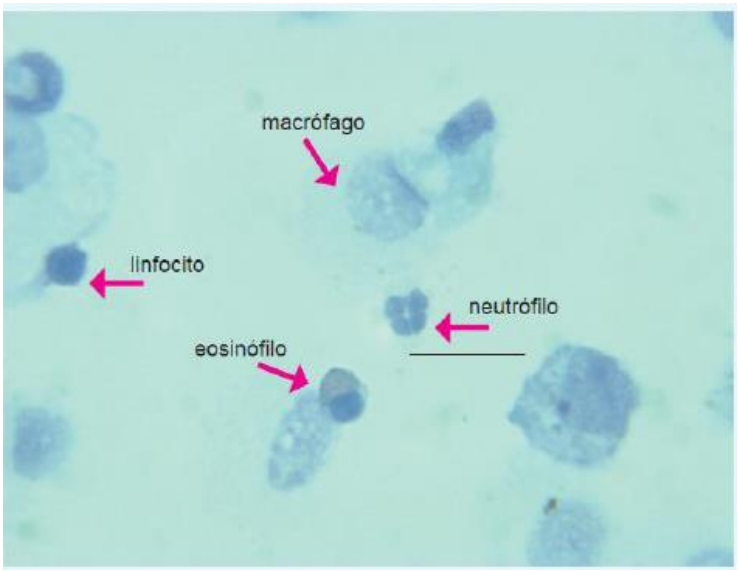

Figura 3. Granulocitos identificables en una muestra de esputo inducido. Imagen procedente del "Manual de Esputo Inducido" del Hospital de la Santa Creu i Sant Pau, Barcelona.

El sedimento se tiñe mediante solución de Wright-Giemsa y sobre él se realiza el recuento diferencial de leucocitos que permite identificar linfocitos, neutrófilos, eosinófilos y macrófagos (figura 3). El valor de eosinófilos y neutrófilos determina la clasificación en los cuatro fenotipos inflamatorios descritos por la *European Respiratory Society* (22):

- Paucigranulocítico: eosinófilos <3% y neutrófilos <64%
- Neutrofílico: eosinófilos <3% y neutrófilos ≥64%
- Eosinofílico: eosinófilos ≥3% y neutrófilos <64%
- Mixto: eosinófilos ≥3% y neutrófilos ≥64%

El valor de eosinófilos en EI puede verse alterado por diferentes factores, como el tratamiento con glucocorticoides, las infecciones, la edad, el tabaquismo, el tiempo que transcurre entre la obtención de la muestra y su procesado e incluso los profesionales que intervienen en el proceso (23). En el asma, los eosinófilos en el EI se correlacionan con la gravedad de la enfermedad, el control, el riesgo de exacerbaciones y el riesgo de hospitalización (24). Este procedimiento permite además realizar otras mediciones, como citocinas en el sobrenadante (25), estudios de microbioma (26), proteómica (27), etc.

Aparte del recuento celular por microscopía el uso de la citometría de flujo se está estandarizando para realizar recuentos celulares automáticos (28). Esta técnica se basa en el uso de una luz láser a través de la cual pasan, de una en una, las células suspendidas en un fluido (figura 4). La transmisión o dispersión de la luz del láser es recogida por detectores, lo que da información sobre el tamaño y complejidad de las células. Además, se pueden utilizar marcadores de membrana específicos de tipos o subtipos celulares que emiten su propia fluorescencia o reaccionan a su vez con el haz láser.

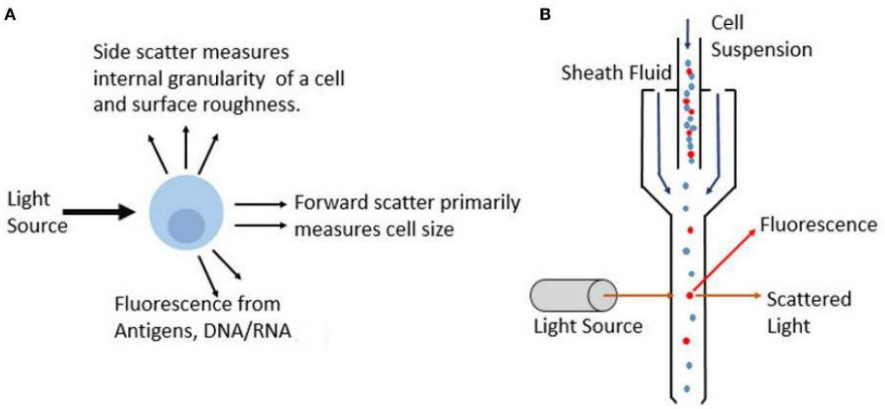

Figura 4. Mecanismo y bases del funcionamiento de la citometría de flujo (107). A) el haz de luz incide en la célula permitiendo medir diferentes parámetros como la extinción, la dispersión y la fluorescencia, y esta interacción proporciona información sobre las propiedades ópticas y la composición de la célula. B) esquema que muestra las células alineadas individualmente y cómo interactúan con la luz láser.

1.2. LOS EOSINÓFILOS

Los eosinófilos son los principales granulocitos implicados en las respuestas inmunes innatas de tipo T2 y, clásicamente, se les ha relacionado con la defensa contra helmintos y las reacciones alérgicas. Su característica principal es la presencia de gránulos citoplasmáticos acidófilos cargados de mediadores inflamatorios. Estos gránulos son los responsables de su nombre, ya que permitieron que fueran identificados al teñirse con eosina (29). Son células conocidas desde finales del siglo XIX, pero antiguas evolutivamente, ya que están presentes en todos los vertebrados y algunos invertebrados, probablemente desde hace más de 600 millones de años (30).

Los eosinófilos se forman en la médula ósea procedentes de precursores mieloides, inducidos por GM-CSF, IL-5 e IL-3 (31). Son

liberados a la sangre como células maduras y su vida media dura entre 3 y 18 horas aproximadamente, suponiendo menos del 5% de linfocitos circulantes en situación de homeostasis. Desde la sangre son reclutados a los diferentes tejidos a través de quimiocinas de la familia de la eotaxina (32), donde pueden sobrevivir hasta varias semanas.

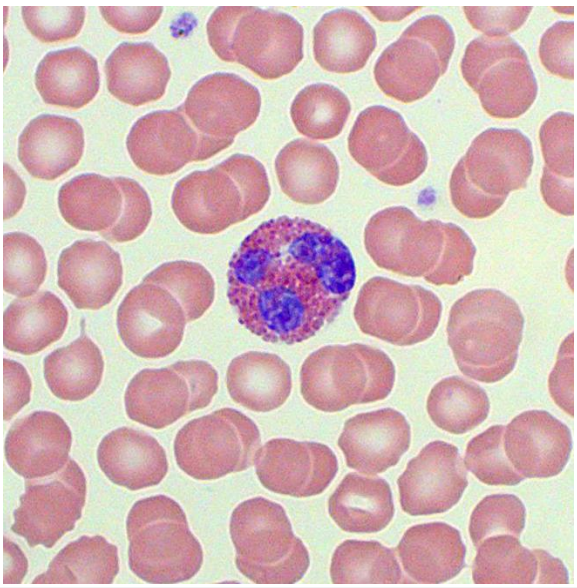

Figura 5. Eosinófilo rodeado de hematíes. Fotografía procedente de www.medschool.co

Estos granulocitos se caracterizan por tener un núcleo polilobulado y las vesículas acidófilas previamente mencionadas (figura 5), que contienen principalmente cuatro proteínas: peroxidasa eosinofílica (MPO), proteína básica mayor (MBP), la proteína catiónica eosinofílica (ECP) y la neurotoxina derivada del eosinófilo (EDN). Además, pueden contener citocinas, enzimas y factores de crecimiento. En el citoplasma de los eosinófilos también hay gránulos primarios formados por cristales de Charcot-Leyden y cuerpos lipídicos. En la membrana celular se encuentran receptores para las moléculas que median en el

crecimiento, maduración, quimiotaxis y degranulación (como por ejemplo IL-5Rα, CCR3 o SIGLEC-8) (figura 6) (33).

Cada vez se conocen mejor los factores que intervienen en el desarrollo, maduración, activación y supervivencia de los eosinófilos. Entre ellos destacan la IL-5 y la eotaxina (ligando del CCR3), y en menor medida, la linfopoietina estromal tímica (TSLP), la IL-25 y la IL-33(34).

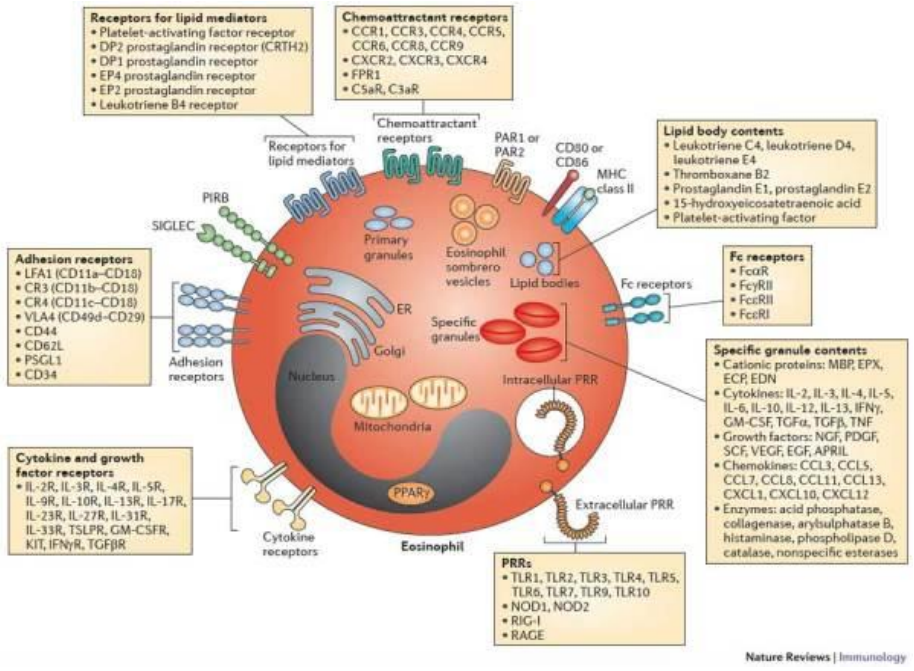

Figura 6. Características celulares de los eosinófilos (33)

La degranulación (35) de los eosinófilos puede producirse a través de tres mecanismos:

- Citólisis: la muerte celular produce rotura de la membrana lipídica y la liberación de los gránulos citoplasmáticos íntegros.
- Exocitosis: la membrana de las vesículas se fusiona con la membrana celular, liberando al exterior su contenido.

- Degranulación fragmentada, con una liberación selectiva y controlada del contenido de los gránulos con la ayuda de pequeñas vesículas, que no requieren fusionarse con otros gránulos ni con la membrana celular.

Además, de forma similar a los neutrófilos, los eosinófilos pueden liberar trampas extracelulares compuestas de DNA mitocondrial, MBP y ECP en el proceso conocido como ETosis (36).

1.2.1. EOSINÓFILOS EN SALUD Y PATOLOGÍA

La función inmune más atribuida a los eosinófilos es la defensa contra helmintos (especialmente nematodos). Los gránulos excretados por los parásitos y el daño epitelial que produce su presencia provocan la liberación de TSLP, IL-25, e IL-33 y por tanto la afluencia y activación de eosinófilos. Estos se unen a los parásitos mediante anticuerpos o complemento y liberan sus gránulos, que son directamente citotóxicos para estos organismos. Más recientemente se ha explorado la posibilidad de que los eosinófilos tengan también actividad bactericida (por ejemplo, la ECP tiene afinidad específica por bacterias Gram negativas (37)), y virucida (algunos estudios muestran cierto efecto protector hacia el virus respiratorio sincitial (38,39)). En prácticamente cualquier órgano o tejido se ha descrito una patología relacionada con la acumulación de eosinófilos (ver tabla 1). Entre ellas destacan las relacionadas con la respuesta alérgica, el asma y los síndromes hipereosinofílicos primarios.

Tabla 1. Enfermedades relacionadas con eosinofilia en órganos y tejidos (33)

Supplementary information S1 | **Disorders associated with eosinophilia and/or eosinophil accumulation in organs and tissues**

Category	Disorder	Description / Comments
Respiratory	Allergic bronchopulmonary aspergillosis (ABPA)	Hypersensitivity to aspergillus observed in patients with pre-existing asthma
	Allergic rhinitis	Allergic inflammation of the nasal passages; commonly known as "hay fever"
	Asthma	Chronic inflammatory disease of the airways; may be allergic or non-allergic
	Chronic rhinosinusitis	Inflammation of the membrane lining the paranasal sinuses; lasting more than 12 weeks
	Eosinophilic bronchitis	Eosinophils in the airway associated with chronic cough; unlike asthma, no airway hyperresponsiveness
	Eosinophilic pneumonia	Loeffler's syndrome; eosinophils in the alveoli from any known or unknown cause
	Nasal polyposis	Eosinophilic inflammation of the mucosae of the nasal and paranasal sinuses typically associated with rhinitis and asthma
Gastrointestinal	Eosinophilic gastroenteritis	Rare condition; patchy or diffuse eosinophilic infiltration of gastrointestinal tissue associated with non-specific symptomatology
	Eosinophilic esophagitis	Allergic inflammatory disorder of the esophagus; eotaxin-3 is prominent biomarker
	Inflammatory bowel disease	Complex heterogeneous inflammatory disorders with impact on small intestine and colon
Dermatologic	Atopic dermatitis	Non-contagious, chronic pruritic skin disorder
	Bullous pemphigoid	Autoimmune skin disorder
	Eosinophilic cellulitis	Wells' syndrome; recurrent granulomatous skin disease with eosinophilia
	Eosinophilic folliculitis	Ofuji disease; papules associated with hair follicles, seen most commonly in association with HIV disease
Hypereosinophilic syndromes (HES)	Myeloproliferative HES	Chronic eosinophil leukemia; frequently associated with FIP1L1/PDGFRA and other gene fusions
	Lymphocytic variant HES	Results from aberrantly activated $CD3^{-}CD4^{+}$ T lymphocyte clone
	Gleich's syndrome	Episodic angioedema associated with eosinophilia
	NERDS syndrome	Nodules–eosinophilia–rheumatism–dermatitis–swelling syndrome; some features similar to HES
Vascular	Kawasaki's disease	Arteritis associated with eosinophilia
	Churg-Strauss syndrome	Autoimmune vasculitis associated with eosinophilia and granulomata
Infection	Helminth	Eosinophils recruited by Th2 cytokines elicited in response to helminth infection
	Fungus	Notable eosinophilia in response to Coccidiomycosis
	Virus	Respiratory Syncytial virus (RSV) in infants, HIV at end stages in association with low $CD4^{+}$ T cells
Immunologic / neoplastic	Omenn syndrome	Autosomal recessive severe combined immunodeficiency; autoreactive T cells
	Kimura's disease	Inflammation of the skin, cervical lymph nodes, and salivery glands
	Hodgkin's lymphoma	Lymphoma; can include prominent eosinophilia in primary lymph node lesions
Muscular / connective tissue	Eosinophilic fasciitis	Shulman's syndrome; eosinophilic inflammation of the fascia and skin, typically of arms and legs
	Inflammatory myopathic syndromes	Eosinophilic inflammation of muscle tissue; related to trauma, helminth, or idiopathic
	Eosinophil-myalgia syndrome	Muscle pain, eosinophilic inflammation correlated to ingestion of L-tryptophan
	Toxic oil syndrome	Pulmonary pathology and myalgias associated with ingestion of a tainted commercial rapeseed oil product
	Calpain-3 mutations	Mutations in the gene encoding calpain-3 lead to muscle tissue dysfunction and eosinophilia
Ocular	Allergic conjunctivitis	Atopic keratoconjunctivitis, a severe form of this disorder, can cause blindness
Iatrogenic	Cytokine infusion therapy	Examples include interleukin-2 (melanoma and renal cancer) and GMCSF (myeloid reconstitution after transplant)
	Drug-reaction	DRESS syndrome; Drug Reaction/ Rash with Eosinophilia and Systemic Symptoms; also characterized by long latency after receiving etiologic agent; associated with significant mortality
	Graft-vs.-host disease (GvHD)	Complication of allogeneic transplant; transplanted cells attack host tissue
	Vaccine hypersensitivity reaction	Pulmonary eosinophilia observed in response to formalin-fixed RSV antigen vaccine

1.2.2. Nuevas funciones y tipos de eosinófilos

Durante los últimos años se han descubierto nuevas funciones y características de los eosinófilos, que van más allá del papel citotóxico tratado previamente.

La hipótesis LIAR (*Local Immunity And/or Remodelling/Repair*) (40) defiende que estos granulocitos no solo actúan como citotóxicos/defensivos, sino que también tienen un papel relevante como células reguladoras. Esta teoría pone en duda el papel de los eosinófilos en la defensa contra helmintos, ya que en las cepas de ratón deplecionadas de eosinófilos las larvas de *T. spiralis* son incapaces de sobrevivir (41) y no se ha observado un riesgo aumentado de infección por *S. mansoni* (42) Estos autores reinterpretan situaciones en diferentes especies y tejidos en las que se dan fenómenos de remodelado o reparación tisular que requieren la presencia de eosinófilos. Un ejemplo es la metamorfosis de anfibios, en los que las modificaciones necesarias a nivel intestinal para la alimentación propia de las fases evolutivas tardías no tienen lugar si no se produce una infiltración eosinofílica (43). En humanos, los eosinófilos también parecen influenciar modificaciones tisulares reguladas hormonalmente, como la morfogénesis de los conductos mamarios en la adolescencia (44), y las modificaciones del endometrio uterino en la menstruación y el embarazo (45). Además, también se han relacionado con patologías que implican remodelado tisular, como precisamente ocurre a nivel bronquial en el asma (46) pero también a nivel de la circulación colateral en la enfermedad cardiovascular (47) o en la esofagitis eosinofílica (48). Esta teoría ha dado lugar a diferentes investigaciones con el objetivo de identificar dos tipos de eosinófilos: los eosinófilos citotóxicos clásicos, inflamatorios o iEOS, y los eosinófilos homeostáticos, reguladores o residentes (rEOS).

1.2.3. LOS EOSINÓFILOS RESIDENTES

El área mejor conocida y con mayor abundancia de rEOS es el tracto gastrointestinal, donde suponen entre un 20 y un 30% de los leucocitos locales. El epitelio intestinal es una barrera en contacto constante con el exterior (alimentos, microorganismos, etc.) y su mucosa tiene un sistema inmune complejo debido a que no solo debe defenderse contra posibles patógenos si no también identificar y tolerar sustancias inocuas y microorganismos comensales. Los rEOS intestinales contribuyen a mantener la integridad de la barrera epitelial mediante la estimulación de secreción mucosa y regulan la microbiota induciendo el cambio de isotipo hacia la producción de IgA en las células plasmáticas (49). También actúan regulando la respuesta linfocitaria, inhibiendo las respuestas Th17(50) y promoviendo la diferenciación hacia células T reguladoras (51). Aunque en menor medida que en el intestino, los rEOS están presentes también en las glándulas mamarias, el timo, el tejido adiposo, la piel y el pulmón.

Las características de los rEOS se han descrito principalmente en estudios realizados en ratones. Aparte de las proteínas citoplasmáticas y de membrana referidas previamente, expresan CD11b, F4/80, CD69 y CD44. Su reclutamiento y desarrollo es independiente o solo parcialmente dependiente de IL-5 (52) y tienen una vida media más prolongada en los tejidos que en el torrente sanguíneo.

1.2.4. TIPOS DE EOSINÓFILOS EN EL ASMA

Entre el 40 y el 60% de los asmáticos presentan inflamación eosinofílica o T2. Como en otros órganos y tejidos, se ha explorado la

posibilidad de que haya rEOS e iEOS implicados en la patogenia del asma.

Los primeros estudios se realizaron a finales del siglo XX y se basaron en el uso de un gradiente de Percoll para separar los eosinófilos circulantes según su densidad. Mediante este gradiente se detectaron eosinófilos hipodensos (<1.082 g/ml) y normodensos (>1.082 g/ml). Los pacientes con síndrome hipereosinofílico primario y los asmáticos presentaban valores significativamente más elevados que los pacientes sanos (95% y 35% respectivamente) (53). Posteriormente otros investigadores mostraron que la población de estos eosinófilos aumentaba tras la broncoprovocación de forma directamente proporcional al descenso de FEV_1 alcanzado (54), y detectaron una correlación significativa con la gravedad del asma y la hiperrespuesta bronquial (55).

Los estudios más actuales se basan, sin embargo, en la citometría de flujo, una técnica que permite el conteo celular al pasar las células de una en una por un haz de luz. Hay dos estudios especialmente destacables:

- Mesnil y colaboradores (56) realizaron un estudio en ratones, en los que muestran cómo a lo largo de los primeros siete días de vida adquieren los eosinófilos residentes pulmonares. Estos rEOS pulmonares son de núcleo anillado y expresan Siglec-F^{int} y $CD125^{int}$. En sangre también son capaces de diferenciar iEOS ($CD62L^{-}CD101^{hi}$) y rEOS ($CD62L+\ CD101^{low}$). Tras la provocación con ácaros en ratones sensibilizados, se produce un aumento de iEOS en el lavado broncoalveaolar y en sangre. Si esta provocación se realiza en ratones con la IL-5 bloqueada

produce una inhibición de los iEOS circulantes y de su reclutamiento pulmonar sin afectar a los rEOS. Realizan un estudio de expresión génica que muestra como los iEOS expresan en mayor medida genes proinflamatorios y los rEOS, reguladores. Finalmente, reproducen el estudio en pulmón de pacientes asmáticos y controles sanos, siendo capaces de identificar en ambos iEOS ($CD62L^{-}$ $IL\text{-}3R^{high}$) y rEOS (CD62L+ $IL\text{-}3R^{low}$)

- Januskevicius y colaboradores (57) llevaron a cabo un estudio en seres humanos en el que incluyeron pacientes sanos, pacientes con asma alérgica y pacientes con asma eosinofílica no alérgica. Identificaron iEOS y rEOS mediante la expresión de CD62L y de CD101. En sangre, los pacientes sanos presentaban niveles similares de ambos eosinófilos, mientras que los pacientes con asma alérgica presentaban predominio de iEOS y los pacientes con asma eosinofílica no alérgica, de rEOS. Los rEOS mostraron mayor capacidad de adhesión en los tres grupos, pero menor vida media, salvo que fueran cultivados con células musculares lisas, situación en la que superaban en supervivencia a los iEOS. Los pacientes alérgicos y los controles sanos fueron sometidos a broncoprovocación con *D. pteronyssinus,* tras la cual los pacientes sanos no mostraron diferencias significativas en ninguna de las dos poblaciones, pero los pacientes asmáticos mostraron un descenso de los iEOS circulantes y un aumento de su capacidad de adhesión, que los autores interpretan como señal de su reclutamiento de los iEOS hacia el pulmón. Recientemente han publicado un segundo estudio en la misma línea de investigación en el que muestran como IL-5 y GM-CSF

estimulan las propiedades pro proliferativas sobre el músculo liso de iEOS y rEOS en pacientes con asma eosinofílica y asma alérgica (58).

1.3. La alergia y la inmunoglobulina E

1.3.1. Fisiopatología de la respuesta alérgica

Las reacciones alérgicas se corresponden principalmente con las reacciones de hipersensibilidad tipo I de Gell y Coombs (59,60), que están mediadas por la IgE. Estas reacciones tienen lugar en dos fases: una primera fase de sensibilización, que se produce cuando hay un primer contacto con una molécula que actúa como alérgeno, y una fase de respuesta, que consiste en la activación del sistema inmune adquirido en las exposiciones sucesivas a dicho alérgeno. La respuesta puede ser inmediata o tardía, o bien cronificarse cuando se producen exposiciones repetidas.

En la fase de sensibilización, las células dendríticas procesan el alérgeno y lo presentan a los linfocitos T a través del complejo mayor de histocompatibilidad. Este contacto provoca que los linfocitos T naïve se conviertan en linfocitos Th2, que serán los responsables de estimular el cambio de isotipo de los linfocitos B hacia la producción de IgE (61) a través de la producción de IL-4 e IL-13 y de la unión entre membranas de ambos linfocitos a través de diferentes proteínas (CD40 con CD40 ligando, y CD80/CD86 con CD28) (figura 7).

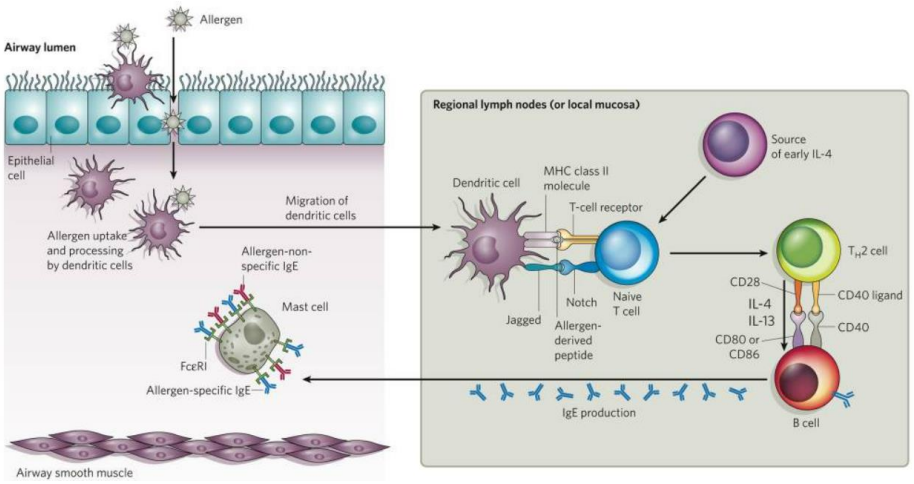

Figura 7. Proceso de sensibilización alérgica (62)

La IgE producida por los linfocitos B puede estar libre en el torrente sanguíneo, en los tejidos, o unida a sus receptores de membrana en diferentes células. En exposiciones sucesivas el alérgeno se une a la IgE previamente formada. La unión del alérgeno con la IgE de la superficie de mastocitos provoca la liberación del contenido de sus gránulos. Estos contienen histamina, proteoglicanos, enzimas, prostaglandinas y leucotrienos, etc., que son las responsables de la aparición de los síntomas inflamatorios, al producir vasodilatación, aumento de permeabilidad capilar, broncoconstricción, hipersecreción mucosa y estimulación de los nervios sensitivos (62).

1.3.2. DIAGNÓSTICO DE ALERGIA

El diagnóstico de alergia se fundamenta en la historia clínica y en la investigación de posibles exposiciones desencadenantes de síntomas. Las exploraciones para confirmarla se basan en la demostración de la existencia de inmunoglobulinas E específicas para uno o varios alérgenos, o en la aparición de sintomatología compatible tras la exposición. Las más utilizadas son:

- **Pruebas cutáneas:** dependiendo del nivel de la piel que se evalúe se denomina *prick-test* (intraepidérmica), intradermorreacción (intradérmica) o la colocación de parches (epicutánea). En el caso de los aeroalérgenos se utiliza el *prick-test*, que consiste en la colocación de una gota del extracto alergénico sobre la piel a través de la cual se realiza una punción con una lanceta cuya punta solo alcanza la epidermis. El contacto del extracto con los mastocitos de la capa intraepidérmica, que tienen IgE adherida a su membrana, provoca su degranulación y una tumefacción local. A los 15-20 minutos se realiza una lectura de la reacción provocada, que se considera positiva si aparece un habón con un diámetro mínimo de 3 mm (63). Los extractos alergénicos utilizados deben variar según la zona geográfica para incluir los más habituales en la región. El *prick-test* incluye un control positivo (histamina) y un control negativo (habitualmente suero fisiológico) ya que, aunque es una prueba sensible y específica, puede dar lugar a falsos negativos, como sucede recientemente en el caso de tratamientos con antihistamínicos, o falsos positivos, como en el caso de afectaciones cutáneas que provoquen una reactividad exagerada, como el dermografismo.

- **IgE total y específica en suero:** es posible determinar la IgE total, la IgE específica (sIgE) para un alérgeno concreto o un panel que incluya un grupo de alérgenos. No existe un valor de IgE total normal establecido, aunque normalmente se consideran valores elevados cuando supera las 150 kU/L (64). La IgE total puede elevarse en otras patologías, como algunas enfermedades inmunológicas (65,66), infecciones parasitarias, algunas neoplasias (67) y por influencia del tabaco(68) y el

alcohol (69). Sin embargo, los valores normales tampoco excluyen la posibilidad de padecer una enfermedad alérgica, por lo que su utilidad radica en su capacidad como herramienta de cribaje, mientras que la IgE específica, en cambio, tiene mayor valor diagnóstico (70) al mostrar una mayor correlación con la probabilidad y la gravedad de los síntomas (71) en la mayoría de los casos. Hoy en día es posible evaluar un gran número de alérgenos simultáneamente mediante técnicas de *micro o macroarrays* (72), pero también es posible evaluar alérgenos concretos, habitualmente cuando se trata de sensibilizantes poco frecuentes, mediante *inmunoblot* (73). La mayoría de laboratorios consideran una IgE específica positiva cuando sus valores superan los 0,35 kUA/L, pero los niveles causantes de síntomas dependen de cada alérgeno, por lo que este punto de corte debe ser interpretado contextualmente.

- **Pruebas de provocación:** en el caso concreto del asma existen las pruebas de broncoprovocación específica (74). Esta consiste en la inhalación de concentraciones progresivas del alérgeno en estudio bajo control espirométrico. Se considera una prueba positiva cuando se produce una caída del FEV_1 ≥20% en las primeras tres horas, o ≥15% en las siguientes 3-7 horas (respuesta tardía). Debido a sus riesgos y requerimientos técnicos se utilizan únicamente en centros especializados y en el ámbito de la investigación, o en los casos de asma ocupacional.

1.3.3. ALERGIA LOCAL

Dentro de la patología alérgica, durante los últimos años se ha extendido el concepto de “alergia local” (75), en la que no es posible

realizar el diagnóstico mediante la detección de IgE específica elevada en sangre o *prick-test* positivo ya que la inflamación se da solo en los tejidos afectados y no a nivel sistémico. Este fenómeno está especialmente descrito en la rinitis, de la que supone una cuarta parte de los casos (76). Debe ser sospechada en un paciente con clínica de rinitis relacionada con una exposición sugestiva, pero con pruebas negativas, y se confirma mediante pruebas de provocación nasal (77). La medición de IgE específica nasal ha obtenido resultados inconsistentes y menor sensibilidad que la provocación específica (78,79). Esta patología es característica de mujeres no fumadoras, con historia familiar de atopia y síntomas graves de inicio temprano (76). Durante la evolución presentan un empeoramiento progresivo y la aparición de conjuntivitis y asma (80). Durante los últimos años también se han publicado evidencias sobre fenómenos equivalentes a nivel bronquial, reportándose una prevalencia de asma confirmada de forma objetiva en el 50-70% de pacientes con rinitis alérgica local y síntomas compatibles (81,82).

1.3.4. Estructura de la IgE

La IgE es uno de los cinco isotipos de anticuerpos presentes en el ser humano y la última inmunoglobulina en ser descubierta. Como las demás inmunoglobulinas, está formada por cuatro cadenas polipeptídicas: dos ligeras y dos pesadas (figura 8).

La región constante (Fc) está formada por las dos cadenas pesadas (Cε) y es la zona de unión a los receptores celulares en la membrana de mastocitos. La región variable (Fab) es la donde se produce el reconocimiento y la unión al antígeno, y está formada por parte de las cadenas pesadas y las dos cadenas ligeras. A su vez, estas cadenas ligeras contienen regiones variables cuyas secuencias de aminoácidos pueden alterarse para alcanzar la afinidad por cada antígeno. Esta estructura le confiere la posibilidad de unirse a dos antígenos. Como peculiaridades, la IgE es la única inmunoglobulina que no tiene una región bisagra en el centro de la molécula, y está más glicosilada que los demás isotipos.

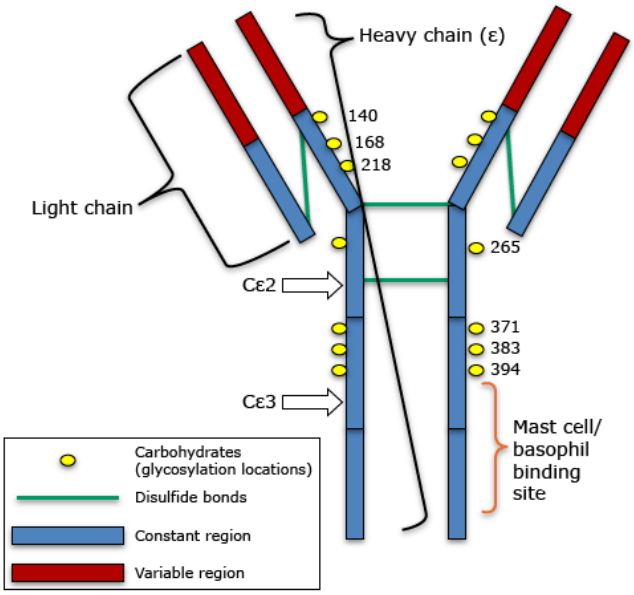

Figura 8. Estructura de la IgE. Imagen procedente de www.uptodate.com

La IgE tiene dos tipos de receptores que se expresan en las membranas celulares: el receptor de alta afinidad (FcεRI) presente en los mastocitos y basófilos, y el de baja afinidad (FcεRII) que se expresa

en un gran número de células, como los linfocitos, células dendríticas y epiteliales.

1.3.5 La IgE en el asma

Las células plasmáticas productoras de IgE se encuentran en la médula ósea, los ganglios linfáticos y la mucosa respiratoria (83). Algunos estudios muestran que, tras la exposición respiratoria al alérgeno, la mayor parte de la sIgE se produce a nivel de la mucosa nasal y bronquial(84), y que posteriormente esta pasa al torrente circulatorio, donde se une a los basófilos. Solo una vez saturados los receptores de los basófilos, la IgE vuelve a los tejidos (incluyendo la piel) donde se une a los mastocitos residentes, y es posible su detección en fluidos biológicos (85). Además, estudios recientes sugieren que también hay una producción continua de IgE en ausencia de exposición al alérgeno (86) .

La IgE total puede encontrarse elevada en algunos casos de asma no alérgica (87). En biopsias bronquiales de pacientes con y sin alergia se han encontrado similitudes entre la expresión de su receptor de alta afinidad y la producción de IL-4 e IL-13 (88,89). Una de las teorías más extendidas es la de la producción de sIgE contra las enterotoxinas de *S. aureus,* relacionándose con la incidencia y la gravedad del asma, incluso en ausencia de otras sensibilizaciones alergénicas (90). Los niveles de IgE total sérica se correlacionan con la presencia y gravedad del asma y la hiperrespuesta bronquial (91), y se utilizan en la indicación y monitorización del tratamiento con omalizumab (92).

2. HIPÓTESIS Y JUSTIFICACIÓN

HIPÓTESIS Y JUSTIFICACIÓN

Teniendo en cuenta los conocimientos disponibles sobre el asma y la eosinofilia planteados en la introducción, concluimos que todavía existen muchos interrogantes respecto a los mecanismos implicados en la inflamación bronquial. Cada vez es mayor la evidencia de que en el asma las determinaciones sistémicas son insuficientes para describir adecuadamente la inflamación local y que hay epifenómenos locales que pasan desapercibidos a las exploraciones diagnósticas utilizadas habitualmente. Entre ellos se encontraría la IgE local, que podría estar mediando una respuesta T2 de tipo alérgico limitada a la vía aérea o alterando la cascada T2 no alérgica bronquial. Otra cuestión relevante sería la presencia de diferentes tipos de eosinófilos en la vía aérea. Si bien se ha descrito su detección en sangre de pacientes asmáticos y en pulmón de ratón, todavía existen muchos interrogantes por resolver, como por ejemplo si están presentes o no en el esputo inducido, cómo se relacionan con los diferentes fenotipos de asma, y cuál sería la metodología más adecuada para su identificación en esta muestra biológica.

En este escenario, el esputo inducido como técnica no invasiva, reproducible y segura, puede proporcionar un buen medio donde identificar y cuantificar estos y otros nuevos biomarcadores que nos permitan describir en detalle la fisiopatología local de la vía aérea de los pacientes con asma. Este conocimiento tiene especial relevancia en el actual escenario terapéutico por la mayor oferta de fármacos biológicos, algunos de ellos similares entre sí. En algunos casos la selección del más adecuado puede no ser sencilla, por lo que es necesario desarrollar nuevas herramientas que nos permitan discriminar entre pacientes y personalizar esta decisión.

En base a ello, en esta tesis se plantean las siguientes hipótesis:

1. Es posible identificar eosinófilos inflamatorios y homeostáticos en el esputo inducido de pacientes con asma. Probablemente cada fenotipo inflamatorio bronquial tiene una distribución característica de estas poblaciones de eosinófilos.

2. Es posible detectar IgE total y específica en el esputo inducido. Los niveles de esta inmunoglobulina pueden discriminar pacientes con asma alérgica, no alérgica y controles sanos.

3. OBJETIVOS

3.1 Objetivo principal

El objetivo principal de esta tesis fue ampliar los conocimientos sobre la inflamación local en el asma mediante la detección de los diferentes tipos de eosinófilos y la medición de la IgE total y específica en el esputo inducido de pacientes asmáticos.

3.2 Objetivos secundarios

1. Identificar eosinófilos inflamatorios y homeostáticos en el esputo inducido, determinar la metodología más adecuada para su caracterización mediante citometría de flujo y determinar la distribución de ambos tipos en los cuatro fenotipos inflamatorios bronquiales.

2. Medir la IgE total y específica en el suero y en el esputo inducido de pacientes sanos, con asma alérgica y asma no alérgica e identificar las diferencias entre ellos.

4. COMPENDIO DE PUBLICACIONES

4.1. ARTÍCULO 1

IDENTIFICATION OF TWO EOSINOPHIL SUBSETS IN INDUCED SPUTUM FROM PATIENTS WITH ALLERGIC ASTHMA ACCORDING TO CD15 AND CD66B EXPRESSION

Elena Curto, Éder F. Mateus-Medina, Astrid Crespo-Lessmann, Rubén Osuna-Gómez, Cristina Ujaldón-Miró, Alba García-Moral, Paula Galván-Blasco, Lorena Soto-Retes, David Ramos-Barbón y Vicente Plaza

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Article

Identification of Two Eosinophil Subsets in Induced Sputum from Patients with Allergic Asthma According to CD15 and CD66b Expression

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Abstract: Two subsets of eosinophils have been described: resident eosinophils with homeostatic functions (rEOS) in healthy subjects and in patients with nonallergic eosinophilic asthma, and inflammatory eosinophils (iEOS) in blood and lung samples from patients with allergic asthma. We explored if it would be possible to identify different subsets of eosinophils using flow cytometry and the gating strategy applied to induced sputum. We conducted an observational cross-sectional single-center study of 62 patients with persistent allergic asthma. Inflammatory cells from induced sputum samples were counted by light microscopy and flow cytometry, and cytokine levels in the supernatant were determined. Two subsets of eosinophils were defined that we call E1 (CD66b-high and CD15-high) and E2 (CD66b-low and CD15-low). Of the 62 patients, 24 were eosinophilic, 18 mixed, 10 paucigranulocytic, and 10 neutrophilic. E1 predominated over E2 in the eosinophilic and mixed patients (20.86% vs. 6.27% and 14.42% vs. 4.31%, respectively), while E1 and E2 were similar for neutrophilic and paucigranulocytic patients. E1 correlated with IL-5, fractional exhaled nitric oxide, and blood eosinophils. While eosinophil subsets have been identified for asthma in blood, we have shown that they can also be identified in induced sputum.

Keywords: allergic asthma; eosinophil subsets; flow cytometry; induced sputum

1. Introduction

Asthma clinical practice guidelines [1,2] recommend phenotyping patients with severe asthma to guide decisions on biological treatment. The most widely used method to establish phenotype is blood eosinophil count based on a 300 cells/μL cut-off. In patients with severe asthma and <300 eosinophils/μL in blood (around 30% of cases), induced sputum can be used to diagnose bronchial eosinophilia [3,4], coexistent or isolated neutrophilic inflammation, and pauci-inflammatory profiles. While counting induced sputum cells using light microscopy is an effective means of identifying bronchial leukocytes, this approach is both laborious and requires specially trained professionals.

Flow cytometry, using specific markers for membrane proteins, can also be used to automatically count cells in induced sputum. Although this application has been

described [5,6], it has not yet been fully standardized. In blood and other biological fluids, flow cytometry can classify leukocytes and correctly identify eosinophils [7] and can even differentiate between eosinophil subsets with different functions in several organs and systems [8–10]. In addition to the eosinophils known to play an inflammatory role in defense against helminths (called inflammatory eosinophils, or iEOSs), there are other eosinophils with homeostatic functions and a long half-life that reside in tissues (called resident eosinophils, or rEOSs) [11,12]. These two eosinophil subsets have been identified in the mouse lung, where they are recruited after exposure to an allergen [13]. Both subsets have also been identified in human samples, with iEOS predominating in nonallergic eosinophilic asthma, and rEOS in allergic asthma and healthy controls. While rEOSs generally have a greater adhesion capacity, both subsets show greater survival in nonallergic eosinophilic patients than in allergic or healthy patients [14]. These two subsets have been also identified in blood and nasal polyps of patients with severe eosinophilic asthma plus concomitant chronic rhinosinusitis with nasal polyps [15]. All those studies used CD62L to differentiate between eosinophil subsets [13–15]. CD62L, an adhesion molecule present in multiple blood cells, is shed from the eosinophil membrane after passage through the endothelium. Since CD62 detection in induced sputum is predictably low [16], a different gating strategy is needed.

Evidence on the existence of different eosinophil subsets may have clinical implications for phenotype definition, patient prognosis, and treatment. Identifying eosinophil subsets in patients with severe, uncontrolled asthma is likely to enable judicious selection of biological treatments; it may even help identify targets for the development of new molecules and treatments specifically focused on pathological eosinophils, which would avoid the deleterious effects of concomitantly eliminating eosinophils with homeostatic functions.

The objective of this study was to explore the possibility of distinguishing between eosinophil subsets in the induced sputum of patients with allergic asthma and to determine the distribution of these subsets in different bronchial inflammatory phenotypes.

2. Materials and Methods

In this single-center cross-sectional observational study, patients monitored for persistent allergic asthma were recruited from Hospital de la Santa Creu i Sant Pau outpatient clinics between September 2016 and January 2019. All patients met diagnostic criteria for persistent asthma according to the current version of the Global Initiative for Asthma (GINA) [1], were in maintenance treatment with inhaled corticosteroids (ICS), and signed a written informed consent. Excluded were patients with other serious respiratory diseases, on chronic corticosteroid or monoclonal antibody treatment for asthma, or who did not provide informed consent. Allergic asthma was defined as a positive skin prick test for common aeroallergens or a positive allergen-specific immunoglobulin E (IgE) test and respiratory symptoms after exposure to the allergen. Data compiled from the latest available analysis included blood eosinophil count and total IgE, exacerbations in the previous year that required systemic corticosteroids, and any maintenance treatment administered. Spirometry and fractional exhaled nitric oxide (FeNO) using Evernoa BASE (EverSens) were performed. The Spanish-validated version of the Asthma Control Test (ACT) [17] was administered to all patients. Patients' induced sputum was analyzed by microscopy and flow cytometry, and cytokines present in the supernatant were analyzed.

2.1. Sputum Induction and Processing

Sputum induction was performed according to the European Respiratory Society (ERS) standardized methodology [18]. Premedication with 200 mg of inhaled salbutamol was followed by nebulization of a hypertonic solution with an ultrasonic nebulizer (NE-007, Omron, Kyoto, Japan). Nebulizations lasted seven minutes each and consisted of hypertonic saline at increasing concentrations (3%, 4%, and 5%) until an adequate amount of sputum was obtained. The sputum was processed within two hours of collection, and mucus plugs were selected for treatment with dithiothreitol (Sputalysin, Calbiochem

Corp., San Diego, CA, USA) and phosphate buffered saline (PBS). The cell suspension was then filtered to remove detritus. Number of cells per gram of sputum, cell viability, and total squamous cells from contaminated upper respiratory tracts were calculated using a hemocytometer and trypan blue staining. Cell preparations were centrifuged to obtain cell pellets and supernatant. The cells' pellets were used for both differential cell counting (using Wright–Giemsa staining) and population analysis (using flow cytometry), and the supernatant was frozen at −80 °C until further analysis. Following ERS recommendations [18], patients were classified by bronchial inflammatory phenotype as follows: paucigranulocytic (eosinophils < 3%, neutrophils < 65%), neutrophilic (eosinophils < 3%, neutrophils ≥ 65%), eosinophilic (eosinophils ≥ 3%, neutrophils < 65%), and mixed (eosinophils ≥ 3%, neutrophils ≥ 65%) [19].

2.2. Flow Cytometry

Leukocytes were identified using autofluorescence (FITC), side scatter (SSC), and CD45 expression [5]. Expression of eosinophil surface markers, activation markers, and characteristic cell function markers was determined (CD66b, CD16, CD15, CD62L, CD63, CCR3, CD123, CD125, and Siglec-8-9). Cells were incubated at 4 °C in darkness with different combinations of antibodies. After 20 min, they were washed with PBS + 0.5% bovine albumin and then resuspended in 200 µL of PBS. Samples in which cell viability was ≥80% and >10,000 cells could be analyzed per determination were analyzed in a MACSQuant 10 cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany). FlowJoX v10.7 software was used to analyze expression levels and the percentage of positive cells for each marker in viable eosinophils, identified based on size and granularity parameters in combination with CD45+, CD66b+, CD16−, and CD15+ expression.

Flow cytometry gating defined the eosinophil population that coexpressed CD66b+ and CD15+. Two eosinophil subsets, which we call E1 and E2, were characterized by high expression of CD66b and CD15 and low expression of CD66b and CD15, respectively. Figure 1 shows flow cytometry examples of eosinophil populations in the induced sputum of patients with allergic asthma.

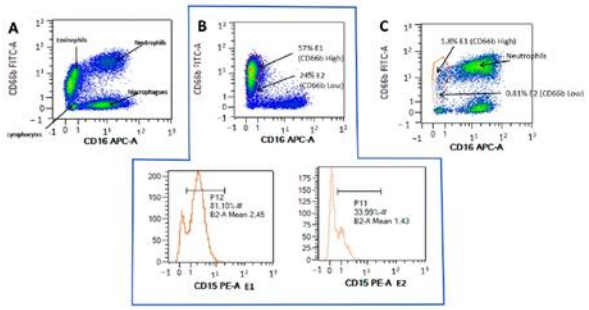

Figure 1. (**A**): Flow cytometry for a patient presenting all cell lines, based on CD16 APC-A and CD66B FITC-A. (**B**): A highly eosinophilic patient with E1 predominance over E2 (57% vs. 24%), with side plots showing CD15 expression of 81% for E1 and 33.99% for E2. (**C**): A neutrophilic patient, with few E1 eosinophils (1.8%) or E2 eosinophils (0.8%).

2.3. Supernatant Cytokine Analysis

Cytokines present in the induced sputum supernatant were analyzed, using LEGENDplex panels (BioLegend, San Diego, CA, USA), to determine levels of the following: eotaxin; immunoglobulins IgA, IgD, IgE, IgG1, IgG2, IgG3, IgG4, and IgM; interferon alpha

and gamma (IFNα and IFNγ); interleukins IL-1B, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-17A, IL-18, IL-23, and IL-33; monocyte chemoattractant protein 1 (MCP-1); matrix metalloproteinase 2 and 9 (MMP2 and MMP9); and tumor necrosis factor alpha (TNFα).

2.4. Statistical Analysis

For normally distributed continuous variables, results were expressed as means and standard deviations (SD), and qualitative variables were expressed as frequencies and percentages. Differences between phenotypes were determined using analysis of variance (ANOVA) for the quantitative variables and Pearson's chi-squared for the qualitative variables. Posthoc analyses were performed using the Sheffe and Games Howell tests for homogeneity and nonhomogeneity of variances, respectively. Pearson's correlation coefficient was used to determine dependence between quantitative variables. In all cases, the level of statistical significance was set to 5% ($\alpha = 0.05$). Statistical analysis was performed using SPSS V. 24.0 for Windows.

3. Results

Clinical characteristics for the 62 recruited patients with allergic asthma are summarized in Table 1. Mean (SD) values in induced sputum were as follows: by microscopy, eosinophils 12.27% (16.71%), and neutrophils 58.85% (18.32%); and by flow cytometry (Table 2), eosinophils 18.59% (22.56%), and neutrophils 63.05% (25.21%). Flow cytometry measured a mean (SD) of 14.24% (19.79%) for E1 and of 4.71% (6.54%) for E2. E1 correlated with FeNO ($r = 0.357$; $p = 0.050$) and blood eosinophils ($r = 0.382$; $p = 0.003$), and E2 with the ACT score ($r = 0.388$; $p = 0.021$). E1 and E2 correlations with exacerbations did not reach statistical significance.

Table 1. Clinical, functional, and light microscopy characteristics based on the induced sputum phenotype.

Variables Mean (SD)/*n* (%)		All (*n* = 62)	Paucigranulocytic (*n* = 10)	Neutrophilic (*n* = 10)	Eosinophilic (*n* = 24)	Mixed (*n* = 18)	*p*
General characteristics	Age, years	51.40 (10.79)	41.80 (12.06)	53.30 (11.02)	52.38 (9.94)	54.39 (8.69)	**0.017**
	Sex, female	29 (48.3%)	7 (70%)	6 (60%)	9 (37.5%)	8 (44.4%)	0.300
	BMI, kg/m^2	28.60 (5.52)	25.57 (5.62)	27.76 (5.44)	30.05 (6.19)	28.82 (4.06)	0.177
	Never smoked	42 (70%)	7 (70%)	4 (40%)	19 (79.2%)	14 (77.8%)	**0.044**
	Active smoker	4 (6%)	2 (20%)	0	1 (4.2%)	1 (5.6%)	
	ICS dose, low	18 (30)	6 (60%)	0 (0%)	6 (25%)	6 (33.3%)	
	ICS dose, medium	27 (45%)	3 (30%)	5 (50%)	12 (50%)	8 (44.4%)	**0.033**
	ICS dose, high	15 (25%)	1 (10%)	5 (50%)	6 (25%)	4 (22.2%)	
	Exacerbations in the previous year	1.15 (1.55)	0.90 (0.99)	1.00 (1.05)	0.83 (0.91)	1.78 (2.41)	0.236
	ACT score	18.82 (7.52)	17.67 (6.68)	14.60 (5.63)	20.50 (8.47)	19.00 (7.04)	0.490
Comorbidities	Rhinitis	44 (73.3%)	9 (90%)	5 (50%)	19 (79.2%)	14 (75.8%)	0.178
	Nasal polyps	16 (26.7%)	1(10%)	0 (0%)	7 (29.2%)	8 (44.4%)	**0.042**
Blood test	Eosinophils (cells/mm^3)	352.5 (328.8)	142 (137.5)	255 (228.78)	430.4 (300.37)	428.1 (438.77)	0.062
	Total IgE (U/L)	269.56 (415.38)	141.43 (138.18)	169.04 (138.10)	250.90 (306.41)	436.61 (683.06)	0.282

Table 1. *Cont.*

Variables Mean (SD)/*n* (%)		All (*n* = 62)	Paucigranulocytic (*n* = 10)	Neutrophilic (*n* = 10)	Eosinophilic (*n* = 24)	Mixed (*n* = 18)	*p*
Lung function	FEV_1/FVC	66.50 (13.78)	74.80 (8.70)	62.54 (14.11)	69.41 (15.00)	64.05 (12.81)	0.143
	FEV_1 (% ref)	80.36 (22.73)	93.70 (13.31)	80.83 (22.55)	78.97 (21.88)	75.44 (25.44)	0.212
	FEV_1 (L)	2.49 (0.88)	2.99 (0.75)	2.30 (0.75)	2.50 (0.94)	2.36 (0.87)	0.252
	FeNO (ppb)	37.58 (33.03)	37.21 (30.64)	26.22 (11.82)	42.14 (35.08)	38.04 (39.92)	0.479
Microscopy induced sputum	Eosinophils, %	11.71 (16.48)	0.80 (0.63)	1.15 (0.66)	22.70 (21.66)	9.00 (5.07)	**0.000**
	Neutrophils, %	58.47 (18.60)	42.00 (15.92)	76.65 (10.19)	48.45 (16.68)	70.88 (4.17)	**0.000**
	Macrophages, %	27.44 (17.33)	54.80 (15.58)	20.00 (9.95)	26.04 (15.14)	18.22 (4.64)	**0.000**
	Bronchial cells, %	2.06 (0.62)	2.30 (0.48)	1.60 (0.69)	2.25 (0.44)	1.94 (0.72)	**0.017**
	Lymphocytes, %	1.79 (0.72)	1.90 (0.56)	1.65 (0.66)	1.87 (0.74)	1.72 (0.82)	0.787

Abbreviations: ACT, asthma control test; BMI, body mass index; FeNO, exhaled fraction of nitric oxide; FEV_1, forced expiratory volume in the first second; FVC, forced vital capacity; ICS, inhaled corticosteroids; IgE, immunoglobulin E; SD, standard deviation. The bold numbers are the ones that reached statistical significance.

Table 2. Flow cytometry results: eosinophil populations, phenotypes, and cytokines in induced sputum supernatant.

Variables Mean (SD)/*n* (%)		All (*n* = 62)	Paucigranulocytic (*n* = 10)	Neutrophilic (*n* = 10)	Eosinophilic (*n* = 24)	Mixed (*n* = 18)	*p*
Flow cytometry	Eosinophils, %	17.75 (22.30)	5.73 (10.21)	6.78 (9.08)	25.93 (27.16)	18.94 (20.53)	**0.036**
	Neutrophils, %	64.36 (25.26)	63.05 (27.49)	78.82 (13.66)	56.52 (27.99)	67.45 (22.72)	0.115
	Macrophages, %	0.42 (0.48)	0.51 (0.44)	0.40 (0.30)	0.46 (0.64)	0.34 (0.34)	0.812
	Lymphocytes, %	6.46 (6.94)	12.49 (7.91)	3.17 (1.53)	6.65 (8.19)	5.01 (4.56)	**0.016**
Phenotypes	E1, %	13.57 (19.51)	3.25 (7.58)	3.85 (7.59)	20.86 (24.91)	14.42 (16.11)	**0.034** *
	E2, %	4.52 (6.44)	2.46 (3.13)	2.51 (2.58)	6.27 (8.00)	4.31 (6.52)	0.302
	E1 CD15	55.13 (39.92)	66.61 (46.05)	57.70 (35.98)	50.94 (39.29)	53.54 (41.85)	0.791
	E2 CD15	9.69 (17.63)	25.48 (31.61)	6.22 (16.01)	6.65 (11.43)	7.76 (12.60)	**0.032** **
Supernatant	IL-5 (pg/mL)	6.83 (4.80)	***	7.44 ****	3.59 (1.86)	8.80 (6.28)	0.604
	IL-4 (pg/mL)	15.50 (17.40)	6.27 (4.92)	9.61 (10.09)	21.90 (23.53)	15.97 (14.15)	0.447
	IL-13 (pg/mL)	8.77 (6.79)	6.13 (3.77)	7.38 (5.12)	11.54 (8.99)	7.21 (4.53)	0.320
	Eotaxin(pg/mL)	15.33 (20.52)	13.80 (13.29)	28.66 (47.42)	12.31 (9.92)	12.67 (7.90)	0.487

Abbreviations: IgE, immunoglobulin E; IL, interleukin; SD, standard deviation. * Differences found between paucigranulocytic and eosinophilic patients ($p = 0.020$) and between neutrophilic and eosinophilic patients ($p = 0.024$). ** No differences found in the posthoc analysis. *** Undetectable. **** Detectable only in one patient. The bold numbers are the ones that reached statistical significance.

Bronchial inflammatory phenotype distribution, according to induced sputum cellularity as measured by microscopy, were as follows (Table 1): eosinophilic, 24 patients (37.5%); mixed, 18 patients (28.12%); and neutrophilic or paucigranulocytic, each 10 patients (15.62%). Neutrophilic patients showed a higher incidence of smokers and exsmokers ($p = 0.040$); paucigranulocytic patients, lower ICS requirements ($p = 0.033$); and eosinophilic and mixed patients, a higher incidence of nasal polyposis ($p = 0.042$).

All four phenotypes had detectable E1 and E2 subsets. E2 was similar across the different phenotypes ($p = 0.302$), whereas E1 differed ($p = 0.034$). E1 predominated over E2 in eosinophilic (20.86% vs. 6.27%) and mixed (14.42% vs. 4.31%) patients, while E1 and E2 distributions were similar for neutrophilic (2.51% vs. 3.85%) and paucigranulocytic (3.25% vs. 2.46%) patients (Figure 2). Posthoc analysis of E1 confirmed differences between neutrophilic and eosinophilic patients (3.85% vs. 20.86%; $p = 0.024$) and between paucigranulocytic and eosinophilic patients (3.85% vs. 20.86%; $p = 0.020$).

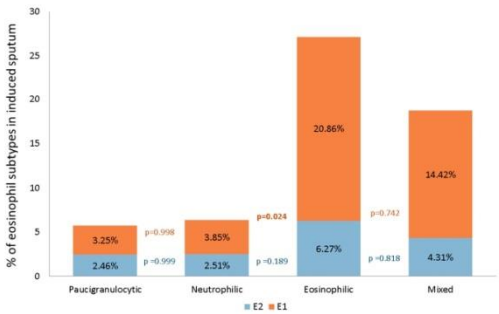

Figure 2. E1 and E2 distributions according to different bronchial inflammatory phenotypes.

No statistically significant differences between phenotypes were evident from supernatant cytokines (Table 2). There were significant correlations in the overall sample for IL-5 with certain parameters, specifically, eosinophils by microscopy and flow cytometry ($r = 0.885$; $p = 0.019$ and $r = 0.975$; $p = 0.001$, respectively), inversely with neutrophils by microscopy ($r = -0.858$; $p = 0.029$), and with E1 ($r = 0.971$; $p = 0.001$; see Supplementary Material Figure S1) but not E2 ($r = 0.571$; $p = 0.232$).

4. Discussion

In this exploratory study we demonstrate that, using flow cytometry, it is possible to identify two subsets of eosinophils in induced sputum in patients with persistent allergic asthma. Subset E1, characterized by high expression of CD66b and CD15, predominates over subset E2 in allergic asthma with bronchial eosinophilic inflammation. E1 levels are correlated with FeNO, blood eosinophil count, and IL-5 levels in induced sputum supernatant, which would suggest that they play more of an inflammatory than a homeostatic role.

As this is the first study that defines subsets of eosinophils in induced sputum, there were no precedents available. For this reason, our gating strategy relied on the expression of CD66b and CD15. The role of CD66b, a membrane protein present in granulocytes that indicates cell activation, in eosinophils has only recently been described. Its activation by monoclonal antibodies or its usual ligand, galectin-3, induces cell adhesion, superoxide molecule production, and eosinophil degranulation [20]. CD15 (also called Lewis X antigen or SSEA-1) is a carbohydrate forming part of the adhesion molecules present in cell membranes, mainly neutrophils but also eosinophils [21]. Its role in eosinophils is not, as yet, well-understood, although a study of patients with hypereosinophilic syndrome has shown that CD15 induces eosinophil cationic protein release and therefore plays a role in tissue damage associated with this syndrome [22]. Expression of CD66b and CD15 was elevated in the E1 subset, probably reflecting an activated cellular state. In studies performed in blood [13,14], iEOS was shown to decrease rapidly after specific bronchoprovocation14, theoretically due to recruitment to the airways. E1 predominance in asthma with eosinophilic inflammation, molecule expression in its membrane, and the link with FeNO and IL-5 may lead to the conclusion that E1 are the iEOS referred to in other studies, even if the latter are defined by other flow cytometry cell markers; however, such a conclusion requires further investigation.

Studies comparing techniques such as induced sputum and bronchoalveolar lavage for evaluation of airway eosinophils have reported weak correlations [23,24], probably because the techniques evaluate different airway zones (bronchial lumen, subepithelial

compartment, and intraepithelial compartment). Since a recent study shows that induced sputum eosinophils correlate with subepithelial eosinophils concentrations [25], it would be interesting to complement that research with bronchial biopsies to assess whether the two subsets are also present and correlated in the subepithelium and the bronchi. Although it may be thought that the two subsets correspond to different maturation stages, irrespective of location, bronchial eosinophils show a certain degree of activation as a consequence of exposure to inflammatory mediators and expression of membrane proteins as required to migrate [26].

Except for a recently published study in mice [27], the available evidence points to iEOS being the only subset dependent on IL-5 [13]. This is important, as treatment with anti-IL-5 or anti-IL-5Ra can influence the two subsets in a different way, probably eliminating iEOS without affecting rEOS. Understanding the functions of these eosinophil subsets and identifying them in patients before starting biological therapies may be decisive in the choice of monoclonal antibody [28] or the evaluation of lack of therapeutic response.

For a large series of patients with differing asthma severity levels, the distribution of inflammatory phenotypes in induced sputum was reported as eosinophilic 37–43%, paucigranulocytic 37–45%, neutrophilic 15–16%, and mixed 3–4% [29]. However, it is not known whether those percentages are reflected in allergic asthma populations such as ours. Allergic asthma is usually accompanied by elevated FeNO and blood eosinophil levels and is generally considered to be eosinophilic [30], yet classifications according to blood eosinophils show that allergic asthma may be noneosinophilic in up to 50% of patients with mild–moderate asthma [31], and also in patients with severe asthma who are candidates for omalizumab [32]. Furthermore, some studies report that induced sputum lymphocyte and neutrophil values for allergic asthma are higher than for nonallergic asthma [33] and that, after specific bronchoprovocation, both neutrophils and eosinophils increase significantly in the airways [34]. In our series, a third of allergic patients were noneosinophilic and 45% had raised neutrophil levels. We found this finding novel and interesting, as it may have therapeutic and prognostic implications for patients with allergic asthma.

The absence in our patients of significant differences in the level of cytokines present in induced sputum is probably explained by the small number who presented detectable levels and by the effect of dithiothreitol [35,36].

Some limitations of this study are its single-center nature and limited number of patients, indicating the need for external validation to confirm the results. As investigations for the future, it would be useful to conduct exploratory studies to correlate our findings with data from blood or bronchial samples, and even to perform the analyses before and after bronchoprovocation with allergens. The relationship between chronic ICS treatment and eosinophil populations was not analyzed since we had no data on asthma treatment adherence, not to mention the fact the acknowledged high level of nonadherence means that such data could introduce bias. It would be interesting to compare how E1 and E2 are distributed in the induced sputum of healthy patients and in patients with nonallergic asthma.

Note that the aim of this study was not to relate eosinophil subsets to clinical variables, but to describe the gating strategy applied to induced sputum and the distribution of eosinophil subsets in different types of allergic patients. The population on which this study was based is a pool of patients with persistent asthma of different degrees of severity, and although mainly moderate, we consider this subpopulation capable of reflecting the same local inflammatory processes as severe asthma.

Although different populations of eosinophils have already been defined for many organs and systems, lung and airway studies are scarce, despite both the important role played by these organs in asthma and the availability of targeted treatments. Our study, by providing new evidence on the possibility of identifying eosinophil subsets in induced sputum using a noninvasive technique, opens doors to new lines of research in asthma and other respiratory diseases.

5. Conclusions

Different eosinophil subsets can be identified in various organs and systems. For asthma, these subsets have already been identified in blood, but we have shown that they can also be identified in induced sputum. The E1 subset (CD66b-high and CD15-high) predominates in patients with allergic asthma and eosinophilic inflammation and is correlated with blood eosinophils, FeNO, and IL-5 in sputum supernatant.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijerph192013400/s1, Figure S1: Correlation between IL-5 (pg/mL) and E1 levels (% on total induced sputum eosinophil levels measured by flow cytometry).

Author Contributions: All the authors contributed significantly to the design and development of the study and have given their approval for this manuscript. Specifically, E.C. collected clinical data from the patients, performed the statistical analysis, and wrote the draft. É.F.M.-M. contributed to the design of the study, processed samples in the laboratory, planned the statistical analysis, and wrote part of the draft. R.O.-G. and C.U.-M. processed samples in the laboratory. A.C.-L. contributed to patient recruitment and data collection and made major corrections to the manuscript. A.G.-M., P.G.-B. and L.S.-R. contributed to patient recruitment and data entry in the database. D.R.-B. and V.P. designed and supervised the study and revised the final manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and was approved by the Clinical Research Ethics Committee of the Hospital de la Santa Creu i Sant Pau (Barcelona, Spain; code EC/14/207).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Conflicts of Interest: E.C. reports nonfinancial support from ALK, AstraZeneca, Novartis, and Menarini, personal fees from Boehringer-Ingelheim and TEVA, and personal fees/nonfinancial support from Chiesi outside the submitted work. A.C.-L. reports personal fees from Novartis, personal fees from Chiesi España, personal fees from Sanofi, grants and personal fees from GlaxoSmithKline, personal fees from Ferrer, personal fees from Gebro, personal fees from Boehringer Ingelheim, personal fees from Bial, personal fees from Teva, personal fees from MSD, grants and personal fees from AstraZeneca, and personal fees from Orion Pharma, outside the submitted work. É.F.M.-M., R.O.-G., C.U.-M., A.G.-M., P.G.-B. and D.R.-B. declare no conflict of interests. L.S.-R. has received fees in the last three years for talks at meetings sponsored by AstraZeneca, Diater, Chiesi, and GlaxoSmithKline, has received travel and attendance expenses for conferences from Sanofi, Allergy-Therapeutics, Hal Allergy, and FAES Farma, has acted as a consultant for Sanofi, Stallergennes-Greer, GlaxoSmithKline, and AstraZeneca, and has received funds/grants for research projects from the nonprofit foundation Spanish Allergy and Clinical Immunology Society (SEAIC). V.P. has received fees in the last three years for talks at meetings sponsored by AstraZeneca, Boehringer-Ingelheim, Merck Sharp & Dohme, and Chiesi, has received travel and attendance expenses for conferences from AstraZeneca, Chiesi, and Novartis, has acted as a consultant for ALK, AstraZeneca, Boehringer, Merck Sharp & Dohme, MundiPharma, and Sanofi, and has received funds/grants for research projects from several state agencies and nonprofit foundations and from AstraZeneca, Chiesi, and Menarini.

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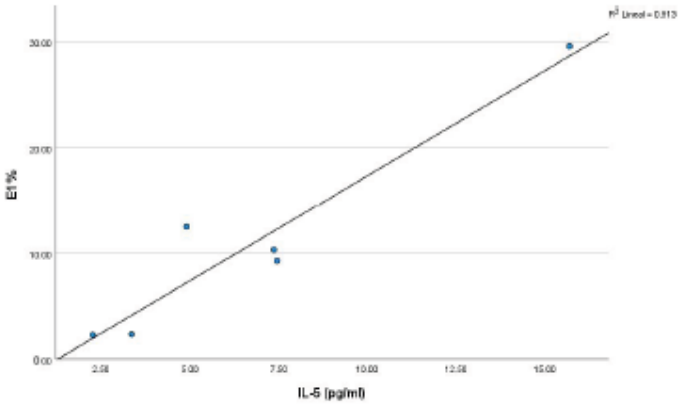
Supplementary material

Figure S1. Correlation between IL-5 (pg/mL) and E1 levels (% on total induced sputum eosinophil levels measured by flow cytometry).



4.2. ARTÍCULO 2

TOTAL AND SPECIFIC IMMUNOGLOBULIN E IN INDUCED SPUTUM IN ALLERGIC AND NON-ALLERGIC ASTHMA

Astrid Crespo-Lessmann, Elena Curto, Eder Mateus, Lorena Soto, Alba García-Moral, Montserrat Torrejón, Alicia Belda, Jordi Giner, David Ramos-Barbón, Vicente Plaza

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RESEARCH ARTICLE

Total and specific immunoglobulin E in induced sputum in allergic and non-allergic asthma

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Data Availability Statement: The dataset supporting the conclusions of this article is available for consultation at www.figshare.com (DOI 10.6084/m9.figshare.11499162.v1).

Abstract

Background

Most patients with nonallergic asthma have normal serum immunoglobulin E (IgE) levels. Recent reports suggest that total and aeroallergen-specific IgE levels in induced sputum may be higher in nonallergic asthmatics than in healthy controls. Our objective is to compare total and dust-mite specific (Der p 1) IgE levels in induced sputum in allergic and nonallergic asthmatics and healthy controls.

Methods

Total and Der p 1-specific IgE were measured in induced sputum (ImmunoCAP immunoassay) from 56 age- and sex-matched asthmatics (21 allergic, 35 nonallergic) and 9 healthy controls. Allergic asthma was defined as asthma with a positive prick test and/or clinically-significant Der p 1-specific serum IgE levels.

Results

Patients with allergic asthma presented significantly higher total and Der p 1-specific serum IgE levels. There were no significant between-group differences in total sputum IgE. However, Der p 1-specific sputum IgE levels were significantly higher ($p = 0.000$) in the allergic asthmatics, but without differences between the controls and nonallergic asthmatics. Serum and sputum IgE levels were significantly correlated, both for total IgE ($rho = 0.498$; $p = 0.000$) and Der p 1-specific IgE (rho, 0.621; $p = 0001$).

Conclusions

Total IgE levels measured in serum and induced sputum are significantly correlated. No significant differences were found between the differents groups in total sputum IgE. Nevertheless, the levels of Der p 1-specific sputum IgE levels were significantly higher in the allergic asthmatics, but without differences between the controls and nonallergic asthmatics. Probably due to the lack of sensitivity of the test used, but with the growing evidence for local

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allergic reactions better methods are need to explore its presence. The Clinical Trials Identifier for this project is NCT03640936.

Introduction

Although the causes of allergic asthma are well-understood, much less is known about the pathophysiology of nonallergic asthma. Non-allergic asthma is generally defined as nonatopic asthma with or without normal serum levels of immunoglobulin E (IgE) antibodies. Unlike allergic asthma, which is triggered by a specific allergen, nonallergic asthma may be triggered by a wide range of factors, including smoke, viruses, and other nonspecific stimuli[1].

Despite their differences, these two types of asthma may share many similarities, including airway inflammation with eosinophilia, increased Th2 cytokine production (interleukin [IL]-4, IL-5, and IL-13), hyperreactivity, and airway-induced exacerbations. Although most patients with nonallergic asthma present normal total serum IgE levels, in some cases IgE may be elevated when compared to healthy controls [2,3], with some reports suggesting that approximately 30% of asthmatic patients with a negative skin prick test have high total circulating IgE (>150 U/mL) [4,5]. These shared features suggest that unidentified environmental allergens could be involved in "nonallergic" asthma, causing a local allergic reaction in these patients[6]. Mouthuy et al. previously demonstrated that patients with nonallergic asthma present elevated levels of total IgE and *Dermatophagoides pteronyssinus* (Der p 1) specific IgE in induced sputum versus healthy controls[7]. Those findings suggest that nonallergic asthma patients may present localized allergic inflammation. This hypothesis is further supported by several studies that have demonstrated symptom relief after the administration of omalizumab—an anti-IgE treatment—in nonatopic patients[8–10]. The positive effect of omalizumab in these patients could be explained by the presence of unidentified allergens[11]. Additional support for this hypothesis comes from the relatively recent identification of a new phenotype of rhinitis—denominated local allergic rhinitis (LAR)—in patients with chronic rhinitis [12–16], characterized by local production of specific IgE with a nasal cellular Th2 immune response to nasal allergen provocation test but with negative skin prick test and undetectable serum IgE. Nevertheless, more research is needed to confirm the existence of this potential new asthma phenotype characterized by a local allergic reaction.

In this context, we hypothesized that patients with nonallergic asthma would present higher levels of IgE in induced sputum than healthy controls. To test this hypothesis, we measured total IgE and Der p 1-specific IgE in serum and induced sputum in three different groups: 1) patients with a confirmed diagnosis of allergic asthma, 2) patients diagnosed with nonallergic asthma, and 3) healthy controls. Secondary aims were to assess the correlation between total and Der p 1-specific IgE levels in serum and induced sputum and to establish a preliminary estimate of total IgE and Der p 1-specific IgE in the induced sputum of healthy individuals.

Materials and methods

Study design and participants

This was a comparative cross-sectional study to measure and compare total IgE and Der p 1-specific IgE levels in the induced sputum of asthmatics and in a group of healthy volunteers. Patients and controls were matched for age, sex, and disease severity; and asthma control for allergic and non-allergic groups. Patients were consecutively enrolled from the outpatient

asthma unit of our institution, a tertiary referral university hospital in Spain, between January and December 2013.

Total and Der p 1-specific IgE were measured in both serum and induced sputum. The IgE levels in serum and sputum were compared to determine the correlation between IgE levels in these two fluids.

Definition of allergic and nonallergic asthma

We defined asthma as a history of variable respiratory symptoms and evidence of variable expiratory airflow limitation. All patients had a positive bronchodilator test or a document positive methacholine challenge test. Asthma severity was defined according to the Global Initiative for Asthma Management (GINA)[17].

Allergic asthma was defined as asthma with 1) positive skin prick test to aeroallergens and/or 2) clinically-significant Der p 1-specific IgE according to the recommendations of the Committee of Skin Tests of the European Academy of Allergy and Clinical Immunology (EAACI) international task force[18]. Patients sensitized to various allergens were included only if dust mite allergy was the only clinically relevant one; if they showed symptoms in relation to other exposures they were excluded. Nonallergic asthma was defined as asthma with: 1) negative prick test, 2) negative Der p 1-specific IgE in serum; and 3) negative Phadiatop test (ImmunoCAP immunofluoroassay; Phadia ThermoFisher Scientific)[19]. *D. pteronyssinus* was the selected allergen because is the perennial allergen more prevalent in our geographic area.

Inclusion and exclusion criteria

Inclusion criteria were: 1) age 18–70 years; 2) continuous residence (> 4 years) in the geographic region of the study; 3) diagnosis of stable bronchial asthma according to GINA criteria [17]; 4) non-smoker; 5) no respiratory infections in the month prior to enrolment; 6) no oral corticosteroids in the last month; 7) no biological treatment with anti-IgE monoclonal antibodies; 8) no allergenic immunotherapy.

Exclusion criteria were: 1) pregnancy; 2) moderate to severe active alcohol use; 3) severe atopic dermatitis; 4) presence of any lung disease, autoimmune disease or systemic inflammatory disease, or cancer.

Control group

The control group consisted of healthy, non-smoking volunteers age 18 to 79 years, without rhinitis, allergic asthma, or other allergic symptoms (GINA criteria) or other lung disease. Controls were recruited from among staff members at our hospital. Participation was completely voluntarily. All controls were required to present a negative prick test for aeroallergens and Der p 1-specific IgE, and negative Phadiatop test.

Assessments and study procedures

Upon enrolment, demographic and clinical variables were assessed and recorded for all participants. On the same day, the following assessment were performed: Fe_{NO} (exhaled nitric oxide test); forced spirometry; inflammatory cell count in induced sputum; eosinophil count in peripheral blood; total serum IgE levels; and skin prick test for common aeroallergens. Patients also completed the validated Spanish-language version of the Asthma Control Test (ACT)[20].

Fe_{NO} was measured before spirometry using an electrochemical equipment (NO Vario Analyzer; FILT Lungen and Thorax Diagnostic GmbH, Berlin, Germany) and an expiratory maneuver providing a sustained 50 mL/s flow from total lung capacity, following the 2005

recommendations of the American Thoracic Society/European Respiratory Society[21]. Spirometry was performed using a Daptospir-600 spirometer (Sibelmed, S.A., Barcelona, Spain) in accordance with the 2003 recommendations of the Spanish Society of Pneumology and Thoracic Surgery (SEPAR), with FEV_1 (forced expiratory volume in 1 second) in the reference range when 80% of the predicted value [22,23].

Skin prick testing was performed according to standard procedures, with wheal diameters $\geq$ 4 mm considered positive. Allergic asthma was defined as the presence of asthma symptoms for one or more allergens, with positive skin prick tests for these allergens. Well-controlled asthma was defined as ACT $\geq$ 20.

Induction and analysis of sputum

Induced sputum was evaluated following the procedures previously described by our group [24] and by Pizzichini et al.[25]. Briefly, mucus plugs were manually selected and weighed, and incubated at room temperature for 15 min in 4 times the weight (in ml) of the selected plug (in mg) in 0.1% dithiothreitol (Calbiochem, San Diego, Calif., USA), washed with 4 times the plug weight (in ml) in Dulbecco's PBS, and gravity filtered through a 41-μm-pore nylon net filter (Millipore Inc; Billerica, Mass., USA). Each specimen was homogenised and aliquoted into two equal volumes. A Neubauer hemocytometer was used to determine total cell count; visually identifiable squamous epithelial cells were not included in the total cell count. Samples with insufficient sputum cell numbers ($<1000 \times 10^6$ cells/g) were excluded. Cell viability was determined by light microscopic assessment using trypan blue exclusion staining. The cells underwent centrifugation to obtain a cell pellet and a supernatant. The cell pellet was used for differential cell counts (macrophages, eosinophils, neutrophils, lymphocytes, and bronchial epithelial cells) performed on May-Grünwald-Giemsa-stained preparations. A differential leukocyte analysis of nonsquamous cells (Diff-Quik stained) was performed on a minimum of 400 cells. Differential cell counts are expressed as the percentage of total nonsquamous nucleated cells. Reference values for the cell counts were performed as described in other publications[26].

Measurement of total and specific IgE antibodies

Total and specific IgE in induced sputum supernatants were measured by the ImmunoCAP fluoroenzyme immunoassay (Phadia ThermoFisher Scientific) following the manufacturer's instructions. The test was considered positive at $>$ 2kU/L for total IgE and $>$ 0.35kU/L for specific IgE, according to manufacturer's recommendation.

Statistical analysis

Categorical variables were expressed as absolute and relative frequencies and quantitative variables as mean and standard deviation (SD). Groups were compared using ANOVA or chi-square test as appropriate. Given that the distribution of total and specific IgE values in serum and sputum were not normal, non-parametric tests (Mann-Whitney or Kruskal-Wallis for independent samples) were applied; the values were expressed as medians with interquartile ranges and ranges. The significance values in the case of non-parametric tests were adjusted by the Bonferroni correction. For the correlation analyses, Spearman's Rho test was used. Statistical significance was set at $p<0.05$. Statistical analysis was performed with Statistical Package for the Social Sciences version 18.0 (SPSS, Chicago, IL).

Ethics approval and consent to participate

The study design complied with the principles of the Declaration of Helsinki and was approved by the Clinical Research Ethics Committee at the Hospital de la Santa Creu i Sant Pau in Barcelona (COD 26/2012). All participants provided written informed consent. All patient-related data were anonymised, with the identity of the participating known only by the treating physicians. The clinicaltrials.gov identifier is NCT03640936.

Results

A total of 56 asthmatic patients—21 (37.5%) with allergic asthma and 35 (62.5%) with nonallergic asthma—met all inclusion criteria and completed the study. The control group consisted of nine healthy volunteers. Table 1 shows the clinical, functional, and inflammatory characteristics of the patients and controls.

There were no significant differences between the three groups with regard to age, sex, or body mass index (BMI). There were significant differences between the groups in: FEV1 and need for more than one round of oral corticosteroids last 12 months. FEV_1 values in the allergic (83.4%) and nonallergic (80%) patient groups were similar, but substantially higher (100.2%) in the healthy controls ($p = 0.013$) and the need for more than one round of oral corticosteroids was significantly higher ($p = 0.022$) in the nonallergic group. As for other not clinically relevant sensitizations detected by prick test in the group of patients with allergic asthma, the most frequent were polysensitized patients (17), sensitized to D. farinae (8) and pet epithelia (4). A single patient had a positive prick test for molds.

Table 2 shows the total and Der p 1-specific levels of IgE in serum and sputum for all three groups. Significant between-group differences in serum IgE levels were observed for both total and Der p 1-specific IgE, with the allergic asthma group presenting significantly higher levels of total IgE (mean, 1702.3 KU/L) and Der p1-specific IgE levels (15.5 KU/L) than the nonallergic group and healthy controls. In the sputum samples, no significant between-group differences in total IgE were observed. However, Der p 1-specific IgE levels were significantly higher

Table 1. Clinical, functional, and inflammatory characteristics of patients and controls.

Variables	Nonallergic asthma (n = 21)	Allergic asthma (n = 35)	Healthy controls (n = 9)	P
Age (years)	54.8 (14.8)	51.7(13.6)	41.88	0.076
Sex (% females)	57.1%	51.4%	88.8%	0.125
BMI (kg/m^2)	28.7 (4.1)	27.3 (5.9)	25.5 (6.48)	0.353
FEV_1 (%)	83.4 (13.9)	80 (20.8)	100.2 (10.74)	0.013
Serum eosinophils ($x10^9/L$)	0.302 (0.310)	0.271 (0.239)	0.095 (0.068)	0.113
Sputum eosinophils (%)	10.33 (19.54)	11.2 (13.01)	0.77 (1.09)	0.166
Rhinitis	61.9%	82.8%	n/a	0.080
Nasal polyposis (%)	28.5%	20%	n/a	0.462
Severe persistent asthma (%)	42.8%	42.8%	n/a	0.870
GINA 4.0 scale, grade 5–6 (%)	52.4%	48.5%	n/a	0.590
Good asthma control (ACT >20%)	19%	20%	n/a	0.357
Emergency room visits last 12 months (%)	28.5%	11.4%	n/a	0.182
>1 round of oral corticosteroids last 12 months	38%	8.5%	n/a	0.022
Inflammatory phenotype in induced sputum (%)	Paucigranulocytic: 28.6%	Paucigranulocytic: 22.4%	n/a	0.425
	Mixed: 9.5%	Mixed: 8.6%		
	Eosinophilic: 33.3%	Eosinophilic: 54.3%		
	Neutrophilic: 28.6%	Neutrophilic: 14.3%		

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Table 2. Total and dust-mite specific IgE n serum and sputum in patients with allergic asthma, non-allergic asthma, and healthy controls.

VARIABLE	NONALLERGIC ASTHMA (N = 21)	ALLERGIC ASTHMA (N = 35)	HEALTHY CONTROLS (N = 9)	p
High quality induced sputum (%)*	61.9%	69.7%	44%	0.294
Total IgE (KU/L) in serum, median (IQR)	55.5 (177.93)	233.5 (368.25)	14.25 (38.30)	<0.0001
Total IgE (KU/L) in serum, average range	423.85	1702.3	87.78	
Der p-specific IgE (KU/L) in serum, median (IQR)	0.01 (0.03)	15.45 (32.68)	0.02 (0.04)	<0.0001
Der p-specific IgE (KU/L) in serum, average range	0.08	99.97	0.10	
Total IgE (kU/L) in sputum, median (IQR)	2.69 (4.55)	4.5 (2.18)	3.16 (1.41)	0.188
Total IgE (kU/L) in sputum, average range	8.11	7.45	4.12	
Der p-specific IgE in sputum (kU/L), median (IQR)	0.055 (0.03)	0.095 (0.09)	0.06 (0.02)	<0.0001
Der p-specific IgE in sputum (kU/L), average range	0.04	0.54	0.05	

*High quality defined as > 40% viability, <20% epithelial cells, > $1x10^6$ cells

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(p<0.0001) in the allergic asthma group compared to the other two groups. Fig 1 shows the total sputum IgE levels in the three groups, with no significant differences between the groups.

Fig 2 shows the specific Der p 1-specific IgE levels in sputum for each group. The allergic asthma group presented significantly higher Der p 1-specific IgE levels than both the nonallergic patients (p<0.0001) and the healthy controls (p = 0.006). There were no significant differences between the nonallergic patients and the healthy controls. In the group of patients with non-allergic asthma, a subgroup analysis was performed among those with total sputum IgE >2 or <2, with no statistically significant differences either at clinical characteristics or regarding the other IgE measurements.

Correlations

Using data from the whole sample (patients and controls), we calculated the correlations between total and Der p 1-specific IgE levels in sputum and serum. Table 3 shows a matrix with all significant correlations (p<0.0001) in descending order from strongest to weakest. As that table makes clear, the strongest correlation was between total IgE and Der p 1-specific IgE in serum.

Discussion

In the present study, we sought to test the hypothesis that the airways of patients with nonallergic asthma exhibit a local inflammatory response by determining total and dust mite-specific IgE antibody levels in induced sputum. Our results showed that patients with allergic asthma presented significantly higher total and Der p 1-specific IgE levels in serum compared to both nonallergic asthmatics and healthy controls. However, contrary to our expectations, there were no significant differences among the three groups in total sputum IgE levels. Moreover, there were no differences in Der p 1-specific sputum IgE levels between healthy controls and nonallergic asthma patients. Overall, the lack of significant differences in total sputum IgE levels between the three groups was surprising. However, diverse factors could explain this unexpected finding, as we discuss in detail below.

The hypothesis that patients with nonallergic asthma may present a local allergic response in the airways derives from the mounting evidence for a new phenotype of local allergic

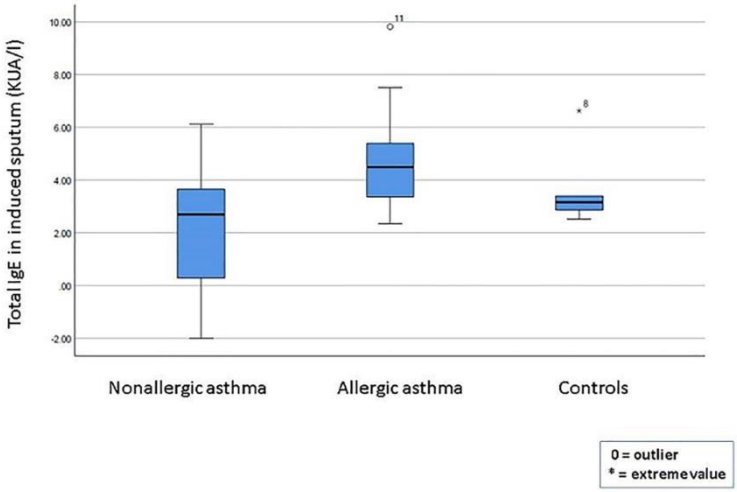

Fig 1. Total IgE in sputum in patients with allergic asthma, non-allergic asthma, and healthy controls.

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rhinitis in nonatopic rhinitis[12–15]. Studies have demonstrated local production of specific IgE in the nostrils of patients with negative skin prick test and undetectable serum IgE[12]. Given the similarities between asthma and rhinitis, it seems highly plausible that patients with nonatopic asthma could also present a local allergic response, particularly considering the lack of a clear physiopathologic explanation for nonallergic asthma. Although there are differences between allergic and nonallergic asthma, these two clinical entities share many similarities, including airway inflammation with eosinophilia, increased Th2 cytokine production, airway-induced exacerbations[5,27]. In addition, up to 30% of patients with nonallergic asthma may present elevated total serum IgE levels[4,5,16]. These shared features suggest that unidentified environmental allergens—which stimulate a local allergic reaction—may be responsible for the symptoms experienced by patients with nonallergic asthma, a hypothesis supported by a small but growing body of evidence showing local airway synthesis of IgE[27], even in patients without any allergen-specific serum IgE[7]. Moreover, the findings from multiple studies that the anti-IgE treatment omalizumab provides symptom relief in nonatopic patients implies that an inflammatory reaction does, in fact, play a role in these patients[8,9,10]. Mouthuy et al.[7] found that nonallergic asthmatics present elevated levels of total and Der p 1-specific IgE in induced sputum compared to healthy controls, a finding that supports the concept of local airway inflammation in those patients. However, we were unable to confirm those findings, as we found no significant differences between the nonallergic asthmatics and healthy controls in IgE levels (both total and Der p 1-specific) in induced sputum or in serum. Our findings were

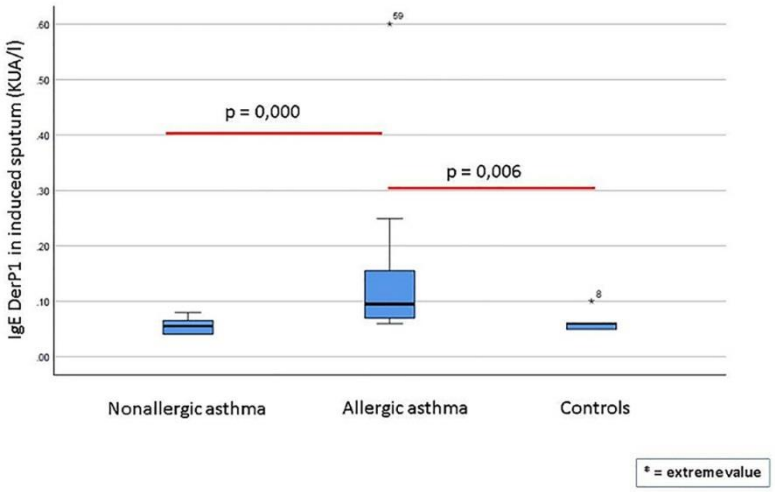

Fig 2. Der p 1-specific IgE levels in sputum in patients with allergic asthma, non-allergic asthma and healthy controls.

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closer to those reported by Manise et al.[28], who—in contrast to Mouthuy et al.—found that total sputum IgE levels were higher in atopic than in nonatopic asthmatics and that there were no differences between nonatopic asthmatics and nonatopic healthy subjects. In view of the heterogeneous findings of these three studies, it is clear that more work will be needed to clarify whether or not there are truly differences among nonatopic and atopic asthmatics and healthy controls with regard to IgE levels in sputum.

There are several reasons that could explain the discrepancy between our results and those reported by Mouthuy et al. First, the lack of any significant differences in IgE levels between

Table 3. Significant correlations between IgE values in serum and induced sputum.

Serum		Sputum		Correlation†
Total IgE	*Der-p* IgE	Total IgE	*Der-p* IgE	
X	X			0.658
	X		X	0.621
X			X	0.538
X		X		0.498
		X	X	0.454

†Spearman's rho; P = 0.000 for all correlations

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the nonallergic asthmatics and healthy controls in our study could potentially be attributed to the size of the control group in our study (n = 9) versus the large control group (n = 25) in the study by Mouthy and colleagues. Another explanation could be related to the ImmunoCAP method, which may not be sufficiently sensitive to detect very small differences in IgE levels. In our sample, although the test did detect higher Der p 1-specific IgE levels in the sputum of allergic asthmatics verus both nonallergic asthmatics and healthy controls, this is probably because there were large differences in Der p 1-specific IgE levels in the allergic asthmatics versus the nonallergic asthmatics and healthy controls given that the study inclusion criteria for those latter two groups specifically required that they have a negative skin prick and negative serum Der p 1. By contrast, the differences in total IgE may have been less marked, making it more difficult to detect using the ImmunoCap technique.

Local IgE production has been documented previously in nonallergic asthma patients using bronchial biopsy[16,29,30]. In this regard, the lack of a significant difference between the three groups in our study with regard to total sputum IgE was surprising, especially considering that —at the very least—allergic asthmatics would be expected to present substantially higher IgE levels than healthy controls. Although the reason for this unexpected finding is not clear, it could be due to the relatively small sample size or to the limited capacity of the ImmunoCap technique to detect small differences in total IgE. In this regard, larger studies will be needed, perhaps using alternative methods to measure sputum IgE.

Correlation between IgE in sputum and serum

In the present study, we used the sputum induction and analysis methods described by Araujo et al.[31], who validated laboratory measurements of total and Der p 1-specific IgE in induced sputum supernatant versus serum levels, but in a heterogeneous sample involving patients with diverse pathologies, not only asthma. In recent years, several studies have correlated total IgE levels in induced sputum and serum, with sometimes contradictory findings[32]. In the present study, we found a highly significant correlation (rho, 0.498; p = 0.000) between total IgE levels in serum and sputum in our overall sample, a finding that is consistent with the results described by Manise et al. in asthmatic patients[28]. By contrast, other authors, including Mouthuy et al.[7] and Ahn et al[33] have not found any correlation between total IgE levels in sputum and serum. These contrasting findings raise further doubts about the sensitivity of the ImmunoCap technique used to measure IgE in sputum, potentially providing an additional explanation for the differences between our results and those of Mouthuy et al.

Data from a recent study conducted by Pillai et al.[34] could help to explain why we were unable to detect significant between-group differences in total IgE in induced sputum. Those authors suggest that IgE produced in the bronchial mucosa of nonatopic asthmatic patients may remain confined to the mucosa, bound to cells that carry those receptors. If this hypothesis is correct, it would explain why IgE is not readily detectable in induced sputum. Pillai and colleagues posited that both atopic and nonatopic asthmatics would have greater total IgE concentrations in the airways than in serum. To test this, they determined IgE levels in the blood and bronchial mucosa of 10 atopic and 10 nonatopic asthmatic patients and 10 nonatopic controls, finding that median total IgE levels were significantly elevated in both the atopic and nonatopic asthmatic patients versus controls. These data are consistent with the hypothesis that IgE synthesis, sequestration, or both are ongoing in the bronchial mucosa of both nonatopic and atopic asthmatic patients. Interestingly, Pillai et al. also suggest, as other several authors have previously proposed[10,35,36], that increased bronchial mucosal IgE production in nonatopic asthmatic patients may be directed against targets other than allergens, including

possible "autoallergens", or that there are allergen-independent roles for IgE in the pathophysiology of asthma.

Overall, the findings reported in studies which use more invasive but more sensitive methods (e.g., bronchial biopsy) strongly suggest the presence of elevated total IgE production in the airways of both allergic and nonallergic asthma patients[10,34–36]. The fact that we were unable to confirm the findings reported by Mouthuy et al. with regard to detecting significant differences in sputum IgE levels between nonatopic asthmatics and healthy controls, together with the contradictory data reported to date regarding the correlation between IgE levels in serum and induced sputum, suggests that more sensitive methods of measuring IgE in sputum may be required.

Total and Der p-specific IgE in induced sputum in healthy individuals

A secondary aim of this study was to determine, on a pilot basis, the standard levels of total and Der p 1-specific IgE in the induced sputum of healthy controls. We found the following values: median (IQR) total IgE in sputum in the controls was 3.16 (1.41) KUA/L. The Der p 1-specific values were 0.06 (0.02). Evidently, the small sample (n = 9) of healthy controls are insufficient to define standard levels, but these data provide an initial estimate. Nevertheless, more data from larger studies will be needed to confirm these initial levels, especially considering the small sample and the potential limitations in the assay technique used to measure IgE in the sputum supernatant.

Study strengths and limitations

The main limitation of the present study is the limited number of healthy controls. Another limitation may be related to the lack of significant differences between the groups in total IgE in induced sputum, which points to limitations in the measurement technique that may have influenced our findings. An important strength of the study is that this is, to our knowledge, only the second study conducted to date to specifically determine total and Der p-specific IgE in induced sputum of allergic, nonallergic, and healthy controls. Given the conflicting results of our study and those reported by Mouthuy et al., additional studies are needed. Finally, another important strength is the well-selected and well-defined sample of patients and controls; we used very strict diagnostic criteria (based on the most recent clinical guidelines) to define both allergic and nonallergic asthma, as well as for the healthy controls.

Conclusions

The findings of this study show that total IgE levels measured in serum and induced sputum are significantly correlated. The significantly higher levels of Der p 1-specific IgE detected in the induced sputum of the allergic asthmatics underscores the potential value of measuring aeroallergen-specific IgE in induced sputum. Nevertheless, the lack of significant between-group differences in total sputum IgE levels suggests that the ImmunoCAP immunoassay technique used in this study may not be sufficiently sensitive to detect small differences in total sputum IgE. To support the results of this work, a larger sample size would be necessary for future studies.

A growing body of evidence indicates that both allergic and nonallergic asthmatics present elevated airway inflammation. Measuring IgE levels in induced sputum is a non-invasive, cost-effective approach that could provide valuable clinical data to help individualize the treatment of nonallergic asthma. However, more sensitive methods are needed to measure IgE levels in induced sputum. Nonetheless, there exists a clear potential to use total and/or aeroallergen-specific IgE levels measured in induced sputum as a marker of treatment efficacy.

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5. RESUMEN GLOBAL DE RESULTADOS

RESUMEN GLOBAL DE RESULTADOS

El conjunto de investigaciones llevadas a cabo muestra la posibilidad de detectar IgE y dos tipos de eosinófilos en el esputo inducido de pacientes asmáticos.

En el primer estudio se muestra cómo mediante citometría de flujo es posible definir dos poblaciones de eosinófilos llamadas E1 ($CD66b^{High}$, $CD15^{High}$) y E2 ($CD66b^{Low}$, $CD15^{Low}$) y su distribución por fenotipos. Se analizaron 62 pacientes de los cuales 24 eran eosinofílicos, 18 mixtos, 10 neutrofílicos y 10 paucigranulocíticos. Las diferencias significativas en variables clínicas analizadas entre los fenotipos fueron una mayor incidencia de tabaquismo o extabaquismo en pacientes neutrofílicos (p=0,040), menores requerimientos de glucocorticoides inhalados en pacientes paucigranulocíticos (p=0,033) y mayor incidencia de poliposis nasal en pacientes eosinofílicos y mixtos (p=0,042).

En la población total se encontró, de media, un 14,24% de E1 (desviación estándar 19,79%) y un 4,71% de E2 (desviación estándar 6,54%) sobre el total de leucocitos medidos por citometría de flujo. La distribución de cada tipo de eosinófilos por fenotipo puede verse en la tabla 2.

Tabla 2. Distribución de cada población de eosinófilos (E1 y E2) en los fenotipos inflamatorios de esputo inducido. *Se encontraron diferencias entre paucigranulocíticos y eosinofílicos (p=0,020) y entre neutrofílicos y eosinofílicos (p=0,024).

	Paucigranulocíticos (n =10)	**Neutrofílicos** (n=10)	**Eosinofílicos** (n=24)	**Mixtos** (n=18)	**p**
%E1 (DE)	3,25 (7,58)	3,85 (7,59)	20,86 (24,91)	14,42 (16,11)	**0.034***
%E2 (DE)	2,46 (3,13)	2,51 (2,58)	6,27 (8,00)	4,31 (6,52)	0.302

En el estudio de correlaciones, E1 se correlacionó con la FeNO (r=0,357 p=0,05) y los eosinófilos en sangre (r=0,382 p=0,003), y E2 con la puntuación del ACT (r=0,388 p=0,021). Los niveles de IL-5 en el sobrenadante se correlacionaron con los eosinófilos en microscopía o citometría de flujo (r=0,885, p=0,019; y r=0,975, p=0,001 respectivamente), de manera inversa con los neutrófilos por microscopía (r=-0,858 p=0,029), y directa con E1 (r=0,971 p=0,001) pero no mostraron correlación con E2 (r=0,571 p=0,232).

En el segundo estudio se analizaron 56 pacientes asmáticos (21 alérgicos y 35 no alérgicos) y 9 controles sanos. Al analizar las diferencias entre grupos solo se detectó mejor función pulmonar medida por FEV_1 en los controles respecto a los asmáticos (0=0,013) y mayores requerimientos de glucocorticoides orales en el año previo en los pacientes no alérgicos respecto a los alérgicos (p= 0,022).
Tras analizar los niveles de IgE total y específica para Der p 1, los pacientes alérgicos presentaron niveles más elevados en sangre que los otros dos grupos. En esputo inducido no se encontraron diferencias estadísticamente significativas entre los grupos en la IgE total, pero la IgE específica fue más elevada en el grupo de alérgicos que en los otros dos (tabla 3)

Tabla 3. IgE total y específica (sIgE) para Der p 1 en sangre y en esputo en cada uno de los grupos analizados.

		Asma no alérgica (N=21)	Asma alérgica (N=35)	Controles (N=9)	p
Sangre (kU/L)	**IgE total**	55.5 (177.93)	233.5 (368.25)	14.25 (38.30)	**<0.001**
	sIgE Der p 1	0.01 (0.03)	15.45 (32.68)	0.02 (0.04)	**<0.001**
Esputo inducido (kU/L)	**IgE total**	2.69 (4.55)	4.5 (2.18)	3.16 (1.41)	0.188
	sIgE Der p 1	0.055 (0.03)	0.095 (0.09)	0.06 (0.02)	**<0.001**

Las correlaciones más consistentes se encontraron entre la IgE total y específica en sangre, y entre la IgE específica en sangre y esputo (ver tabla 4).

Tabla 4. Correlación entre mediciones de IgE en sangre y en esputo, total y específica para Der p 1. En todas las correlaciones, p<0,001.

			Esputo inducido	
			Total	Específica
			0.454	
Sangre	Total	0.658	0.498	0.538
	Específica		NS	0.621

6. Resumen global de la discusión

6.1. Discusión general

Esta Tesis Doctoral muestra que los pacientes con asma alérgica presentan dos tipos de eosinófilos diferentes en esputo inducido y que sus niveles de IgE específica local son mayores que en los pacientes no alérgicos.

En el primer estudio se muestra la metodología de citometría de flujo que permite identificar los dos tipos de eosinófilos, E1 y E2, en esputo inducido. Se ha usado una nomenclatura independiente de iEOS y rEOS debido a que los marcadores utilizados para su discriminación son diferentes a los utilizados en otras publicaciones que los identifican en sangre. Habitualmente estos estudios se basan en la expresión de CD62L (56,57) pero esta molécula se pierde una vez que el eosinófilo atraviesa el endotelio (93). Un estudio reciente realizado en ratones fue incapaz de detectar CD62L en eosinófilos residentes pulmonares, reforzando esta teoría (94) y otro estudio realizado sobre eosinófilos residentes en pólipos nasales mostró un predominio de baja expresión de CD62L (95). Nuestro método se basa en la expresión de CD66b (96) y CD15 (97), moléculas implicadas en la activación y adhesión de los eosinófilos.

Los niveles de E2 fueron bajos y similares entre los diferentes fenotipos, pero los niveles de E1 estaban elevados en los pacientes con eosinofilia bronquial, es decir, en los fenotipos eosinofílico y mixto. Los niveles de E1 se correlacionaron con IL-5 en el sobrenadante, la FeNO y los eosinófilos en sangre. Estos hechos nos llevan a teorizar que E1 se corresponde con los iEOS de otros trabajos, que también han mostrado relación con los niveles sanguíneos de IL-5 y son los predominantes en el asma alérgica (56). Conocer la relación de iEOS y rEOS con los diferentes fenotipos puede tener implicaciones

terapéuticas, especialmente porque hoy en día se dispone anticuerpos monoclonales dirigidos contra diferentes interleucinas eosinofílicas (anti IL-5 o anti IL4/IL-13) con efectos probablemente diferentes sobre cada tipo de eosinófilo.

El segundo estudio se basa en la medición de IgE total y específica para Der p 1 en sangre y en sobrenadante de esputo inducido en pacientes con asma alérgica, asma no alérgica y controles sanos. Los niveles séricos están bien descritos en otros estudios y nuestros hallazgos coinciden con los previamente reportados (98,99). A nivel de esputo inducido, sin embargo, hay dos hallazgos relevantes.

El primero es que los niveles de IgE total en esputo inducido fueron similares entre voluntarios sanos, asmáticos no alérgicos y alérgicos. Estudios previos han mostrado niveles similares entre sanos y asmáticos no alérgicos, pero menores que en pacientes alérgicos (100) o niveles similares entre asmáticos independientemente de la alergia, pero superiores a los de los controles sanos (101).

El segundo hallazgo es que, en nuestra muestra, la IgE específica sí fue más elevada en los pacientes alérgicos que en los otros dos grupos. Esta comparación también ha sido reportada por otro grupo de investigadores, pero ellos detectaron niveles más elevados en los asmáticos, ya fueran alérgicos o no, que en los controles sanos. En este estudio, sin embargo, en los pacientes asmáticos no alérgicos la provocación con extracto de ácaros del polvo no desencadenó una respuesta clínica ni inflamatoria detectable (101).

Estas discrepancias muestran la necesidad de ampliar los conocimientos sobre las dinámicas de exudación, producción local de IgE, su posible bloqueo por unión a los receptores celulares (102) y su significado clínico. Los niveles elevados de IgE total y específica en pacientes alérgicos parecen intrínsecos a la patología, pero *a priori* en

pacientes no alérgicos sería esperable detectar niveles similares a pacientes sanos, como ocurre en nuestro estudio. Sin embargo, hay algunos indicios de la implicación de la IgE en el asma no alérgica que justificarían las evidencias de respuesta al tratamiento con omalizumab (103), como la capacidad de esta inmunoglobulina de modular el cambio de isotipo de células B (104), la mayor expresión del receptor de alta afinidad por la IgE en las células plasmáticas en relación a las infecciones víricas (105), o la activación de los basófilos por la IgE (101), en el asma no alérgica. Además, en esta situación suelen quedar dudas sobre la posible implicación de un alérgeno poco frecuente, o la posibilidad de que se trate de un caso de alergia local. Ambas situaciones pasarían desapercibidas como asmáticos no alérgicos con los estudios diagnósticos de alergia recomendados en la práctica clínica habitual y engrosarían el grupo de pacientes catalogados en los estudios como “no alérgicos”.

6.2. Aportaciones e implicaciones clínicas

Este trabajo aporta nuevos conocimientos sobre factores determinables en esputo inducido y de características fenotípicas específicas de la inflamación en el asma alérgica.

Se describe por primera vez la presencia en esputo inducido de los dos tipos de eosinófilos ya descritos en otras muestras biológicas y se detalla la metodología necesaria para su identificación en citometría de flujo, de manera que otros equipos de investigadores puedan aplicarla en las situaciones que consideren convenientes, tanto para replicar externamente nuestros resultados como para utilizar este método en otras patologías o situaciones clínicas. La investigación sobre tipos de eosinófilos en el asma es muy incipiente y quedan muchos detalles por

describir, por lo que creemos que será una vía de investigación clave para comprender, por ejemplo, el porqué del fracaso terapéutico de los fármacos biológicos en algunos pacientes.

De manera similar, ampliar los conocimientos sobre la IgE local en la vía aérea aporta información sobre la cascada inflamatoria bronquial, tanto en asmáticos alérgicos como no alérgicos, ayudando a comprender un eslabón más de la compleja fisiopatología del asma. Con el concepto de alergia respiratoria local cada vez más aceptado es necesario poder discriminar con precisión pacientes alérgicos de no alérgicos para seleccionar el tratamiento más adecuado, y para ello la determinación de biomarcadores locales será clave en la consolidación de esta teoría.

Estos trabajos ratifican la importancia del esputo para identificar la eosinofilia, difícil de predecir por otros métodos, y su utilidad para realizar otras mediciones. Hoy en día existen tratamientos muy eficaces para tratar el asma eosinofílica, pero sin el esputo inducido habrá pacientes que nunca tendrán la posibilidad de a recibirlos si la fenotipación se limita a métodos sistémicos.

6.3. LIMITACIONES

Las limitaciones principales de esta Tesis Doctoral se derivan de su diseño metodológico, que es de tipo unicéntrico y observacional. Este diseño restringe la posibilidad de extrapolar los resultados obtenidos y extraer conclusiones de tipo causa-efecto. Debido al coste y la complejidad del esputo inducido, los tamaños muestrales son pequeños, y algunas determinaciones solo pudieron ser realizadas en los escasos pacientes que alcanzaron la calidad de la muestra

necesaria. Sería recomendable reproducir los análisis en un grupo externo de validación para confirmar los resultados obtenidos.
Debido a que en dos de los estudios se realizan mediciones poco habituales o validadas (fenotipos de eosinófilos e IgE local), sería recomendable disponer de controles sanos, o un número mayor de ellos en el caso del segundo estudio, de cara a compararlos con los pacientes asmáticos.

Las mediciones de la IgE local en mucosa nasal, mucho más accesible que las muestras bronquiales, por el momento tampoco ha dado resultados útiles en el estudio diagnóstico de pacientes con rinitis, por lo que son necesarios otros métodos más sensibles para detectar esta inmunoglobulina.

En los estudios realizados en pacientes asmáticos siempre subyace la duda del posible efecto que pueda estar teniendo el tratamiento inhalado y su influencia sobre los biomarcadores medidos. El alto grado de incumplimiento y la dificultad para valorarlo de forma objetiva pueden dar lugar a sesgos de interpretación a este respecto.

7. CONCLUSIONES

CONCLUSIONES

1. La citometría de flujo, mediante la expresión de CD66b y CD15, permite identificar dos poblaciones de eosinófilos, que hemos llamado E1 y E2.

2. E1 probablemente denomina a eosinófilos inflamatorios y E2 homeostáticos. E1 predomina en asma alérgica eosinofílica y se correlaciona con los niveles de IL-5 en el sobrenadante, la FeNO y los eosinófilos en sangre.

3. Es posible detectar IgE total y específica en sobrenadante de esputo inducido. Los pacientes con asma alérgica presentan niveles más elevados de IgE específica, pero no de IgE total, que los pacientes no alérgicos o los controles sanos.

4. Los niveles de IgE específica local se correlacionan con la IgE total en sangre y en esputo.

8. LÍNEAS DE FUTURO

LÍNEAS DE FUTURO

La presente Tesis Doctoral se basa en mediciones novedosas realizadas sobre las muestras obtenidas a través de la inducción de esputo. Por ello, abre la posibilidad de aplicar estas técnicas para valorar, tanto la IgE local como los eosinófilos inflamatorios y residentes, en otras situaciones, como diferentes poblaciones de pacientes asmáticos o escenarios terapéuticos. En el momento actual ya se está trabajando en la redacción de un manuscrito que compara la distribución de los fenotipos de eosinófilos en asma alérgica y no alérgica, con una metodología similar a la detallada en el primer trabajo, con resultados prometedores.

Estas determinaciones necesitan estudios de validación y correlación entre sangre y tejidos en diferentes órganos y sistemas, y comparaciones entre pacientes sanos y situaciones patológicas. Debido a su novedad, se desconoce la estabilidad temporal de estas mediciones y los efectos de factores internos o externos, como las exposiciones, los tratamientos (glucocorticoides inhalados, anticuerpos monoclonales…), la diferencias entre géneros, la coexistencia de otras patologías, el envejecimiento, etc.; por lo que serían necesarios estudios prospectivos con mediciones repetidas y comparadas.

9. BIBLIOGRAFÍA

BIBLIOGRAFÍA

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10. Anexo

10.1. ARTÍCULO ADICIONAL

CHARACTERISTICS OF INDUCED-SPUTUM INFLAMMATORY PHENOTYPES IN ADULTS WITH ASTHMA: PREDICTORS OF BRONCHIAL EOSINOPHILIA.

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ORIGINAL RESEARCH

Characteristics of Induced-Sputum Inflammatory Phenotypes in Adults with Asthma: Predictors of Bronchial Eosinophilia

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Purpose: The objectives of this study were, for patients attending a specialist asthma clinic at a tertiary care hospital, to determine, from sputum induction (SI), proportions of bronchial inflammatory phenotypes, demographic, clinical and functional characteristics of each phenotype, and the most accessible non-invasive inflammatory marker that best discriminates between phenotypes.

Patients and Methods: Included were 96 patients with asthma, attending a specialist asthma clinic at a tertiary care hospital, who underwent testing as follows: SI, spirometry, fractional exhaled nitric oxide (FeNO), blood eosinophilia, total immunoglobulin E (IgE), and a skin prick test.

Results: SI phenotypes were 46.9% eosinophilic, 33.3% paucigranulocytic, 15.6% neutrophilic, and 4.2% mixed. No significantly different clinical or functional characteristics were observed between the phenotypes. A positive correlation was observed between SI eosinophilia and both emergency visits in the last 12 months ($p = 0.041$; $r = 0.214$) and FeNO values ($p = 0.000$; $r = 0.368$). Blood eosinophilia correlated with SI eosinophilia ($p = 0.001$; $r = 0.362$) and was the best predictor of bronchial eosinophilia, followed by FeNO, and total blood IgE (area under the receiver operating characteristic curve (AUC-ROC) 72%, 65%, and 53%, respectively), although precision was only fair.

Conclusion: In consultations for severe asthma, the most frequent phenotype was eosinophilic. Peripheral blood eosinophilia is a reliable marker for discriminating between different bronchial inflammatory phenotypes, is useful in enabling doctors to select a suitable biologic treatment and so prevent asthma exacerbation, and is a better predictor of bronchial eosinophilia than FeNO and IgE values.

Keywords: asthma, sputum induction, phenotype, eosinophilia

Introduction

Sputum induction (SI), the gold standard for evaluating bronchial inflammation in patients with asthma, is a non-invasive, standardized, and validated test[1,2] that distinguishes between 4 bronchial inflammatory phenotypes: eosinophilic, paucigranulocytic, neutrophilic, and mixed.[3] This technique, however, is not available in all hospitals as it requires trained personnel and a suitable infrastructure; therefore, other more accessible markers are used in current clinical practice, such as eosinophil count in peripheral blood and fractional exhaled nitric oxide (FeNO) measurement.

Especially important for severe asthma is phenotype identification, as it enables an individualized approach to treatment.[4,5] Several studies have confirmed that eosinophilic airway inflammation predicts response to anti-inflammatory treatment with both inhaled corticosteroids[6,7] and biologics.[8–13] Indeed, the main clinical guidelines for asthma management propose using SI to evaluate severe asthma[14,15] and to manage severe uncontrolled asthma treatment

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if the patient is clinically followed up in suitably equipped centers.[14] However, the fact that SI is laborious and requires experienced personnel explains current attempts to identify non-invasive markers that discriminate between different bronchial inflammatory phenotypes in a simple and cost-effective way. While peripheral blood eosinophilia is a marker that predicts airway eosinophilia,[16] a common cut-off point has not been established, and correlations between blood and SI eosinophilia vary widely.[16–18] Studies have also reported a relationship between blood eosinophilia and the risk of severe exacerbations, decreased lung function, responsiveness to corticosteroid treatment, and predicted efficacy of some biologic treatments.[10–13,19–21] While FeNO is a non-invasive marker that reflects eosinophilic inflammation,[22,23] certain variables may modify its value,[24,25] and its correlation with bronchial and peripheral blood eosinophilia is also variable.[26–29]

Our study objectives, for a population of patients with asthma attending a specialist asthma clinic attached to a tertiary care hospital, were as follows: (a) to determine, from an SI test, the different proportions of bronchial inflammatory phenotypes and their demographic, clinical, and functional characteristics and (b) to identify an accessible non-invasive inflammatory marker used in routine clinical practice that discriminates between the different bronchial inflammatory phenotypes.

Materials and Methods

Our cross-sectional descriptive study included 96 patients, aged 18–80 years old. Patients were consecutively enrolled from our tertiary care university hospital's severe asthma outpatient unit (located in Spain) for evaluation in 2018 and 2019. All the patients complied with asthma diagnostic criteria according to Global Initiative for Asthma (GINA) guidelines.[15] Excluded were smokers and patients who had experienced respiratory infections or required oral corticosteroids in the previous month.

Demographic, clinical, and functional data were collected for the 96 patients, and on the same day, the following procedures were carried out: skin prick test, total blood immunoglobulin E (IgE), absolute eosinophil count, SI inflammatory cell count, and forced expiratory volume in 1 second (FEV1), forced vital capacity (FVC), and FeNO measurements. Patients were also asked to complete the Asthma Control Test (ACT)[30] and Asthma Quality of Life Questionnaire (AQLQ).[31] The skin prick test, performed for common local aeroallergens according to the standard procedure,[32] was considered positive when papule diameter was >3 mm. Total blood IgE was determined using the enzyme-linked immunosorbent assay (ELISA) method (UNICAP, Pharmacia, Uppsala, Sweden) and was considered increased for values >160 IU/mL. Used to assess asthma control was the ACT, a self-assessment questionnaire validated in Spanish,[30] with good control considered to be >20 points. Quality of life (QoL) was assessed using the self-administered AQLQ, likewise validated in Spanish.[32] Spirometry measurements were made with a Datospir-600 device (Sibelmed SA, Barcelona, Spain) by an experienced technician and following the 2005 recommendations of the American Thoracic Society/European Respiratory Society (ATS/ERS);[33] FEV1 >80% was considered to be in the reference range of the theoretical value.[34] FeNO, following ATS/ERS 2005 recommendations[35] and using an electrochemical analyzer (NO Vario Analyzer, Filt Lungen- und Thoraxdiagnostik GmbH, Berlin, Germany), was measured at a flow of 50 mL/s and was considered to be significantly increased when values were >50 ppb.[36] Sputum samples were obtained and processed according to the method described by Djukanović et al,[1] and patients were classified according to cell counts as follows: eosinophilic if eosinophils ≥3%, neutrophilic if neutrophils ≥61%, paucigranulocytic if neutrophils <61% and eosinophils <3%, and mixed if neutrophils ≥61% and eosinophils ≥3%.[6] An absolute eosinophil count of ≥300 cells/μL was taken to define blood eosinophilia.[14]

Ethical and Legal Aspects

The study complied with the principles of the Declaration of Helsinki (18th World Medical Assembly, 1964) and was approved by the Clinical Research Ethics Committee of Hospital Santa Creu i Sant Pau in Barcelona. The patients provided their written consent prior to participation in the study and all study data were anonymized.

Statistical Analysis

Categorical variables were expressed as absolute and relative frequencies and quantitative variables as mean and standard deviation (SD) values. Between-group comparisons were analyzed using analysis of variance (ANOVA) for quantitative variables, and the chi-square or McNemar test for categorical variables, as appropriate. The area under the receiver operating characteristic curve (AUC-ROC) of the biomarkers used to detect the eosinophilic inflammatory phenotype was calculated using a combined impact model (general linear model; GLM), and Pearson's test was used for correlation analyses of the studied population.

The results were considered significant for $p < 0.05$. The analysis was performed with SPSS version 26.0 (SPSS Inc., Chicago, IL, USA).

Results

SI Inflammatory Phenotype Proportions and Characteristics

Of the 96 patients who underwent the SI test, almost half were eosinophilic (n = 45; 46.9%), around a third were paucigranulocytic (n = 32; 33.3%), and the remainder were neutrophilic (n = 15; 15.6%) or mixed (n = 4; 4.2%).

Demographic, clinical, and functional characteristics are summarized in Table 1. Overall mean age was 50 years. No significant differences were observed regarding sex, body mass index (BMI), asthma severity, disease control, emergency

Table 1 Demographic, Clinical, and Functional Characteristics for 4 Inflammatory Phenotypes Identified by SI (n = 96)

	Eosinophilic (n=45)	Paucigranulocytic (n=32)	Neutrophilic (n=15)	Mixed (n=4)	p
Age, mean (SD) years	52.1 (14.7)	51.2 (14)	53.2 (17.9)	57 (26.2)	0.900
Women, %	71.1%	62.5%	46.7%	25%	0.143
Childhood asthma diagnosis, %	24.4%	21.9%	20%	25%	0.984
Severe persistent asthma, %	53.3%	34.4%	33.3%	50%	0.674
GINA 2021 asthma treatment steps, %	STEP 1–2: 6.7%	STEP 1–2: 12.9%	STEP 1–2: 15.9%	STEP 1–2: 50%	0.671
	STEP 3: 28.9%	STEP 3: 22.5%	STEP 3: 21%	STEP 3: 0%	
	STEP 4: 35.5%	STEP 4: 38.8%	STEP 4: 21%	STEP 4: 0%	
	STEP 5: 28.8%	STEP 5: 25.8%	STEP 5: 21%	STEP 5: 50%	
Poor asthma control (ACT <20), %	15.6%	12.5%	6.7%	50%	0.371
ED visits in previous 12 months, mean (SD)	1.4 (2.2)	1.1 (1.7)	1.3 (2.6)	3.5 (5.7)	0.291
AQLQ, mean (SD)	3.3 (2.3)	3.1 (3.2)	1.8 (2.1)	8.1	0.162
BMI, mean (SD) kg/m²	27.1 (4.2)	27.6 (5.6)	24.8 (3.6)	24.8 (5.7)	0.201
Nasal polyposis, %	24.4%	9.4%	13.3%	0%	0.250
Rhinitis, %	68.9%	68.8%	53.3%	25%	0.243
FEV1, mean (SD) %	83.7 (21)	103.9 (98.9)	80.9 (17.5)	77.5 (16)	0.433
BDT, %	26.7%	15.6%	33%	50%	0.327
Prick test +, %	68.9%	71.9%	60%	100%	0.476
FeNO, mean (SD) ppb	46.1 (37.2)	33.4 (26.5)	29.8 (27.4)	30.2 (12.9)	0.207

(*Continued*)

Table 1 (Continued).

	Eosinophilic (n=45)	Paucigranulocytic (n=32)	Neutrophilic (n=15)	Mixed (n=4)	p
Total IgE, mean (SD) IU/mL	460.4 (770.8)	276 (301.4)	241.9 (503)	239.2 (349.1)	0.454
Blood eosinophils, mean (SD) cells/μL	360 (300)	230 (100)	230 (100)	320 (800)	0.057[a] 0.027[b]
Eosinophils, mean (SD) %	5.4 (4)	3.5 (2.1)	3.5 (1.9)	3.7 (1)	0.041[a] 0.021[b]
Dose of inhaled corticosteroids, %	Medium 37.8% and high 28.9%	Medium 37.5% and high 28.1%	Medium 20% and high 40%	High 75%	0.587
Eosinophils, mean (SD) %	12.4 (12.3)	0.79 (0.7)	1.17 (0.9)	9.2 (5.8)	0.000
Neutrophils, mean (SD) %	38 (17.7)	36.6 (18)	76.2 (7.6)	72 (3.4)	0.000
Macrophages, mean (SD) %	48.35 (18.7)	59.8 (17.8)	19.8 (8.7)	17 (8.5)	0.000
Lymphocytes, mean (SD) %	0.89 (0.6)	0.93 (0.6)	0.91 (0.4)	1.58 (1.4)	0.207

Notes: [a]Significance comparing the 4 groups. [b]Significance comparing eosinophilic, paucigranulocytic, and neutrophilic phenotype groups.
Abbreviations: ACT, asthma control test; AQLQ, Asthma Quality of Life Questionnaire; BDT, bronchodilator test; BMI, body mass index; ED, emergency department; FeNO, fractional exhaled nitric oxide; FEV1, forced expiratory volume in 1 second; GINA, Global Initiative for Asthma; IgE, immunoglobulin E; SD, standard deviation; SI, sputum induction.

department (ED) visits in the past 12 months, QoL as measured by AQLQ, bronchial obstruction (FEV1), associated rhinitis or nasal polyposis, total blood IgE, FeNO, or inhaled corticosteroid dose. In contrast, significant differences were observed between the different phenotypes in peripheral blood eosinophil percentages and counts, which were significantly higher in the eosinophilic group.

Asthma Severity

In the analysis by asthma severity, the predominant inflammatory phenotypes were as follows: paucigranulocytic with intermittent asthma, 46.6%; eosinophilic with mild persistent asthma, 45%; eosinophilic with moderate persistent asthma, 36%; and eosinophilic with severe persistent asthma, 57.14% ($p = 0.674$).

Variable Correlations for the Studied Population

For the 96 patients, positive correlations were observed between SI eosinophilia and ED visits in the previous 12 months ($p = 0.041$; $r = 0.214$), between SI eosinophilia and FeNO values ($p = 0.000$; $r = 0.368$), and between SI eosinophilia and peripheral blood eosinophilia ($p = 0.001$; $r = 0.362$). We interpreted discriminatory capacity as follows (see Figure 1): $r = 0.5$, equivalent to a coin toss; $r = 0.5–0.6$, poor; $r = 0.6–0.75$, fair; $r = 0.75–0.9$, good; and $r = 0.9–0.97$, very good.

SI Eosinophilia ≥3% Detection in the Combined Model

The AUC-ROC values that detected SI eosinophilia ≥3% were as follows: absolute blood eosinophils (EOS), 72% ($p = 0.000$); FeNO, 65% ($p = 0.014$); and total blood IgE, 53% ($p = 0.590$).

Discussion

In patients attending our specialist asthma clinic at a tertiary care hospital, the eosinophilic bronchial inflammatory phenotype was predominant, and there were no significant differences in clinical and functional characteristics for the various bronchial inflammatory phenotypes. Furthermore, our study supports the following findings: (a) peripheral blood eosinophilia is a marker that enables the eosinophilic inflammatory phenotype to be differentiated from other bronchial inflammatory phenotypes, although note that precision is only fair according to the AUC-ROC; and (b) while positive

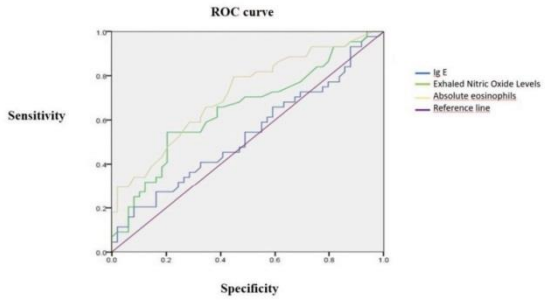

Figure 1 AUC-ROC plot from EOS, FeNO, and total IgE values in a combined model (n=96).
Abbreviations: AUC-ROC, area under the receiver operating characteristic curve; EOS, absolute eosinophils; FeNO, fractional exhaled nitric oxide; IgE, immunoglobulin E.

correlations exist between SI eosinophilia and both FeNO and blood eosinophil values, they were not strong, suggesting a possible activation of various inflammatory pathways in patients with asthma. This would point to the need for a more comprehensive approach to asthma management that goes beyond mere biomarker threshold positivity.

Our AUC-ROC value for detecting SI eosinophilia ≥3% was slightly higher than documented in the literature. Hastie et al[39] reported a value of 69% for detection of SI eosinophilia ≥2% and concluded that while there was an association between blood eosinophilia and SI eosinophilia, precision in terms of correct classification of patients with and without an eosinophilic phenotype was poor, and, furthermore, that this poor precision persisted when biomarkers such as FeNO and IgE were also considered.

The usefulness of blood eosinophilia is supported by several studies. Wagener et al[16] showed that blood eosinophilia was an accurate biomarker of eosinophilic airway inflammation in 2 independent cohorts of patients with asthma (AUC 89%; $p < 0.001$; sensitivity 78% and specificity 91%), while Schleich et al[3] reported that blood eosinophilia ≥220/mm3 enabled SI eosinophilia ≥3% to be detected with 77% sensitivity and 70% specificity (AUC 0.79, $p < 0.0001$).

There is no consensus regarding the blood eosinophilia cutoff value to define the eosinophilic phenotype, and, although current evidence points to 150–300 cells/μL, that range of values is still a matter of debate.[16,18] Note that, in patients with severe asthma, part of the variability reported in different studies is explained by possible variations in blood and SI eosinophilia depending on doses of inhaled or systemic corticosteroids.[4,37] Variability may also result from factors such as smoking (OR = 6.44; $p = 0.013$) and having had a recent asthma exacerbation (OR = 5.84; $p = 0.022$).[38]

While we found positive correlations between SI eosinophilia and both ED visits in the previous 12 months ($p = 0.041$; $r = 0.214$) and FeNO values ($p = 0.000$; $r = 0.368$) and between SI eosinophilia and peripheral blood eosinophilia ($p = 0.001$; $r = 0.362$), none of those correlations were sizeable, indicating no linear relationship between the variables. Another study found better correlation for patients with asthma when the comparison was based on ≥300 cells/μL in peripheral blood and SI eosinophilia ≥2% ($p = 0.0002$; $r = 0.5235$).[40]

The growing importance attached to blood eosinophilia is because it is the most relevant marker for both the choice of, and response to, biologic treatments for severe uncontrolled eosinophilic asthma. The fact that the vast majority of studies use peripheral blood eosinophilia and not SI eosinophilia as the biomarker of choice for a biologic is because not all hospitals have the facilities necessary for SI cell counting.[9,41]

Peripheral blood eosinophilia has been demonstrated to be a marker of a better response to biologics. For instance, it is the key biomarker for measuring response to mepolizumab, a monoclonal antibody against interleukin-5 (IL-5), with exacerbations greatly reduced (73%) in patients with blood eosinophils ≥500/μL.[9,41] Efficacy of another intravenously administered antibody against IL-5, reslizumab, has also been demonstrated for patients with blood eosinophilia ≥400/

μL.[42] Finally, benralizumab, an antibody that acts against the IL-5 receptor through apoptosis of eosinophils and basophils, has been shown to reduce exacerbation rates in patients with blood eosinophilia ≥300/μL.[9,20]

Blood eosinophilia can also predict response to treatment with both inhaled and systemic corticosteroids[14] and can help adjust oral corticosteroid dosage for patients with severe asthma, as demonstrated by Wark et al,[43] who reported that blood eosinophilia maintained at <200 cells/μL prevented exacerbations, improved asthma control, and enabled lower oral corticosteroid doses.

In our series, the FeNO value was less correlated with SI eosinophilia (AUC-ROC 65%) than in the review by Korevaar et al,[44] which included 12 studies with a combined sensitivity of 66% and specificity of 76% in detecting SI eosinophilia ≥3% (AUC 0.74; 95% confidence interval (CI): 0.70–0.78). This difference is possibly explained by the multiple factors that modify FeNO values, such as allergic rhinitis, upper and lower respiratory tract viral infections, age, tobacco use, and atopy;[25–28] it may also be due to the higher sensitivity but lower specificity of FeNO, resulting in high negative predictive values but low positive predictive values for eosinophilic inflammation.[45]

However, it is important to understand the complexity of inflammatory mechanisms in type 2 inflammation in different patients, so it is recommended to simultaneously measure several biomarkers (EOS, IgE, FeNO) to identify potential targets for treatment with biologics. A post-hoc analysis of the QUEST study[46] that assessed dupilumab efficacy by biomarker subgroups, as defined by GINA,[15] found that reference blood eosinophilia and FeNO levels clearly pointed to similar disease severity at the outset in all subpopulations (EOS ≥150 cells/μL, FeNO ≥20 ppb, and both EOS ≥150 cells/μL and FeNO ≥20 ppb).[47] In a study of 110 patients published by our working group, we reported a dissociation between increased FeNO (≥50 ppb) and SI eosinophilia in 42% of patients; that study identified 2 groups with discordant values: a younger group mainly associated with a paucigranulocytic phenotype and atopy, with high FeNO and no SI eosinophilia, and with better FEV1, and an older group mainly associated with a non-allergic eosinophilic phenotype, with low FeNO and high SI eosinophilia, and accounting for more ED visits in the previous 12 months.[48] Those data support the existence of different activation patterns in underlying inflammatory pathways in patients with asthma, suggesting the need for a more comprehensive and more personalized approach to management that goes beyond mere biomarker threshold positivity.

Use of IgE as a biomarker of eosinophilia is poorly supported. In our study, total IgE was the weakest predictor of SI eosinophilia, corroborating other studies[45–47] reporting low sensitivity, specificity, and AUC values for IgE. Demarche et al,[49] in particular, indicated that IgE alone does not adequately predict SI eosinophilic status. Westerhof et al[17] proposed joint use of blood eosinophilia and FeNO to improve airway predictions of eosinophilia (AUC 0.87; p = 0.027), while Demarche et al[49] proposed the joint use of blood eosinophilia, FeNO, and IgE, as an approach that, in their study, identified 58% of patients with a high or low probability of having SI eosinophilia ≥3%, and that correctly classified 87% of those patients.

Regarding the distribution of inflammatory phenotypes in our study, the eosinophilic phenotype predominated (almost half), followed by the paucigranulocytic phenotype (around a third); this finding corroborates another large series,[3] but contradicts other studies that reported predominance of the paucigranulocytic phenotype.[50] The difference is possibly explained by the fact that the studies in which the paucigranulocytic phenotype predominated were of patients whose asthma was less severe than that of patients recruited in specialist asthma clinics.

We found no significant differences in clinical and functional characteristics between the bronchial inflammatory phenotypes. This finding differs from that of Schleich et al,[3] who reported that the eosinophilic phenotype was associated with atopy, bronchial hyperresponsiveness, poorer control, a reduced FEV1/FVC ratio, increased FeNO and IgE values, and nasal polyposis.[51] The difference may reflect sample size: 96 in our series compared to 508 in the study by Schleich et al.[3] Note that there is probably a significant overlap in biomarker positivity in patients with asthma[52] that may suggest no differentiating characteristics according to inflammatory phenotype. This issue needs to be addressed through more studies, as relevant pathogenic knowledge is required for an era of biologic monoclonals and more personalized medicine.

Another result to highlight from our study was the positive correlation between SI eosinophilia and ED visits in the previous 12 months, possibly comparable to the poorer asthma control of the eosinophilic phenotype reported by

Schleich et al,[3] and in line with the established fact that eosinophilia is a predictor of exacerbations in patients with asthma.[52]

As limitations, our cross-sectional descriptive study may incur possible selection bias; all our patients were required to have undergone specific testing for inclusion, and were patients with predominantly moderate-severe persistent asthma attending a specialist clinic in a tertiary care hospital. Another limitation is the small sample size compared to other studies (for instance, those by Schleich et al[3] and Abdo et al[53]), and, within our sample, the fact that the patients with a mixed inflammatory phenotype were so few that we were unable to characterize them; note, however, that the mixed phenotype prevalence rate in our study reflects that of other studies.[3] A strength of our study is that inflammatory phenotypes were identified on the basis of SI, and comparisons were possible with biomarkers used in typical asthma consultations, such as peripheral eosinophilia and FeNO.

The main conclusions of our study of 96 patients with asthma attending our specialist asthma clinic at a tertiary care hospital are as follows: (a) the predominant bronchial inflammatory phenotype was eosinophilic, and there were no significant differences in clinical and functional characteristics between the 4 different bronchial inflammatory phenotypes; and (b) peripheral eosinophilia detected SI eosinophilia ≥3% with greater diagnostic accuracy than markers such as FeNO and total IgE and was also the only marker that distinguished the eosinophilic phenotype from the other inflammatory phenotypes.

While the SI cell count is the gold standard for non-invasive evaluation of bronchial inflammation in patients with severe asthma and a useful test to guide the choice of biologic treatment, our study would suggest peripheral blood eosinophilia as an alternative when this test is not available, given that, as a good marker for detection of the eosinophilic inflammatory phenotype, it can be potentially useful for doctors to select a suitable biologic treatment and prevent asthma exacerbations.

Abbreviations

ACT, Asthma Control Test; ANOVA, analysis of variance; ATS, American Thoracic Society; AQLQ, Asthma Quality of Life Questionnaire; AUC, area under the curve; BMI, body mass index; CI, confidence interval; ED, emergency department; ELISA, enzyme-linked immunosorbent assay; EOS, absolute eosinophils; ERS, European Respiratory Society; FeNO, fractional exhaled nitric oxide; FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity; GEMA, Spanish Asthma Management Guidelines; GINA, Global Initiative for Asthma; IgE, immunoglobulin E; IL-5, interleukin-5; QoL, quality of life; ROC, receiver operating characteristic; SD, standard deviation; SI, sputum induction.

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Disclosure

AC-L has received fees in the last 3 years for talks at meetings sponsored by AstraZeneca, Bial, Boehringer Ingelheim, Chiesi, Ferrer, GlaxoSmithKline, MSD, Novartis, Orion Pharma, and Sanofi, has received travel and attendance expenses for conferences from Bial, Gebro, GlaxoSmithKline, Novartis, and TEVA, and has received funds/grants for research projects from several state agencies, non-profit foundations, and AstraZeneca and GlaxoSmithKline. EC reports non-financial support from ALK, AstraZeneca, Novartis, and Menarini, personal fees from Boehringer-Ingelheim and TEVA, and personal fees/non-financial support from Chiesi outside the submitted work. This paper is part of the doctoral thesis of EC. EP has received travel and attendance expenses for conferences from Gebro Pharma, Chiesi, FAES Farma, Rovi, GlaxoSmithKline, and Sanofi, and has received funds/grants for research projects from state agencies, non-profit foundations, and Alpha Bioresearch. LS-R has received fees in the last 3 years for talks at meetings sponsored by AstraZeneca, Diater, Chiesi, and GlaxoSmithKline, has received travel and attendance expenses for conferences from Sanofi, Allergy-Therapeutics, Hal Allergy, and FAES Farma, has acted as a consultant for Sanofi, Stallergenes-Greer, GlaxoSmithKline, and AstraZeneca, and has received funds/grants for research projects from Spanish Allergy and Clinical Immunology Society (SEAIC), a non-profit foundation. VP has received fees in the last 3 years for talks at meetings sponsored by AstraZeneca, Boehringer-Ingelheim, Merck Sharp & Dohme, and Chiesi, has received travel and

attendance expenses for conferences from AstraZeneca, Chiesi, and Novartis, has acted as a consultant for ALK, AstraZeneca, Boehringer, Merck Sharp & Dohme, MundiPharma, and Sanofi, and has received funds/grants for research projects from several state agencies, non-profit foundations, and AstraZeneca, Chiesi and Menarini. EFMM and ABS and SSM declare no conflicts of interest in this work.

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10.2. Comunicaciones a congresos internacionales

Curto E, Crespo-Lessman A, Mateus E, Soto L, García Moral A, Torrejón M, Belda A, Ramos D, Giner J, Plaza V. Total IgE and Der p 1 (D1) specific IgE in induced sputum (IS) in patients with allergic and non-allergic asthma. European Respiratory Journal Sep 2018, 52 (suppl 62) PA1095

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Total IgE and Der p 1 (D1) specific IgE in induced sputum (IS) in patients with allergic and non-allergic asthma

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Introduction: Nowadays, asthma is considered an heterogeneous disease, and the phenotype of non-allergic asthma is not yet well defined. It has been described that this group of patients has severe asthma, persistent eosinophilia and poor response to conventional treatments. The role of local IgE is one theory currently being considered in its pathogenesis.

Objectives: Validate the measurement technique and standardize the levels of total and specific IgE to *Dermatophagoides pteronyssinus* in induced sputum in allergic and non-allergic asthma patients, and healthy volunteers. Correlate total IgE and D1-specific IgE levels in IS and peripheral blood in patients with allergic asthma and non-allergic asthma.

Methods: A total of 56 asthma patients (21 with allergic asthma and 35 non-allergic) and 9 healthy volunteers were studied. Total IgE and D1-specific IgE were measured in the EI supernatant using the ImmunoCAP immunofluoroassay technique.

Results: D1-specific IgE mean in IS was higher in allergic asthmatics than non allergic, and in these higher than healthy subjects. An intense positive correlation was observed between: 1) total IgE in IS and D1-specific IgE in IS r=0.574 (p=0.000), 2) total IgE in IS and total IgE in blood r=0.743 (p=0.000), 3) total IgE in blood and D1-specific IgE in IS r=0.404 (p=0.010). There was no significant correlation between D1-specific IgE in blood and IS.

Conclusions: This work confirms the local production of total and specific IgE in the induced sputum of asthmatic patients with both allergic and non-allergic asthma. The role and clinical significance of this local production has yet to be defined.

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